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Unraveling the Genotypic Insight of Bedaquiline Resistant Isolates of *Mycobacterium tuberculosis* in Pakistan

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Faiqa Rashid

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To my Beloved Parents and Respected

Professor Zhao Yanlin for being my inspiration

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This is to certify that the research work presented in the dissertation, entitled "**Unraveling the Genotypic Insight of Bedaquiline Resistant Isolates of Mycobacterium Tuberculosis in Pakistan**" was conducted under the supervision of **Dr. Shaukat Iqbal Malik**. No part of this dissertation has been submitted anywhere else for any other degree. This dissertation is submitted to the **Department of Bioinformatics & Biosciences, Capital University of Science and Technology** in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of **Biosciences**. The open defence of the dissertation was conducted on **September 10, 2025.**

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List of Publications

It is certified that following publication(s) have been made out of the research work that has been carried out for this thesis:-

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Abstract

Tuberculosis (TB) remains a significant global health challenge, exacerbated by the rise of drug-resistant TB (DRTB). The World Health Organization (WHO) reports that DR-TB accounts for approximately 13% of all antimicrobial resistance-related deaths worldwide. In 2022, an estimated 410,000 individuals developed multidrug-resistant or rifampicin-resistant TB (MDR/RR-TB), with the highest burden (47%) in the Southeast Asian region. A Knowledge Attitude and Practice (KAP) study elucidates the knowledge, attitudes, and practices of variety of social as well professional groups regarding diverse health care issues. KAP studies on tuberculosis are typically conducted on a global scale. Literature indicates that the KAP points concerning tuberculosis among HCWs remains constantly low, especially regarding diagnosis and treatment. Bedaquiline (BDQ), a key drug targeting mycobacterial ATP synthase, is an essential component of the WHO-recommended Bedaquiline, Pretomanid, and Linezolid (BPaL) regimen for MDR/XDR-TB treatment. However, its extensive and improper use has led to the rapid emergence of BDQ resistance. Pakistan, ranked as the sixth highest RR/MDR-TB burden country, introduced BDQ into its treatment regimen in 2015. Although limited, existing literature from Pakistan has highlighted the presence of BDQ-resistant TB strains, emphasizing the urgent need for continued surveillance and appropriate treatment strategies. This study investigated the role of the knowledge, attitudes, and practices (KAP) of healthcare workers (HCWs) in evaluating their understanding and approach toward TB/DR-TB detection and management. Moreover, the molecular mechanism of BDQ resistance and its associated mutations using phenotypic drug susceptibility testing (pDST) and whole-genome sequencing (WGS), phylogenetic analysis to determine the distribution of BDQ-resistant strains across different Mycobacterium tuberculosis (MTB) lineages, and protein dynamics and molecular docking were performed to assess the impact of key mutations on BDQ binding affinity in MTB strains from Pakistan. The KAP survey revealed substantial knowledge and practice gaps, particularly regarding TB transmission, diagnostic techniques, and resistance detection. The mean scores were calculated as knowledge 15.05 (SD = 3.96), attitude 83.68 (SD

= 15.74), and practices 6.31 (SD = 2.21). Knowledge was significantly associated with education and occupation, with moderate correlations between knowledge and both attitude (r = 0.28) and practice (r = 0.40). Most HCWs lacked formal TB-related training. The study also identified key mutations in Rv0678 (T133P, D89N), atpE (A63P), and pepQ (V244M) genes, which were significantly associated with BDQ resistance. Phylogenetic analysis revealed that BDQ-resistant strains were predominantly found in lineage 2 (Beijing) and lineage 3 Central Asian (CAS), with the Beijing lineage exhibiting a higher frequency of MDR/XDR-TB cases. Structural modeling and molecular docking studies demonstrated that BDQ resistance mutations reduce drug-binding affinity, potentially compromising treatment efficacy. esXo is presented to be an important marker for future BDQ resistance detection. From the Rv0678 marker mutations, we found stabilizing effect of Ala99Asp (0.122 kcal/mol) and Pro48Leu (0.296 kcal/mol) on protein residue while all other mutations including Met139Ile, Gly87Ar, Val120Met and Leu32Ser got destabilizing effect. Whole genome sequencing of 50 phenotypically bedaquline-resistant and 50 BDQ-sensitive isolates revealed 29% BDQ heteroresistance in our study. No significant association of patient variables (age, gender, region and history of anti-tuberculosis treatment [ATT]) was found, while drug resistance pattern among MDR + BDQ and XDR patterns (OR, 0.53 [0.01–0.26]; $P \leq 0.001$ and OR, 0.09 [0.19–0.50]; P = 0.006) were significantly different to BDQ heteroresistance. Higher proportion of BDQ heteroresistant cases with no history of BDQ containing treatment was found. Most bedaquiline heteroresistant strains (n = 19) were from lineage 3, none of the strain bear mixed lineage, with Rv0678 mutations (95%) being the most prevalent genetic marker. We identified both new mutations (n = 17) and reported mutations (n = 21) that contribute to BDQ heteroresistance. The strains with missense variants had the highest percentage of heteroresistance (56%). BDQ heteroresistance is an important indicator of emerging BDQ resistance, predominantly observed in previously treated cases without mixed infections, suggesting a higher likelihood of acquired resistance. Our findings accentuate the complexity of BDQ heteroresistance and the need for better diagnostic and appropriate therapeutic treatment approaches for drug-resistant TB with BDQ-containing regimens. The KAP survey revealed significant gaps

in HCWs' knowledge and adherence to BDQ resistance detection and treatment protocols. This study highlights the increasing threat of BDQ resistance in Pakistan, particularly in high-risk MTB lineages, emphasizing the need for enhanced molecular surveillance and early detection strategies. The findings suggest that BDQ resistance mutations impact drug efficacy through structural alterations in target proteins. Moreover, the gaps identified in HCW knowledge and practices stress the urgent need for improved training programs and adherence to standardized TB treatment guidelines. Addressing these challenges is crucial to ensuring the long-term efficacy of BDQ-based treatment regimens. Conducting larger-scale genomic studies will further elucidate emerging mutations and their impact on BDQ resistance. Investigating the functional consequences of Rv0678, atpE, and pepQ mutations through in vitro and in vivo models, exploring alternative therapeutic strategies including combination regimens, and strengthening molecular diagnostic tools for early detection of BDQ resistance in clinical settings are essential steps forward. This study highlights the need for a multidisciplinary approach to combat BDQ-resistant TB by integrating molecular diagnostics, phylogenetic analysis, and healthcare worker training. Incorporating WGS into surveillance, developing computational resistance models, and strengthening clinical practices can guide policymakers and clinicians in designing effective strategies to improve treatment outcomes in high-burden settings like Pakistan.

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Abbreviations

ADP	Adenosine Diphosphate
AFB	Acid Fast Bacilli
AMK	Amikacin
AMR	Antimicrobial Resistance
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
ATT	Anti-tuberculosis Treatment
BCG	Bacillus Calmette-Guérin
BD	Bectec Decson
BDQ	Bedaquiline
BpaLM	Bedaquiline, pretomanid, linezolid and Moxifloxacin
BSC	Biosafety Cabinet
CFZ	Clofazimin
COPD	Chronic Obstructive Pulmonary Disease
CTAB	Cetyltrimethylammonium Bromide
DLM	Delamanid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DRTB	Drug Resistant Tuberculosis
DST	Drug Sensitivity Testing
ECOFF	Epidemiological cut-off value
EUCAST	European Committee on Antimicrobial Susceptibility Testing

FM	Fluorescence Microscopy
FQ	Fluoroquinolones
GC	Growth Control
GWAS	Genome Wide Association Studies
HCWs	Health Care Workers
HIV	Human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
ICT	Islamabad Capital Territory
INH	Isoniazid
KAP	Knowledge Attitude and Practices
KPK	Khyber Pakhtunkhwa
LED	Light Emitting Diode
LFX	Levofloxacin
LJ	Lewenstein Jensen
LPA	Line Probe assay
LZD	Linezolid
MDR-TB	Multi Drug Resistant TB
MFX	Moxifloxacin
MGIT	Mycobacterium Growth Indicator tube
MIC	Minimum Inhibitory Concentration
MTB	Mycobacterium Tuberculosis
MTBC	Mycobacterium tuberculosis Complex
NAAT	Nucleic Acid amplification Test
NALC	N-Acetyl L-Cysteine
NGS	Next Generation Sequencing
NRL	National Reference Laboratory
NTM	Non-Tuberculosis Mycobacterium
NTP	National TB Control Programme
PANTA	Polymyxin B, Amphotericin-B, Nalidixic Acid, Trimethoprin, Azilocillin
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction

pDST	Phenotypic Drug Susceptibility Testing
PMDT	Programatic Management Drug Resistance
PZA	Pyrazinamide
REMA	Resazurin Microtitre Assay
RIF	Rifampicin
RMR-TB	Rif Mono Resistance TB
RR-TB	Rifampicin Resistance Tuberculosis
SD	Standard Deviation
SIRE	Streptomycin, Isoniazid, Rifampicin, Ethambutol
SLD	Second Line Drug
STBs	Smooth Tuberculosis Bacilli
TAE	Tris Acetate EDTA
TB	Tuberculosis
UN	United Nations
USFDA	United states Fedral drug authority
WGS	Whole Genome Sequencing
WHO	World Health Organization
WT	Wild Type
XDR-TB	Extensively Drug Resistance Tuberculosis
ZN	Ziehl Neelesen

Chapter 1

Introduction

1.1 Background of the Study

1.1.1 Tuberculosis

Tuberculosis (TB) is considered as a primeval infectious malady and a global public health issue that is caused by a single causative agent *Mycobacterium tuberculosis* (MTB) [1, 2]. It is estimated that approximately 25% of people worldwide have contracted tuberculosis [2]. The chance of contracting tuberculosis is highest within the first two years following infection (about 5%), after which it is significantly reduced [3]. Some patients will recover from the infection [4, 5]. Men are more likely than women to get tuberculosis (TB), with approximately 90% of cases occurring in adults. Although pulmonary tuberculosis usually affects the lungs, it can also infect other body regions [5]. TB still affects more than 10 million people year, and since 2021, the number has been increasing. In order to eradicate the worldwide tuberculosis epidemic by 2030, the World Health Organization (WHO) and each of United Nations (UN) collaborators got consent for taking prompt action to end it. In 2023, however, TB most possibly reclaimed its status as the predominant infection which is caused by infectious agent to cause death after being displaced for three years by coronavirus disease (COVID-19) [6].

1.1.2 Global Burden of TB

As stated in Global TB Report 2024, TB persists a main health concern internationally, however after the COVID-19 pandemic caused disruptions, a slight incidence stabilization has been achieved. More prompted actions are needed to meet the End TB Strategy's objectives by 2035. Globally, the rise in TB event cases—the number of people who become ill has slowed and started to level out. In 2023, about a population of 10.8 million, there were somewhat more people than in 2022 (10.7 million), but still significantly higher than in 2021 (10.4) and 2020 (10.1 million) (Figure 1.1). Population growth is mostly responsible for the global increase in incident instances in 2022–2023. Occurrence rate of tuberculosis increased by a relatively small (0.2%) to 134 (95% UI: 125–145) in 2023 [6]. A global estimate describes that out of 10.8 million TB sufferers in 2023, 87% of the total TB burden is bear by thirty high burden countries worldwide, highlights the WHO's study of a modest but steady increase in TB incidence worldwide. India was the highest contributor (26%), Indonesia (10%), China and Philippines (6.8% each) and Pakistan at 6.3%. It is significant to mention that 56% of the worldwide TB burden is bear by the five nations. (Figure 1.2) Geographically, South-East Asia (45%), Africa (24%), and the Western Pacific (17%) accounted highly of TB cases in 2023, with lower percentages in the Eastern Mediterranean (8.6%), the Americas (3.2%), and Europe (2.1%). While there is a decline in mortality level due to tuberculosis globally, it still ranks higher cause of death in comparison to COVID. The main cause of TB mortality is complications from poorly managed or untreated TB infections [6, 7].

1.1.3 Drug Resistance Tuberculosis

The degree of resistance to important medicines in anti-TB medication regimens is what defines drug-resistant TB (DR-TB). Rifampicin (Rif), a bactericidal drug which shortened the course of regimen, is the most significant of these agents [9]. *M. tuberculosis* resistant to both Rifampicin as well as isoniazid (Inh) is known to be multidrug-resistant TB (MDR-TB) [10]. Because Rifampicin resistance TB

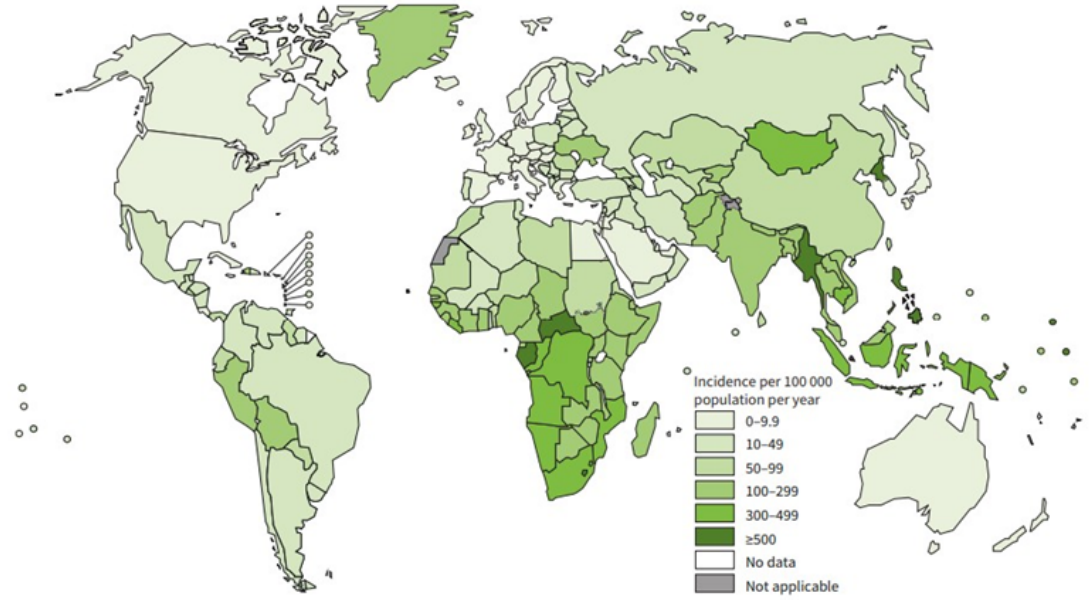

FIGURE 1.1: TB Incidence in world wide population [8]

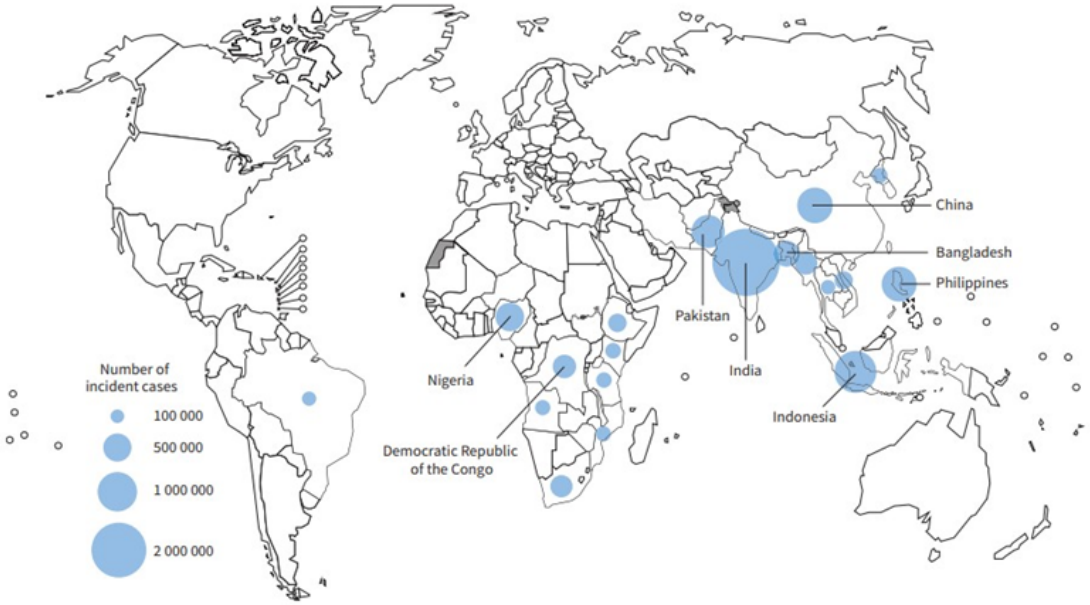

FIGURE 1.2: TB Incidence in high burden countries [8]

RR-TB patients get similar treatment regimens as MDR-TB patients, independent of Inh resistance, RR-TB is regarded as the preliminary stage for second-line TB treatment regimen [11]. Since the beginning of the decade, enhanced surveillance and diagnosis of RR-TB have been made possible by nucleic acid amplification tests (NAATs), particularly extensively used Xpert MTB/Rif assay [12]. Due to the less sensitive molecular diagnostics for Inh and the associated expenses, diagnostic testing for Inh resistance is less than that for Rif [13]. Nonetheless,

MDR cases are the second highest prevalent type of DR-TB in various regions of the globe, while Inh-resistant and Rif-susceptible TB (Hr-TB) is the very frequent prevalent type [14]. Inh is a powerful and well-tolerated first-line treatment for tuberculosis [15–17]. Evidence regarding the influence of Inh resistance on treatment failure rates, however, points to a less impact than that of MDR or RR-TB [18, 19]. For HR-TB, same treatment is recommended as for Drug susceptible tuberculosis while replacing inh with fluoroquinolones [20–22]. The last ten years have seen the development of novel medications to treat MDR or RR-TB, such as bedaquiline (Bdq), pretomanid and delamanid (Dlm), as well as a greater understanding of the effectiveness of linezolid (Lzd) and late-generation fluoroquinolones. The WHO announced a change to the definition of extensively drug resistant tuberculosis (XDR-TB) and pre-XDR in 2021. It is currently defined as MDR-TB with resistance to fluoroquinolones (fq) and at least one additional group A medication (Bdq or Lzd), while pre-XDR-TB is redefined as MDR tuberculosis plus resistant to any fluoroquinolone [10, 15]. Only amikacin and streptomycin are still advised as toxic, second-line injectable medications in situations where all-oral compositions are not feasible or where rescue treatment is required. These amendments also declare the clinical influence of such progressive types of drug resistance [10, 20].

1.1.4 Drug Resistance Burden

In 2022, 410,000 (95% UI 370,000–450,000) persons were estimated by the WHO to have developed MDR/RR-TB, making up 3.9% out of 10.6 million projected TB incidence that year [8]. It is estimated that 13% (95% UI 10–19%) of all deaths globally attributed to antimicrobial resistance are caused by multidrug resistance, exclusive of XDR TB [23]. It was unknown what percentage of individual were encountered MDR-TB. It was estimated that approximately 19 million people are at risk of activating a latent multi drug tuberculosis infection. 30 countries with a high DR-TB burden are listed by the WHO [8]. These are declared as high burden on the basis of both estimated cumulative MDR/RR-TB cases and population wise occurrence rates. Five countries represented half proportion of

MDR/RR-TB in 2023: India (27%), the Russian Federation (7.4%), Indonesia (7.4%), China (7.3%) and the Philippines (7.2%) [24] (Figure 1.3). Prior to the development of new techniques for estimating RR-TB in 2022, which resulted in a significant downward amendment for RR-TB incidence estimates, Pakistan was regarded as one of the three nations with the maximum RR-TB cases [8].

It is an estimation that there is an increase in the prevalence of Rif mono-resistant TB from 12% of total MDR/RR-TB patients in the year 2014 to 22% in the year 2019 [10]. RMR-TB and HIV infection may be linked in specific contexts [25, 26].

Additionally, there is some evidence that the rise in RR-TB is being driven by Rif mono drug resistance TB. An estimated 1.3 million TB cases were Inh-resistant in 2022, including individuals belonged to MDR-TB and HR-TB groups [8].

According to an analysis of survey data from 156 various locations between 2003 and 2017, the prevalence of HR-TB among TB patients who have never had treatment is 7.4%, while the prevalence among those who have received treatment is 11.4% [27].

Since Rif resistance is typically the starting point for additional drug susceptibility testing (DST), the majority of such cases are implausible to be identified.

FIGURE 1.3: Incidence of MDR/RR [8]

1.1.5 Drug Resistance Tuberculosis Diagnosis

The WHO released a standard in 2023 for molecular testing of presumptive TB cases as well as comprehensive Drug Susceptibility Testing (DST) for all bacteriologically confirmed TB cases, at least for Rif resistant (RR) and fq for those with proven RR-TB [28]. But 60% of drug resistant cases worldwide go undiagnosed [8]. Improving treatment results and DR-TB management requires easily approachable testing that can fully as well as reliably determine drug resistance [29, 30]. The main obstacles to widespread access to DST are limited infrastructure and resources as well as a lack of practice implementation [31] (Figure 1.4).

1.1.5.1 DST on Culture

Despite being the standard for years, its use is restricted by its availability and lengthier turnaround time (two to eight weeks) [32]. Resistance is defined as less than equal to one percent of growth seen at a particular critical concentration of the drug while comparing to an inoculum on a control plate using the standard method for solid culture DST; a 42 days procedure [33]. Although globally standardized liquid culture techniques such as BACTEC Mycobacterium Growth Indicator Tube (MGIT) can reduce the duration to about 10 days only, they are unable to identify clinically substantial resistance in some RR-TB cases, which has a considerable impact on treatment choices [34–36].

1.1.5.2 NAATs

In the past ten years several NAATs are currently endorsed by the WHO due to rapid outcome (Figure 1.4). The most popular test in the world is the Xpert MTB/Rif by Cepheid, Sunnyvale, USA [37, 38]. The Xpert MTB/Rif Ultra is another advanced form technique which is more sensitive for TB detection, especially when paucibacillary infection exists, but at the expense of a reduced specificity. The needs for a steady electrical power source, temperature maintained environment, expense, lack of expert staff and maintenance assistance have restricted its

wide use [39, 40]. The 90-minute follow-up test, the Xpert MTB/XDR (Cepheid, Sunnyvale, USA), has a moderate to high diagnostic accuracy on average in comparison to other laboratory-based molecular testing. It can differentiate between high-level and low-level resistance to Inh and fluoroquinolones as well as resistance to second-line injectable (SLI) drugs [41].

Another kind of tests are the moderately challenging automated NAATs, which require more technical know-how and infrastructure and use laboratory-based equipment. Because of the poor infrastructure for sample transportation and result reporting, several countries with high TB burdens have restricted the use of these centralized tests for diagnosis. The WHO has approved them due to comparable performance to Xpert. These assays enable high throughput, test findings in a few hours, and a lower biosafety level (2 versus 3) than phenotypic DST (pDST).

Additionally, the platforms can multiplex and take advantage of economies of scale, which makes them appealing in situations where it is practical to send samples to a central laboratory. The rpoB gene for Rif and the katG and inhA areas for Inh, are the targets of the four suggested tests, which can be used to identify *M. tuberculosis* and first-line TB medications resistance [15].

1.1.5.3 Line probe assays (LPA)

It can be carried out in intermediate laboratories (biosafety levels 2 to 3) and are less complex than the centralized assays mentioned above [15, 42]. The results are obtained in five hours. To detect pyrazinamide resistance, the WHO recently suggested using the LPA Genoscholar PZA-TB II assay to detect resistance against Rif, Inh, fluoroquinolones, ethambutol [43].

Although they are not yet accessible, integrated molecular diagnostics for important DR-TB medications like Bdq and Lzd are being developed as knowledge of the genotypic correlates for drug resistance advances [44]. A consensus mutation library that should serve as the foundation for genotype-based assays was offered by the WHO and a number of expert groups [44].

1.1.5.4 Next Generation Sequencing (NGS)

By identifying reported mutations in targeted genes or the entire genome, NGS offers a flexible method for quick and thorough resistance diagnosis. Four steps are conceptually included in the workflow: Sequencing, data analysis, library preparation, and DNA extraction with quality control [45].

The Illumina MiSeq, ThermoFisher Scientific Ion Torrent Personal Genome, Qiagen GeneReader NGS system, and Oxford Nanopore MinION are commercially available NGS platforms that can be clinically utilized for DR-TB diagnosis. The use of Nanopore for clinical practice has not still been established because there is disagreement over interpretation and there is currently no proof of its effectiveness in practical situations [46].

1.1.5.5 Whole Genome Sequencing (WGS)

WGS can identify all known genotypic markers of drug resistance, including those for new and repurposed drugs. Additionally, it may easily be modified to detect and identify novel resistance mutations, such as the I491F mutation in rpoB, which NAAT tests cannot detect [47]. Moreover, it offers details on lineage, heteroresistance, epistasis, transmission mode and compensatory mutations [48].

Discordant results in phenotypic and genotypic testing may be resolved by using WGS to detect drug resistance in *M. tuberculosis* [49]. WGS is currently carried out using MTB cultured isolates as it requires a comparatively large amount of high-quality DNA to produce WGS data, may prevent wider use because of the culture's delay. However, WGS results can be produced 7% less expensively than current diagnostic procedures and an average nine days faster results than reference laboratory reports [50]. Direct WGS from uncultured sputum has not always been successful, especially when the bacterial load is low [51].

Adding a bait enrichment step has allowed for the successful sequencing of sputa with lower bacterial concentrations [52]. This is still too costly to scale up for clinical usage. Although a phase IV trial is currently examining the WGS use to

direct RR-TB treatment, better sample processing is necessary before WGS can completely replace the phenotypic DST workflow procedure [53].

Methodology	Drug detection	Time	Sample
WHO-endorsed tests			
Xpert, GeneXpert Edge	Rifampicin, isoniazid, second-line injectables, fluoroquinolones	Less than 2 h	Sputum and more than 10 other specimen types
Line probe assay	Rifampicin, isoniazid, second-line injectables, fluoroquinolones, pyrazinamide, ethambutol	5 h to 1 day (GenoScholar PZA-TB[a])	Sputum and culture
Loop-mediated isothermal amplification	Possibility for resistance detection	Less than 1 h	Sputum
Truelab	Rifampicin (isoniazid, second-line injectables, fluoroquinolones and linezolid are under development)	Less than 1 h	Sputum
Centralized drug-sensitivity testing (Roche or Abbott)	Rifampicin, isoniazid, second-line injectables, fluoroquinolones	8 h or less	Sputum, culture, bronchial alveolar lavage, sediment
Targeted sequencing	Rifampicin, isoniazid, second-line injectables, fluoroquinolones, pyrazinamide, ethambutol, linezolid, bedaquiline, clofazimine	2–3 days	Sputum or early positive culture
Emerging technologies[b]			
Point-of-care nucleic acid amplification test	Rifampicin, isoniazid, second-line injectables, fluoroquinolones	Less than 2 h	Sputum
Whole-genome sequencing	Rifampicin, isoniazid, second-line injectables, fluoroquinolones, pyrazinamide, ethambutol, linezolid, bedaquiline, clofazimine, ethionamide	2–3 days	Early positive culture

[a]The specified time is specific for the GenoScholar PZA-TB that detects pyrazinamide resistance. [b]Tests that are not yet endorsed by the WHO but are under development or evaluation.

FIGURE 1.4: WHO recommended tests for TB and DRTB [54]

1.1.6 Drug-resistant TB Treatment

Combating the gaps for diagnosis and enhancing therapy results, there are still RR, MDR-TB; pre-XDR-TB and XDR-TB cases [55]. Bacteriological confirmation of tuberculosis and resistance testing employing quick molecular diagnostic testing, culture methods, or sequencing techniques are necessary for the detection of treatment resistance.

In contrast to earlier regimens that included injectable drugs, WHO has endorsed all-oral treatment regimens for MDR/RR-TB treatment since 2018 [56].

Three main regimen types are included in the most recent recommendations for treating drug-resistant [20, 57]. For patients with MDR/RR-TB (with or without fluoroquinolone resistance), category-I comprises of two SIX month all-oral treatment. MDR/RR-TB patients who do not exhibit fluoroquinolone resistance, the second group comprises a number of all-oral, brief regimens lasting nine months.

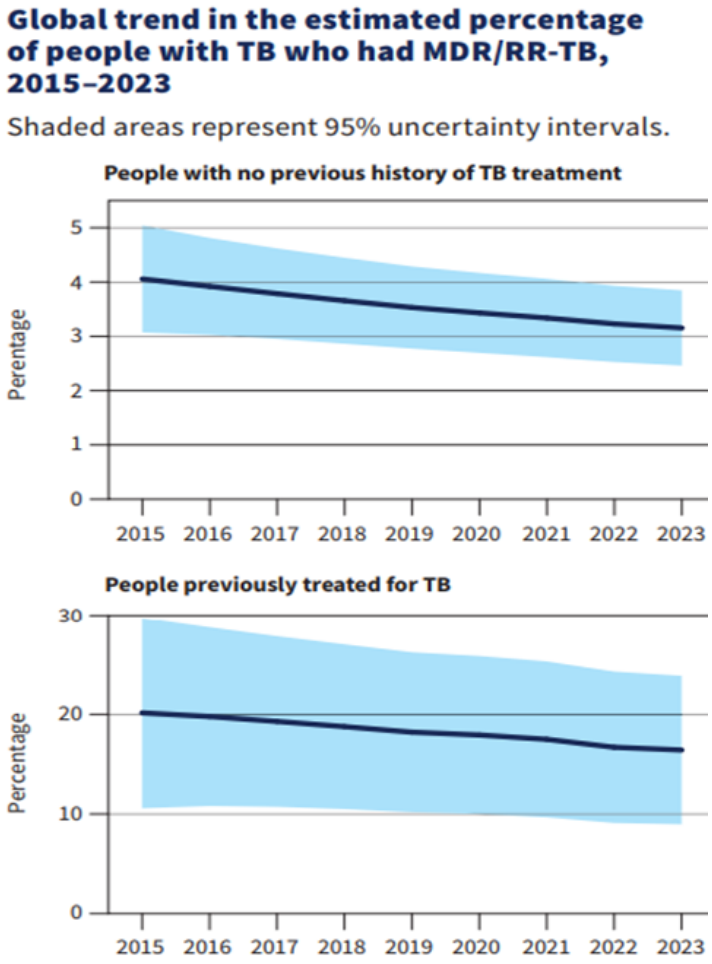

FIGURE 1.5: Global Trend of RR/MDR cases [8]

Longer treatment regimens of 18–20 months that might comprise an injectable medication (amikacin) fall under the third type. The longest regimens are used as a last resort, with 6-month regimens being used first. Rifampicin resistance testing was performed on 79% of individuals (3.4/4.3 million) with bacteriologically approved pulmonary tuberculosis worldwide in 2023. This is significantly higher prior to pendemic (62%) in 2019 and up from 73% (2.9/4.0 million) in 2022 and 69% (2.4/3.5 million) in 2021.

All six WHO areas had improvements; in 2023, the percentage in South-East Asia, Europe in addition to the Western Pacific reached $\geq$80%. Out of those who were tested in 2023, there was only a minor increase (4.6%) from a total of 180 426 individuals tested in 2022 (Figure 1.5). Even though more people were tested in total but fewer persons were found to have MDR/RR-TB in 2022 in contrast to

2019. This aligns with the projected decrease in MDR/RR-TB cases (Figure 1.6).

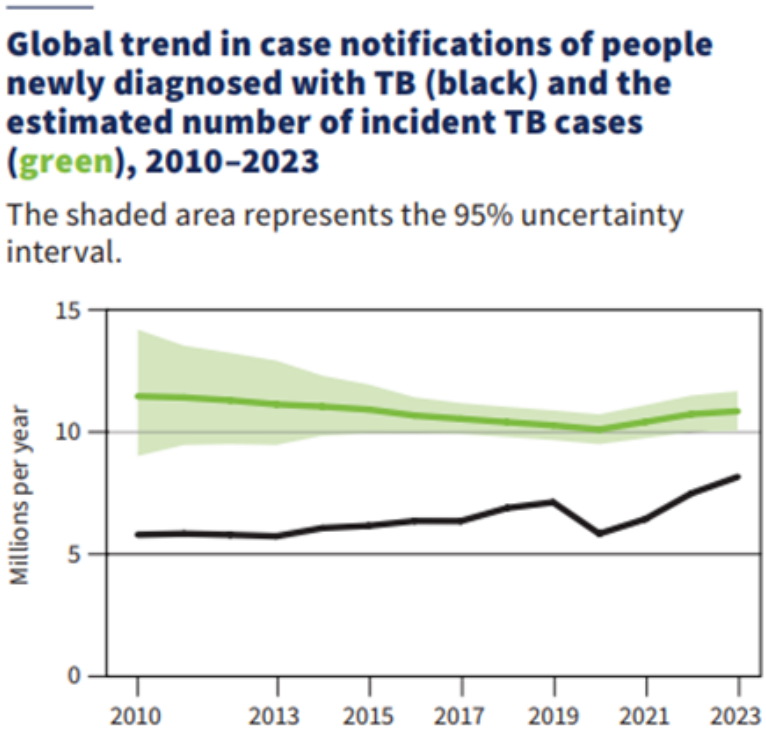

FIGURE 1.6: Global trend in incident TB case notifications [8]

Ten countries presented about 75% of the discordance between the number of patients receiving treatment globally in 2023 and the anticipated number of MDR / RR-TB event cases globally in the same year. India, the Philippines, Indonesia, China, Pakistan, Myanmar, Ukraine, Nigeria, Vietnam, and South Africa were listed according to their respective shares of the gap.

Enhancements in drug resistance testing coverage and treatment access are required in these nations in order to significantly reduce this disparity. The treatment success rates for MDR or RR individuals have significantly improved in last few years (Figure 1.7). It was found that the treatment success rate was about 68% for those who started therapy in the year 2021, which marks significant improvement from 50% in 2012 and up from 64% in 2020 and 60% in 2019. In 2021, the Eastern Mediterranean Region had a 74% treatment success rate, while the European Region had a 61% rate. By the end of 2023, there were 58 countries that preferred the 6-month BPaLM regimen over 41 by the end of 2022 for treating patients with MDR/RR-TB or pre-XDR-TB. Around 100 nations were using the

9-month oral regimens to treat MDR/RR-TB, compared to 95 in 2022 and 93 in 2021 [8].

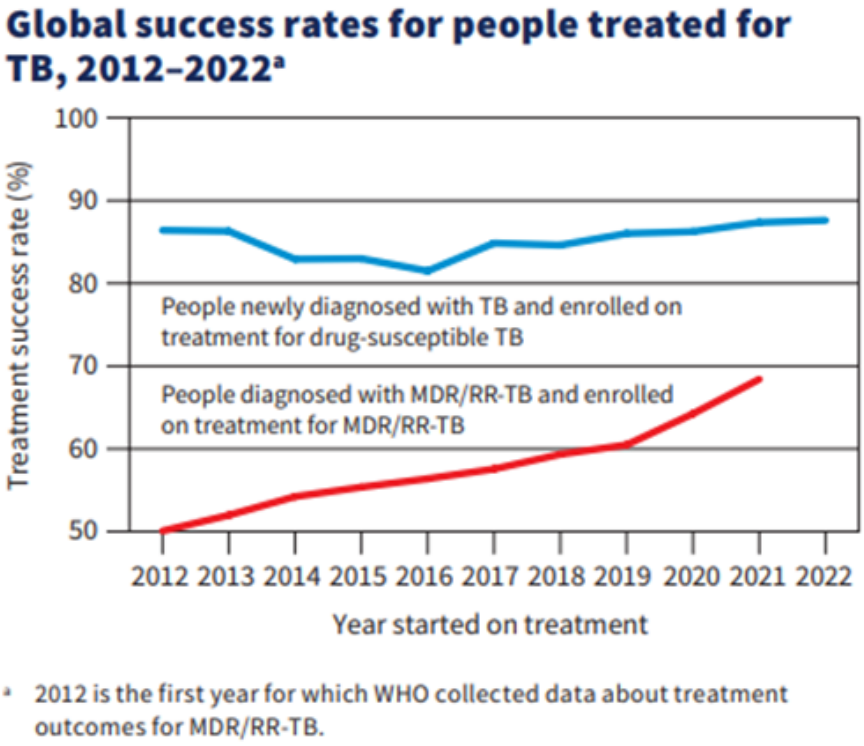

FIGURE 1.7: Global TB treatment success rate [8]

1.1.7 Epidemiology of TB/DRTB in Pakistan

Pakistan ranks fifth internationally for TB occurrence and is declared as one of the high-burden nations for tuberculosis, MDR-TB, and TB/HIV co-infection [58]. Pakistan recorded 611,000 TB cases annually in 2022, including 36,000 MDR-TB cases. This accounts for 5.8% of the global TB burden. As in 2021, Pakistan's TB occurrence rate was 264 patients per 100,000 people, which was much higher than the global average of 134 cases per 100,000 people [59]. Given that Pakistan ranks fourth among nations with a high burden of MDR-TB, representing 7.2% of the worldwide burden and an annual incidence of 28,000 cases, the growing frequency of DRTB strains is another serious concern [60]. In Pakistan, a yearly prevalence and death rates for tuberculosis are 340 and 20, respectively, per 100,000 people. The age range most affected by tuberculosis is 15–49 years old [61].

The TB epidemic in Pakistan is mostly caused by poverty, overcrowding, malnourishment, and restricted access to medical care [62]. Furthermore, the stigma attached to tuberculosis frequently postpones identification and treatment, which exacerbates the disease's transmission [63].

Although Pakistan's National Tuberculosis Control Program (NTP) has made progress in augmented diagnosis and treatment services but issues such a lack of money, a shoddy healthcare system, and a lack of qualified staff still exist.

1.1.8 Bedaquiline

BDQ is a crucial medication endorsed by the WHO to treat MDR and XDR cases [64]. As a part of Bpal (Bedaquiline, Pretomanid and Linezolid) treatment regimen, it indicates its efficacy in achieving successful treatment outcomes for DRTB in a reduced timeframe. Bedaquiline exhibits bactericidal properties by specifically targeting mycobacterial ATP synthase. The extensive and improper use of BDQ has led to a rapid emergence of resistance. Bedaquiline acts as an inhibitor of adenosine triphosphate (ATP) synthase of MTB.

It demonstrates in vitro effectiveness against both drug-sensitive and resistant strains of *Mycobacterium tuberculosis*. By the end of 2015, more than 2,500 patients in 50 countries had been treated with bedaquiline, which had been accelerated approved for use in MDR-TB treatment regimens by 2012 [65]. The application of bedaquiline may significantly benefit a considerable number of patients with MDR-TB; however, expectations for enhanced outcomes and widespread utilization are moderated by concerns regarding the swift emergence of resistance. It is alarming that resistance to bedaquiline appeared so quickly after it was first introduced. In order to tailor anti-TB treatment, it is crucial to make it easier for people to obtain quick and accurate drug susceptibility testing (DST) for all drugs [66]. WHO has warned that using bedaquiline improperly could speed up the emergence of resistance. According to WHO data, bedaquiline was introduced in Pakistan into the treatment regimen for MDR and XDR-TB in November 2015 [64].

1.1.9 Health Care Workers and TB/DRTB KAP Involvement

Health care workers (HCWs) are the primary personnel responsible for managing patients with various disease conditions. Timely diagnosis and effective management of tuberculosis patients by knowledgeable as well as skilled healthcare workers are essential for communicating about this infectious disease. Research has shown that healthcare workers frequently exhibit significant gaps in knowledge concerning tuberculosis diagnosis, treatment, and prevention and control measures. Evaluating the knowledge, attitudes, and practices regarding infectious diseases, particularly tuberculosis, is essential [67]. Additionally, certain healthcare workers exhibit negative attitudes and concerns regarding tuberculosis stigmatization, resulting in suboptimal practices that contribute to increased tuberculosis incidence, both in themselves and in the patients being served. Their level of understanding of TB administration was evaluated to be inadequate with relevance to latest TB training [68]. A number of studies conclusively determined that pre-training in tuberculosis knowledge, supplemented by further education, may improve the current understanding of its management [69–71]. Furthermore, evidence demonstrates that periodic trainings and administration meetings can enhance tuberculosis insight and amenities among healthcare workers [72, 73].

A Knowledge Attitude and Practice (KAP) research is essential for optimizing challenges, limitations, and capacities to improve specific scenarios. These studies elucidate the knowledge, attitudes, and practices of variety of social as well professional groups regarding diverse health care issues. KAP studies on tuberculosis are typically conducted on a global scale [74–76]. Research indicates that the KAP points concerning tuberculosis among HCWs remains constantly low, especially regarding diagnosis and treatment [77, 78]. Several factors, including education, insufficient training related to tuberculosis, limited experience with tuberculosis patients, stigmatization of tuberculosis, inadequate knowledge, implicit attitudes, and misleading practices, may contribute to low knowledge, attitudes, and practices (KAP) scores. A significant number of KAP studies on tuberculosis

have been carried out within the general population [79–83] healthcare workers (HCWs) globally [84–88].

Although numerous TB and DRTB related KAP studies have been conducted within the normal population of Pakistan, there is a significant lack of similar research focused on healthcare workers in the country [89–92]. To address the gaps in research and improve already existing body of knowledge, we conducted a TB-KAP study involving healthcare workers in Islamabad capital territory, Pakistan.

Conclusively, the current study is designed to provide an important aspect of DRTB while providing a brief study of bedaquiline which is part of group A TB treatment regimen and related mutational analysis. Involvement of BDQ in creating drug resistance in MDR/XDR patients is an important aspect to focus on to avoid increase in DRTB prevalence rate. As an additional objective of the study involvement of HCWs was studied in DRTB settings to address the gaps present in TB control programs generally.

1.1.10 Gap Analysis

Some investigations have been reported on genotypic mechanism of drug resistance in response to first and second-line in Pakistan [93–95], bedaquiline has been ignored, a key anti-TB drugs, recommended by WHO for TB treatment specifically for MDR TB. Genotypic mechanism of resistance to bedaquiline is needed to be investigated on broad level including target mutations in addition to geographic specific mutations. Further, studies are also needed to find specific geographic molecular markers for bedaquiline resistance and susceptibility to elucidate its contribution in DRTB prevalence.

1.1.11 Problem Statement

Drug resistance isolates are commonly validated through phenotypic methods. Genotypic approaches are rarely performed to explore the causes of drug resistance. Although very few studies have explored the genotypic level of resistance

to first and second-line drug, bedaquiline has been ignored which is one of the key anti-TB drugs, recommended by WHO for TB treatment. For better management of TB in high burden countries, genotypic methods to unravel the mechanism of drug resistance are time efficient and free of false negative bedaquiline resistance results. Further, molecular methods to investigate the bedaquiline resistance, may not only be useful for diagnostic accuracy but they also offer molecular data in novel drugs designing for better management of TB, drug resistance and novel therapeutic agent in high burden countries including Pakistan.

Furthermore, systemic and behavioral gaps in TB control and prevention programs contribute to drug resistance in addition to microbial evolution. The emergence of DRTB, including BDQ resistance, may be accelerated by selection pressures caused by healthcare personnel' low KAP scores, which can be caused by delayed diagnosis, improper prescription practices, or inadequate treatment adherence monitoring. Consequently, a thorough strategy that combines a measurement of healthcare worker KAP with molecular analysis of BDQ-resistant bacteria is crucial. In addition to improving treatment results and diagnostic precision, closing this twofold gap will offer crucial information for creating new therapeutic drugs and resistance-preventing strategies. Therefore, as interrelated aspects of the larger DRTB problem, the current study investigates both the genotypic features of BDQ resistance and the role of healthcare worker practices.

1.1.12 Objectives of the Study

1. To assess the KAP of HCWs in TB/DRTB settings and explore how these factors impact the management and outcomes of DRTB cases.

2. To explore the molecular mechanism of bedaquiline resistance and underlying mutation conferring genes.

3. To phylogenetically analyze the *Mycobacterium tuberculosis* isolates, prevalent in Pakistan.

4. To evaluate the mutation's effect on bedaquiline target protein dynamics.

1.1.13 Significance of the Study

1.1.13.1 Valuating HCWs' KAP in TB/DRTB Settings

In a country with a significant burden of TB and DRTB, it is essential to comprehend the obstacles to effective TB control. Healthcare workers are essential in overseeing patients undergoing extended TB therapies, such as BDQ and DLM. Assessing their Knowledge, Attitude, and Practices (KAP) is crucial for enhancing treatment adherence, infection control, and patient outcomes. This study aims to evaluate TB-KAP among healthcare workers to improve TB management strategies.

1.1.13.2 Investigating BDQ Resistance Molecular Mechanisms

Determining resistance-associated mutations (*Rv0678*, *atpE*, and *pepQ*) would improve knowledge of BDQ resistance pathways, facilitating early identification and focused therapeutic approaches.

1.1.13.3 Phylogenetic Analysis of Isolates of *M. tuberculosis*

In order to guide regional TB control measures, mapping the genetic diversity of BDQ-resistant strains in Pakistan will assist in monitoring lineage-specific resistance trends and transmission dynamics.

1.1.13.4 Assessing Mutation Effects on BDQ Target Proteins

By analyzing the structural and molecular aspects of BDQ resistance mutations, it will be possible to determine how genetic alterations affect drug binding and bacterial fitness, which will aid in the development of new drugs and treatment adjustments.

Chapter 2

Review of Literature

2.1 TB Pathophysiology

The *Mycobacterium tuberculosis* complex consists of closely related members that have the potential to cause disease in both humans and animals. The additional members from this group include *Mycobacterium africanum*, *Mycobacterium canetti*, *Mycobacterium bovis*, and *Mycobacterium microti*. Other mycobacteria from this group are known as non-tubercle mycobacteria (NTM). MTB is described as asporous, stationary, obligate, aerobic, facultative, catalase-negative, intracellular, non–spore-forming, and nonmotile bacilli [96].

Tuberculosis bacilli are transmitted from one individual to another through airborne droplet nuclei that can remain part of the air for extended periods. The likelihood of transmission increases significantly with extended exposure duration to droplet nuclei. Upon inhalation, droplet nuclei can attach to the airway mucosa, potentially leading to an infection, though this is not guaranteed. (Figure 2.1) The bacillus depends on intricate and inadequately defined pathogen virulence factors alongside host immunomodulatory mechanisms, which may result in its elimination, persistence in latency, or progression to active tuberculosis disease [97].

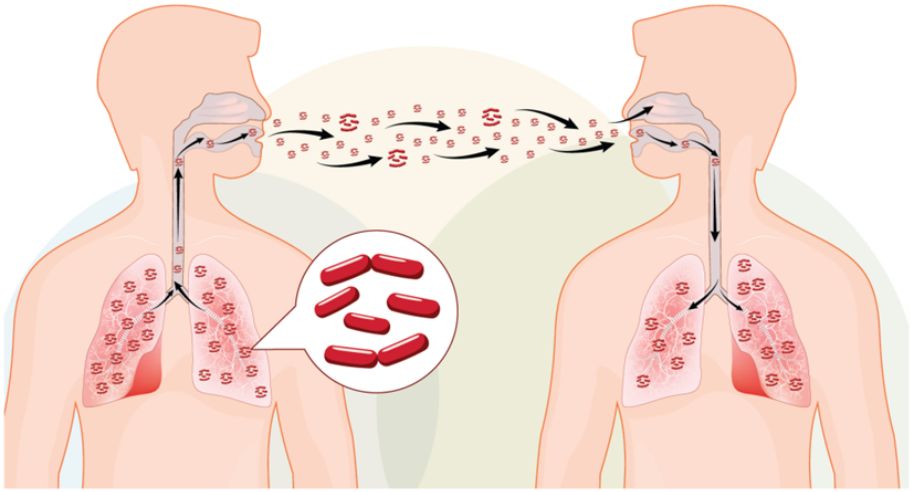

FIGURE 2.1: TB Pathophysiology [4]

2.2 MTB Symptoms

Cough, fever, weight loss, night sweats, and malaise are common constitutional signs of tuberculosis. It is likely to rank highly on the differential diagnostic list for physicians working in regions where tuberculosis is prevalent. On the other hand, the diagnosis could not be given first thought by medical professionals working in environments where tuberculosis is rare. When patients appear with extra-pulmonary tuberculosis, the diagnosis is frequently difficult. Important elements of the history include the routine examination of risk factors, including a previous history of tuberculosis, known contacts with exposure, country of origin, international travel, family history, occupational and residential exposures, immunosuppression, and immunocompromised state. The likelihood of an earlier TB diagnosis will rise if this information is obtained during all initial patient encounters, given the high prevalence of TB worldwide [3].

2.3 MTB Genome and Lineage Distribution

The complete genome sequence of *M. tuberculosis H37Rv* (a virulent strain isolated in 1905 and then propagated in vitro) was published in 1998 [99]. The circular

genome comprises of 4,411,532 bp and has a mean guanine and cytosine content of 65.6% (Figure 2.2).

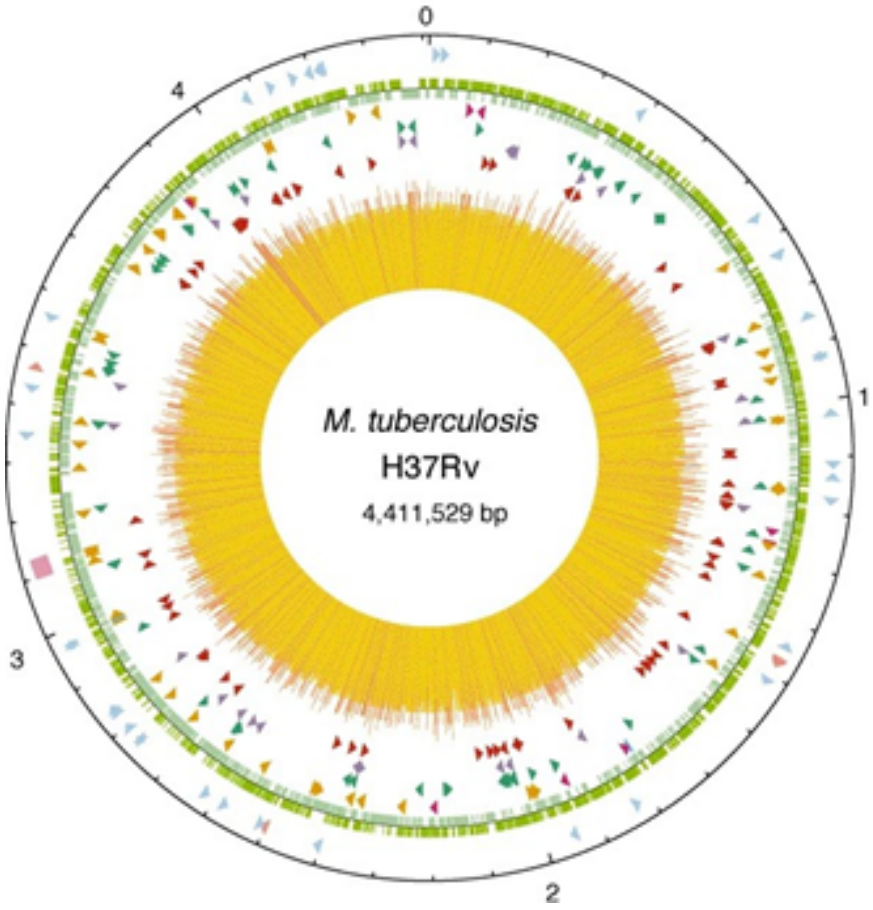

FIGURE 2.2: MTB Genome [4]

Despite the presence of these conventional species of MTBC, it also includes *M. canettii* and the so-called smooth tuberculosis bacilli (STBs). The STBs exhibit notable efficiency in colony formation, alongside various other important characteristics, including horizontal gene transfer, distinguishing them from other MTBC [98]. This group of bacteria infrequently infects humans, with approximately sixty types having been characterized to date [99]. From an evolutionary perspective, one group of MTBC has undergone a deletion of a genomic region known as *TbD1*, which is also referred to as evolutionary modern. While a collective lacking such deletions are recognized as the ancestral form [100]. According to the analyses of WGS, the *Mycobacterium tuberculosis* complex (MTBC) has been categorized into seven lineages that are adapted to humans [101]. Lineage 2 and 4 are regarded as the most dominant groups. Lineage 2 is prevalent in Central and East Asia,

South Africa, and Russia. Lineage 4 of Euro-American origin was the dominant presence across Europe, Asia, Africa, and America. Lineages 1 and 3 are confined to East Africa, Central Asia, South Asia, and South-East Asia. Lineages 5, 6, and 7 are confined exclusively to West Africa, while 1 and 2 are referred to as *M. africanum* [102]. Largely, two more lineages within the classical MTBC have adjusted to various wild and domestic animal species. One of these two lineages includes animal-associated strains identified as *M. bovis* (vaccinal strain BCG) [103].

2.4 Antimicrobial Resistance

TB has long been a widespread disease affecting humans [104]. Currently, tuberculosis continues to be a significant worldwide health issue, since it is the primary cause of mortality from an infectious illness in humans [105, 106]. In 2022, more than 7.5 million cases of tuberculosis were reported, leading to an estimated 1.30 million deaths directly associated with the disease [107].

In humans, the basic TB causative bacteria are part of MTBC [108]. The genetic diversity of the MTBC is relatively constrained when compared to other bacterial species; however, the global populations of MTBC that have adapted to humans can be classified into nine distinct phylogenetic lineages [108–111].

The significance of antimicrobial resistance (AMR) in tuberculosis remain profound, as it stands as the primary cause of death linked to this issue, accounting for nearly 200,000 of the approximately 700,000 fatalities related to antimicrobial resistance in 2014. [112]. AMR presents an escalating global challenge, resulting in elevated rates of treatment failure, extended durations of therapy, increased healthcare costs, and a heightened risk of adverse effects associated with treatment [113–116]. AMR exerts a considerable impact on both economic and social dimensions [112, 117].

AMR presents a considerable obstacle to effectively address TB and other infectious diseases [113, 118]. The emergence of AMR is an complicates phenomenon

shaped by various socioeconomic and behavioral factors [119–122]. The development of antimicrobial resistance in a pathogenic population is essentially an evolutionary process [123, 124]. Multiple factors, including pharmaceutical influences and the genetic makeup of pathogens, play a significant role in this evolutionary process. The specific type of medication and its dosage can impact the traits and frequency of antimicrobial resistance mutations within a particular group of pathogens, often described as the mutational profile for antimicrobial resistance [125–127].

Furthermore, populations of pathogens are composed of genetically differentiated strains and this genetic diversity could impact the development of antimicrobial resistance [128, 129]. Differences in the genetic backgrounds of pathogens may lead to diverse inherent sensitivities to a specific medication [130, 131]. This could potentially influence the outcomes of patient treatment [132]. The genetics is much involved in shaping the emergence and prevalence of antimicrobial resistance, [133, 134] the mutational profile for AMR [128, 129], and the phenotypic contribution of AMR mutations [135, 136].

Investigating a connection between genetic aspect of pathogen and drug pressure is essential for comprehending the ways to mitigate the rise of antimicrobial resistance in pathogen populations. MTB exhibits reduced genetic diversity when compared to other bacterial diseases [136]. A variety of studies indicate that this limited genetic diversity influences the traits and prevalence of antimicrobial resistance [128, 135].

2.5 Emergence of Drug Resistance

Drug resistance created by human actions is a reality. This often arises from inadequate treatment approaches, such as relying on a single medication, administering dosages below the recommended levels, utilizing inferior medications, and lack of adherence to treatment protocols (Table 2.1). Drug-susceptible MTB populations experience selection pressure from TB medications, leading to a reduction in

drug-susceptible bacilli. This creates favorable conditions to yield drug resistant mutants, resulting in the development of drug resistance, a phenomenon referred to as acquired resistance to TB drugs within treatment duration. Primary resistance denotes resistance to strain infection prior to any anti-TB treatment [137].

TABLE 2.1: Causes of DRTB [140]

Health care providers: Improper medical care (Regimen)	**Drugs: Inadequate supply/quality**	**Patients experiencing insufficient medication intake or inadequate response to therapy.**
Unsuitable instructions	Low quality	Insufficient information
Noncompliance with guidelines	Drug shortages	Inability to comply with therapy due to lack of transportation
Lack of guidelines	Inadequate storage scenarios	Social, economic concerns and Myths
Inadequate training and poor KAP of HCWs	Improper dose or combination, treatment Knowledge	Negative occurrences
Lack of proper treatment monitoring	Poor regulation of medicines	Insufficient directly monitored therapy

Typically, a mutation leads to resistance against one or several medications. Sequential mutations across multiple genes lead to the development of resistance against two or more medications. Understanding the origin of drug resistance is of utmost importance. Was it obtained, or was it inherent?

The distinction holds significance as it illustrates that while acquired medication resistance highlights the necessity for enhanced patient management to prevent the progression of resistance, primary drug resistance underscores the imperative for improved tuberculosis control to halt transmission. The basic purpose of tuberculosis control is to halt its transfer via the timely identification of infected individuals and to prevent the development of treatment resistance. Employing the history of care serves as a clear approach to differentiate between the two [137, 138]. Acquisition and transmission of DRTB is elaborated in Figure 2.3.

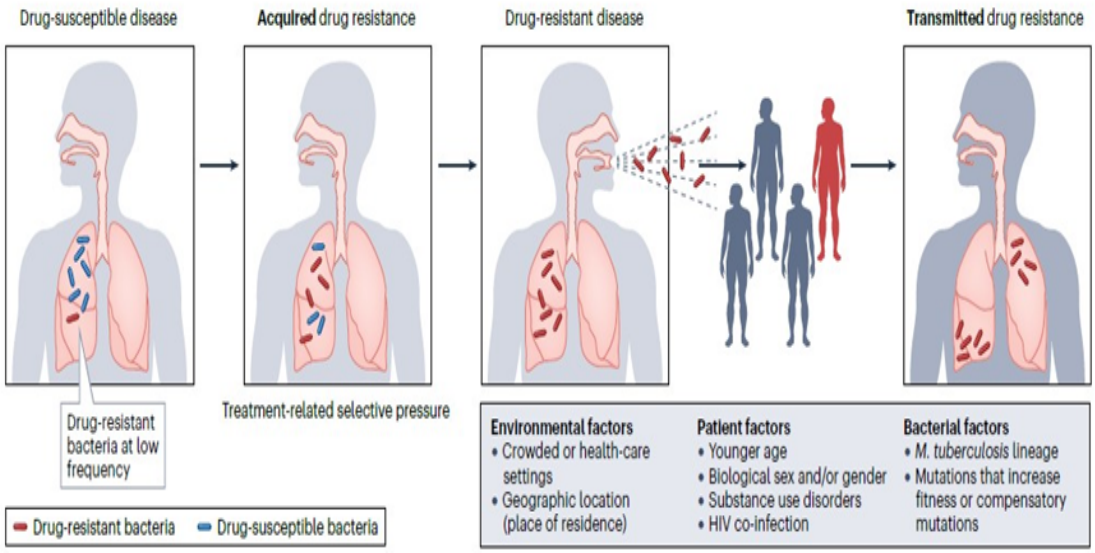

FIGURE 2.3: Acquisition and transmission of DRTB [138]

There is low DRTB bacteria frequency in in Drug susceptible patients. DRTB is acquired due to selection pressure relevant to treatment.

A combination of environmental, patient and bacterial factors are responsible in DRTB acquisition and transmission [138].

2.6 Indicators of Drug Resistance

2.6.1 Intrinsic Resistance

MTB exhibits variability in its innate or intrinsic resistance to a range of drugs. Mycobacteria are somehow more closely linked to Streptomyces species in diversity of self-defense against antibiotic resistance.

This self-defense mechanism is characterized by a hydrophobic waxy cell covering, a minimal count of porins which are water filled and a low lipid profile of the cell membrane, all of which contribute to its resistance against various compounds.

There is significant interest in developing compounds that can effectively counteract antibiotic resistance; however, this goal has yet to be realized.

2.6.2 Acquired Resistance

This is more connected to the clinical aspects of DRTB. The process is facilitated by the participation of variations in genomic DNA, incorporating different mutations or alternative mechanisms. This is regarded as a significant and irreversible process, with extremely low probabilities of reverting any acquired mutation back to the wild type genotype. The predominant mechanism of MTB acquired resistance typically involves genomic mutations in the drug targets, leading to resistance against both first and second line DRTB medications Figure 2.4.

Alterations in genes that serve as drug targets lead to inadequate binding between the drug and its target, as well as ineffective drug action. For illustration purposes, let us examine BDQ and its objectives. The ATP synthetase subunit E, which is coded by atpE, acts as the target for BDQ. Nevertheless, mutations within this gene are rare, probably because of the significant fitness cost associated with them.

The BDQ mechanism that is most commonly employed. The transmembrane transporter MmpL5-S5 (Rv0676c-Rv0677c) is regulated by a transcriptional repressor encoded by the mmpR5 gene (Rv0678), which has mutations linked to resistance. BDQ resistance is nominated as the single mode of acquired resistance linked to efflux in *M. tuberculosis*, which is attributed to the overexpression of MmpL5-S5, probably due to enhanced transport of BDQ through the membrane. There has been a growing body of literature documenting a phenomenon of heteroresistance which describes the coexistence of wild-type and resistance-associated variants within a single clonal TB infection [139].

The heteroresistance identification has been potentially assisted by high-throughput sequencing due to their detection eligibility in low-frequency populations within pathogenic isolates, with its prevalence potentially influenced by the specific drug and its clinical application. Subpopulations that occur at very low frequencies are often viewed as transient. Nevertheless, their frequency can increase due to positive selection, which may result in the fixation of resistance variants in appropriate environments. Nevertheless, the importance of variants with reduced frequencies

and the handling of hetero-resistance noted prior to or during treatment remains ambiguous [139].

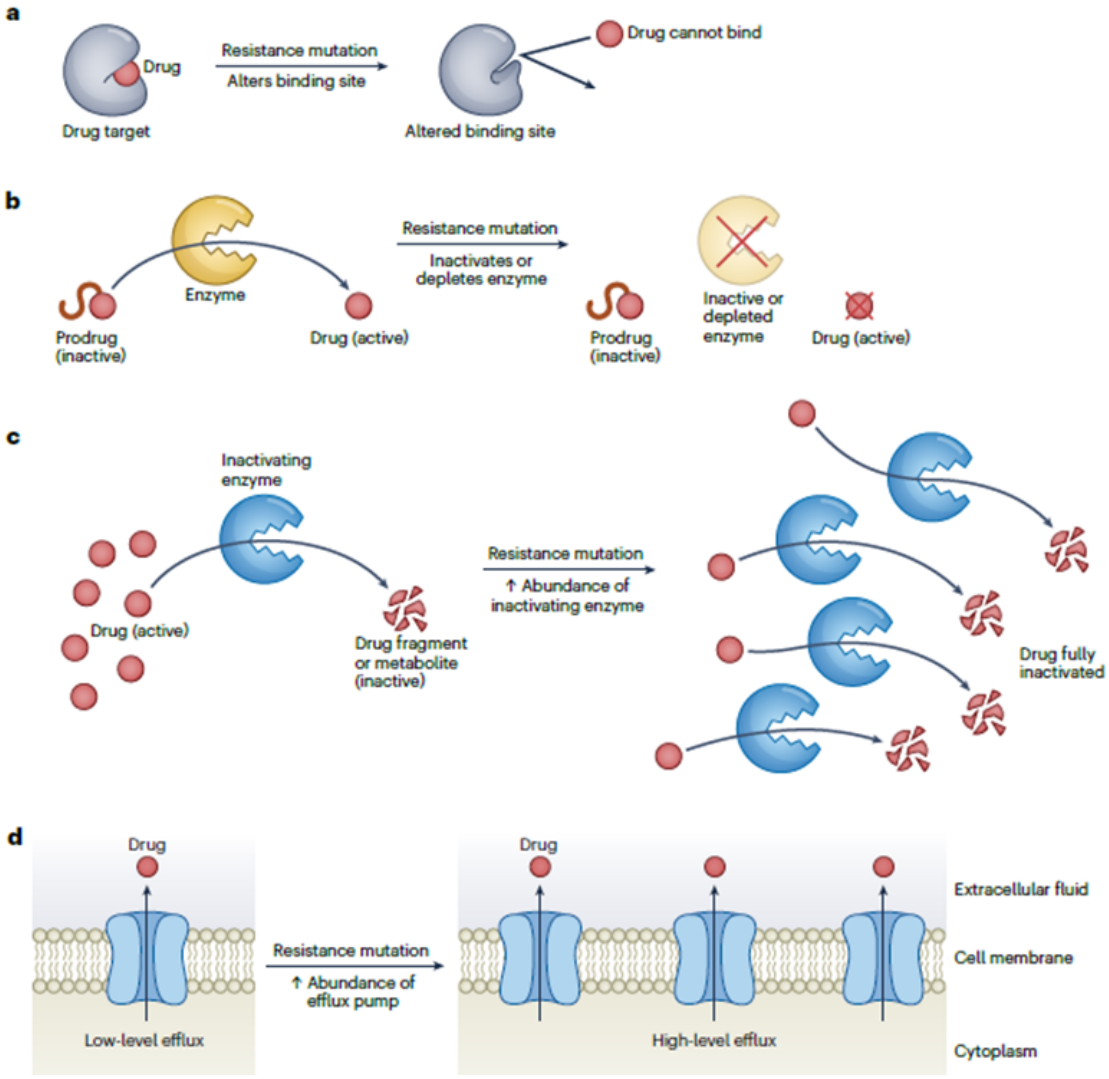

FIGURE 2.4: Mechanisms of DRTB [183]

The figure illustrates key mechanisms of acquired drug resistance in *M. tuberculosis*: (a) Mutations altering drug targets prevent drug binding (e.g., rifampicin, pyrazinamide, linezolid, ethambutol resistance). (b) Loss of prodrug-activating enzymes leads to inactive drugs (e.g., isoniazid, pyrazinamide, ethionamide resistance). (c) Increased production of drug-inactivating enzymes neutralizes the drug (e.g., kanamycin, amikacin resistance). (d) Regulatory mutations enhance drug efflux, reducing drug efficacy (e.g., bedaquiline, clofazimine resistance) [138].

2.7 Antituberculosis Treatment Regimens & Their Genetic Insight

Three groups (A, B, and C) of TB medications for DR-TB have been created by WHO. The ratio of efficacy to safety determines their ranking. (Table 2.2)

2.7.1 Rifampicin

RIF is an important part of TB treatment significantly as it is bactericidal in action. Mutations resistant to RIF are less frequently observed in comparison to those associated with other anti-TB drugs, the occurrence of RR is still increasing due to its extensive application. Rifampicin (RIF) specifically focuses on the *rpoB* gene, responsible for encoding the β component of RNA polymerase. Around 95% of *rpoB* mutations are associated to rifampicin resistance. The maximum mutations identified in the *rpoB* gene are located within an 81 base pair Rifampicin-Resistance Determining Region (RRDR), especially in a hotspot area.

Non-synonymous mutations occur with greater frequency compared to insertions, deletions, and frameshift mutations among the various types of mutations. [140]. Drug resistance primarily develops due to genetic variations rather than through gene transfer from other bacteria. It has been posited that the drug resistance in MTB isolates is due to spontaneous mutations [141]. The *rpoB* mutations were observed across different regions worldwide, coinciding with a significant prevalence of tuberculosis [142]. *rpoB* gene variation have been demonstrated to correlate with elevated Minimum Inhibitory Concentration (MIC) to rifampicin in countries across Asia [143].

In a study involving 1080 TB isolates from Pakistan, 63 exhibited resistance, with 24 classified as MDR-TB. DNA sequencing via PCR was performed on 19 of the 24 MDR-TB samples to identify mutations in the 81bp hotspot regions of the *rpoB* gene linked to rifampicin resistance.

In the investigation, eleven isolates exhibited the S531L SNP in the *rpoB* gene, marking it as the most prevalent finding. The second most frequent mutation was identified at codon 516, featuring two SNPs: Asp516Val and Asp516Tyr.

The study revealed changes Ser512Ile and Leu533Pro, while Tyr528Tyr was noted as a synonymous mutation. A strain of MDR-TB exhibiting dual mutations at codon 512 and 516 has been initially documented in Pakistan [144].

In Punjab, Pakistan, the most common non-synonymous variants observed in the *rpoB* gene included the Ser531Leu SNP and alterations at codon 516 involving two SNPs [145]. The SNP Ser512Ile was initially identified in MDR-TB isolates from Pakistan, although it had been previously noted in a study conducted in Poland [146]. The Leu533Pro SNP has been previously documented in Turkey [140].

The isolate that was published earlier exhibited mutations at amino acid positions 512 and 516, with a twofold increase [147]. Five isolates of MDR-TB demonstrated phenotypic resistance to RIF, despite the absence of any mutation in the *rpoB* hotspot region.

The potential cause could be linked to global genetic alterations or the emergence of mutations beyond the 81 base pair region. The possibility exists for additional rare *rpoB* mutations or different resistance mechanisms to Rifampicin to be present [148].

2.7.2 Isoniazid

INH plays a crucial role as an antibiotic in addressing active TB infection. The CDC recommends four treatment plans that include the use of isoniazid for drug-sensitive strains of tuberculosis. The treatment protocols consist of an initial phase lasting two months, succeeded by a continuation phase that may extend for either 4 or 7 months [149]. Recent guidelines now advocate for rifampin-based regimens as the preferred approach for treating latent TB infection, replacing isoniazid. Rifampin-based treatments show comparable effectiveness while offering a reduced duration and improved completion rates [150].

Isoniazid has been a fundamental medication in tuberculosis treatment protocols since 1952. The compound operates as a prodrug, becoming activated via the enzyme catalase-peroxidase *KatG*. This process generates a range of radicals and adducts that impede the synthesis of mycolic acids, which are essential components of the mycobacterium's cell wall. This action makes it an exceptionally potent bactericidal agent.

It seems to operate alongside other substances generated by *KatG* and different drugs employed in the management of TB [151]. Changes in the *katG*, *inhA*, *kasA*, and *ahpC* genes can lead to resistance against isoniazid treatment. The emergence of resistance in MTB happens more rapidly while the treatment is limited to isoniazid monotherapy [151].

2.7.3 Ethambutol (EMB)

EMB has been employed in the management of TB since the 1960s. The initial formulation of EMB consisted of a racemic mixture of its L and D enantiomers. The D enantiomer of ethambutol is recognized for its therapeutic benefits, whereas the L enantiomer has been associated with toxicity, resulting in its cessation of use [152]. Ethambutol serves as a treatment for pulmonary tuberculosis.

This medication must be administered alongside at least one other antituberculosis agent, such as isoniazid, and should not be used in isolation. EMB demonstrates efficacy against strains of *Mycobacterium tuberculosis*; however, it lacks effectiveness against viruses, fungi, or other bacterial forms.

Ethambutol is a fundamental treatment for TB, typically given in conjunction with rifampicin, isoniazid, and pyrazinamide. Ethambutol functions as a bacteriostatic agent by disrupting the synthesis of arabinogalactan within the cell wall, which consequently inhibits the growth of bacilli [153]. However, the essential molecular pathways remain elusive [154]. It has been suggested that ethambutol augments the effect of INH against MTB by inhibiting a transcriptional repressor of the *inhA* gene.

2.7.4 Pyrazinamide (PZA)

The chemical synthesis of PZA, a derivative of nicotinamide, was initially carried out in 1936. It was not until 1952 that its potential as an antituberculosis (anti-TB) agent was acknowledged. PZA plays an essential role in minimizing the length of TB treatment regimen.

PZA is essential part of Drug sensitive and drug resistant TB treatment as it is effective in targeting non-replicating markers that other treatments fail to combat with. PZA operates through unique mechanisms that set it apart from traditional antibiotics, as it successfully inhibits various targets for energy generation and trans-translation [155].

Variations in *pncA* gene, which encodes pyrazinamidase, are the main source of resistance to PZA. This enzyme is essentially required for the conversion of the prodrug PZA into pyrazinoic acid which is its active form. Certain PZA-resistant bacteria display mutations in the target of the medication, ribosomal protein S1 (*RpsA*).

The recent discovery of *panD* mutations in certain PZA-resistant strains, alongside the absence of *pncA* or *rpsA* mutations, suggests the presence of a third PZA resistance gene and indicates a potential new target for PZA. The existing PZA DST might produce false resistance results, which compromises its reliability. Sequencing is more promising mode to be used in this scenario [156].

2.7.5 Fluoroquinolones

According to the latest WHO guidelines, fluoroquinolones, are recognized as the best second-line agents for tuberculosis treatment [157]. The recommendations align with our predictions regarding moxifloxacin and gatifloxacin, derived from an analysis of the pharmacokinetics and pharmacodynamics of 14 fluoroquinolones for tuberculosis [158]. Despite the fact that moxifloxacin was not endorsed until the WHO guidelines were revised in 2011, its significance in the treatment of

DRTB, potentially at a dosage of 600 or 800 mg once daily, was undervalued due to the significant infiltration of moxifloxacin in alveolar macrophages and other body fluids [158].

Fluoroquinolones demonstrate in vitro efficacy against MTB and exhibit remarkable penetration into macrophages, an essential characteristic enabled mycobacteria for its survival and proliferation within phagocytic cells [159]. Recent studies indicate that novel fluoroquinolones like levofloxacin and moxifloxacin demonstrate greater efficacy against *Mycobacterium tuberculosis* in comparison to ciprofloxacin and ofloxacin [160]. FQ resistance primarily develops due to point mutations in the Quinolone Resistance Determination Region (QRDR) of the DNA gyrase A and B genes [161]. Resistance levels are dependent on specific mutations in *GyrA* results in high level mutation while in *Gyr B* vice versa [162].

2.7.6 Diarylquinolines: Bedaquiline (BDQ)

BDQ represents a groundbreaking advancement in the treatment of MTB, being the first medication in four decades to receive FDA expedited approval due to its unique method of action. This compound, a diarylquinoline, inhibits ATP synthase and proves fatal to both actively dividing and non-dividing mycobacteria [163]. In light of the resistance mechanism in reported region *atpE*, *RV0678* and *pepQ*, it is crucial to consider the possibility of cross-resistance to CFZ when formulating the MDR TB treatment strategy. Furthermore, due to the extended half-life of BDQ, patients who do not achieve culture conversion upon the cessation of BDQ-containing treatment may experience the development of resistance to BDQ [164].

2.7.7 Nitroimidazoles: Delamanid (DLM) and Pretomanid (PTM)

DLM and PTM function as pro-drugs that require metabolic activation through coenzyme F420. Mutations in the genes fgd1, ddn, fbiA, fbiB, and fbiC have

been recognized as resistance mutations associated with prodrug activation and the F420 biosynthesis pathway [164, 165]. DLM resistance has been witnessed in patients undergoing MDR treatment [166, 167]. Individuals diagnosed with multidrug-resistant tuberculosis, who have not previously undergone treatment with delamanid, exhibit resistance to delamanid as indicated by their observable characteristics. The occurrence of pre-existing DLM resistance differs based on the MIC threshold, with a notable percentage of 9.76% observed in Korea when the threshold was established at 0.2 mg/dL as advised by the manufacturer [168].

2.7.8 Oxazolidinones: Linezolid (LZD)

In 1978, a category of synthetic antibacterial agents known as oxazolidinones was first developed for use in agriculture. During the 1980s, the first efforts toward human application began [169]. Nonetheless, their progress was obstructed by intolerable toxicity until the advent of LZD a decade later [170]. LZD received FDA approval in 2000 for the treatment of Gram-positive infections; however, its usage in tuberculosis remains restricted. The FDA has accelerated the conditional approval process for the use of LZD in combination with BDQ and PTM for cases of non-responding MDR TB or XDR TB, based on the findings from the Nix-TB study [170].

Oxazolidinones inhibit protein synthesis by obstructing the formation of the initiation complex through their binding to the 50S ribosomal subunit, located near the interface with the 30S subunit [170]. The lack of cross-resistance can be explained by two main factors: (1) The drug-binding domain is located far from the domains of other antibiotics that impede drug production; (2) Oxazolidinones interfere with an initiation step that occurs before the elongation phase targeted by other protein synthesis inhibitors [171].

TABLE 2.2: Recommended classification of medications for the DR-TB treatment regimen [175]

Group	**Medicine**	**Abbreviation**
Group A	Levofloxacin OR Moxifloaxacin	LFX or MFX

Table 2.2 continued from previous page

Group	Medicine	Abbreviation
Include all three medications unless contraindicated.	Bedaquiline	BDQ
	Linezolid	LZD
Group B	Clofazimine	CFZ
Combine both medications unless contraindicated.	Cycloserine OR Terizidone	TRD
Group C	Ethambutol	EMB
Include in the treatment plan when medications from Group A and B are contraindicated.	Delamanid	DLM
	Pyrazinamide	PZA
	Impenem-cilastatin OR Meropenem	Ipm-Cln
		Mpm
	Amikacin (OR Streptomycin)	AMK
		(S)
	Ethionamide OR Protemanid	ETH OR PTO
	p-aminosalicylic acid	PAS

2.8 Bedaquiline and its Antimicrobial Properties

2.8.1 Structure of BDQ

BDQ is classified as a diarylquinoline, representing a new category of treatments for tuberculosis. BDQ features a quinolinic central heterocyclic nucleus complemented by alcohol and amine side chains, which contribute to its anti-TB activity.The structural formula of BDQ reveals two critical components: (i) a hydrophobic segment featuring $N(CH_3)_2$, crucial for its interaction with ATP synthase; and (ii) an H2-bonding acceptor/donor that contributes to its stability (Figure 2.5). The anti-TB activity of BDQ is linked to the diarylquinoline ring, the side chain

featuring the N,N-dimethyl amino terminus, the hydroxyl group, and the naphthalene moiety. Previous investigations indicate that the molecular weight of BDQ is roughly 555.51 Da, defined by the molecular formula $C_{32}H_{31}BrN_2O_2C_4H_4O_4$. The chemical formulation is composed of fumaric acid in a 1:1 ratio, identified as BDQ fumarate. BDQ, an enantiopure compound featuring two adjacent chiral centers, was successfully isolated from a mixture of four isomers through high-performance liquid chromatography (HPLC).

BDQ consists of several inactive components, such as croscarmellose sodium, lactose monohydrate, polysorbate 20, microcrystalline cellulose, hypromellose 2910 (15 mPa s), colloidal silicon dioxide, corn starch, magnesium stearate, and purified water, as noted in sources.

Concerning the structural complexity of BDQ, efforts have been directed towards enhancing the anti-TB efficacy of this drug, along with its pharmacokinetic and pharmacodynamic characteristics.

A range of investigative initiatives have concentrated on optimizing the structure while preserving its efficacy against TB. While these thorough investigations resulted in the discovery of new related compounds, none have advanced to the stage of clinical evaluation. Consequently, the identification of BDQ analogues is essential for providing potential new leads [172].

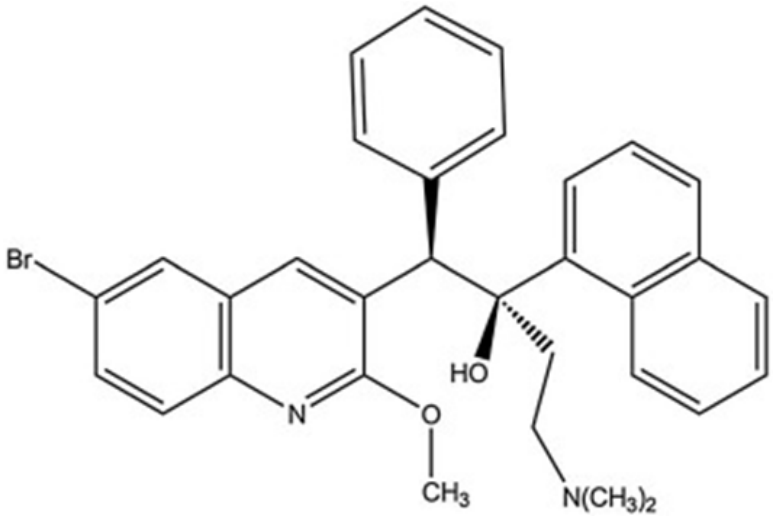

FIGURE 2.5: Structure of BDQ [176]

2.8.2 Mechanism of Action of Bedaquiline

BDQ is closely related to the fluoroquinolones; however, it does not exhibit inhibitory effects on DNA gyrase, and its mechanism of action is distinct from that of the fluoroquinolones. BDQ stands out as the sole FDA-approved anti-TB medication that specifically targets adenosine triphosphate (ATP) through the inhibition of the proton pumping mechanism. ATP is generated by ATP synthase and serves as an essential molecule for all eukaryotic and prokaryotic cells, encompassing mycobacteria in both their extracellular and intracellular forms, irrespective of their replication status, whether they are active or dormant.

ATP synthase, an essential enzyme found in the inner membrane of mycobacterial mitochondria, plays a crucial role in generating energy that supports the catabolic and anabolic processes in dividing mycobacterial cells. This intricate enzyme consists of two distinct regions: the cytoplasmic region F1, comprising five subunits (a3, b3, g, d, and e), and the membrane region F0, which includes three subunits (a, b2, and c10-15). The c subunits of F0 are organized into disks, serving as an ion-conducting pathway, while F1 comprises three catalytic sites that facilitate the combination of one ADP with a phosphate (Pi) to produce ATP.

Evidence indicates that the proton motive force via F0 revitalizes the rotation of the cylindrical ring made up of subunits c, which subsequently propels the coordinated rotation of the catalytic b subunit within the F1 domain, leading to ATP synthesis. It is thoroughly established that BDQ has the capability to bind to the oligomeric/proteolipidic subunit c within the F0 domain of the ATP synthase complex, thereby inhibiting its function. BDQ has demonstrated the ability to inhibit mycobacterial F-ATP synthase by specifically targeting the enzyme's e subunit, in addition to its interaction with the c subunit. The inhibitory effect of BDQ on ATP synthase is particular to mycobacteria.

It is important to highlight that the ATP synthase complex in humans exhibits a sensitivity to BDQ that is 20,000 times lower than that of *M. tuberculosis*, suggesting that target-based toxicity and interaction with human ATP synthase are unlikely [172]. Mechanism of action of BDQ is presented in Figure 2.5 [173].

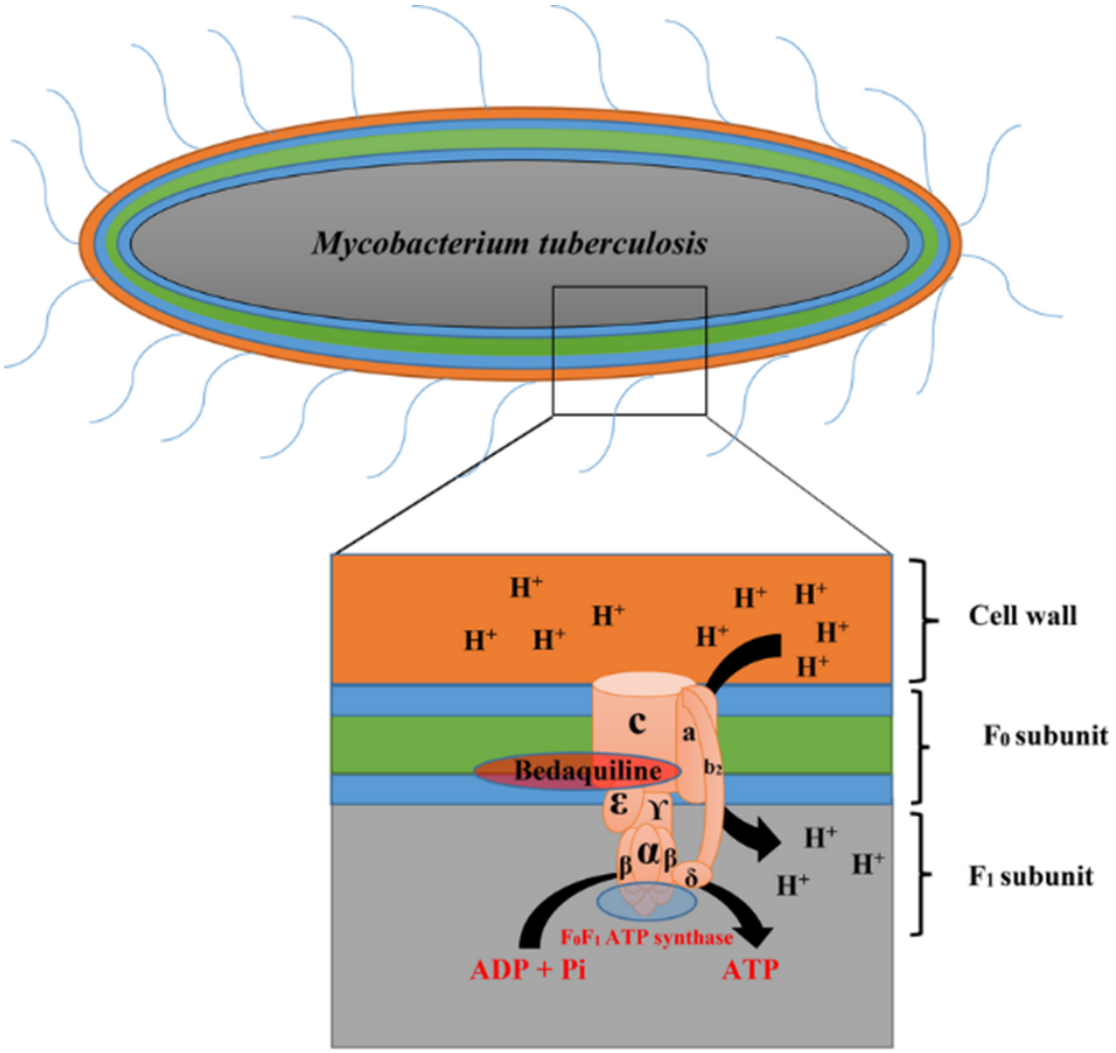

FIGURE 2.6: Mode of action of BDQ [175]

ATP synthesis can be inhibited by BDQ's ability to attach to the oligomeric subunit c in the F0 domain of mycobacterial ATP synthase and prevent the ring-shaped assembly of the c-subunit from rotating [80].

2.8.3 Bedaquiline Resistance

A significant challenge faced by individuals with TB is the extended length of treatment and the use of various anti-TB regimens, which can result in the development of drug resistance and its spread among mycobacteria. Additional treatment could potentially decrease the frequency of antibiotic-susceptible isolates, thereby enabling the rise of highly resistant isolates. The rising resistance

to anti-TB agents, along with the emergence of MDR and XDR-TB strains, poses a significant health challenge in various regions worldwide and necessitates comprehensive investigation. Following the introduction of BDQ for MDR-TB treatment, there has been a notable rise in resistance to this antibiotic. As of the conclusion of 2015, a total of 50 countries reported utilizing BDQ in the treatment of over 2500 patients. According to reports from the WHO, by the conclusion of 2017, 68 countries had either implemented or initiated the use of BDQ for treating MDR/XDRTB. While BDQ is advised to enhance the treatment effectiveness of MDR-TB, improper or inadequate application could result in the swift development of resistant strains. The WHO has highlighted that the emergence of resistance to BDQ could stem from improper use of this antibiotic, underscoring the need for vigilant monitoring. The WHO has highlighted the critical need to enhance the accuracy and consistency of drug susceptibility testing for BDQ. It was suggested that, in the absence of targeted drug susceptibility testing, sequential MIC assessments should be utilized to monitor BDQ resistance.

A range of studies conducted globally has highlighted the ongoing discussions regarding the definition of BDQ resistance. Regrettably, a consensus on the establishment of standardized drug susceptibility testing for BDQ has not been achieved as of now. The standards set forth by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) delineate the MIC breakpoints for BDQ: a susceptibility threshold of 0.25 mg/L and a resistance threshold that surpasses 0.25 mg/L [172].

2.8.4 Mechanisms of BDQ Resistance and Crystal Structure of Drug Targets

At present, three mechanisms of BDQ resistance have been identified at the molecular level [173, 174]. These mechanisms are briefed in table 2.3. The main mechanism consists of variations in target *atpE* gene. This gene encodes the ATP synthase enzyme, as detailed by Andries et al. [173]. The likelihood of BDQ

resistance mutations is 5×10^{-7} at 4-fold of the MIC, whereas it is 5×10^{-8} at 8-fold.

TABLE 2.3: Mechanism of BDQ Resistance [72, 73]

Genetic Marker	**Function**	**MIC Change**	**Comments**
atpE	Transmembrane protein of the ATP synthase	8- to 133-fold increase in Bdq MIC	Mutations in atpE have been isolated in vitro upon exposure to Bdq
Rv0678	Expression of the MmpS5-MmpL5 efflux pump regulation	2- to 8-fold increase in Bdq MIC	Rv0678 mutations have been identified in vitro after being exposed to Bdq
pepQ	Still not reported	4-fold increase in Bdq MIC	Mice given Bdq have been shown to have mutations in pepQ. Low-level Bdq resistance was linked to the pepQ mutations

Sequencing of the BDQ resistant *atpE* gene in in vitro mycobacterium isolates revealed six mutants in the c subunit of the ATP synthase enzyme. The six mutations involved the substitution of one amino acid for another. The combined effect of these substitutions prevents the binding of BDQ to its target site on the c subunit, thereby ensuring the continuation of H+ transfer and ATP synthesis. Consequently, the MIC has risen by 100 folds [175, 176].

The secondary mechanism encompasses mutations in *Rv0678*. These mutations are examined through in vitro studies that involve exposure to Bedaquiline and clofazimine. An increase in MIC of 2-8 folds is observed when patients are treated with BDQ, while a 2-4 fold increase is noted when patients are subjected to CFZ [172].

The role of drug efflux as a secondary mechanism is essential for understanding both innate and acquired resistance to BDQ. The tertiary mechanism encompasses mutations in *pepQ* (putative Xaa-Pro aminopeptidase). *pepQ* point mutations significantly elevates the likelihood of BDQ and CZF occurring by several times.

Variations in *pepQ* were identified as being linked to the lowest levels of BDQ resistance, which impacts the efficacy of BDQ [177]. Crystal Structure of *Rv0678*, *pepQ* and *atpE* from pdb database (https://www.rcsb.org/) is provided below in Figure 2.7.

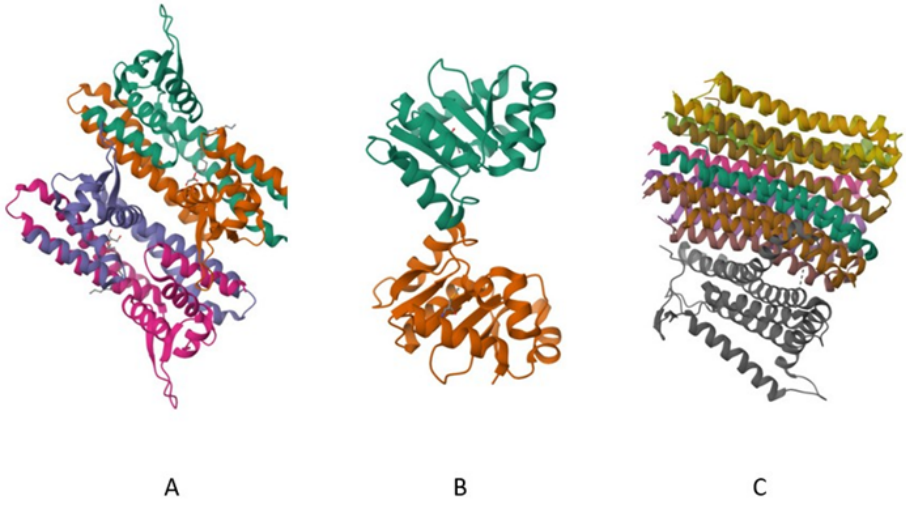

FIGURE 2.7: Crystalline Structure of BDQ Drug targets. A. *Rv0678* [183] B. *pepQ* [184] C. *atpE* [185]

2.8.5 Epidemiology of Bedaquiline Resistance

The initial implementation of BDQ in MDR-TB treatment has resulted in thousands of individuals worldwide receiving this medication [178]. Like other medications, strains resistant to BDQ emerged quickly, prompting serious concerns regarding its further use in anti-TB treatment protocols.

MTB isolates acquire BDQ resistance via instant chromosomal mutations in the *atpE* gene, *Rv0678* and pepQ. Furthermore, mutations have been identified in the intergenic region between *Rv0678* and *Rv0677c*, resulting in the suppression of ATP synthase inactivity [178]. In 2014, the first BDQ resistant multidrug-resistant was reported. After a two year treatment duration and a following relapse, cross-resistant cases of BDQ and CFZ involving Rv0678 mutations were reported in a Tibetan patient [179]. Hence, it is mandatory to evaluate if the patient is sensitive to BDQ in case it was previously treated with CFZ [180].

A study revealed that a 50 year old patient was BDQ resistant at low level while baseline sample had shown eight folds increase in MIC at random weeks from 22 week onwards tested on resazurin microtitre assay (REMA) [181]. WGS revealed that two isolates harbored non-synonymous mutations in mmpl5, whereas one isolate displayed the *rv1979c* M245L mutation [178]. In Pakistan, Ghodousi and colleagues evaluated 70 MTB strains obtained from 30 patients with previous history of BDQ treatment. The baseline isolates were BDQ sensitive, five were BDQ resistant with increase MICs during the treatment period. Furthermore, specific mutations in *Rv0678* were analyzed for their role in the elevation of BDQ MICs observed in instances of treatment failure [182].

The emergence of BDQ resistance is concerning, as it could lead to a rapid decline in the effectiveness of this new antibiotic. Consequently, elucidating the mechanisms underlying BDQ resistance and enhancing standardized antibiotic susceptibility testing will guide treatment decisions and reduce the likelihood of resistance development.

2.8.6 Emerging Role of WGS

WGS has the potential to identify known mutations that confer drug resistance. Recently, methods based on WGS have demonstrated their ability to replace culture-based drug susceptibility testing for first-line medications [183]. Nonetheless, the deployment of this technology remains impractical in nations facing a significant TB burden due to limitations in cost and technical expertise. Consequently, minimal evidence was gathered regarding the relationship between WGS prediction and phenotypic testing outcomes within the context of population-based cross-sectional surveillance. Furthermore, the majority of existing phenotypic and genetics-based drug susceptibility testing methods yield a binary outcome of 'resistant' or 'susceptible,' which may overlook strains that increase the minimum inhibitory concentration but do not reach the threshold to be classified as 'resistant.' The resistance level plays a crucial role in shaping treatment regimens. The implementation of informed high-dose regimens, which aim to enhance the

clinical effectiveness of less toxic and more accessible medications like rifampicin and isoniazid, must be carefully considered. Additionally, strains that fall below the ECOFF threshold can still result in unfavorable clinical outcomes, including incurability or relapse.

Prior investigations have sought to assess the levels of drug resistance among various resistance variants; however, these studies have been constrained by limited sample sizes and a narrow range of drugs examined [183].

2.9 Knowledge, Attitude and Practice of TB in HCWs

Healthcare workers are crucial in addressing pathological conditions such as tuberculosis. Due to increased exposure to patients with TB/DRTB, the likelihood of contracting this highly infectious disease rises significantly [184–186] A recent study indicated that healthcare workers experienced an incidence rate of active tuberculosis that was higher than that of the common population [187]. The WHO promotes effective managerial, administrative, and environmental controls that can be applied in diverse contexts. The suggestions encompass, among other aspects, the creation of an infection control strategy, the prioritization of suspected TB cases, regular TB screening for healthcare personnel, and the improvement of natural ventilation in the facility.

Moreover, the availability and effective use of particular respirators (e.g., the proper and consistent use of N95 respirators) represent an essential strategy for personal protection [188]. A thorough understanding of the disease is essential for effective infection control and management of tuberculosis. Consequently, implementing a survey focused on knowledge, attitude, and practice (KAP) among healthcare professionals can prove advantageous in assessing their awareness of the disease and comprehending the infection control measures that are presently in use.

A firm understanding of TB transmission, pathophysiology, and resistance development is essential not only for effective patient management but also for minimizing nosocomial transmission and guiding rational antibiotic use. Knowledge deficits, negative attitudes, and poor practices among HCWs have been associated to delayed diagnosis, inappropriate treatment regimens and poor adherence monitoring, all of which can possibly add to the emergence and spread of drug-resistant TB strains, including resistance to newer drugs like Bedaquiline [191].

Despite the growing adoption of Bedaquiline in multidrug-resistant TB (MDR-TB) treatment regimens, few studies have examined HCWs' knowledge or preparedness regarding its appropriate use [191, 192]. This is concerning, as lack of familiarity with novel therapeutics can lead to inconsistent administration, poor monitoring of side effects, and unintentional selection pressure for resistance. In countries such as Pakistan, where healthcare infrastructure faces multiple challenges and DR-TB burden is high, assessing HCWs' KAP becomes even more critical [193].

2.10 Integration of Study Objectives

Several KAP studies conducted in countries like India, Ethiopia, and Nigeria have revealed significant gaps in HCWs' understanding of TB transmission, resistance mechanisms, and the rationale behind treatment protocols [191, 192]. However, data from Pakistan remain limited, particularly in the context of second-line and novel drugs such as BDQ. Therefore, this study's inclusion of a KAP survey component provides a valuable lens through which to assess systemic and behavioral contributors to resistance development, complementing the molecular investigation of BDQ-resistant Mycobacterium tuberculosis strains. Together, these approaches aim to provide a more comprehensive understanding of the clinical and environmental factors influencing resistance, informing both policy and practice.

Chapter 3

Material and Methods

This study employed a dual-methodological approach to comprehensively investigate Bedaquiline (BDQ) resistance in Mycobacterium tuberculosis. Molecular methods were used to detect and characterize genetic mutations associated with BDQ resistance and heteroresistance, while a Knowledge, Attitude, and Practice (KAP) survey was conducted among healthcare workers to assess systemic and behavioral contributors to resistance emergence. This integrated strategy reflects the study's central hypothesis: that BDQ resistance is shaped not only by microbial genetics but also by gaps in healthcare delivery and provider practices. Together, these methodologies provide a holistic view of the biological and clinical landscape of BDQ resistance in a high-burden setting.

3.1 Knowledge, Attitude and Practices of Health Care Workers

3.1.1 Study Design, Setting and Ethical Approval

A cross-sectional study of Knowledge, Attitude, and Practice related to TB among Health Care Workers in capital territory of Islamabad, Pakistan was conducted.

KAP data on TB/DRTB were gathered from healthcare workers (HCWs) in Islamabad. The data collection period spanned from June 1, 2023, to July 31, 2023. The participants were affiliated with health facilities in Islamabad that refer patients for TB testing or TB reporting to the National Reference Laboratory of Pakistan.

These health facilities included public sector basic medical units and private sector facilities. This study is approved by the ethics review committee of Capital University of Science and Technology (Ref: Bl&BS/ERC/23-07).

3.1.2 Sampling Method and Sample Size

The present study includes 306 HCWs from the above-mentioned health facilities. The sample size for the KAP survey was calculated using the standard formula for cross-sectional studies, based on a 95% confidence interval, an estimated 50% prevalence of adequate knowledge regarding DR-TB (to maximize sample size), and a 5% margin of error.

This yielded a minimum required sample size of 384 respondents. From all the recruited health facilities, 306 subjects based on the random sampling done from TB departments had shown their interest to be part of the study, which has increased the chances of Health care workers from each occupation from technician to the doctors and researchers involved directly or indirectly in the field of tuberculosis.

3.1.3 Selection Criteria and Recruitment

The study included HCWs aged between 20 and 55 years. Individuals below the specified age range were excluded from participation. All recruited subjects were involved, either directly or indirectly, in the TB control and prevention program. The HCWs which were not part of TB department by any means were excluded. Initially, research team gathered the information about all the health facilities which receive patients for TB testing or TB reporting health facilities of Islamabad affiliated to NTRL. In next step, research team contacted the administration

of these selected health facilities and recruited via electronic email and direct outreach. A clear and concise information material was provided so as to explain the aims and objectives in addition to potential outcomes of the current study.

3.1.4 Pilot Study

To finalize the appropriate questionnaire for collecting TB KAP data, a pilot study was conducted. After the basic introductory session, a subset of 50 healthcare workers from both primary and tertiary TB care health facilities agreed to participate in the pilot study and the original questionnaire printed in English language from another study was distributed to them. Data was collected anonymously and collected the study questionnaires from HCWs once they were filled.

3.1.5 Data Collection and Components of Questionnaire

For conducting current study, a questionnaire from a recent study [202] with slight modifications was followed. We adapted the questions based on the results and recommendations from the pilot study to ensure ease, interest and time efficiency for healthcare workers while gathering maximum data, as per Pakistan's guidelines. These slight changes include the reduction of all explanatory sections which were part of any of the question's multiple choice response included in the questionnaire. Two data collection modes were adopted; online Google forms and printed copies of the same questionnaires and data from both sources was merged in a single Microsoft Excel spread sheet. Along with KAP data, demographic detail of the study participants was also collected. Demographic details encompass essential characteristics such as age, gender, duration of experience with TB patients, profession within the healthcare facility, educational level, participation in TB control activities, history of previous active TB infection, and family history of TB, among other traits. In evaluating tuberculosis knowledge among healthcare workers, participants were presented with a set of twenty-seven questions covering TB fundamentals, diagnosis, and treatment. The attitude section comprised 24 questions addressing TB stigma, the importance of TB training, attitudes toward

TB treatment, as well as concerns among healthcare workers about contracting TB. The practice section involved presenting participants with three different scenarios. (Appendix I).

3.1.6 Statistical Analysis

IBM SPSS statistics 21 was utilized for analysis of the data. All the gathered data was tested for normality and appropriate statistical analysis was done in addition to descriptive stats. Cronbach's α (α =0.86), was adopted to prove the reliability and internal consistency for the KAP questions.

Proportion of each question was calculated at the confidence interval of 95% followed by the categorization of Health Care Workers on the basis of different parameters. One Way Analysis of Variance (ANOVA) followed by Tukey's Multiple Comparison was applied to calculate KAP mean score against different variables. Pearson correlation coefficient was utilized to explore the association between two qualitative variables. The non-parametric tests were applied to evaluate difference of KAP score in relation to demographic covariates individually.

3.2 BDQ Genetic Analysis

3.2.1 Ethical Approval

The present research was ethically approved by the ethical committee of Capital University of Science and Technology Islamabad, Pakistan.

3.2.2 Setting

The current study was being designed and experimented in the laboratories (BSL-2 and BSL-3) of National TB reference Laboratory (NTRL) Islamabad, Pakistan. Patients from NTRL were considered as the target population as NTRL provides

DST services to majority of the country's population originated from various regions. MTB culture isolates from TB patients with a diagnosis of Rifampicin resistance are typically subjected to phenotypic DST at Programmatic Management Drug Resistance (PMDT) sites on GeneXpert MTB/RIF Ultra with uncertain RIF status.

3.2.3 Sampling

The study was conducted from April 2022 to September 2023. For this study positive MTBC culture isolates were evaluated which have undergone comprehensive DST after the initial diagnostic testing. DST is mainly a value for clinical management of individual patients, epidemiological purposes and individual patient management: Comprehensive phenotypic DST is mainly prioritized for the Patients diagnosed as rifampicin resistant (RR) on Xpert /MTB RIF Ultra, Patient with high suspicion of MDR who is MTB+ve but rifampicin resistance is reported "Not detected" or "Indeterminate" on Xpert/MTB Rif Ultra, Patient while on second line drugs treatment, additional DST is recommended for emergence of resistance when patient fails to i) culture CONVERT (culture conversion) at month 3 or after initial conversion, ii) reverts back to positive (culture reversion) and Culture positive patient whose rifampicin resistance status is not known. For this study, MTBC isolates were selected from RR cases with positive MTBC culture and available DST results.

All the samples with poor quality, quantity, rif sensitive, false negative and contaminated cultures and failed DST results were excluded from this study. Four type of drug resistance pattern were selected for the current study. These include XDR TB isolates reported resistant to BDQ and FQ, MDR TB with resistant to BDQ but not FQ. Pre-XDR TB and RR/MDRTB strains without any additional resistant to BDQ.

From both groups 50 Bedaquiline resistant cases would be selected in total. As a control group equal number of BDQ sensitive cases were selected from Rif resistant

group. Both categories BDQ sensitive and BDQ resistant cases were investigated for their genetic insight.

3.2.4 Techniques and Methodology

Recruited samples had undergone GeneXpert MTB/RIF Ultra, smear, culture, Drug susceptibility testing and WGS (Figure 3.1).

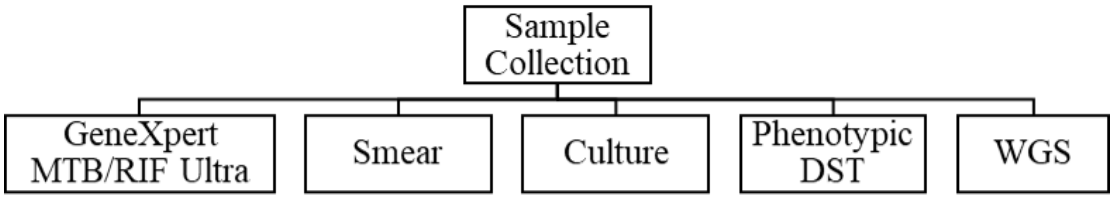

FIGURE 3.1: Methodology

3.2.4.1 GeneXpert MTB/RIF Ultra

A minimum of one milliliter of sputum was obtained, and specimens with visible food particles or other solid particles were not included. If there was more than 4.0 mL of specimen, some was moved to a sterile, clean, labeled Falcon tube, which was then used for testing. Prior to processing, specimens were kept between 2 and 8 degrees Celsius wherever feasible. The specimens were kept at a maximum temperature of 35^{o}C for three days or at 4^{o}C for four to ten days in the event that sample processing was delayed. GeneXpert Instrument was launched. First, the GeneXpert instrument was powered on and then the PC. On the Windows desktop, double-clicked the GeneXpert Dx shortcut icon. Workbenches and the BSC were cleaned. It was determined how many Xpert MTB/RIF cartridges were required, took them out of the packing and labelled each one with the sample identifier (ID).

A portion of the specimen was moved to a sterile, clean, labeled falcon tube, which is used for testing, if the volume exceeded 4.0 mL. After carefully unscrewing the cover of the sputum collection container, use the graduated disposable pipette to

measure and record the volume of sputum. Carefully disposed of the pipette to avoid any aerosol generation.

Gently added Sample Reagent 2:1 (v/v) to the sample using a different plastic disposable pipette, then replace the lid. Shake vigorously 10–20 times back and forth in a single shake. For fifteen minutes, the sample was incubated in the sputum cup at room temperature. Given the specimen another good shake ten to twenty times.

There were no visible sputum clumps in the liquefied specimens following a lengthy 5-minute incubation period. Tubes were shaken well again and incubate for another three to five minutes if there are still clumps of sputum.

Getting the cartridge ready. After placing the samples in the cartridge, began the test within half an hour. Using the included sterile transfer pipette, aspirated the liquefied material into the pipette until the meniscus is above the minimal mark (= 2mL).

The cartridge had not been tampered with, as evidenced by the tearing sound made when the lid was opened. Sample was put into the cartridge. Placed the transfer pipette inside the container for biohazardous trash. The cartridge cover was closed firmly.

Cartridge was places into the GeneXpert bay in GeneXpert Module Machine (Figure 3.2). Depending on the MTB target present in the sputum sample, the MTB result was shown as High, Medium, Low, Very Low, and Trace if MTB target DNA was found. Rif status is represented as detected, not detected and indeterminate.

Based on the melt curve analysis, we will determine the rifampicin status if MTB is found. Particularly in situations where the bacterial burden is insufficient to identify resistance, the rifampicin status can be identified, not detected, or inconclusive.

The DNA target is absent if MTB is not found, but the SPC and probe checks would still be successful. When the probe check is successfully completed and the SPC is outside of the valid range, the result might be identified as invalid.

FIGURE 3.2: GeneXpert Module

3.2.4.2 Culture Processing of Sputum Samples

Sputum samples are decontaminated via the method of NALC-NaOH [194]. Affixed a unique ID to every sample and recorded its physical characteristics and amount. The sample was then homogenized in the reagent by vortexing an equal amount of NALC-NaOH solution for 20 seconds. The sample was then left on the rack for 15 minutes to disinfect. After 15 minutes, add Phosphate Buffer Saline solution (PBS) to neutralize the sample and halt the decontamination process. Samples were centrifuged in a chilled centrifuged machine at 4 degrees Celsius and 3000 g for the next 20 minutes. The supernatant was cautiously decanted into a discard containing 4% phenol following centrifugation. Following a gentle vortex, 1000 μl of PBS was added to the sediment to reconstitute it. Prepared the antibiotic supplement (PANTA) to add in the MGIT tubes in a sterile cabinet and using aseptic techniques, reconstituted MGIT PANTA in 15 mL of MGIT Growth Supplement. Label microscope slides, LJ medium slopes (prepared using SOPs mentioned in Appendix - II), and MGIT 7 mL tubes with the patient's ID and the date for each specimen. Added 0.8 mL of the PANTA mixture with a

sterile pipette tip to each MGIT 7mL tube. After preparation, the aforementioned sediments were inoculated on solid media. Lewenstein-Jensen and liquid media from 7H9 broth.

Scanned the MGIT tubes in the MGIT960 BD machine and loaded them in their respective stations. Place the LJ slopes with one step loose cap in a slanting position for 48 hours and afterward weekly read the slopes and tighten the caps (Figure 3.3 - 3.4).

There are other sample types as well for TB testing which include gastric lavages, and other bodily fluid. All of these fluids were centrifuged in case of more than 5ml in quantity at 3000 g for 20 minutes. Discarded the supernatant and left the sample 5mL in the processing tubes.

Other than fluids and washings, tissue biopsies from different sites were also processed. Transferred tissue specimen in a petri dish. Used a clean, pair of sterile forceps for holding the specimen and cut the tissue sample into small pieces with a sterile surgical blade. Add approximately 1-2 mL of saline or PBS and chop the tissue in saline/PBS solution until a homogeneous suspension is obtained.

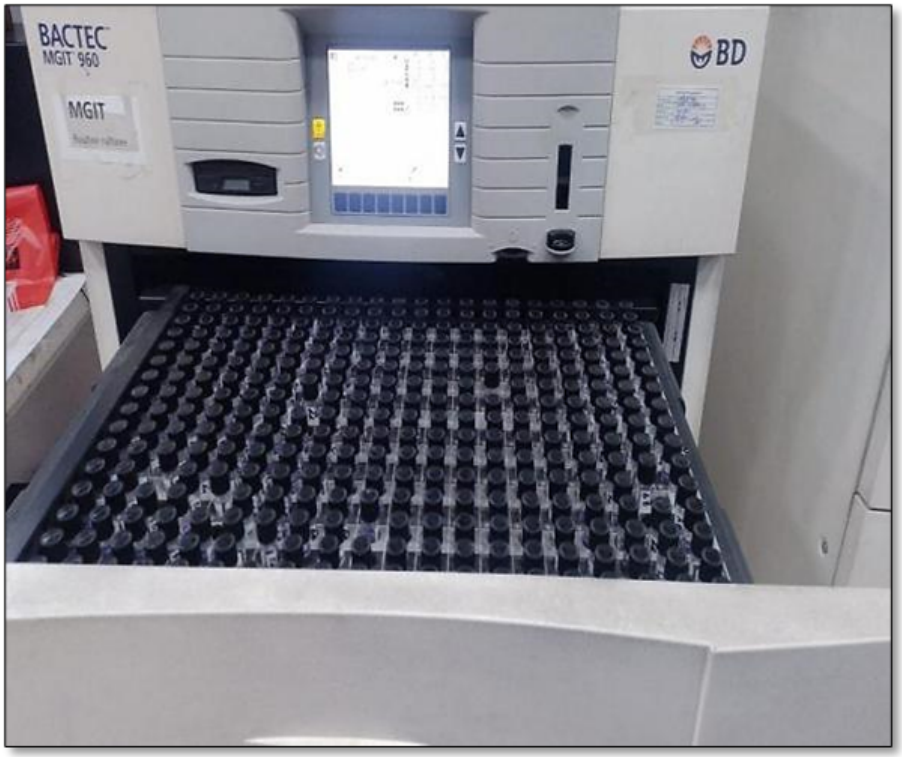

FIGURE 3.3: BECTEC MGIT 960 (Liquid culture)

FIGURE 3.4: Incubations of solid culture

Used a disinfected pipette to transfer suspension into a 50 mL conical tube, make up volume upto at least 5mL with PBS, and mix thoroughly to homogenize the suspension. Centrifuged at 3000g for 15 minutes. Let it stand for 05 minutes before opening the cap. Decanted supernatant and re-suspend the deposit in approximately 3mL of PBS. Vortex, inoculate 0.5 mL suspension on one 7mL MGIT tube and two slopes of LJ medium. Inoculated each LJ slope with approximately 0.2 mL (4-5 drops). Make one drop smear on slide for microscopic inspection. Proceed for GeneXpert testing as per Xpert SOP. Reading, validation of a positive culture, interpretation, recording, and reporting was done accurately.

Colony formation was examined weekly, preferably twice within the first week, to enable prompt contamination reading and, if needed, the timely request for an additional specimen. All the contaminated cultures were eliminated. In three to four weeks, *M. tuberculosis* colonies should be fully grown. Outcomes that were reported right away following identification and detection. Before reporting culture as unfavorable, they should be preserved for up to eight weeks, whereas the following rapidly proliferating colonies visible in < 7 days and contaminated cultures were eliminated. Reporting criteria were followed as given in the table below (Table 3.1):

TABLE 3.1: Culture Reading Criteria

Observation	**Report**
Contamination	Contaminated
No colony	Negative
1-9 colonies	Actual number
10-100	1+
100-200	2+
>200	3+

3.2.4.3 Smear Microscopy and MTBC Identification

Fluorescent Microscopy: One drop of the sediment was used to make a smear on the slide for FM staining and microscopy. For FM microscopy all the slides were settles on a staining rack, including positive and negative control slides. Dropped Auramine O (0.1%) and let it settle for a minimum of 20 minutes.

Rinsed the slide with water. Next added acid alcohol (0.1%) as a decolorizer for two minutes and rinsed again with water.

After draining water from slides, flooded slides with Methylene Blue as a counter-stain (0.3%) to for a minute.

Slides were then rinsed with water, drained and allowed smears to air dry. Avoided exposure to sunlight. Examined smears under a fluorescence microscope at earliest. Switched on the LED microscope (Figure 3.5A).

Placed a dry smear on the microscope's stage. Using the x10 objective, the presence of fluorescence was investigated at lower power fields. Slide was then examined for any fluorescence, observed and verified the presence of AFB using an x40 objective (Figure 3.6).

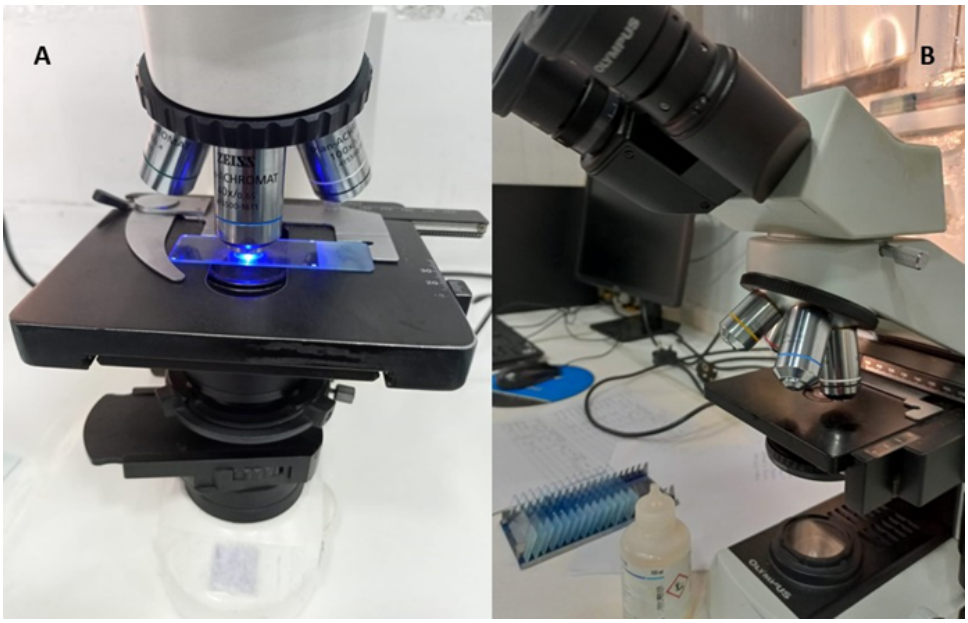

FIGURE 3.5: Microscopy A. FM Microscopy B. ZN Microscopy

No. of AFB found	Record at fields	Report as
	200x (30 fields)	400x (60 fields)
Negative	Zero AFB / 30 fields	Zero AFB / 60 fields
Confirmation required*	1-4 AFB / 30 fields	1-2 AFB / 60 fields
Actual count	5-49 AFB / 30 fields	3-24 AFB / 60 fields
1+	3–24 AFB / fields	1-6 AFB / fields
2+	25-250 AFB / field	7-60 AFB / field
3+	>250 AFB / field	> 60 AFB / field

FIGURE 3.6: Semi-quantification scale for reporting AFB stained with Auramine O

Follow-up Procedure for the Positive Cultures: After an MGIT tube flags positive or an LJ slant shows positive growth, this positive culture was subjected to various tests for identification and sterility testing Liquid cultures were incubated for 24 hours after unloaded from the BECTEC MGIT 960 machine.

Identification of Mtb Using ZN Acid-fast Staining: Positive culture tubes (Figure 3.7) were also identified for MTB using Ziehl Neelsen (ZN) acid fast staining and Microscopy (Figure 3.5B) for the presence of MTBC (Figure 3.7). The next

day, smears were prepared from these cultures and performed their ZN staining. Cultures with negative smear results were considered contaminated and positive cultures were tested for NTM and MTBC identification. The same is the procedure followed for solid media except for one day of incubation. Rapid identification of MTBC using Immuno-chromatic kits MPT64 BD. Positive test for rapid Identification test (MPT64 antigen present) at the Test "T" well and the Control "C" is referred to as MTBC.

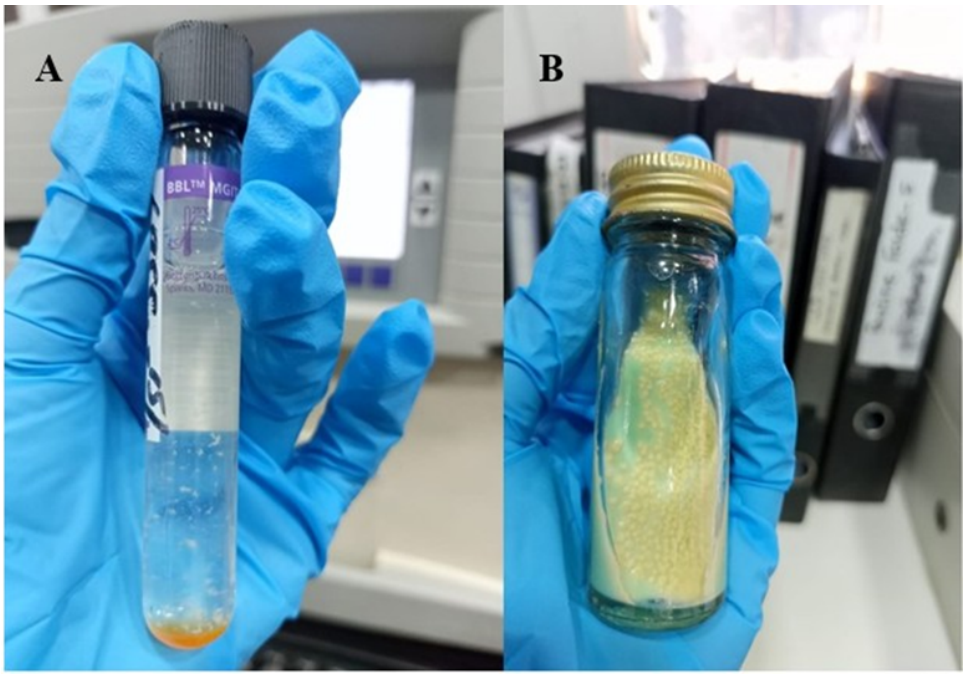

FIGURE 3.7: MTBC colonies A. Positive Growth on Liquid B. Solid Media

3.2.4.4 Drug Susceptibility Testing (Phenotypic DST)

For MTB, accurate drug susceptibility testing (DST) is essential for monitoring drug resistance and guiding therapy [195]. If DST is not used during TB treatment in areas where drug resistance to the disease is common, treatment may not be successful and drug resistance may worsen [196]. After being decontaminated, the samples were injected with liquid media and left to incubate for six and eight weeks at 37°C. For DST, six first-line anti-bacillary antibiotics were used: Ofloxacin (2.0 & μg/mL), Levofloxacin (1.0 & μg/mL), Moxifloxacin (1.0 & μg/mL), Bedaquiline (1.0 & μg/mL), Clofazimine (1.0 & μg/mL), Ethambutol (EMB, 5.0 & μg/mL), Streptomycin (STP, 1.0 & μg /mL), and Pyrazinamide (PZA, 100.0 & μg/mL).

SLI Medications and the BACTECT MGITTM 960 system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) was used to measure the following: amikacin (1.0 & μg/mL), capreomycin (2.5 & μg/mL), and kanamycin (2.5 & μg/mL).

Mycobacterium tuberculosis complex (MTBC)-positive liquid positive cultures that are pure and include acid-fast bacilli (AFB) can be used for susceptibility testing. The MTBC isolate's development in a tube containing drug is used to compare its progress to that of a drug-free growth control (GC) tube. Tubes are continuously checked by the MGIT instrument for increasing fluorescence. The device uses an analysis of the drug-containing tube's fluorescence in comparison to the GC tube's fluorescence to determine susceptible findings. These data are automatically interpreted by the MGIT equipment, which then classifies the test as susceptible or resistant.

Step-by-step procedure of DST: To create the appropriate stock solution, reconstituted each BACTEC MGIT 960 SIRE kit medication vial with 4.0 mL of deionized water. (Table 3.2 - 3.3). To create the appropriate stock solution, reconstituted each BACTEC MGIT 960 PZA kit medication vial with 2.5 ml of deionized water (Table 3.3).

TABLE 3.2: Reconstitution volumes for 1^{st} line TB drugs

Drug lyophilized	**Volume of water added (mL)**	**[C] of the drug after reconstitution (μg/mL)**	**Volume for MGIT tube DST (μl)**	**Final concentration in MGIT tubes (μg/mL)**
STR	4.0	83.0	100.0	1.0
INH	4.0	8.3	100.0	0.1
RIF	4.0	83.0	100.0	1.0
EMB	4.0	415.0	100.0	5.0
PZA	2.5	8000.0	100.0	100.0

MGIT tubes were prepared by adding 0.8 mL of SIRE supplement. Additionally, put 0.8 milliliters of PZA supplement into each PZA tube using the same process. Using a 100ul pipette and sterile tip, opened each MGIT tube for the respective drug one at a time and added 0.1mL to each tube. Change tip between drugs.

Ensured that all functions of the BSC (lights, pressure, smoke checks) were adequate for working. Cleaned BSC thoroughly with freshly prepared disinfectant followed by 70% alcohol. Utilized growth on a solid medium that has not been used for more than 15 days (with positive growth appearing after 15 days). Aseptically added few glass beads in a tube. Aseptically, add 0.1 mL of sterile saline or distilled water. Scoop some of the growth from the Lowenstein Jensen slant with a sterile loop, being careful not to scrape the media. Colonies were added into the tubes by passing the loop through the glass beads. Tightened the tube caps and Vortex for a minute. Matched the suspension with McFarland 0.5 Standard by adding sterile saline or distilled water using a sterile plastic Pasteur's pipette. This suspension was used to inoculate the MGIT tubes as described for liquid media. Loading DST set in MGIT machine. Inserted DST tube set into the lighted space. Unloaded all the positive sets of MGIT tubes. The MGIT machine automatically calculates the results once the GC reads 400GUs. The MGIT machine automatically interprets the results of the test.

TABLE 3.3: Source of Drugs

Drug	**Category**	**Brand**	**Cat #**
Moxifloxacin (CB)	SLD	BD	215349
Amikacin	SLD	BD	215350
Levofloxacin	SLD	Sigma	28266
Bedaquiline	New drug	Janssen pharma (HIV Reagent Program-USA)	116620
Delamanid	New drug	Otsuka (BEI Resources-ATCC)	NR-5636
Clofazimine	Repurposed drug	Sigma	C8895
Linezolid	Repurposed drug	Sigma	PZ0014

Weighing of Drugs Standard units of activity are measured for each antimicrobial agent. The assay units might vary significantly from the powder's actual weight and frequently between lots of drugs produced. As a result, the laboratory needs to standardize the antimicrobial solutions according to the potency of each medication powder lot (Table 3.4).

TABLE 3.4: Weighing of drugs and Solvents

Drug	**Drug weight (μg)**	**Solvent**	**Solvent Volume (mL)**
Moxifloxacin (CB)	498	Distilled Water	6.0
Amikacin	332	Distilled Water	4.0
Levofloxacin	10mg*	0.1M/0.4% NaOH	2.5*
Bedaquiline	10.0mg*	DMSO	2.5*
Delamanid	20.0mg*	DMSO	5.0*
Clofazimine	10.0mg*	DMSO	2.5*
Linezolid	25mg*	Distilled water	2.5*

* Required for preparation of stock dilutions. Further dilutions are made to attain working solutions as indicated below.

All the steps for second-line DST were the same following the reconstitution volumes mentioned in Table 3.5.

TABLE 3.5: Reconstitution volumes for 2^{nd} line TB drugs

Drug	**Working solution (μg/mL)**	**Volume of MGIT (μl)**	**Final conce. (μg/mL)**
AMK	83	100	1.0
LFX	84	100	1.0
MFX	83	100	1.0
BDQ	84	100	1.0
CFZ	84	100	1.0
DLM	5	100	0.06
LNZ	84	100	1.0

Experimental Equipment and Major Reagents: Below is the list of experimental equipment and reagents used for our experimental processing. (Table 3.6 - 3.7).

TABLE 3.6: Experimental Equipment

Equipment Name	**Company Name**
Bacteriological Incubator	Kalstein company
MGIT 960	Bectec company

Table 3.6 continued from previous page

Equipment Name	**Company Name**
Refrigerated Centrifuge Machine	LIC 609 company
Fluorescent LED Microscope	ZEISS company
ZN Microscope	ZEISS company
Water Bath	Labnet International company
Thermal Cycler	Esco Lifesciences company
TwinCubator	Biomedical company
Autoclave Machine	Tuttnauer company

TABLE 3.7: Major reagents

Reagent Name	**Company Name**
Sodium Hydroxide	Sigma-Aldrich
N-acetyl-L-cystein	Sigma-Aldrich
Tri sodium citrate	Sigma-Aldrich
Disodium hydrogen phosphate	Sigma-Aldrich
Monopotassium dihydrogen phosphate	Sigma-Aldrich
L-Asperagin	Sigma-Aldrich
Magnesium Citrate	Sigma-Aldrich
Magnesium Sulphate	Sigma-Aldrich
2% Malachite Green	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Auramine stain	Sigma-Aldrich
Carbol-Fucsin	Sigma-Aldrich
Hydrogen Chloride	Sigma-Aldrich
Sulfuric Acid	Sigma-Aldrich
Phenol	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Methanol	Sigma-Aldrich

3.2.4.5 Whole Genome Sequencing (WGS)

WGS of the recruited samples was being performed in BSL-2 at NTRL Chinese center of disease control and prevention, China (CDC). WGS involved following (Figure 3.8).

a) DNA Extraction: WGS involves the DNA extraction using the Cetyl Trimethylammonium Bromide (CTAB) extraction method of DNA purification [197] refers to the high-throughput sequencing of the entire genome, analyzing inter-individual variations and annotating SNPs along with genomic structures.

In the CTAB method, the samples underwent heat inactivation by at 80^{0}C for 18-24 hours in a dry bath. Using the pipette, a 50 μL volume of 10 mg/mL lysozyme was added and gently mixed.

Vials were cleaned with 1% Virkon before being taken out of the biosafety cabinet and incubated for the entire night at 37^{o}C in a water bath. Set the water bath to 65^{o}C and put the vials back in the biosafety cabinet. The CTAB/NaCl buffer should be preheated to 65^{o}C.

Filled each sample vial with 5 μL of 20 mg/mL proteinase K* and 70 μL of 10% SDS*. Using the pipette tip, stir gently to mix. Vials were cleaned with 1% Virkon, moved to a water bath, and incubated for ten minutes at 65^{o}C. Vials were briefly spun before being put back in the biosafety cabinet.

Filled each vial with 100 μL of pre-warmed CTAB/NaCl buffer* and 100 μL of 5M NaCl. Using the pipette, mix gently. Vials were cleaned with 1% Virkon, taken out of the water bath and incubated for ten minutes at 65^{o}C.

Following a brief spin, the vials were moved to a fume hood. 750 μL of chloroform/isoamyl alcohol (24:1 v/v) was added to the mixture. The tubes were inverted at least ten times to mix. After being taken out of the fume hood, the samples were spun in vials at 10,000 g for five minutes. The upper (aqueous) supernatant layers from the previous step were carefully picked up and put into the properly labeled vials that had been produced earlier.

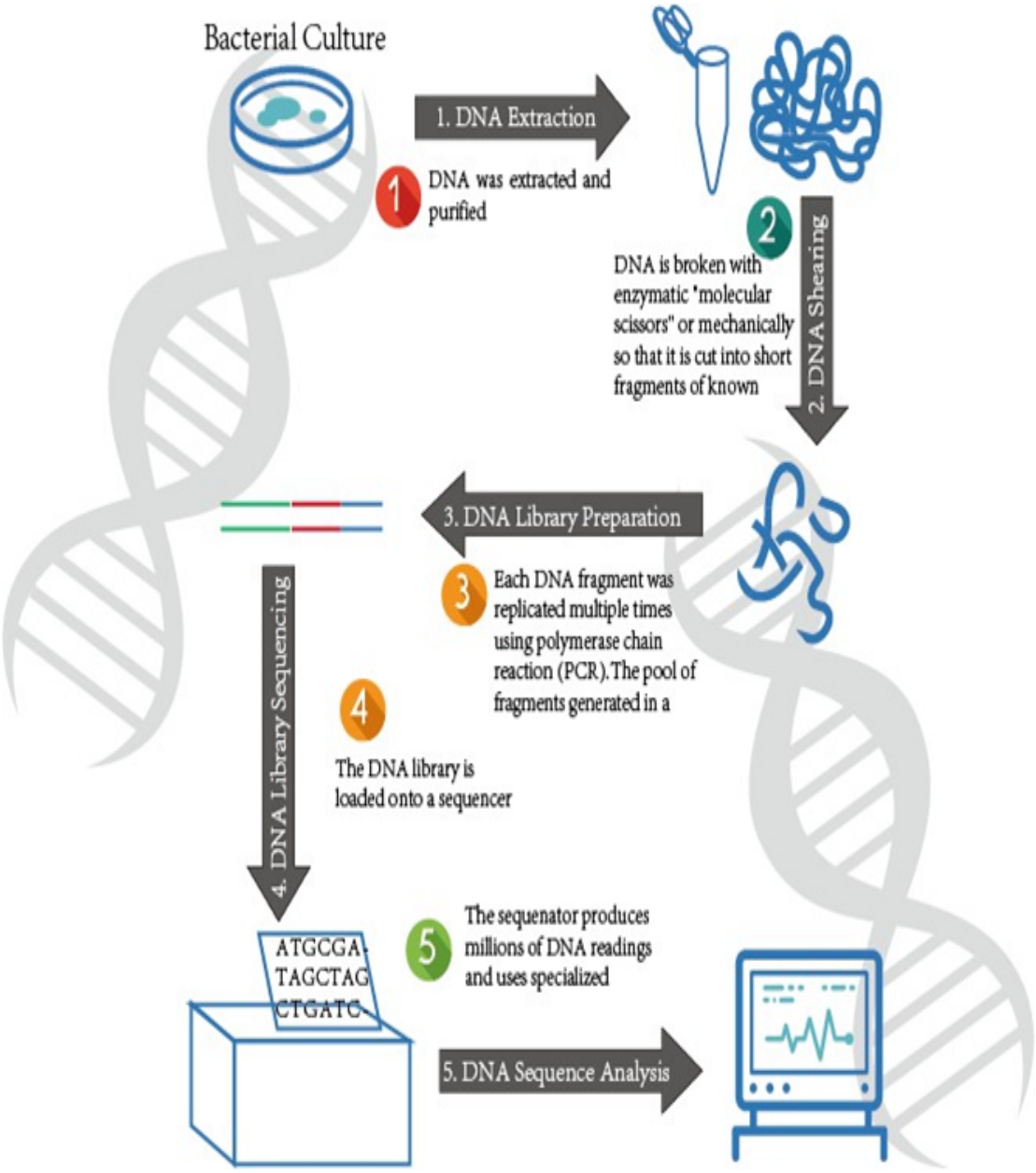

FIGURE 3.8: WGS Pipeline

Each 1.5 mL vial had an aliquot of 450 μL of ice cold (-20^{o}C) 100% isopropanol added to it. Organic layers were discarded, and samples were refrigerated for 30 minutes at -20 ^{o}C. Samples were spun for 15 minutes at room temperature at 10,000 g.

The supernatants were thereafter taken out and thrown away, and the pellets were cleaned with one milliliter of ice-cold (-20^{o}C) 70% ethanol. The vials were gently inverted and then spun at 10,000 g for five minutes at room temperature. As much

ethanol as you can without damaging the pellets should be taken out and thrown away in the very next step.

All of the pellets were dried by letting them sit in a heating block that was set to 65^{o}C with the lids open until the ethanol had completely evaporated. 50 μL of TE buffer was added to the pellets to rehydrate them, and they were then incubated at 4^{o}C for the entire night. The extracts were kept at -20^{o}C.

b) Estimation of DNA quality Using Nanodrop In order to evaluate nucleic acid concentration and DNA quality, the NanoDrop 2000 spectrophotometer from nanodrop technologies was used by using the 'Nucleic Acid' application module.

Selected 'DNA-50' for double-stranded DNA as sample type. Performed a blank run prior to DNA sample testing. For proper working of the instrument, the pedestal was cleaned. The measurement pedestal was filled with 2 μl of molecular grade water. The sampling arm was closed and clicked on the 'Blank' button.

As soon as the measurement was complete, the blanking buffer was wiped. Afterwards DNA (2 μL) was added to the pedestal, the 'Measure' command was given. The quantity of DNA was noted for each sample.

c) DNA Concentration detection through the Qubit Fluorometer Nucleic acid quantification is an important part of genomic techniques. fluorometry is considered as an important tool to quantify DNA concentration precisely. Invitrogen™Qubit™3.0 Flurometer (Life Technologies, CA, USA) was utilized as a rapid and reliable fluorescence-based quantification of nucleic acids with a minute quantity of 1 μL sample. (Figure 3.9)

d) Estimation of DNA Integrity using Agarose Gel Electrophoresis This technique was used for the confirmation and analysis of genotyping. For agarose gel electrophoresis, 2.5 μl of PCR product was used. The gel was composed of 1X TAE buffer (tris acetate EDTA) and agarose. About 4 μl of ethidium bromide was use for staining. The prepared gel was poured in the gel cassette and after solidification it was placed in gel tank. 1kb plus DNA ladder was added in a well of gel as a marker. The electrophoresis was allowed to be done at voltage 120V, time

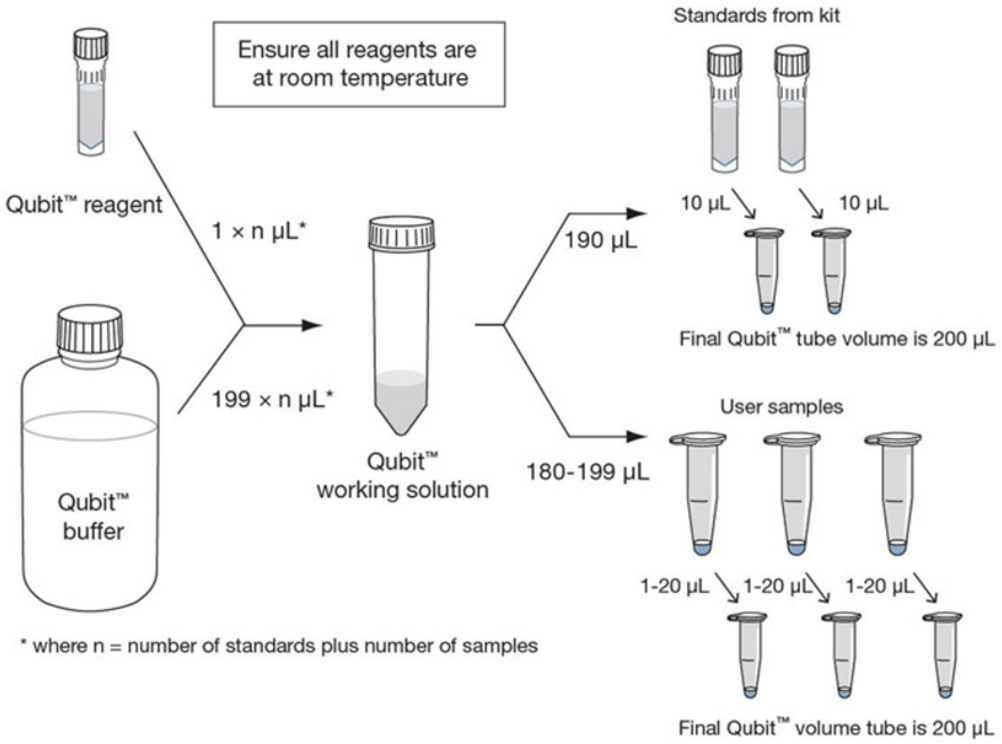

FIGURE 3.9: DNA quantification on Qubit 3

45min. The gel was viewed and images were taken using gel documentation system (Alpha, innotech). Few gel electrophoresis results are provided below (Figure 3.10).

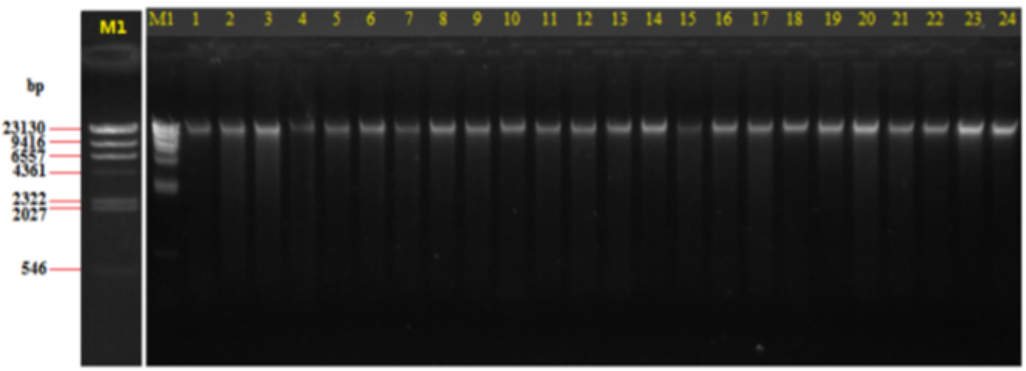

FIGURE 3.10: Gel Electrophoresis Results

e) Library preparation for DNA fragments After passing the test, each sample was selected with 0.2 μg gDNA template, and the library was prepared according to the Watchmaker DNA Library Prep Kit with Fragmentation (7K0019-096).

f) Library examination To perform primary quantification, the DNA concentration of the library was quantified using Qubit®3.0. The Fragment analyzer system was used to assess insert size. Since the insert size complied with the requirements, ABI Quant Studio 12K Flex was used to conduct Q-PCR in order to accurately calculate the library's operative concentration (>10nM) for library quality assurance.

g) Library clustering and sequencing: As directed by the manufacturer, the HiSeq PE Cluster Kit v4-cBot-HS (Illumina) was used to cluster the index-coded samples on the cBot. Following that, 150 bp double-ended sequencing reads were obtained using the HiSeq X Ten Reagent Kit V2.5 and paired end sequencing was carried out on the HiSeq sequencing platform. The cluster generation and sequencing were run on Illumina NovaSeq X Plus platform, using NovaSeq X Series 10B/25B Reagent kit.

h) WGS Analysis FastQC was used to study sequence reads www.bioinformatics.babraham.ac.uk as a preliminary data quality evaluation. Raw standard reads were omitted by utilizing trimmomatic software [198] and sequenced in comparison to the reference genome H37Rv through the BWA-mem alignment package [199]. Single nucleotide polymorphism (SNP) call was made through the BCF/VCF software suite [200], followed by their FASTA format. While analyzing the WGS files of all heteroresistant isolates, the cutoffs of heteroresistance for the current study were defined between 5% and 95%, and the variants of quality ≥30 and depth ≥5 were annotated. This cutoff is consistent with international standards and prior studies, ensuring detection of clinically relevant minority populations while minimizing sequencing noise. According to the WHO mutation catalogue [201], detection of resistant subpopulations down to 25% is critical for accurate Bedaquiline resistance prediction, as rare but impactful mutations—particularly in Rv0678—can exist below fixation thresholds. A 5% lower bound balances sensitivity and specificity in variant calling, while the upper 95% limit distinguishes heteroresistance from fully fixed resistance mutations. This range assists robust identification of mixed bacterial populations, which are significant for understanding early resistance development and treatment failure risks.

i) Evolutionary analysis It was done by using IQ-Tree that is an integrated tool for leading automatic and manual sequence alignment, inferring phylogenetic trees. Geographic distribution of study isolates was done using Qgis 3.36.2. Phylogenetic tree was constructed using Chiplot [201].

j) GWAS Analysis fastQC was used to study sequence reads (www.bioinformatics.babraham.ac.uk/projects/fastqc/) as a preliminary data quality evaluation. Raw standard reads were omitted by utilizing trimmomatic sofware [100] and sequenced in comparison to the *H37Rv* through the BWA-mem alignment package [101]. SNPs calls were made through the BCF/VCF software suite [102] followed by their FASTA format. For Genome Wide Association Studies (GWAS) data analysis, GEMMA software was used [105]. Furthermore, after getting the genotypic profile from GEMMA, it was compared to the DST profile of the study samples by using PLINK software to provide linkage analysis [106].

To investigate the potential interaction between potential new marker for BDQ-R resulted from GWAS and BDQ, we performed molecular modeling studies adopting Glide docking simulations mode in GLIDE (www.schrodinger.com/ platform/products/glide/). Subsequent Molecular Dynamic Simulation was performed using AMBER https://ambermd.org/.

k) Protein Dynamic Analysis The effect of mutation conferring genes on the thermodynamic properties of their protein were evaluated while using Dynamut Tool [201]. This is a valuable tool to predict the impact of mutations on the protein ultimate conformation [201].

3.2.5 Statistical Analysis

We analyzed the data of our current study by using IBM SPSS version 21. Patient demographic variables and History of ATT were analyzed using chi-square test and values were found significant at 0.05. We applied univariate analysis with 95% CI to analyze the patient and bacterial potential factors for Bedaquiline heteroresistance.

Values were considered to be statistically significant at the significance level of 0.05. Heteroresistance percentage is indicated by the number of reads in the BWA-MEM pileup that contain mutations.

3.2.6 Data Availability

The FASTQ files for all 100 strains are uploaded on public repository and can be accessed at BioProject no. 1195559. The data is public repository under the grant by the National Key Research and Development Program of China (2022YFC2305200) provided by China Center of Disease Control and Prevention

Chapter 4

Results

4.1 Knowledge, Attitude & Practices of Health Care Workers

4.1.1 Characteristics of HCWs

In total 306 HCWs participated in this study who were evaluated for their demographic characteristics. Gender wise 77% male and 23% female Health Care Workers were part of the study (Table 4.1). Jobwise, 30% of the participants were working as HCWs for less than one year whereas 14% were working for more than a decade (Table 4.18).

Healthcare workers in Pakistan encompass various designations. Among all the categories, the majority (35%) of healthcare workers were classified as laboratory staff, which may include lower-level positions such as screeners, laboratory attendants, and raiders. Additionally, 27% of the healthcare workers included in the study were doctors by profession (Table 4.1).

Based on the level of education, Maximum number of the TB Health Care Workers were of university level in contrast to 14% those who were trained for technical proficiency (Table 4.1). The age range of the recruited HCWs varied from 20 to

55 years, with the majority of TB HCWs falling within the 26-30 years range. The calculated mean age was 33±8.4 (Table 4.1). The participants were asked if they have ever participated in any TB-related training. It was observed that146 (48%) HCWs have participated in the training while 52% of the participants were not trained for TB. Only 25% of the TB HCWs get their training in last six months (Table 4.1).

As far as direct involvement of HCWs in TB control activities was concerned, 37% of them were directly involved in comparison to 63% indirectly involved (Table 4.18). The participants were also asked if they were diagnosed with TB in the past. Only 5% of the TB HCWs had informed that they suffered from TB in the past. Additionally, the recruited subjects of the study were asked for any close contact which might include friends or family members ever infected with TB. The results showed 33% of the studied population of HCWs were in close contact of TB patients.

TABLE 4.1: Characteristics of Health care workers

Variable	**Category**	**Total**	
		N	**%**
Gender	Male	236	77%
	Female	70	23%
Time Spent Working TB Patients	Less than One Year	92	30%
	One to Five Years	70	23%
	Five to Ten Years	16	5%
	More than Ten Years	42	14%
	Never	84	27%
Profession	Medical Agent	8	3%
	Nurse	30	10%
	Doctor	84	27%
	Laboratory Staff	106	35%
	Lab Manager	24	8%
	Quality Officer	6	2%
	Safety Officer	0	0%
	Internee/Student	18	6%
	Technician	48	16%

Table 4.1 continued from previous page

Variable	Category	Total	
		N	%
Level of Education	Primary School	8	3%
	Secondary School	12	4%
	College	24	8%
	Professional Technical Training	42	14%
	University	220	72%
Age	20-25 Years	58	19%
	26-30 Years	76	25%
	31-35 Years	48	16%
	36-40 Years	68	22%
	41-45 Years	30	10%
	46-50 Years	14	5%
	51-55 Years	12	4%
Participated in TB Training in the Past	Yes	146	48%
	No	160	52%
TB Training in past 6 Month	Yes	76	25%
	No	230	75%
Directly Involved in TB Control Activity	Yes	112	37%
	No	194	63%
Diagnose with Active TB in the Past	Yes	14	5%
	No	292	95%
In Close Contact of Person ever sick with TB (Friends, Family)	Yes	102	33%
	No	204	67%

4.1.2 Knowledge Score of HCWs

Overall mean score of knowledge was computed as 15.05±3.96 (Table 4.2). The present study evaluated the level of knowledge of Health Care Workers related to TB basics, diagnosis and treatment. Data presents some lack of knowledge by TB HCWs in few and far fields. About 15% of the TB related Health Care

Workers are not sure of the exact mode of TB transmission while 85% were sure that this disease spreads through aerosols or droplets (Table 4.2). It was observed that a large portion (41%) of the participants think that TB is more prevalent in patients with Chronic Obstructive Pulmonary Disease (COPD), male gender and those living tropical areas altogether. Maximum percent of these Health Care Workers mentioned that TB is more prevalent in patients with HIV (Table 4.2). There was a random and wrong opinion (69%) related to the likelihood of developing active TB on contracting TB within immediate timeframe while the least subjects (30%) opted for the correct likelihood (10%). There was a large knowledge gap concerning the best diagnostic tool of TB. Only 26% of the participants selected opted culturing as best diagnostic tool. Healthcare workers in the tuberculosis department lacked knowledge (66%) regarding the required number of sputum samples for diagnosing tuberculosis. The majority (71%) of healthcare workers were unaware of the proper storage procedures for sputum samples before testing. Seventy-six percent of healthcare workers were knowledgeable about the tuberculosis vaccine, while seventy-three percent knew the recommended timing for administering the TB vaccine (Table 4.2).

TABLE 4.2: Descriptive Statistics for Knowledge of Health Care Workers

Questions	**N**	**Mean**	**SD±**
1. What is the causative agent of tuberculosis?	306	0.96	0.2
2. Is tuberculosis a transmissible disease?	306	0.93	0.24
3. How does tuberculosis spread?	306	0.85	0.35
4. Which group of people is at higher risk of developing tuberculosis?	306	0.58	0.49
5. If a person contracts tuberculosis, what is the likelihood they will develop active tuberculosis within the immediate time frame?	306	0.3	0.46
6. What type of preventive measures can you take as health care professional if you are dealing with a tuberculosis patient or a suspect of tuberculosis	306	0.06	0.24
7. Have you ever heard of the Tuberculosis Infection Control Plan for your Health Unit?	306	0.73	0.44
8. If yes, have you received training on its content?	306	0.41	0.49
9. What is the most common symptom of pulmonary tuberculosis?	306	0.61	0.48

Table 4.2 continued from previous page

Questions	N	Mean	SD±
10. What is the best diagnostic tool for tuberculosis?	306	0.26	0.44
11. TB diagnosis in children is more difficult than in adults?	306	0.9	0.28
12. How many sputum samples are necessary for diagnosis?	306	0.34	0.47
13. When should the initial sputum sample be taken?	306	0.07	0.26
14. How should a sputum sample be stored before laboratory analysis?	306	0.28	0.45
15. Do you know what Gene Xpert is?	306	0.8	0.39
16. Is tuberculosis a curable disease?	306	0.96	0.19
17. How long is the first line treatment of pulmonary tuberculosis?	306	0.67	0.46
18. Does tuberculosis treatment in children have a longer length?	306	0.41	0.49
19. How many drugs are used in the first line treatment of tuberculosis?	306	0.47	0.50
20. Do you know what direct observed treatment during the initial phase of treatment is?	306	0.69	0.45
21. What is multi-drug resistant tuberculosis?	306	0.62	0.48
22. In which group of people is multi-drug resistant tuberculosis most likely to occur?	306	0.48	0.50
23. When should the first follow up sputum sample be carried out following the commencement of treatment of a confirmed case of tuberculosis?	306	0.35	0.47
24. What is the major element to assess tuberculosis treatment cure?	306	0.14	0.35
25. What are the consequences of incomplete treatment?	306	0.58	0.49
26. Is there a vaccine for TB?	306	0.76	0.42
27. When is the best time for BCG vaccination?	306	0.73	0.44
Overall Knowledge Score	306	15.1	3.96

4.1.3 Attitude Score of HCWs

The score of attitudes towards TB in the HCWs enrolled in the study was evaluated (Table 4.3). Inclusive mean attitude score of the studied population was 83.68 (15.74). The results presented the importance of determining every new TB case so as to control the disease as majority (61%) of the subjects strongly agreed the statement. Equal proportion of HCW (1:1) agreed and strongly agreed the

fact that TB is considered and treated as a stigma in Pakistan. Around 25% of the subjects disagreed with statement that Pakistani population is well aware of the TB as a serious health problem. There was a strange response regarding the question that traditional or other alternative medicine can work for the betterment of TB patients (35%). Maximum participants (48%) strongly agreed with the importance of Directly Observed Therapy (DOT) in disease control. When training of the HCWs regarding TB was inquired, a high percentage of the HCWs (33%) responded disagreement. Number of TB staff adequacy was well enough in the health facilities as responded by the HCWs (43% agreement). As a preventive step for TB prevalence the suspected as well as confirmed TB population accepts to wear mask (47% agreement). A maximum percentage (34%) of HCWs Pakistan agreed the concern of having had TB infection. HCWs strongly disagreed (34%) that if they would get TB infection, they would be allowed to continue working within their present capacity. If any of the HCWs get infected with TB, he or she is confident (42% agreement) that his/her employer would prior the confidentiality.

Table 4.3: Descriptive statistics of Attitude of HCWs

Questions	**N**	**Mean**	**SD**
1. Finding every new case of tuberculosis is essential for control of the disease	306	**4.35**	**1.13**
2. Community engagement is essential for the control of the disease	306	4.35	1.07
3. There is a substantial increase in treatment completion rates if direct observed treatment is used	306	3.98	1.36
4.There is a stigma associated with tuberculosis in Pakistan	306	4.48	0.526
5. The way you interact with tuberculosis patients/regard tuberculosis contributes any possible stigma there is	306	3.38	1.45
6. Tuberculosis as a disease has more stigma associated with it than HIV	306	3.1	1.45
7. Money spent on educating the Pakistan general population is better than money spent on direct observed treatment	306	3.3	1.46
8. In Pakistan, the general population is aware of the tuberculosis services that are available	306	3.19	1.41
9. Public awareness regarding tuberculosis as a health problem in Pakistan is adequate	306	3.07	1.47
10. Multi-drug resistant tuberculosis is a problem in Pakistan	306	3.75	1.3

Table 4.3 continued from previous page

Questions	N	Mean	SD
11. Traditional or alternative medicine assists in wellbeing of tuberculosis patients	306	3.04	1.42
12. First line therapies for tuberculosis are accepted by patients	306	3.77	1.3
13. In Pakistan, there are many barriers to tuberculosis treatment	306	3.38	1.39
14. The majority of staff in your health center have adequate training regarding tuberculosis	306	2.57	1.14
15. The laboratory service that your health center uses is adequate for the diagnosis of tuberculosis	306	3.47	1.42
16. In your health centre, there is a sufficient number of people required to treat the tuberculosis patients seen	306	3.21	1.42
17. Making people with suspected/confirmed pulmonary tuberculosis wear masks in the hospital is acceptable	306	3.85	1.23
18. Teaching tuberculosis patients cough hygiene is not important	306	2.85	1.14
19. Infection control is an important means to prevent contracting tuberculosis	306	3.97	1.28
20. I have been seriously concerned I have had tuberculosis	306	3.17	1.34
21. I should know whether I have got or had tuberculosis	306	3.46	1.36
22. If I contracted tuberculosis, I would be allowed to continue working in my current capacity	306	2.9	1.33
23. My employer would maintain confidentiality if I were to contract tuberculosis	306	3.13	1.41
24. I should know my own HIV status	306	3.87	1.31
Overall Attitude Score	306	83.7	15.74

4.1.4 Practices Score of HCWs

To evaluate practices of HCWs, three scenarios were provided to the subjects and scored (Table 4.4). The overall mean score of HCWs' practices was calculated as 6.31 (SD =2.21). In the first scenario, the first question was the differential diagnosis of the infection on the basis of the symptoms provided in addition to the diagnostic test to be opted for the diagnosed infection and after how long test should be performed. From the studied population 79% of the HCWs responded correctly that the differential diagnosis is TB and GeneXpert test should be performed immediately after diagnosis. The next query with reference to the

first scenario was regarding the drug(s) used to treat TB and treatment duration. Majority of the HCWs were well aware of the drugs employed in TB treatment in initial phase along with their appropriate duration of treatment. These drugs include Pyrazinamide, Isoniazid, Rifampicin and Ethambutol and should be prescribed for 2 months only. The next question of the first scenario was to get the information regarding the drugs in continuation phase and the duration of treatment prescribed. Sixty-two % of the HCWs responded correctly to the option of Isoniazid and Rifampicin for four months. The second scenario included in the study was to evaluate the practice of the HCWs, when the TB patient was sputum positive following four months of treatment. Larger group (63.7%) of HCWs responded correctly to start the first line TB treatment in this situation. Third scenario focused the practice of the HCWs on the side effects of TB treatment that if the patient turns pale on taking the TB medication for three weeks only. Around 47% of the Health Care Workers adopt all the relevant practices in this situation altogether rather than practicing any single of them (52.9%).

TABLE 4.4: Descriptive Statistics of Practices of Health Care Workers

		N	**Mean**	**SD**
1st Scenario	Differential Diagnosis, Technique, Instance of Testing	306	2.35	1.24
	TB Treatment and Duration	306	1.59	0.81
	Treatment in Continuation Phase and duration	306	1.25	0.97
2nd Scenario	Sputum Positive on Fourth Month Treatment Follow-up	306	0.64	0.48
3rd Scenario	Side Effects of TB treatment	306	0.47	0.50
	Overall Practice Mean Score	306	6.31	2.22

4.1.5 Knowledge and Level of Education

As mentioned above, the mean score of knowledge of the HCWs enrolled in the study was 15.05 (SD = 3.96) displayed in Figure 4.1. There was significant difference ($p<0.05$) between Knowledge and various level of education. HCWs with

professional technical training were observed to have more TB knowledge (16.33) in comparison to those with university, college, secondary and primary level of education.

It was observed that primary school group have the least TB knowledge in comparison to professionally technical HCWs by 5.01 ($p < 0.001$).

Also, knowledge score of university level HCWs is significantly different ($p<0.05$) to technically proficient HCWs and the primary level knowledge. Knowledge of technically proficient staff was different from university, college and primary level knowledge ($p<0.001$). College level HCWs' knowledge was only significantly different from that of university level ($p<0.001$).

Knowledge of secondary level HCWs was only significantly different to primary level knowledge mean ($p=0.03$). The mean knowledge score turned to be statistically different to that of university, technically trained and secondary group ($p=0.04$, $p<0.001$and $p=0.03$ respectively).

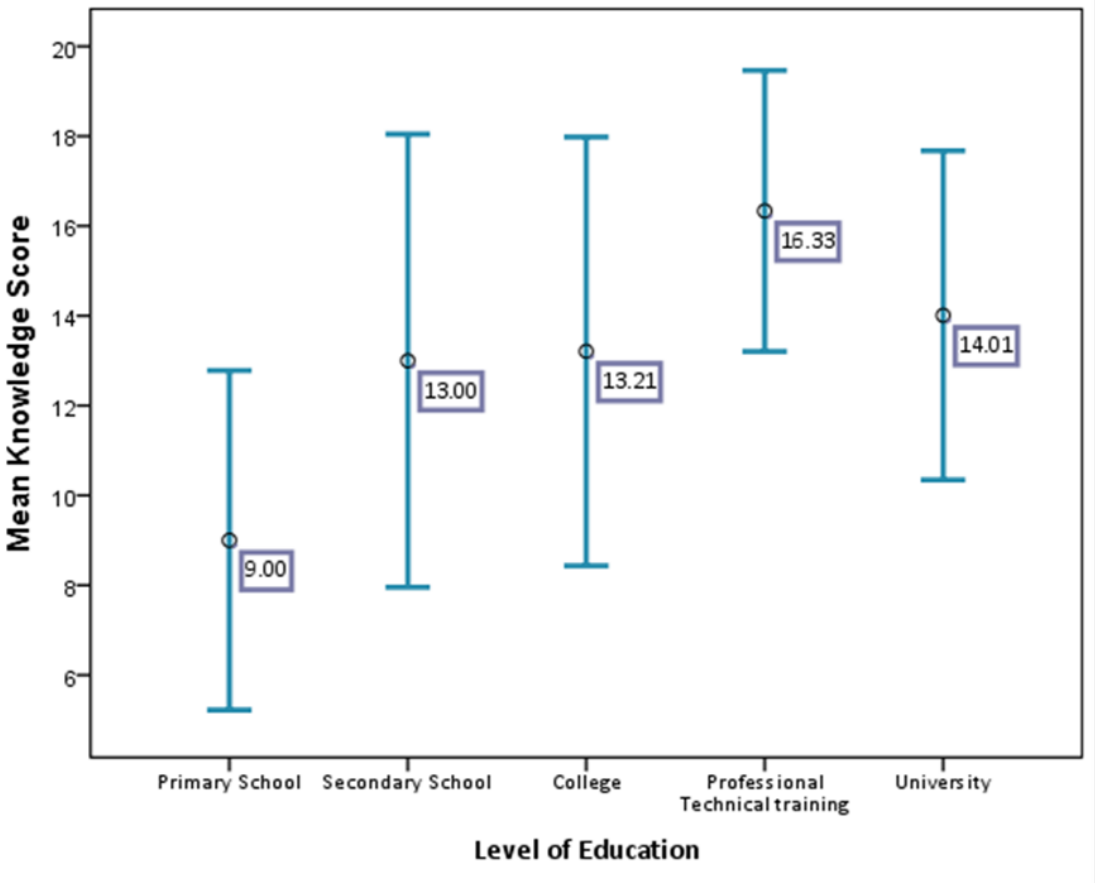

FIGURE 4.1: Mean Knowledge Score with Level of Education of HCWs

4.1.6 Knowledge and Profession as a HCWs

Current study also stratified the knowledge of the HCWs according to their profession within the TB department as shown in Figure 4.2.

It can be seen from the graph that quality officer and lab manager both are better in knowledge of TB in comparison to other professions (17.00 and 15.13 respectively). Doctors have less technical information in comparison to nurses (14.26 and 14.57 respectively).

ANOVA results of our data exhibited statistically significant variation between knowledge and profession of health care worker in the said TB referring health facility (p=0.02).

Tukey's post hoc results informed that only knowledge mean score of nurses and the internee/student group was proved to be statistically different from each other (p=0.03).

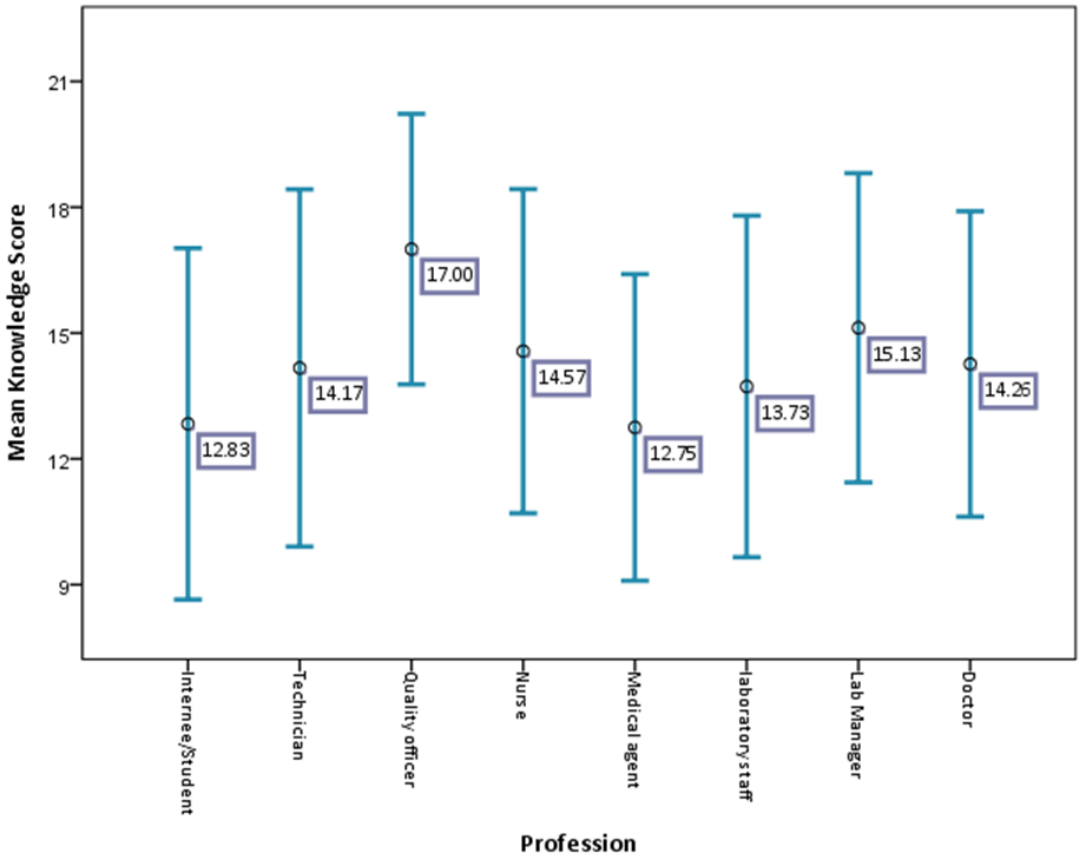

FIGURE 4.2: Mean Knowledge Score with Profession of HCWs

4.1.7 Knowledge of HCWs Relevant to Working Duration as a TB Staff

The present study tried to unveil the importance of working duration of HCWs in the TB department on their mean knowledge score depicted in Figure 4.3. There was no significant association present between mean knowledge of HCWs with their duration of working in TB department.

There was no significant association present between mean knowledge of HCWs with their duration of working in TB department.

Maximum mean knowledge was calculated for the HCWs who worked in TB department for less than one year (15.68) followed by the mean values of the HCWs who worked for five to ten year and one to five year (15.19 & 15.16 respectively).

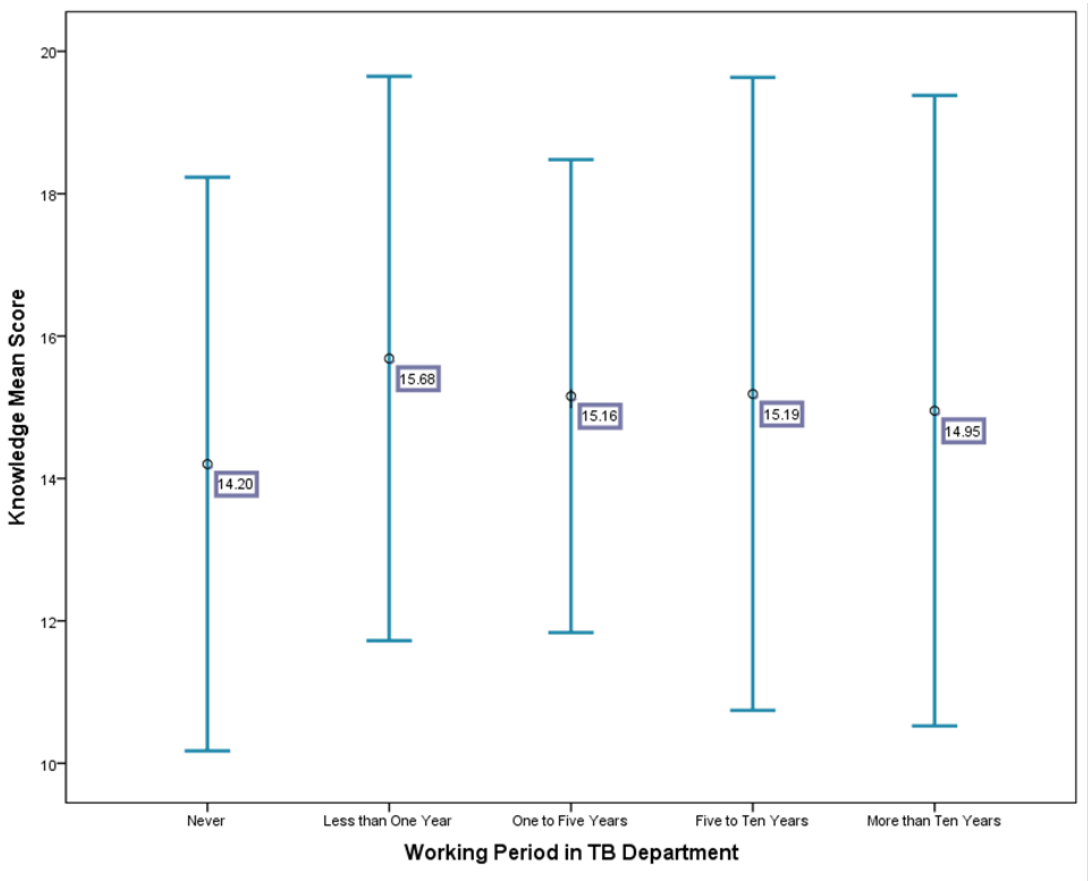

FIGURE 4.3: Mean Knowledge Score with Working Period in TB Departments

4.1.8 Correlation of KAP Score

The present KAP data was evaluation for Pearson correlation displayed in Table 4.5.

Pearson coefficient (r) was positively correlated for all the three categories. Hyphen represented the direct correlation of the three variables with each other. Pearson coefficient of attitude score with knowledge of Health Care Workers was 0.28.

Practice mean score was correlated to knowledge mean score with r = 0.40. On the other hand, practice score was r =0.29 with attitude mean score Figure 4.4.

4.1.9 Demographic Variables and KAP Scores

All the demographic parameters along with their variables were studied in comparison to descriptive of knowledge, attitude and practices (Table table:4-15).

Knowledge score was calculated as significantly different with age, level of education attainment, profession within health facility, involvement in TB control activities, TB specific training and status of TB infection.

There was a statistically significant difference in attitude score and age group, gender, level of education attainment, profession within health group, duration of working in health center, working duration with TB patients and TB infection status.

In practice score of HCWs, age, education and working duration with TB patients were statistically different with knowledge score except health care professional status within family, involvement in TB control activities and family history of TB.

TABLE 4.5: Correlations Matrix of Knowledge, Attitude and Practice Mean Score

		Knowledge	**Attitude**	**Practice**
Knowledge	Pearson Correlation	-		
	Sig. (2-tailed)			

Table 4.5 continued from previous page

		Knowledge	Attitude	Practice
	N	306		
Attitude	Pearson Correlation	.280**	-	
	Sig. (2-tailed)	0		
	N	306	306	
Practices	Pearson Correlation	.400**	.295**	-
	Sig. (2-tailed)	0	0	
	N	306	306	306

** Correlation is significant at the 0.01 level (2-tailed)

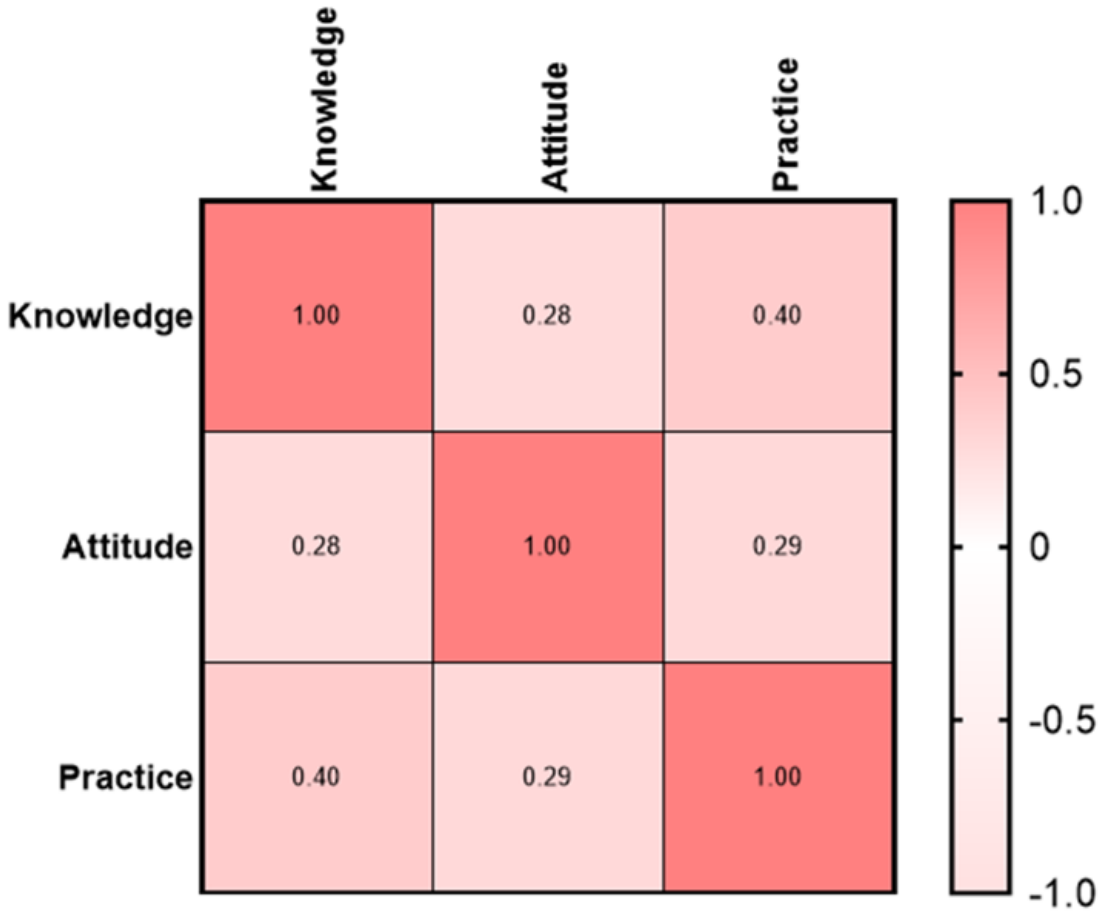

FIGURE 4.4: Pearson Correlation of KAP score

Table 4.6: Demographic variables and TB KAP HCWs

VARIABLE Categories	**N**	**KNOWLEDGE**			**ATTITUTDE**			**PRACTICES**		
		MEAN	SD	p-Value	MEAN	SD	p-Value	MEAN	SD	p-Value
Age (Years)				<0.001*			<0.001*			<0.001*
20-25	58	12.19	3.56		74.2	18.45		4.83	2.08	
26-30	76	16.21	3.25		84.86	15.16		6.47	2.26	
31-35	48	16	3.27		90.04	10.65		6.5	1.7	
36-40	68	14.23	4.39		85.73	14.66		6.78	2.41	
41-45	30	16.13	2.99		82.86	17.14		7.07	1.91	
46-50	14	16.57	4.56		79.28	11.99		6.29	1.63	
51-55	12	18	3.07		92.16	3.58		7.17	1.64	
Gender				0.66			0.09			0.07
Male	236	14.81	4.15		83.49	18.17		6.26	2.34	
Female	70	15.31	4		81.87	13.03		6.3	2.04	
Level of Educational Attainment				<0.001*			<0.001*			<0.001*
University	220	14.63	4.03		82.2	16.92		6.2	2.26	
Professional Technical Training	42	17.52	2.6		86.64	13.78		6.76	2.29	
College	24	13.83	4.31		86.08	18.25		6.21	2.08	
Secondary School	12	16	5.18		94.5	21.76		7.58	1.62	
Primary School	8	11	4.53		64	9.94		3.75	2.05	
Health Care Professional Within Family				0.48			0.34			0.57
No	64	14.98	4.61		80.06	19.7		5.56	2.47	

Table 4.6 continued from previous page

VARIABLE Categories	N	KNOWLEDGE			ATTITUTDE			PRACTICES		
Yes	242	14.91	3.99		83.93	16.32		6.45	2.18	
Profession Within Health Center				0.02*			0.08			0.09
Doctor	84	15.13	4.58		80.4	20.55		6.81	2.13	
Lab Manager	24	14.04	3.08		78.75	12.09		6.13	2.15	
Lab Staff	106	14.58	4.21		83.5	17.84		5.67	2.44	
Medical Agent	8	13.62	3.62		83.12	4.94		5.25	2.96	
Nurse	30	16.76	3.7		89.06	10.61		7.27	1.7	
Quality Officer	6	15	3.89		80.33	4.13		7.33	1.03	
Technician	30	15.86	3.94		86.66	14.27		6.27	2.27	
Internee/ Student	18	13.11	2.54		84.44	18.08		5.89	1.77	
Duration of Working in Health Center				0.64			0.02*			0.05*
Less than One Year	66	15.03	4.31		83.34	17.03		6.05	2.27	
One to Five Year	106	14.96	3.86		86.75	13.31		6.25	2.26	
Five to Ten Year	50	15.62	3.45		83.74	17.45		6.74	1.66	
More than Ten Year	84	14.39	4.61		77.98	20.07		6.19	2.57	
Working Duration with TB Patients				0.14			0.04*			0.08
Never	84	14.2	4.02		84.02	16.1		6.12	2.05	
Less than One Year	92	15.68	3.96		85.01	15.46		6.37	2.3	
One to Five Year	70	15.15	3.32		84.91	15.28		6.53	2.02	
Five to Ten Year	16	15.18	4.44		77.81	16.17		6.38	2.82	
More than Ten Year	44	14.27	5.34		76.52	23.18		5.89	2.75	

Table 4.6 continued from previous page

VARIABLE Categories	N	KNOWLEDGE			ATTITUTDE			PRACTICES		
Involved in TB Control Activities				0.02*			0.21			0.27
No	194	14.5	4.17		81.92	17.77		6.33	2.28	
Yes	112	15.67	3.94		85.18	15.79		6.16	2.26	
Experienced TB-specific Training				<0.001*			0.56			0.69
No	160	14.3	4.07		82.75	18.3		6.26	2.21	
Yes	146	15.61	4.07		83.52	15.78		6.28	2.34	
Ever been Sick with TB				0.03*			<0.001*			<0.001*
No	274	15.08	4.07		84.3	17.32		6.41	2.22	
Yes	32	13.62	4.36		73	11.09		5.09	2.34	
Family History of TB				0.62			0.5			0.41
No	204	14.92	4.01		83.63	17.24		6.45	2.21	
Yes	102	14.93	4.35		82.08	16.93		5.9	2.35	

*p-value for the Mann–Whitney U or Kruskal-Wallis test

N; number of observations SD; standard deviation, TB tuberculosis

In this study, the KAP survey was conducted independently among healthcare workers across various facilities and was not linked at the individual level to specific patients or their molecular data.

As such, statistical integration of KAP scores with patient-level clinical or genomic findings of BDQ study was not feasible due to the lack of a one-to-one mapping between HCWs and the patient cohort.

4.2 BDQ Genetic Analysis

Current Study is designed to highlight the significance of BDQ in MDR/XDR-TB treatment regimen. The study includes the MTB isolates from various regions of Pakistan (Figure 4.5) which is declared as 5th highest burden country for TB and 6th highest RR/MDR-TB burden country by WHO [203].

For the present study, 50 BDQ sensitive and 50 BDQ resistant strains were screened from RR/MDR, Pre-XDR and XDR group. All the samples were received and processed at NTRL, Islamabad Pakistan to elucidate the genotypic insight of BDQ resistance in MTB strains from Pakistan.

4.2.1 Characteristics of Recruited Patients

The demographic distribution of MTB cases which were part of this study revealed that males (57%) outnumber females (43%) (Figure 4.6). Age-wise, adults (46%) form the largest group, followed by young individuals (30%) and older individuals (24%) (Figure 4.7).

Geographically, Punjab accounts for the highest number of cases (78%), whereas Balochistan (2%) and KPK (4%) reported the lowest. Sindh (11%) and ICT (5%) contributed moderately. (Figure 4.8) Regarding treatment history, the majority of cases were new (80%), while 20% are previously treated or being followed up

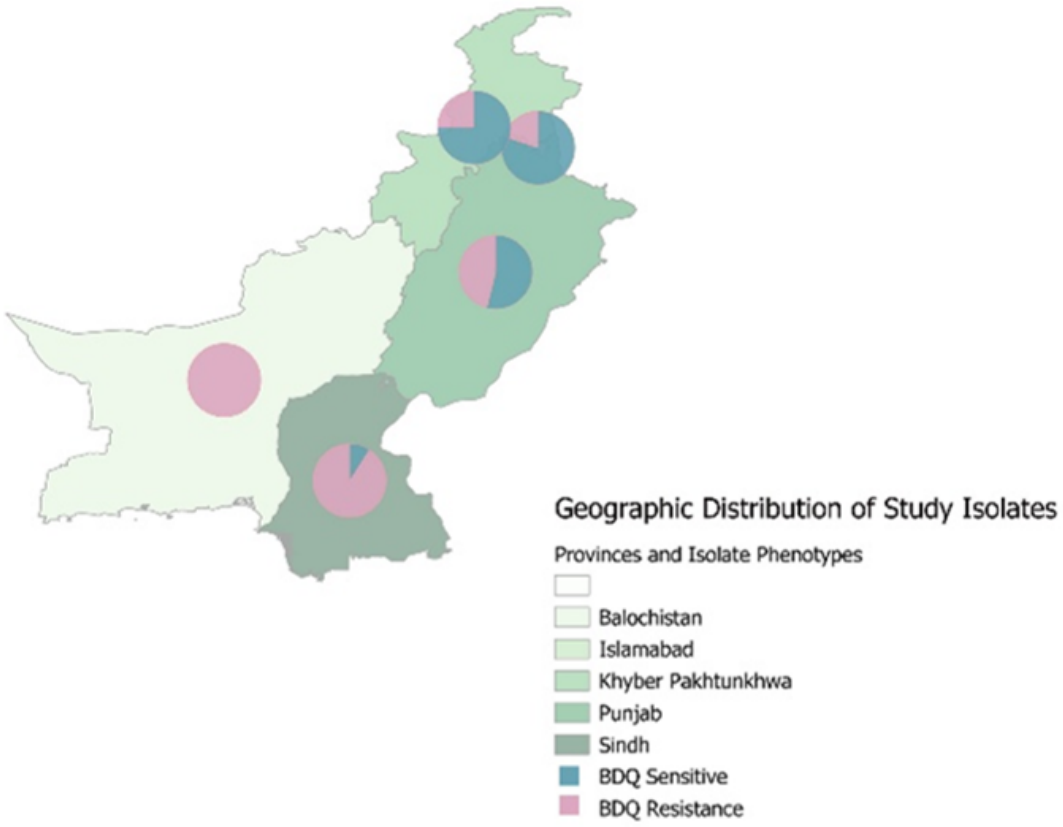

FIGURE 4.5: Distribution of study MTB Isolates from Pakistan

for TB treatment (Figure 4.9). These findings indicate significant regional and demographic variations in case distribution, which may inform targeted intervention strategies.

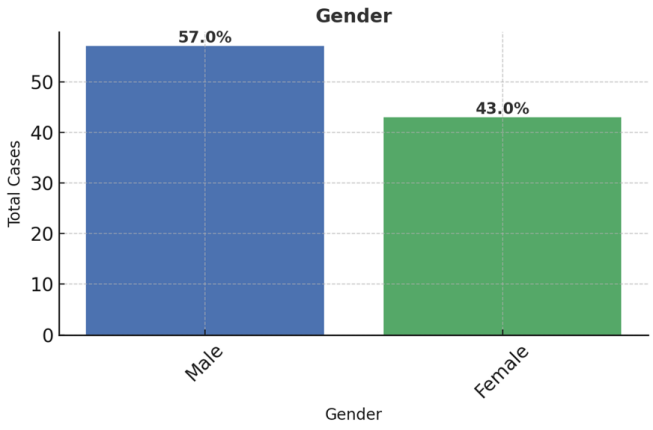

FIGURE 4.6: Patient Characteristics: Gender Distribution

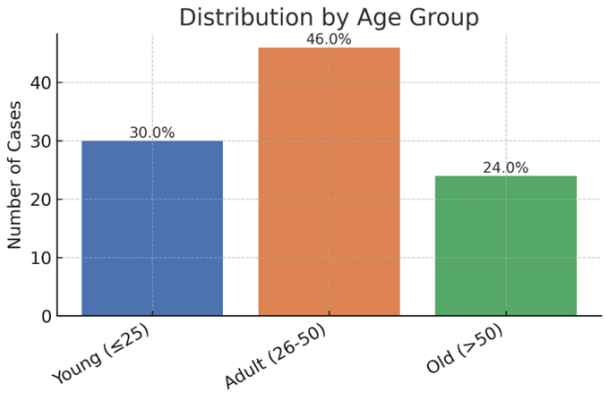

FIGURE 4.7: Patient Characteristics: distribution in age groups

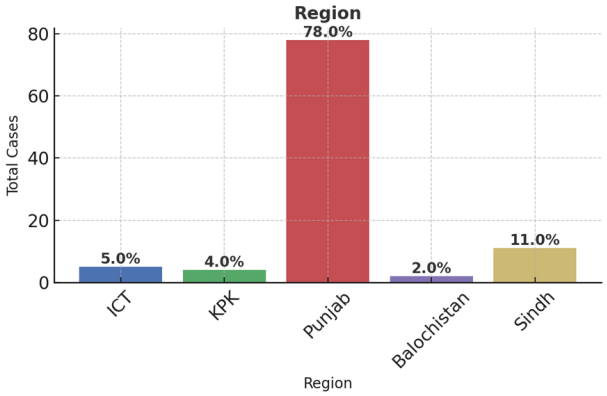

FIGURE 4.8: Patient Characteristics: distribution in regions

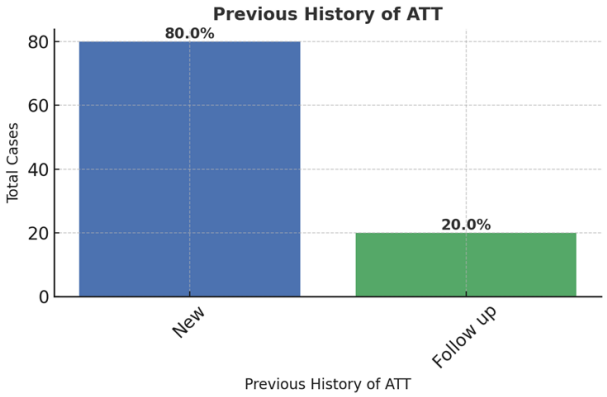

FIGURE 4.9: Patient Characteristics: distribution in new and previously treated cases (Follow up)

4.2.2 Patient Demographic Variables and History of ATT

Table 4.7 presents the distribution of recruited MTB cases based on gender, age, and region with relevance to history of anti-tuberculosis treatment; new and previously treated cases. Among the 100 total MTB cases, 57% were male and 43% were female, with no statistically significant difference between genders ($X^2 = 2.34$, $p = 0.12$). Age-wise, adults (46%) were the most affected group, followed by young individuals (30%) and older individuals (24%), with a significant association between age and treatment history ($X^2 = 6.25$, $p = 0.04$). Regionally, Punjab had the highest number of cases (78%), while Balochistan (2%) and KPK (4%) had the lowest, though the regional differences were not statistically significant ($X^2 = 7.88$, $p = 0.09$). Among the 46 new cases, 65.21% were male, and 58.69% were adults, while previously treated cases (54%) had a more even gender distribution. These findings specify that TB cases are more prevalent in certain demographics, particularly among males, adults, and individuals from Punjab, which may help in designing targeted interventions.

TABLE 4.7: Patient demographic variables and History of ATT

Variable	**Total no. of cases**	**New Cases (n=46)**	**Previously Treated (n=54)**	**X2**	**P value**
Patient	100	n (%)	n (%)		
Gender				2.34	0.12
Male	57	30 (65.21)	27 (50.00)		
Female	43	16 (34.78)	27 (50.00)		
Age				6.25	0.04*
Young	30	9 (19.56)	21 (38.88)		
Adult	46	27 (58.69)	19 (35.18)		
Old	24	10 (21.73)	14 (25.92)		
Region				7.88	0.09
ICT	5	2 (4.34)	3 (5.55)		
KPK	4	0 (0.00)	4 (7.40)		
Punjab	78	39 (84.78)	39 (84.78)		
Balochistan	2	2 (4.34)	0 (0.00)		
Sindh	11	3 (6.52)	8 (14.81)		

Table 4.7 continued from previous page

Variable	Total no. of cases	New Cases (n=46)	Previously Treated (n=54)	X2	P value

*P≤0.05

4.2.3 Mutations Present in BDQ Sensitive and Resistant Isolates in *Rv0678* Region

Rv0678 is considered as the hotspot for the mutations responsible for bedaquiline resistance. Table 4.8 presents nucleotide mutations and corresponding amino acid changes in the *Rv0678* gene among BDQ-sensitive (BDQ S) and BDQ-resistant (BDQ R) *M. tuberculosis* strains. Mutations associated with BDQ resistance are predominantly frameshift (e.g., Asp47fs, Ile67fs, Asn70fs), missense (e.g., Met139Ile, Ala99Asp), and nonsense (e.g., Glu147*) mutations, primarily found in lineage 3 and 4 strains. In contrast, BDQ-sensitive strains exhibit fewer mutations, including missense variations such as Asn142Lys and Gly87Arg.

These findings highlight the genetic diversity in the *Rv0678* gene contributing to BDQ resistance in *M. tuberculosis*. Asp47fs was found to be most frequent mutation which was present in three strains. In two strains it involved a duplication of guanine at 139 nucleotide position.

In another strain, the same mutation existed where there was a duplication of adenine at 140 nucleotide position.

TABLE 4.8: Mutational present in BDQ sensitive and resistant Isolates in *Rv0678* region

BDQ Status	Strain Name	Nucleotide Position	Amino Acid Change	Gene	Lineage
BDQ R	BDQ_FR_001	139dupG	Asp47fs	*Rv0678*	3
BDQ R	BDQ_FR_012	417G>T	Met139Ile	*Rv0678*	3
BDQ R	BDQ_FR_013	493dupG	Asp165fs	*Rv0678*	3
BDQ R	BDQ_FR_016	198dupG	Ile67fs	*Rv0678*	4
BDQ R	BDQ_FR_002	139dupG	Asp47fs	*Rv0678*	3

Table 4.8 continued from previous page

BDQ Status	Strain Name	Nucleotide Position	Amino Acid Change	Gene	Lineage
BDQ R	BDQ_FR_021	209delA	Asn70fs	*Rv0678*	3
BDQ R	BDQ_FR_022	144dupC	Glu49fs	*Rv0678*	3
BDQ R	BDQ_FR_026	143C>T	Pro48Leu	*Rv0678*	3
BDQ R	BDQ_FR_003	140dupA	Asp47fs	*Rv0678*	3
BDQ R	BDQ_FR_035	296C>A	Ala99Asp	*Rv0678*	4
BDQ R	BDQ_FR_037	417G>T	Met139Ile	*Rv0678*	3
BDQ R	BDQ_FR_038	151C>G	Gln51Glu	*Rv0678*	4
BDQ S	BDQ_FR_041	426C>G	Asn142Lys	*Rv0678*	2
BDQ S	BDQ_FR_047	370G>C	Ala124Pro	*Rv0678*	3
BDQ S	BDQ_FR_067	259G>C	Gly87Arg	*Rv0678*	1
BDQ S	BDQ_FR_070	23A>G	Asp8Gly	*Rv0678*	3
BDQ R	BDQ_FR_008	201dupC	Ser68fs	*Rv0678*	3
BDQ R	BDQ_FR_081	144dupC	Glu49fs	*Rv0678*	3
BDQ R	BDQ_FR_083	18_19delGG	Val7fs	*Rv0678*	3
BDQ R	BDQ_FR_084	358G>A	Val120Met	*Rv0678*	3
BDQ R	BDQ_FR_089	439G>T	Glu147*	*Rv0678*	3
BDQ S	BDQ_FR_097	95T>C	Leu32Ser	*Rv0678*	2

4.2.4 Mutation Present in BDQ Sensitive and Resistant Isolates in *pepQ* Region

WGS analysis of the isolates present in our dataset deciphered another genetic marker for BDQ resistance named *pepQ*. All recorded mutations involve a single nucleotide substitution (20G>A), resulting in the amino acid change Arg7Gln. These mutations are consistently found in lineage 3 strains. Unlike mutations in Rv0678, which are commonly associated with BDQ resistance, *pepQ* variations in BDQ-sensitive strains suggest potential background polymorphisms rather than direct involvement in resistance. Mutations identified in *pepQ* were detected across both phenotypically resistant and susceptible isolates. Given the lack of strong evidence supporting its standalone role in BDQ resistance, these mutations are interpreted as background polymorphisms rather than primary resistance determinants in this study. With reference to the WHO mutation catalogue [204], *pepQ*

is not presented as the primary genetic marker for BDQ resistance, hence it is a non-primary polymorphism.

TABLE 4.9: Mutations present in *pepQ*

BDQ Status	**Strain Name**	**Nucleotide Position**	**Amino Acid Change**	**Gene**	**Lineage**
BDQ S	BDQ_FR_004	20G>A	Arg7Gln	*pepQ*	3
BDQ S	BDQ_FR_030	20G>A	Arg7Gln	*pepQ*	3
BDQ S	BDQ_FR_049	20G>A	Arg7Gln	*pepQ*	3
BDQ S	BDQ_FR_062	20G>A	Arg7Gln	*pepQ*	3

4.2.5 Mutations Present in BDQ Sensitive and Resistant Isolates in *atpE* Region

Another important genetic marker of BDQ resistance is atpE region. Mutational analysis of 100 MTB strains resulted in a single mutation, present in a single BDQ resistant isolate.

This table highlights a single mutation in the atpE gene among BDQ-resistant (BDQ R) *M. tuberculosis* strains. The mutation (187G>C) results in an amino acid change from Ala63Pro and is observed in a lineage 4 strain.

Since atpE encodes a subunit of ATP synthase, mutations in this gene are known to confer resistance to BDQ by altering the drug's binding site, reducing its inhibitory effect on bacterial energy metabolism.

TABLE 4.10: mutations present in *atpE*

BDQ Status	**Strain Name**	**Nucleotide Position**	**Amino Acid Change**	**Gene**	**Lineage**
BDQ R	BDQ_FR_032	187G>C	Ala63Pro	*atpE*	4

4.2.6 Mutations Present In BDQ Sensitive and Resistant Isolates In *Rv1979c* Region

This section presents the mutational analysis of Rv1979c in BDQ-resistant (BDQ R) and BDQ-sensitive (BDQ S) M. tuberculosis strains. The most recurrent mutation (857A>G, Asp286Gly) appears in both BDQ-resistant and BDQ-sensitive strains across lineages 1 and 4, suggesting it may not be directly linked to BDQ resistance.

Other mutations, including 733A>C (Met245Leu), 524C>T (Ser175Leu), and 112G>A (Glu38Lys), are found only in BDQ-resistant strains, indicating a possible role in BDQ resistance. However, the presence of 112G>A in both resistant and sensitive strains suggests further investigation is needed to determine its impact. (Table 4.11)

TABLE 4.11: Mutational Analysis of BDQ sensitive and resistant strains in *Rv1979c* region

BDQ Status	**Strain Name**	**Nucleotide Position**	**Amino Acid Change**	**Gene**	**Lineage**
BDQ R	BDQ_FR_005	857A>G	Asp286Gly	*Rv1979c*	1
BDQ S	BDQ_FR_069	857A>G	Asp286Gly	*Rv1979c*	1
BDQ S	BDQ_FR_072	857A>G	Asp286Gly	*Rv1979c*	1
BDQ R	BDQ_FR_028	857A>G	Asp286Gly	*Rv1979c*	4
BDQ R	BDQ_FR_088	857A>G	Asp286Gly	Rv1979c	4
BDQ R	BDQ_FR_001	733A>C	Met245Leu	Rv1979c	3
BDQ R	BDQ_FR_005	524C>T	Ser175Leu	Rv1979c	1
BDQ R	BDQ_FR_031	112G>A	Glu38Lys	Rv1979c	3
BDQ S	BDQ_FR_042	112G>A	Glu38Lys	Rv1979c	3

4.2.7 Potential Markers for BDQ Heteroresistance among MTB Isolates

Our study investigated the BDQ heteroresistance in 100 MTB strains and among these isolates, 29 BDQ heteroresistance isolates were witnessed. We evaluated

the potential patient and bacterial factors that could be associated to BDQ heteroresistance. None of the patient variables (gender, age, region, previous history of anti-tuberculosis treatment) were statistically significant for Bedaquiline heteroresistance. The proportion of BDQ heteroresistance in the patients with the previous history of anti-tuberculosis treatment (n=20) is higher (85%) than that in new patients (15%). MDR+BDQ R and XDR pattern tested through pDST were the only bacterial variables that were computed statistically significant to BDQ heteroresistance [OR, 0.53 (0.01-0.26); p=<0.001 & OR, 0.09 (0.19-0.50); p=0.006]. Majority of the study isolates belonged to lineage 3 (n=73), likewise there were highest number of isolates that originated from lineage 3 (n=19).

We found less no. of BDQ heteroresistance cases in lineage 1 (n=,2), 2 (n=4) and 4 (n=4) (Table 4.12). These 19 cases comprise of 16 BDQ resistant and 3 BDQ sensitive cases on phenotypic DST. None of the heteroresistant strain was detected with mix lineage.

TABLE 4.12: Potential markers for Bedaquiline heteroresistance among MTB isolates

Variable	**Total no. of cases**	**No. of Heteroresistance cases**	**Univariate Analysis**		
Patient Variable			OR	*95% CI*	P value
Gender					
Male	57	14	1.20	*0.38-3.78*	0.74
Female	43	15			Ref
Age					
Young (≤25 Yrs)	30	9	0.65	0.12-3.40	0.61
Adult (26-55 Yrs)	46	15	0.77	0.17-3.37	0.73
Old (≥55 Yrs)	24	5			Ref
Region					
ICT	5	0	-	-	-
KPK	4	1	1.26	0.06-26.67	0.87
Balochistan	2	1	1.04	0.48-22.46	0.97
Sindh	11	3	5.18	0.84-31.63	0.07
Punjab	78	24			Ref
History of ATT					

Table 4.12 continued from previous page

Variable	**Total no. of cases**	**No. of Heteroresistance cases**	**Univariate Analysis**		
New Cases	80	12	1.15	0.33-3.97	0.82
Previously Treated	20	17			Ref
Bacterial Variables					
pDST pattern					
Rif Mono	3	1	0.08	0.00-1.94	1.22
Rif R+BDQ R	1	0	-	-	-
MDR +BDQ R	19	11	0.53	0.01-0.26	<0.001*
Pre-XDR	4	0	-	-	-
XDR	30	13	0.09	0.19-0.50	0.006*
MDR	43	4			Ref
MTB lineage					
Lineage 1	5	2	0.88	0.81-9.54	0.91
Lineage 2	7	4	0.31	0.03-2.73	0.29
Lineage 4	15	4	0.29	0.05-1.68	1.17
Lineage 3	73	19			Ref

ICT, Islamabad Capital Territory; KPK, Khyber Pakhtunkhwa; ATT, Anti Tuberculosis Treatment

4.2.8 Genetic Profile of BDQ Heteroresistant Isolates

We represented the genetic profile of BDQ heteroresistance isolates in the form of a multilayer donut plot (Figure 4.10). The outermost layer of the donut plot shows pDST patterns of BDQ heteroresistant isolates, with extensively drug-resistant strains being the most prevalent (43%), followed by Rif+BDQR & MDR+BDQR (37%), MDR (13%), and Rif Mono (7%).

The middle layer highlights genetic markers associated with BDQ resistance, with mutations in *Rv0678* being the most common (95%), followed by *Rv1979c* (2%) and *pepQ* (3%). The innermost layer depicts mutation types, showing that 56%

of heteroresistant strains had missense variants, 39% had frameshift variants, and 5% had nonsense variants.

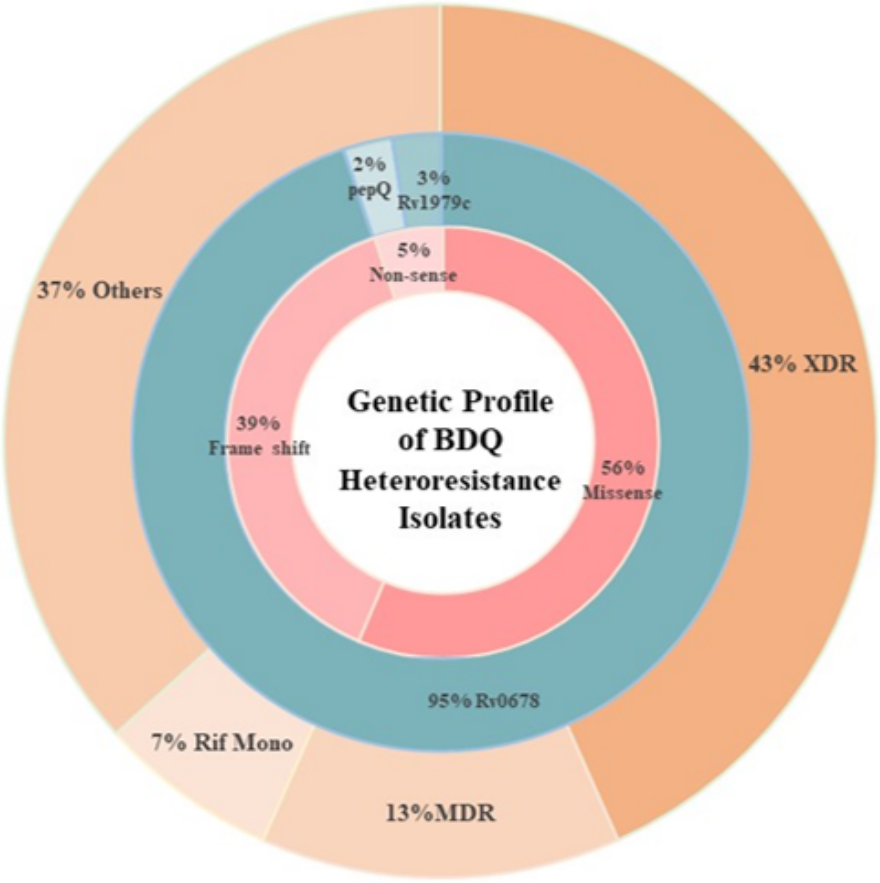

FIGURE 4.10: Genetic profile of BDQ heteroresistant Isolates.

4.2.9 Mutational Analysis of BDQ Heteroresistance

The WGS of the 50 phenotypically BDQ sensitive and 50 BDQ resistant isolates provided a variety of heteroresistance harboring mutations. (Table 4.13) From all BDQ resistant isolates, 24 BDQ resistant and 5 BDQ sensitive strains had heteroresistance mutations (Table 4.14), accounting for 29% heteroresistant population cumulatively. Mutations that existed in *Rv0678* (95%) include already reported (n=20) as well as novel mutations (n=16) (Table 4.13). We have only one mutation in *Rv1979c* region (Phe300Leu) that was already reported while a single unreported heteroresistance mutation was present in *pepQ* gene of MTB genome. The WGS data of current study isolates demonstrated that none of the

strains was having heteroresistance mutation in *atpE* region (Table 4.14). The proportion of heteroresistance caused by each mutation was also computed. Among the reported BDQ resistance mutations, p.Ile67fs displayed heteroresistance phenomenon in maximal 5 BDQ resistant strains while majority of the novel mutations were present in a single isolate either from BDQ case or control group (Table 4.13). These BDQ heteroresistance harboring mutations revealed different heteroresistance percentage from all three genetic markers ranging from 9% to 91%.

TABLE 4.13: Mutational analysis of BDQ heteroresistance

Gene	**Mutation**	**No. of Isolates**	**TB Profiler**	**Source**	**Confidence with BDQ R**
Rv0678	Arg34 Gln	1	Unreported	-	-
Rv0678	Asp88fs	1	Unreported	-	-
	Ile108fs	1	Unreported	-	-
Rv0678	Asp47fs	1	reported	*WHO catalogue v2*	Assoc with R
	Arg89Leu	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Gly87fs	1	Unreported	-	-
	Pro129fs	1	reported	WHO catalogue v2	Assoc w R - Interim
Rv0678	Arg72Trp	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Glu55*	1	Unreported	-	-
Rv0678	Ile67fs	5	reported	WHO catalogue v2	Assoc with R
Rv0678					
Rv0678					
Rv0678					
Rv0678					
Rv0678	Leu142Arg	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Leu32fs	2	reported	WHO catalogue v2	Assoc with R
Rv0678					
Rv0678	Ala124fs	2	Unreported	-	-

Table 4.13 continued from previous page

Gene	Mutation	No. of Isolates	TB Profiler	Source	Confidence with BDQ R
Rv0678					
Rv0678	Met10Lys	1	Unreported	-	-
Rv0678	Arg94Trp	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Phe19Ser	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Asn70Asp	1	reported	WHO catalogue v2	Assoc w R - Interim
Rv0678	Thr58fs	1	Unreported	-	-
Rv0678	Arg50Trp	1	reported	WHO catalogue v2	Uncertain significance
	Gln115fs	1	Unreported	-	-
Rv0678	Leu43Val	1	Unreported	-	-
Rv0678	Arg34Gln	1	reported	WHO catalogue v2	Uncertain significance
	Leu35Phe	1	Unreported	-	-
Rv0678	Tyr26Asp	1	reported	WHO catalogue v2	Uncertain significance
Rv0678	Glu49fs	1	reported	WHO catalogue v2	Assoc with R
Rv0678	p.Glu55fs	1	reported	WHO catalogue v2	Assoc w R - Interim
Rv0678	Gln51Glu	1	Unreported	-	-
Rv0678	Leu83Pro	1	reported	WHO catalogue v2	Uncertain significance
	Ala110fs	1	reported	WHO catalogue v2	Assoc w R - Interim
Rv0678	Leu83Phe	1	Unreported	-	-
Rv0678	Tyr145*	1	reported	WHO catalogue v2	Assoc w R - Interim
Rv0678	Gly78Trp	1	reported	WHO catalogue v2	Uncertain significance
	Ala86Val	1	reported	WHO catalogue v2	Uncertain significance
	Ala124fs	2	Unreported	-	-

Table 4.13 continued from previous page

Gene	Mutation	No. of Isolates	TB Profiler	Source	Confidence with BDQ R
	Gly65Val	1	Unreported	-	-
	Arg96Gln	1	Unreported	-	-
Rv1979c	Phe300Leu	1	reported	WHO catalogue v2	Uncertain significance
pepQ	Arg160Ser	1	Unreported	-	-

TABLE 4.14: Heteroresistance strains and proportion of heteroresistance

Study #	BDQ pDST Profile	Gene	Mutation	Heteroresistance (%)
BDQ_FR_004	BDQ-R	*Rv0678*	Arg34 Gln	*11%*
BDQ_FR_005	BDQ-R	*Rv0678*	Asp88fs	*21%*
			Ile108fs	*44%*
BDQ_FR_006	BDQ-R	*Rv0678*	Asp47fs	*44%*
			Arg89Leu	49%
BDQ_FR_009	BDQ-R	*Rv0678*	Gly87fs	57%
			Pro129fs	39%
BDQ_FR_010	BDQ-R	*Rv0678*	Arg72Trp	20%
BDQ_FR_011	BDQ-R	*Rv0678*	Glu55*	22%
	BDQ-R		Ile67fs	11%
	BDQ-R		Leu142Arg	26%
BDQ_FR_017	BDQ-R	*Rv0678*	Leu32fs	28%
BDQ_FR_018	BDQ-R	*Rv0678*	Ala124fs	24%
BDQ_FR_020	BDQ-R	*Rv0678*	Met10Lys	31%
BDQ_FR_023	BDQ-R	*Rv0678*	Ala124fs	51%
	BDQ-R	*Rv0678*		55%
BDQ_FR_024	BDQ-R	*Rv0678*	Phe19Ser	23%
	BDQ-R	*Rv0678*		41%
BDQ_FR_025	BDQ-R	*Rv0678*	Thr58fs	15%
BDQ_FR_027	BDQ-R	*Rv0678*	Arg50Trp	16%
				29%
BDQ_FR_029	BDQ-R	*Rv0678*	Leu43Val	33%
BDQ_FR_030	BDQ-R	*Rv0678*	Arg34Gln	55%
				20%
BDQ_FR_031	BDQ-R	*Rv0678*	Ile67fs	9%
BDQ_FR_033	BDQ-R	*Rv0678*	Tyr26Asp	13%

Table 4.14 continued from previous page

Study #	BDQ pDST Profile	Gene	Mutation	Heteroresistance (%)
	BDQ-R	*Rv0678*		18%
BDQ_FR_034	BDQ-R	*Rv0678*	Leu32fs	27%
	BDQ-R	*Rv1979c*	Phe300Leu	28%
BDQ_FR_036	BDQ-R	*Rv0678*	Glu55fs	74%
BDQ_FR_039	BDQ-R	*Rv0678*	Gln51Glu	20%
BDQ_FR_059	BDQ-S	*Rv0678*	Gly78Trp	10%
	BDQ-S		Ala86Val	9%
BDQ_FR_060	BDQ-S	*Rv0678*	Ala124fs	9%
BDQ_FR_066	BDQ-S	*Rv0678*	Gly65Val	67%
	BDQ-S	*pepQ*	Arg160Ser	9%
BDQ_FR_079	BDQ-R	*Rv0678*	Ile67fs	91%
	BDQ-R		Leu83Pro	11%
			Ala110fs	40%
BDQ_FR_080	BDQ-R	*Rv0678*	Ile67fs	81%
BDQ_FR_086	BDQ-R	*Rv0678*	Leu83Phe	70%
	BDQ-R	*Rv0678*	Tyr145*	12%
BDQ_FR_087	BDQ-R	*Rv0678*	Ile67fs	11%
BDQ_FR_091	BDQ-S	*Rv0678*	Arg96Gln	15%
BDQ_FR_099	BDQ-S	*Rv0678*	Ala124fs	34%

*Stop gained

4.2.10 Evolutionary Analysis

4.2.10.1 Evolutionary Analysis of BDQ Sensitive and Resistant MTB strains

The bar chart illustrates the distribution of Bedaquiline (BDQ) resistant and BDQ sensitive *Mycobacterium tuberculosis* isolates across four lineages.

Lineage 3 shows the highest number of both BDQ-resistant (34) and BDQ-sensitive (39) isolates, suggesting a significant presence of BDQ resistance within this lineage.

Lineages 1 and 2 have BDQ-resistant isolates (5 and 7, respectively) but no BDQ-sensitive isolates, indicating a possible strong association of these lineages with BDQ resistance.

In contrast, Lineage 4 has fewer BDQ-resistant isolates (4) and a relatively higher count of BDQ-sensitive isolates (11). This distribution suggests potential lineage-specific variations in BDQ resistance, emphasizing the need for further investigation into genetic factors influencing drug susceptibility (Figure 4.11).

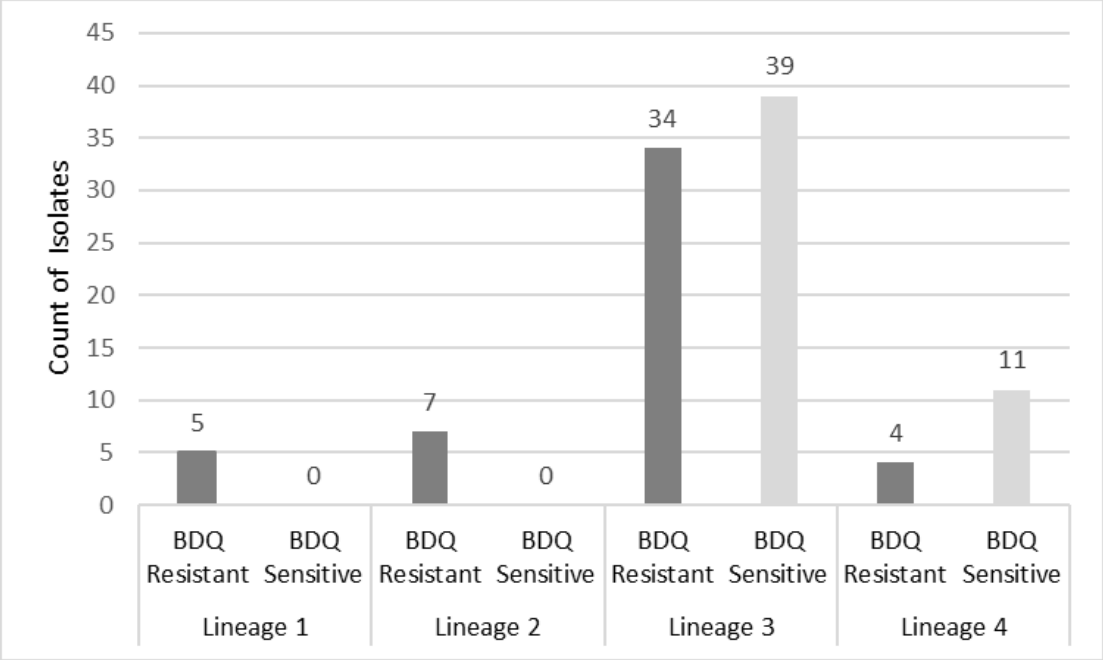

FIGURE 4.11: Lineage distribution among BDQ sensitive and BDQ resistant strains

4.2.10.2 BDQ Heteroresistant Strains Evolutionary Analysis

The MTB lineage distribution was done in phenotypically BDQ sensitive and BDQ resistant strains that were stated heteroresistant on WGS analysis. The amount of heteroresistance in each lineage was shown in numbers.

Our results indicated that the maximum number of heteroresistant strains were part of lineage 3 as 16/19 resistant while 3/19 heteroresistant BDQ sensitive strains resided in this lineage. All the BDQ resistant strains from lineage 1 and lineage 2 were heteroresistant (Figure 4.12).

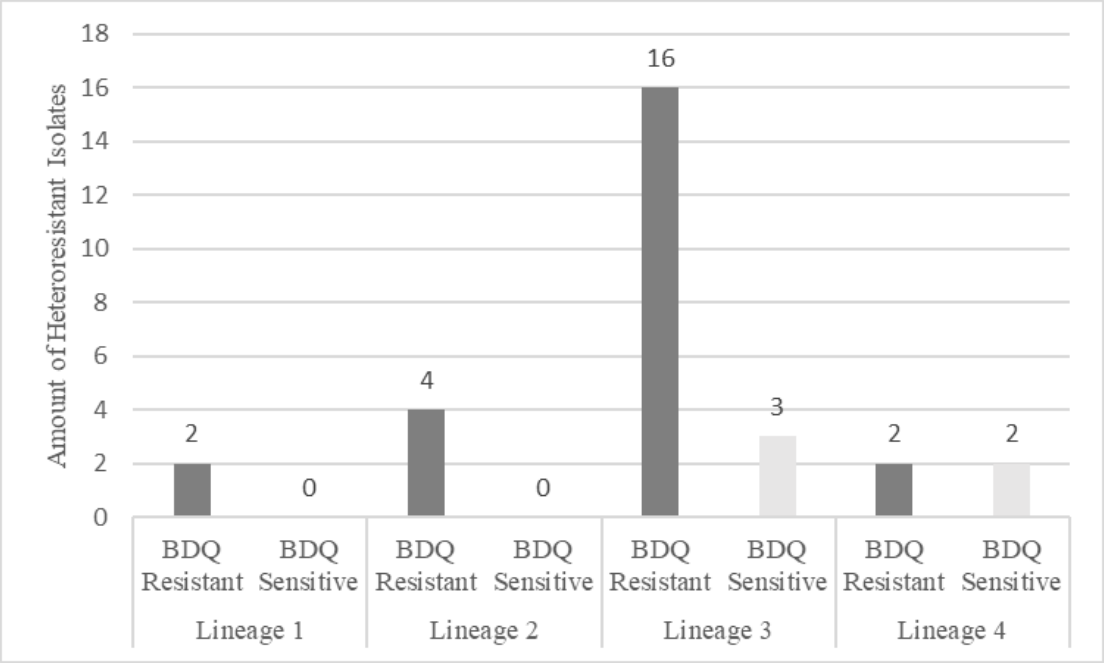

FIGURE 4.12: Lineage distribution of BDQ heteroresistance among BDQ sensitive and BDQ resistant strains

The figure 4.13 represents a phylogenetic tree of *Mycobacterium tuberculosis* isolates, with multiple concentric rings depicting various phenotypic and genotypic characteristics.

The tree structure shows the evolutionary relationships between BDQ-resistant isolates, with branch colors likely indicating different lineages (Lineage 1–4).

The outermost ring highlights Bedaquiline (BDQ) resistance (red) and sensitivity (green), suggesting a focus on BDQ-resistant isolates. The inner rings represent resistance (R), sensitivity (S), or non-available (NA) data for multiple TB drugs, including Isoniazid, Rifampicin, Pyrazinamide, Fluoroquinolones (Levofloxacin, Moxifloxacin), Clofazimine, Delamanid, Linezolid, and Amikacin.

The color-coded legend categorizes isolates based on their pDST profiles (MDR, Pre-XDR, XDR, Rifampicin-monoresistant) and their geographical origins (Punjab, ICT, Sindh, KP, Balochistan).

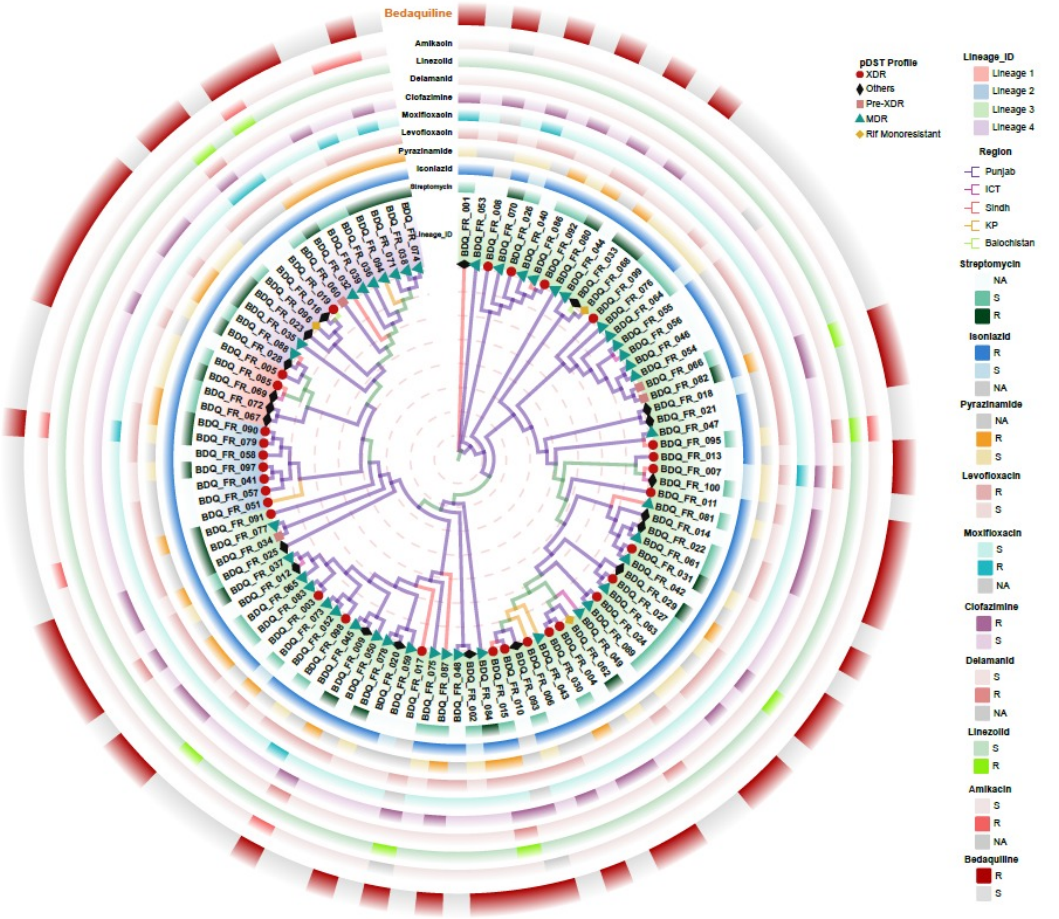

FIGURE 4.13: Phylogenetic analysis

This visualization provides insights into the genetic diversity and drug resistance patterns of BDQ-resistant *M. tuberculosis* isolates in Pakistan, helping to identify lineage-specific resistance trends and potential genetic markers associated with BDQ resistance.

Maximum number of isolates belong to lineage 3, followed by 4, 2 and lineage 1. BDQ resistant strains variedly belong to four lineages. Maximum number of BDQ resistant isolates belong to lineage 3 (n=34), to lineage 2 (n=7), lineage 1 (n=5) and lineage 4 (n = 4) (Figure 4.13).

The MTB isolates' phenotypic drug susceptibility testing (pDST) profiles across the four main lineages are shown in this table 4.15. With all isolates resistant to fluoroquinolones, clofazimine, and bedaquiline, and 100% identified as XDR-TB, Lineage 2 (Beijing) had the highest level of treatment resistance.

The highest percentage of MDR-TB cases (47%) and notable resistance to isoniazid (92%) and bedaquiline (47%), respectively, are found in lineage 3 (CAS). While Lineage 1 (East-African Indian) has the fewest isolates but considerable resistance to bedaquiline (100%) and XDR-TB (40%), Lineage 4 (Euro-American) exhibits moderate MDR-TB prevalence (60%) but lower XDR-TB rates (7%).

These results emphasize the necessity for individualized treatment strategies by highlighting lineage-specific differences in medication resistance. A 100% BDQ resistance pattern was observed in lineage 1 and 2.

TABLE 4.15: Lineage distribution and pDST pattern

Lineage Type	**Lineage 1**	**Lineage 2**	**Lineage 3**	**Lineage 4**
Total	5	7	73	15
EMB n(%)	2 (40)	2 (29)	5 (7)	3 (20)
STR n(%)	0 (0)	1 (14)	22 (30)	4 (27)
INH n(%)	4 (80)	4 (57)	67 (92)	14 (93)
PZA n(%)	0 (0)	1 (14)	19 (26)	6 (40)
LFX n(%)	2 (40)	7 (100)	45 (62)	7 (47)
MFX n(%)	1 (20)	1 (14)	6 (8)	1 (7)
AMK n(%)	0 (0)	1 (14)	4 (5)	2 (13)
DLM n(%)	0 (0)	3 (43)	3 (4)	1 (7)
CFZ n(%)	4 (80)	7 (100)	21 (29)	2 (13)
LNZ n(%)	0 (0)	0 (0)	7 (10)	1 (7)
BDQ n(%)	5 (100)	7 (100)	34 (47)	4 (27)
MDR n(%)	0 (0)	0 (0)	34 (47)	9 (60)
XDR n(%)	2 (40)	7 (100)	20 (27)	1 (7)

4.2.11 GWAS Analysis

4.2.11.1 Prediction for potential BDQ Resistance Marker

After running genome wide association analysis on 50 case and 50 control isolates, genetic markers which were found to be associated with BDQ resistance at significance level of 0.05 were stratified. In total 453 SNPs resulted from GWAS analysis and 39 were significantly associated to BDQ resistance at p-value of 0.05.

Out of 39 markers, all the PE/PPEs, non-sense mutations, upstream and downstream SNPs were excluded, leaving missense and frameshift markers behind (Table 4.16).

Currently we selected esXo as the only potential marker in relation to BDQ resistance to analyze its potential relation to BDQ resistance as well as its potential structure prediction for molecular dynamics.

TABLE 4.16: GWAS resulting candidate markers for BDQ Resistance

Position	**Reference Allele**	**Alternate Allele**	**Gene**	**Mutation**	**Function**
1788570	GTTGT	TTTGC	*Rv1587c*	Gly184Asp	Function unknown
2338810	CG	TA	*Rv2082*	Lys35Glu	Function unknown
2494430	C	G	*Rv2223c*	Gly324Arg	To hydrolyze proteins
2606163	A	C	*mez*	Asn352Lys	Catalyzes the oxidative decarboxylation of malate into pyruvate, important for a wide range of metabolic pathways
2614909	G	GC	*mmpl9*	Ala73Ser	Thought to be involved in fatty acid transport.
2626026	GGCCG	AGCCA	*esxo*	Val48Ala	Function unknown
3296843	G	A	*pks15*	Val333Ala	Polyketide synthase possibly involved in lipid synthesis
3851887	CC	AT	*Rv3433c*	Ser443Ala	Function unknown
4053050	A	G	*Rv3611*	Asn34Ser	Function unknown
4155516	T	C	*leuA*	Pro593Ser	Involved in leucine biosynthesis and catalyzes condensation of acetyl-CoA and 2-oxoisovalerate

4.2.11.2 Structural Prediction Report: BDQ - Resistance Mutant of esXO

The esXO protein is a membrane protein (Figure 4.14), and its mutant form confers resistance to BDQ. Currently, only the monomeric structure of this protein is available in the PDB database. However, in physiological environments, esXO exists as a polymeric structure, potentially forming transmembrane assemblies similar to those observed in other membrane proteins.

Based on prior knowledge, BDQ targets the membrane-bound region of ATP synthase, which exhibits structural characteristics analogous to esXO. Specifically, seven monomeric subunits, each approximately 81 amino acids in length (similar in length but not sequence to esXO), form a ring-shaped structure that functions as a pump-like system (Figure 4.15). BDQ binds to the cytoplasmic side of ATP synthase, interacting with regions on every pair of adjacent monomers (Figure 4.16).

```
MTINYQFGDVDAHGAMIRAQAGLLEAEHQAIVRDVLAAGDFWGGAGSVACQGFITQLGRNFQVIYEQANAHGQKVQAAGNNMAQTDSAVGSSWA-WT
MTINYQFGDVDAHGAMIRAQAGLLEAEHQAIVRDVLAAGDFWGGAGSAACQGFITQLGRNFQVIYEQANAHGQKVQAAGNNMAQTDSAVGSSWA-Mut
```

FIGURE 4.14: Sequence alignment of wild-type (WT) and mutant (V48A) esXO

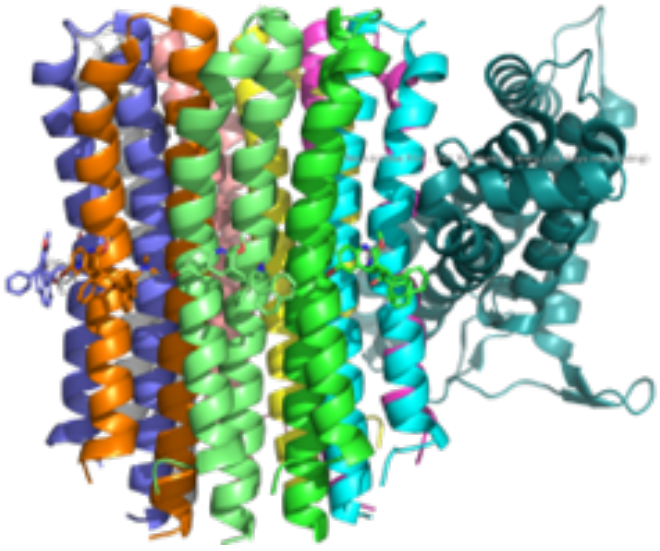

FIGURE 4.15: Binding mode of ATP synthase transmembrane region with BDQ

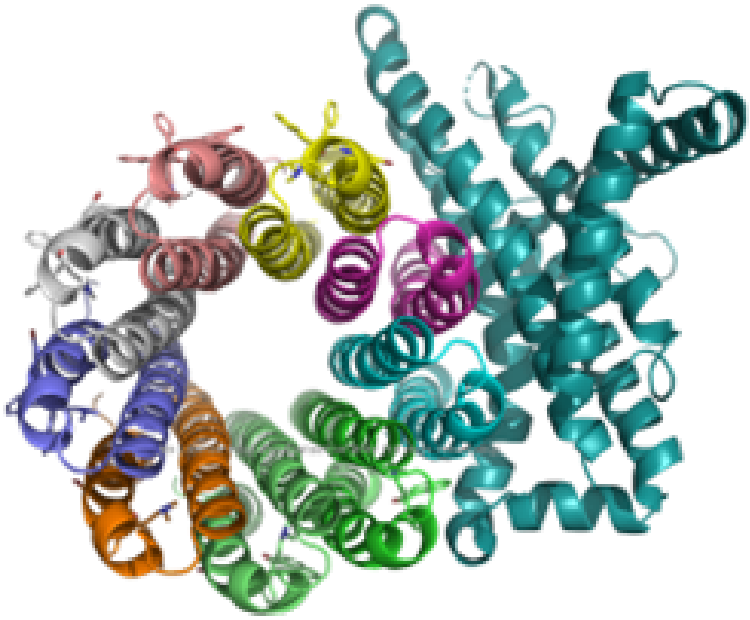

FIGURE 4.16: Overview of BDQ binding to the ATP synthase transmembrane region.

To investigate the potential interaction between esXO and BDQ, we performed molecular modeling studies. Initially, Glide docking simulations [204] were conducted to predict the binding mode of BDQ to the esXO monomer (Figure 4.17). Subsequently, MD simulations were performed using Amber [205] with a solvent model to refine the docking results for 400ns of both WT and mutant systems.

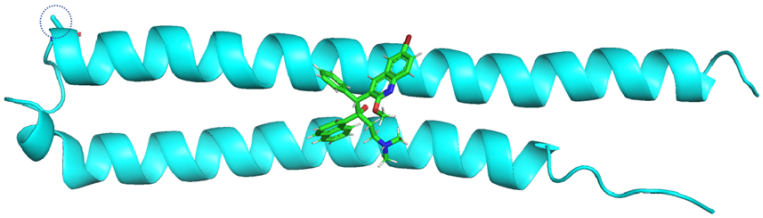

FIGURE 4.17: Glide docking results of esXO monomer with BDQ.

The binding free energies of BDQ to WT and mutant esXO were calculated (Table 4.17). For GBSA calculations, the two binding affinities are ∼27 kcal/mol, for GBSA calcualtion, they are around 24 to 25 kcal/mol. Surprisingly, no significant differences in binding energy were observed between the two forms. This outcome suggests that the mutation does not directly affect the binding interface but may influence higher-order structural features.

TABLE 4.17: Binding free energies of BDQ to wild-type (WT) and mutant esXO (units: kcal/mol)

WT				**Mutant**		
Terms of GBSA	Average	SD	SE	Average	SD	SE
VDWAALS	-34.7462	2.7516	0.4351	-35.0468	3.4926	0.5522
EEL	-67.817	9.3883	1.4844	-66.6977	8.7254	1.3796
EGB	78.8456	8.8488	1.3991	78.0386	8.1042	1.2814
ESURF	-3.963	0.3257	0.0515	-4.0139	0.341	0.0539
DELTA G gas	-102.5631	10.5671	1.6708	-101.7445	9.9519	1.5735
DELTA G solv	74.8826	8.7776	1.3879	74.0248	8.0038	1.2655
DELTA TOTAL	-27.6805	3.202	0.5063	-27.7197	3.8272	0.6051
Terms of PBSA	Average	SD	SE	Average	SD	SE
VDWAALS	-34.7462	2.7516	0.4351	-35.0468	3.4926	0.5522
EEL	-67.817	9.3883	1.4844	-66.6977	8.7254	1.3796
EPB	81.2282	8.7256	1.3796	80.043	8.0676	1.2756
ENPOLAR	-3.5225	0.1826	0.0289	-3.5331	0.2127	0.0336
EDISPER	0	0	0	0	0	0
DELTA G gas	-102.5631	10.5671	1.6708	-101.7445	9.9519	1.5735
DELTA G solv	77.7057	8.7095	1.3771	76.5098	8.0288	1.2695
DELTA TOTAL	-24.8575	3.5457	0.5606	-25.2347	3.6664	0.5797

Based on these findings, we hypothesize that esXO may form a polymeric structure analogous as to the ATP synthase transmembrane region, and BDQ binding occurs within this polymerized state (Figure 4.18).

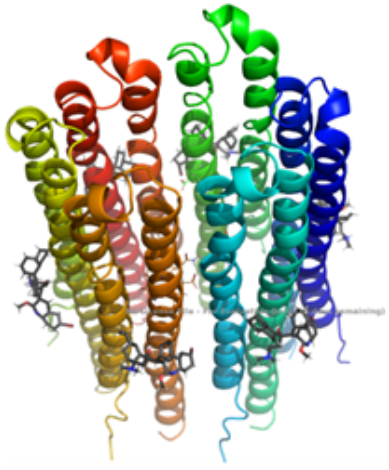

FIGURE 4.18: Manually constructed model of esXO with BDQ

Given the limited sample size (50 resistant vs. 50 susceptible isolates), the GWAS findings presented here should be considered exploratory. These preliminary associations serve as a basis for subsequent in silico analyses and warrant further validation in larger cohorts.

4.2.12 Protein Dynamic Analysis

Proteins are dynamic macromolecules, with their function closely connected to their biological motions. Previous studies have demonstrated that both drug-resistant mutations and genetic disease mutations can influence protein conformational equilibria and dynamics. To comprehensively grasp the molecular implications of a mutation, it is essential to take into account alterations in protein dynamics [204]. This study also details the impact of mutations in BDQ sensitive and resistant strains on protein dynamics. As WGS results of our data set provided maximum number of mutations in *Rv0678*, so a number of mutations are analyzed for their impact on protein dynamics of this target protein. All mutations are destabilizing except the two. Destabilizing mutations are indicated by the negative ΔΔG values (Table 4.18). Mutations impacting protein dynamics are given below and wild-type and mutant residues are colored in light-green and are also represented as sticks alongside with the surrounding residues which are involved on any type of interactions.

TABLE 4.18: Structure guided prediction of *RV0678* region mutations

#	**Wild Type Amino Acid**	**Position of Amino Acid**	**Mutant Chain Amino Acid**	**Prediction Outcome ΔΔG ENCoM**	**NMA Based Predictions ΔΔG ENCoM**	**Δ Vibrational Entropy Energy Between Wild-Type & Mutant ΔΔSVib ENCoM:**
1	ALA	124	PRO D	-0.047 kcal/-mol (Destabilizing)	0.169 kcal/mol (Destabilizing)	-0.211 $kcal.mol^{-1}.K^{-1}$ (Decrease of molecule flexibility

Table 4.18 continued from previous page

#	**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 & Mutant ΔΔSVib ENCoM:**
2	MET	139	ILE	A	-0.777 kcal/mol (Destabilizing)	-0.449 kcal/mol (Destabilizing)	0.561 $kcal.mol^{-1}.K^{-1}$ (Increase of molecule flexibility)
3	GLY	87	ARG	D	-1.616 kcal/mol (Destabilizing)	-0.091 kcal/mol (Destabilizing)	0.114 $kcal.mol^{-1}.K^{-1}$ (Increase of molecule flexibility
4	ALA	99	ASP	B	0.122 kcal/mol (Stabilizing)	0.003 kcal/mol (Destabilizing)	-0.003 $kcal.mol^{-1}.K^{-1}$ (Decrease of molecule flexibility)
5	VAL	120	MET	*A*	-0.053 kcal/mol (Destabilizing)	-0.096 kcal/mol (Destabilizing)	0.119 (Increase of molecule flexibility)
6	PRO	48	LEU	*B*	0.295 kcal/mol (Stabilizing)	-0.518 kcal/mol (Destabilizing)	0.648 $kcal.mol^{-1}.K^{-1}$ (Increase of molecule flexibility)
7	LEU	32	SER	*A*	-0.912 kcal/mol (Destabilizing)	-0.522 kcal/mol (Destabilizing)	0.652 $kcal.mol^{-1}.K^{-1}$ (Increase of molecule flexibility)

Table 4.18 continued from previous page

#	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 & Mutant ΔΔSVib ENCoM:
8	GLN	51	GLU	*D*	-0.785 kcal/-mol (Destabilizing)	-0.096 kcal/mol (Destabilizing	0.120 $kcal.mol^{-1}.K^{-1}$ (Increase of molecule flexibility)

4.2.12.1 Alanine124Proline

The Ala124Pro mutation introduces a proline residue, which is known for its rigid structure and tendency to disrupt α-helices or β-sheets.

This mutation is predicted to be destabilizing (-0.047 kcal/mol) and decreases molecular flexibility (-0.211 $kcal.mol^{-1}.K^{-1}$).

The rigidity of proline may lead to local structural perturbations, potentially affecting protein stability and function. The predicted structure change due to this mutation is shown in Figure 4.19.

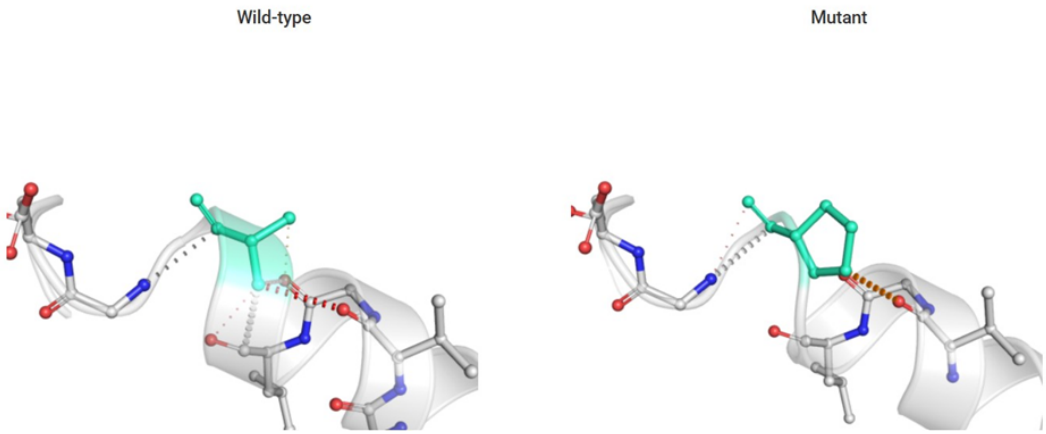

FIGURE 4.19: Ala124Pro

4.2.12.2 Methionine139Isoleucine Mutation

The Met139Ile mutation involves the substitution of methionine with isoleucine, which has a branched hydrophobic side chain. This mutation is destabilizing (-0.777 kcal/mol) but increases molecular flexibility (0.561 kcal.mol^{-1}.K^{-1}).

The increased flexibility may allow structural shifts that impact the regulatory function of *Rv0678*, potentially influencing its interaction with other molecules (Figure 4.20).

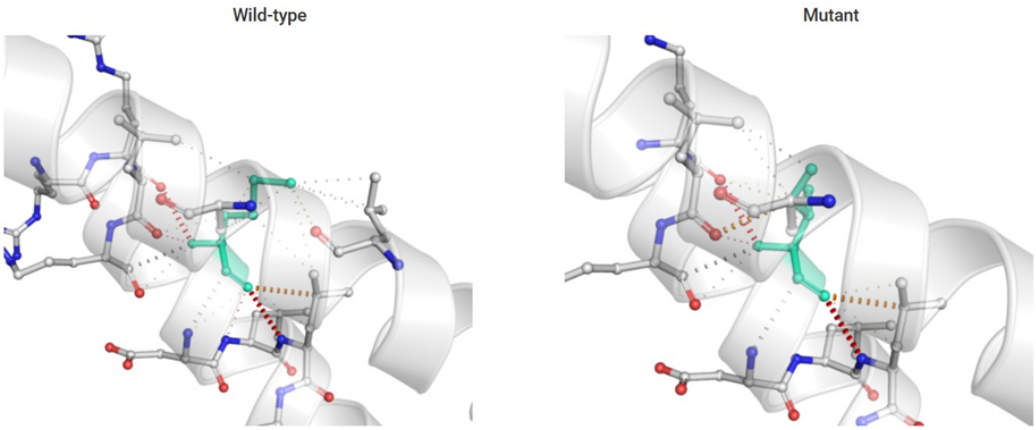

FIGURE 4.20: MET139ILE

4.2.12.3 Glycine87Arginine Mutation

The Gly87Arg mutation replaces a small, flexible glycine with a bulkier, positively charged arginine.

This change is highly destabilizing (-1.616 kcal/mol) due to steric clashes and charge repulsion.

It also slightly increases flexibility (0.114 kcal.mol^{-1}.K^{-1}), potentially leading to misfolding or impaired function of *Rv0678*, which could enhance drug resistance (Figure 4.21).

FIGURE 4.21: GLY87ARG

4.2.12.4 Alanine99Aspartic Acid Mutation

The Ala99Asp mutation substitutes a nonpolar alanine with a negatively charged aspartic acid. This mutation is slightly stabilizing (0.122 kcal/mol), though it has a negligible destabilizing effect in an alternative prediction (0.003 kcal/mol). The change may introduce new electrostatic interactions that could alter protein conformation or functional interactions (Figure 4.22)

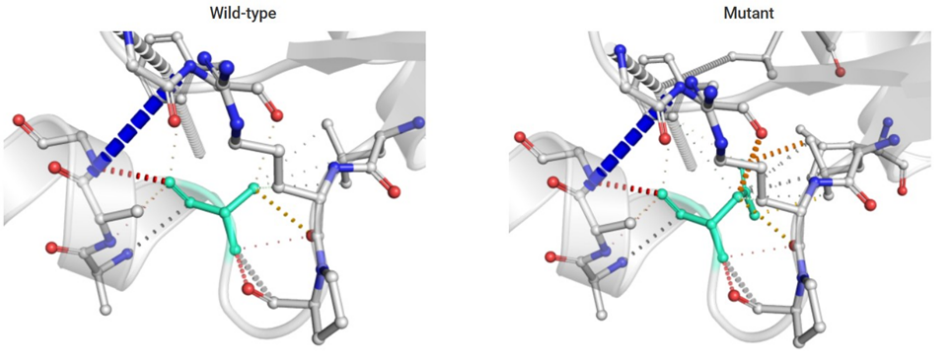

FIGURE 4.22: Ala99Asp

4.2.12.5 Valine120Methionine Mutation

The Val120Met mutation replaces valine with methionine, which has a larger, more flexible side chain. This mutation is mildly destabilizing (-0.053 kcal/mol) and increases molecular flexibility (0.119 kcal.mol^{-1}.K^{-1}). Such flexibility may affect

the structural integrity of the protein and its ability to interact with regulatory elements. (Figure 4.23).

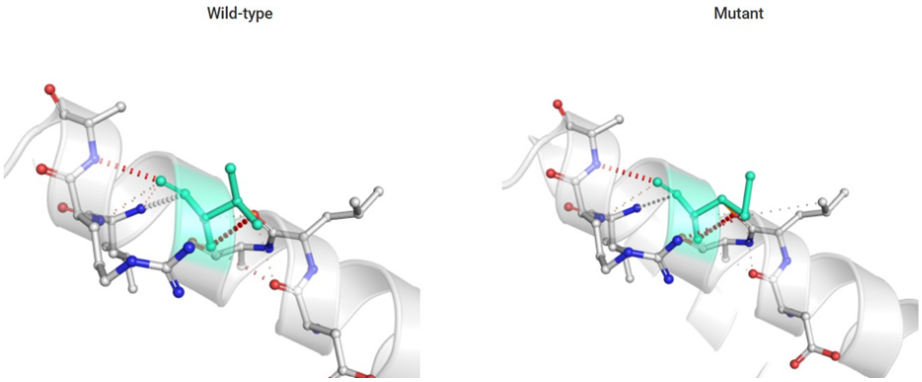

FIGURE 4.23: Val120Met

4.2.12.6 Proline48Leucine Mutation

The Pro48Leu mutation substitutes proline, a rigid and structurally constrained residue, with the more flexible leucine. While it is slightly stabilizing (0.295 kcal/-mol) according to one prediction, it is also destabilizing (-0.518 kcal/mol) in another, with a significant increase in flexibility (0.648 kcal.mol^{-1}.K^{-1}). This change could impact protein folding and function, possibly affecting drug resistance mechanisms (Figure 4.24).

FIGURE 4.24: Pro48Leucine

4.2.12.7 Leucine32Serine Mutation

The Leu32Ser mutation replaces the hydrophobic leucine with a polar serine, which disrupts the local hydrophobic environment. This mutation is highly destabilizing (-0.912 kcal/mol) and increases flexibility (0.652 kcal.mol^{-1}.K^{-1}), which may

weaken the structural integrity of the protein and impair its regulatory function, potentially leading to enhanced efflux pump activity (Figure 4.25).

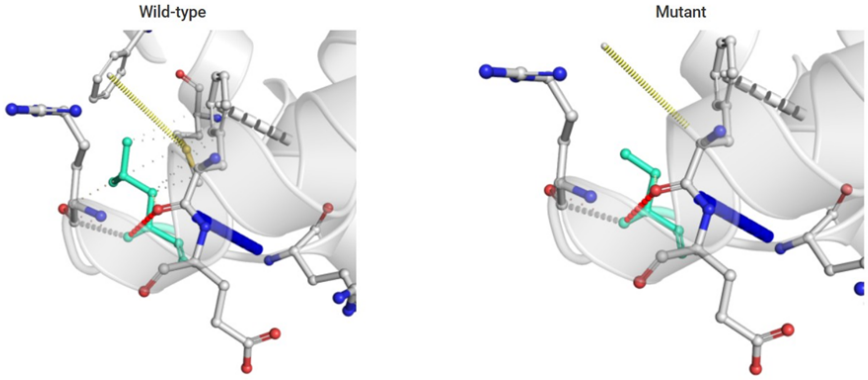

FIGURE 4.25: Leu32Ser

4.2.12.8 Glutamine51Glutamic acid Mutation

The Gln51Glu mutation involves replacing glutamine with glutamic acid, introducing a charge change that may disrupt electrostatic interactions. This mutation is destabilizing (-0.785 kcal/mol) and increases flexibility (0.120 kcal.mol^{-1}.K^{-1}). Such changes may impair *Rv0678*'s structural stability and regulatory function, leading to altered drug resistance mechanisms (Figure 4.26).

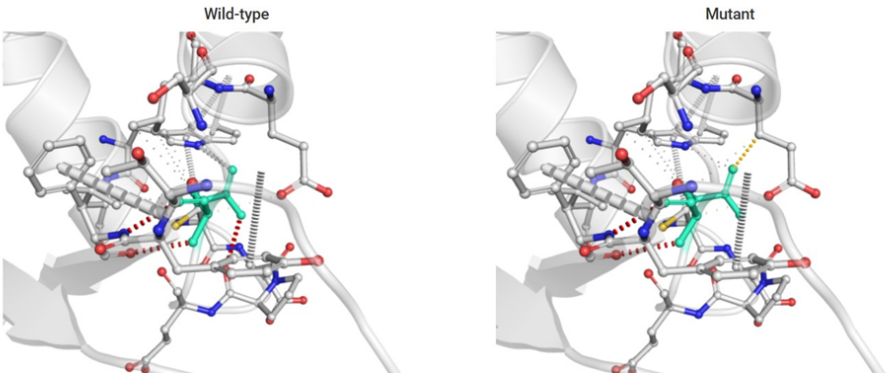

FIGURE 4.26: Gln51Glu

Chapter 5

Discussion

Health Care Workers are the key players in any health department. Our study focused on the knowledge of the Health Care Workers working in TB department. It is mandatory for the HCWs dealing with TB infected material or serving the TB patients should have high caliber knowledge in all aspects whether it is related to TB diagnostics or regarding TB treatment. Demographic characteristics of our study cohort presented that majority of the relevant HCWs were fresh graduates from the university and in spite of lack of any proper training, their knowledge mean score is good enough to work in this field. Still, due to lack of TB training, their attitude and knowledge towards few aspects of TB like diagnostic sampling and disease contraction on exposure to infected persons, needs to be reconsidered. Among all educational levels included in the study, the maximum mean score was studied in those HCWs who were having professional technical training in comparison to others. This result highlighted the significance of professional training which enhances the knowledge of the trainee thoroughly. University level is usually considered as the highest educational level, HCWs from this level had shown second highest knowledge mean score. This data provided the necessity of introducing courses and diplomas which can increase the knowledge of the students for public health issues like dealing with infectious diseases such as TB. Mean Knowledge score of university level HCWs was computed to be statistically different from technically professional and secondary Health Care Workers. Hence, higher education level HCWs had the highest mean knowledge score.

These results were in line with other studies [50–52]. Attitude of HCWs Pakistan towards TB questions was recorded in terms of 5-point Likert scale. Our studied cohort strongly agree that there should be an actual and emergent diagnosis of every new case. Considering TB, a stigma in high burden countries like Pakistan can results in increase in TB incidence with a decrease of TB treatment ratio. People who are infected with TB usually turn insecure and become phobic if others come aware of their infection, they will start avoiding them [53]. As TB symptoms also resemble to those of other diseases like lung cancer, there are the chances of misdiagnosis of both diseases. There is another perception among the public to treat TB symptomatically by using other traditional medicine or antibiotics used for common allergy and infections leading to antimicrobial resistance. Mean practice score valuated in HCWs of ICT, Pakistan was good (70%) as the mean score was close to the possibly correct scoring point which was 9. These results show that HCWs were good at TB practices, might be because they regularly follow the consolidated TB guidelines for both Drug sensitive TB and Drug resistant TB. Literature reported the same good percentages of practices of TB [51, 54].

Knowledge of HCWs can vary from one profession to another depending upon their terms of responsibility but altogether both are significantly different from each other as described by the current results. Our data presented that out of all other profession, quality officer and lab manager got an improved knowledge regarding TB while in comparison, those directly dealing with patients or testing in labs were inferior in knowledge. Doctors were supposed to have much higher knowledge score than other fields but that is not the case in our study and this result was discordant to that cited by Noe [51].

The working experience of HCWs with TB play a vital in augmenting their knowledge. But in our study the Pakistan who are naive in tb field were superior in knowledge in comparison to those working for many years. The only reason which seemed to be relevant is that the new comers are more focused in preparing, revising and experiencing the TB like infectious disease and hence, they were proved to be more serious in applying their knowledge on the patients around. Our results were on opposing the results by other studies from Mozambique and KSA [51, 55].

Correlation analysis of KAP mean score was analyzed with each other. There was weak correlation computed for attitude mean score with knowledge mean score of Health Care Workers working with TB patients. A significantly positive and modrate correlation was calculated between practice mean score and knowledge mean score. Practices mean score and attitude mean scores of the HCWs exhibit positive but weak correlation to each other. Data from the TB KAP study done in KSA had shown similar results of weak correlation for attitude with knowledge score and practice score with attitude score among HCWs [55]. On contrary, in other studies it was concluded that Knowledge, attitude and practices share a complex relationship with each other [52][55, 57, 58] The difference of knowledge, attitude and practice score with demographic covariates was evaluated using non-parametric tests as appropriate. The Knowledge score was significantly different with various demographic variables including age, gender, education, profession, TB control activities and TB specific training. These results appear to be concordant with other studies from Mozambique, Iraq, Iran. China, India, Mexico, Ethiopia, Russia, Peru, Uganda in addition to a study done in KSA while Hajj where HCWs from a variety of nations were appointed to serve the pilgrims [50, 55] [58–65]. Attitude of HCWs part of our study displayed a significance difference with age, gender, educational level, occupation, working experience with TB patients. Results were more or less comparable in the TB KAP research done in Mozambique. Practice score was highly associated with age and educational attainment. The attitude and practice score in our study follow the result of a study done the HCWs of Mozambique [66].

Conclusively, TB KAP study among HCWs provides us the opportunity to identify all the gaps in knowledge, to assess the TB related attitudes like stigmatization, evaluating practices for treatment adherence. It has further elaborated its importance in improving the patient treatment outcomes, policy defining for HCWs and for the TB patients as well. HCWs are the role models in the field of medical, their improved KAP score can lead to impact positively and deeply influential for the TB/DRTB patients.

The rising incidence of DRTB is the major hindrance in attaining the global goal

of TB control. To improve the understanding of DRTB mechanics, it is decisive to unravel the demographic factors in addition to treatment history that contribute to the resistance of MTB strains against the anti-tuberculosis drugs [207]. Among all anti-tuberculosis drugs, new drugs including BDQ are of great significance due to their addition in MDR/XDR-TB treatment regimen. Resistance against any particular drug occurs due to mutation in the drug targets, mixed stain infection involving different MTB strains and heteroresistance in drug target [208, 209].

While considering the patient's characteristics, age, gender, region and history of anti-tuberculosis treatment were evaluated. All of our MTB isolates were from the patients suffering from drug resistance tuberculosis. Gender distribution in our study revealed that majority of the cases were male in contrast to female (Figure 4.6). Previous literature tell us the random trend where in few studies females outnumber males [210, 211] while in others male exceed in percentage to bear DRTB conditions [212, 213]. Age group is an important risk factor in any disease progression and prevalence. As there are different health conditions in each age group depending upon the other genetic and environmental factors including life style. Well the association of DRTB and resistance against some particular drugs is still ambiguous due to varied distribution of age groups in different studies. In present study we distributed our patients into three groups young ($\leq$25 years), adults (26-50 years) and old age group which includes individuals with age greater than 50 years.(Figure 4.7) We found that adult age group is more prone to contract DRTB in comparison to other age groups. Same results are observed in a number of studies [212, 214–216]. Regional distribution of our studied population presented highest proportion of the DRTB candidates belonged to Punjab province which is the biggest province of Pakistan by population. These results are in line with other study done in Pakistan [214] (Figure 4.8). History of ATT is considered as one of the most important indicator of DRTB as suggested by many other studies [217–219]. In current study as it is designed to decipher the occurrence and mutational analysis of BDQ, new cases here poses a history of first line ATT majorly but these cases are without DRTB treatment involving BDQ. (Figure 4.9) Hence we found more individuals with new cases who are prone to BDQ resistance in contrast to previously treated cases with BDQ drug in their treatment. These results are in

line with another study [216]. In present study patient's characteristics are linked to history of ATT and found that age is the only risk factor which is associated with history of ATT in contributing to DRTB in our patients.

Bedaquiline is one of the most important new drug which is part of MDR/XDR-TB. Literature described the mechanism of BDQ resistance due to mutations in *Rv0678*, *atpE*, *pepQ* and *Rv1979c*. BDQ functions to inhibit ATP synthesis and many mutations in these reported regions provide additional resistance causing more prevalence of BDQ resistance. We found higher proportion of BDQ mutations in *Rv0678* target region just like many other studies [220] Higher proportion of mutations in *Rv0678* results in low level BDQ resistance both clinically as well as in vitro. Mutations in *Rv0678* along with *pepQ* and *Rv01979c* altogether contribute to BDQ and clofazimine cross-resistance as reported in literature. WGS data of our dataset revealed that Asp47fs and Glu49fs are the most frequent mutation in *Rv0678*. These mutations are presented as uncertainly significant to WHO mutation catalogue. Same results are presented by another study in the country of Georgia [221].

WGS information from our dataset reveals a single mutation Arg7Gln present in *pepQ* region that is being present only in BDQ sensitive isolates and upon literature mining we found no clear mechanism of this mutation and its involvement in BDQ resistance. We found a single mutation in *atpE* region Ala63Pro in a single isolate from our dataset belonging to lineage 4. As BDQ inhibits ATP formation while deforming subunit c, mutations in *atpE* are highly involved in conferring BDQ resistance. It is clearly proved in the literature that mutations in *atpE* are no doubt low in frequency but are responsible for causing high level of BDQ resistance in comparison to *Rv0678* which involves very frequent mutations for BDQ resistance [220, 222]. Another important marker of BDQ resistance is *Rv1979c* which is also responsible for CFZ cross resistance. We found Asp286Gly as the most frequent mutation in this region in both BDQ sensitive as well as in BDQ resistant isolates. This mutation is also found in another study from Indonesia [220]. This reference study had also reported this mutation in phenotypically BDQ sensitive isolates [220].

As mentioned in the introductory paragraph of the discussion that resistance to any drug also occurs due to an important phenomenon of heteroresistance at drug target. Heteroresistance is the phenomenon of the coexistence of drug-susceptible and drug-resistant isolates. For instance, it turns complicated to detect MTB drug-resistant isolates due to heteroresistance that causes the dominance of drug-sensitive isolates over drug-resistant ones. It is the transitioning stage towards drug resistance and is possibly caused by superinfection (infection with a resistant and susceptible strain simultaneously) or antibiotic selection pressure (a susceptible isolate converts to resistant because of any genomic mutation). In heteroresistance, bacteria can significantly utilize growth opportunities even in antibiotic environments, and this scenario makes the MTB isolates more vulnerable to drug resistance while undermining the treatment success [223]. Previous studies deciphered the heteroresistance in various DRTB drugs including Bedaquiline but there is an inadequate literature for the mutational analysis of BDQ heteroresistance that is being addressed in the current study.

The findings of the present study indicated 29% BDQ heteroresistance in our population of interest. Previously, in two relevant studies, the percentage of BDQ heteroresistance was computed as 21.01% [209] and 60% [224]. The majority of the patients suffering from heteroresistance infection were adult in age group for current study which is considered as the most productive age group [225]. Higher proportion of this age group cannot be ignored as it acts as an important potential factor for heteroresistance in our results. Although history of ATT was not significantly associated to BDQ heteroresistance but higher proportion of previously treated heteroresistant cases needs to be investigated for their previous treatment regimen and treatment outcome. This information can be useful to stratify the respective strains whether they are suffering from primary or acquired resistance. Our data represents 20 patients with previous treatment, out of which 17 cases were presented as heteroresistant. We found 4 of these cases were previously cured with BDQ in their treatment regimen and rest of the cases were only having previous history of first line ATT. Hence, our results report that BDQ heteroresistance was more common in pretreated cases. Our research findings were somehow little different than another study in that mix infection and heteroresistance among

DRTB isolates were more prevalent in young age group, that is indeed a growing age [226] The number of XDR in addition to MDR +BDQ resistant strains, was statistically significant in relation to the presence of heteroresistance which represents the significance of Bedaquiline in MDR and XDR patients' treatment. Though MDR group is the largest group in our study, but there are very fewer number of heteroresistance cases present in this group (0.09%). Concurrently it was noticed that high proportion of these heteroresistant cases were suffering from MDR-TB. Punjab is the most populated province of Pakistan [225] and the maximum number of heteroresistance patients belonged to this region. These results indicate MDR is highly prevalent in Punjab as described in another study [225] and MDR patients who take BDQ as an integral part of their treatment are more prone to BDQ resistance because they are BDQ heteroresistant that means half way to resistance. This scenario is another evidence that signifies the participation of BDQ heteroresistance in BDQ resistance occurrence.

The phylogeny of *M. tuberculosis* comprises of four major lineages (L1–L4), each containing discrete strain types that may vary in their transmission action, resulting in severe disease manifestation [227] Lineage 3 was the most frequent lineage present in our dataset, particularly in BDQ heteroresistant isolates. Lineage 3 is the Central Asian lineage and there are many studies designed to characterize MTB on the base of their genetic diversity while adopting whole genome sequencing. These studies concluded that MTB isolates from Pakistan belong to central Asian lineage which is included in lineage 3 of the MTB genome [228, 229]. A study from Pakistan on representation of drug resistant MTB mutations and its transmission concluded with same results in that 74.2% strains belonged to lineage 3 [230]. Another investigation from Karachi, Pakistan also represented lineage 3 as the most prevalent lineage [231]. The maximum prevalence of Lineage 3 in our Pakistani isolates reveals the molecular epidemiology of MTB in Pakistan [232]. Many other studies also nominated CAS as the most common MTB lineage [228, 229, 233–236]. A study from South Africa that focused on Bedaquiline heteroresistance, and presented Beijing as the most predominant lineage [224]. As mentioned earlier, in heteroresistant cases the susceptible MTB stains mask the resistant strains [31], so we can see that many of the BDQ sensitive strains in

present research was proved phenotypically BDQ sensitive but heteroresistant on WGS analysis. There was no heteroresistant strain detected with mix lineage. This indicates that acquired resistance is more likely to occur. Hence, it can be added that heteroresistance phenomenon was more concentrated and frequent in previously treated cases. Bedaquiline is a core drug used in the treatment of rifampicin-resistant tuberculosis. Several candidate BDQ resistance genes have been identified, but only a few genomic variants *atpE*, *pepQ*, *Rv1979c*, and *Rv0678* have been statistically linked to Bedaquiline resistance [32].

Our results provided us the mutation data in *Rv0678*, *pepQ* and *Rv1979c* for Bedaquiline heteroresistant MTB species but those mutations were not enough to be computed for statistical analysis as the majority of these mutations were harbored by a single strain. With relevance to our results, there was some evidence of BDQ heteroresistance reported only in *Rv0678* [33] but none of the study presented heteroresistance caused by mutations in *pepQ*, *atpE* and *Rv1979c* gene targets. A study in 2023 used published phenotype data for BDQ resistance variants in *Rv0678*, *atpE*, *pepQ* and *Rv1979c* genes in 756 Mycobacterium tuberculosis isolates and applied Bayesian methods to estimate the posterior probability of Bedaquiline resistance and concluded that the role of *Rv0678*, and *atpE* is evident in BDQ resistance, but the role of *pepQ* and *Rv1979c* variants is uncertain. There was a high level invitro BDQ resistance observed for *atpE* as it can cause a loss of binding affinity of BDQ with its hotspot [34], but there was not a definite evidence of its involvement for clinical isolates [35]. However, the accelerated expression of the MmpS5/MmpL5 efflux system resulted by the mutations in *Rv0678* gene is the main cause of clinical resistance to BDQ. Last of all, there was a poor evidence of BDQ resistance in the *pepQ* and *Rv1979c* genes because of mutations, but still they exist as the biomarker of BDQ resistance [36]. In a recent systematic review the linkage between phenotypic BDQ resistant and genomic variants in *atpE*, *Rv0678*, *Rv1979c*, and *pepQ* was analyzed [37], but only 0.006% (2/313) variants were statistically significant to BDQ resistance [38]. According to WHO mutation catalogue 2021, no mutations fulfilled the criteria for BDQ resistance [39], while the recent mutation catalogue published in 2023 reported six mutations fulfilled this criteria of association with Bedaquiline resistance. [40]. Therefore, amount

of evidence on BDQ genotype-phenotype association failed to affirm the use of gDST but the data from WGS and pDST results added in the second addition of WHO mutation catalogue somehow signified these techniques as a better mode for patient care. Variant type of our study isolates were also stratified and missense mutation exceed their involvement for BDQ heteroresistance in contrast to frame shift and non-sense mutations. Literature review provided the evidence that there was a low probability for synonymous mutations in *Rv0678* (3.3%), high (55.1%) for nonsense mutations in *Rv0678*, comparatively low for missense (31.5%) mutations and frameshift (30.0%) in *Rv0678* and low for missense mutations in *pepQ* (2.6%) and *Rv1979c* (2.9%) [36].

Current study involved the mutational analysis of our BDQ case control isolates. We screened the WGS data to highlight all the mutations that were part of BDQ target genes and underwent mutations in these regions from our dataset. As mentioned in Table 4.13, maximum mutations causing the heteroresistance phenomenon were located in *Rv0678* region. Among the reported mutations, Asp47fs existed in a single isolate but possess its significance in BDQ resistance and is being discussed in a variety of DRTB studies previously [18][41–43]. This mutation was proved to be involved in both primary as well as acquired BDQ resistance in addition to cross resistance to clofazimine and poor treatment outcome [44]. Another mutation Arg89Leu was studied recently in an epidemiological study of MTB from Georgia and was present with a frequency of 99.1% while in our dataset it presented its heteroresistance with 49% [15]. Ile67fs as one of most ancient and commonly occurring mutational marker existed in 5 stains with a heteroresistance level of 9 – 91% [45], Various other mutations Arg72Trp, Leu142Arg, Arg94Trp, Phe19Ser, Arg50Trp, Glu49fs, Ala86Val and Arg96Gln were part of our MTB strains but thee mutations confer BDQ resistance in *Rv0678* region as reported in past experiments in other countries [32][46–49]. The presence of these mutations in relation to BDQ heteroresistance in our population emphasize their upcoming fixation probability as confirmed BDQ conferring mutations in Pakistani population enduring Bedaquiline added treatment.

All the novel mutations (n=17) discovered in this study were present in single isolates for all three markers for Bedaquiline resistance (*Rv0678*, *Rv1979c* and *pepQ*) with a varied percentage of heteroresistance and need to be further investigated in diverse and larger DR-TB population for their fixation probability as a definite resistant markers. The mutations found in non-heteroresistance (Table 4.8 - 4.11) isolates as well as in heteroresistant strains are providing the significance that on acquiring possible true resistance in near future these mutations would harbor as true resistant biomarkers. As a core part of DR-TB treatment especially Bpal (bedaquiline, pretomanid, and linezolid), BDQ can improve the treatment outcomes for drug-resistant TB, but challenges in predicting BDQ heteroresistance and optimizing its use remain areas of concern.

From GWAS analysis of 100 MTB strains, we got few suitable novel candidates and evaluated for the protein thermodynamic analysis. Manhattan Plot for all the significant candidates of GWAS was not added due to low number of samples being run for association study. We only selected *esXo* for its analysis for molecular dynamics (MD) and simulations.

If the mutation (V48A) affects BDQ resistance, it is likely through an indirect mechanism. Specifically, the mutation may alter the polymerization mode of *esXO*, thereby disrupting the interaction between adjacent monomers and BDQ. However, this hypothesis is limited by our inability to resolve the complete three-dimensional structure of *esXO*, particularly its polymeric form under physiological conditions.

The MD simulations and structure prediction of gene *esXo* faces a significant limitation: the lack of structural information for *esXO* in its native state, including details about its oligomeric assembly and inter-subunit angles. These gaps hinder precise modeling of BDQ binding to the polymerized form and complicate efforts to predict drug interactions or identify key resistance residues accurately.

Present study also includes effect of mutation in BDQ target genes harboring mutation on the protein dynamics. We presented a number of protein structures for

a variety of mutations involved in true resistance in *Rv0678* region. For mutation Ala124Pro mutation, proline introduces rigidity due to its cyclic structure, which can disrupt the proteins secondary structure. This minor destabilization suggests that the mutation slightly reduces protein flexibility without abrupt affecting function. (Figure 4.19) With regard to Met139Ile mutation, both Met and Ile are hydrophobic, but methionine has a larger side chain with sulfur, while isoleucine is branched.

The loss of sulfur may alter local interactions, leading to moderate destabilization. (Figure 4.20) In Gly87Arg variant, glycine provides flexibility, while arginine is Bigger and positively charged. This substitution disrupts the local structure, increasing steric hindrance, and electrostatic interactions, causing destabilization. (Figure 4.21)The A99D mutation introduces a negatively charged aspartic acid in place of the nonpolar alanine, leading to slight structural stabilization (0.122 kcal/mol) with negligible destabilization. This substitution reduces molecular flexibility (-0.003 kcal·mol^{-1}·K^{-1}), potentially making the protein more rigid at this position. The introduction of charge may affect local interactions, possibly forming new hydrogen bonds or altering protein function if the site is involved in enzymatic activity, ligand binding, or structural integrity. However, if the mutation occurs in a non-critical region, its overall impact may be minimal. (Figure 4.22) Val120Met mutation results in destabilization of protein residue.

Both residues are hydrophobic, but methionine is biggest molecule and has a sulfur atom. The minor destabilization suggests small structural rearrangements rather than complete disruption. (Figure 4.23) The Pro48Leu mutation introduces leucine in place of proline, leading to a mixed effect on protein stability and flexibility. While it shows a slight stabilizing effect (0.295 kcal/mol), it is also more destabilizing (-0.518 kcal/mol), suggesting a net reduction in protein stability. Additionally, the mutation increases molecular flexibility (0.648 kcal·mol^{-1}·K^{-1}), which could impact structural integrity, especially if the site is functionally or structurally important. Since proline is a rigid residue that often induces structural constraints, replacing it with leucine may disrupt local folding, potentially

affecting protein function. (Figure 4.24) In Leu32Ser substitution, leucine is hydrophobic, whereas serine is polar. This substitution likely disrupts hydrophobic interactions and alters protein folding, leading to notable destabilization. (Figure 4.25) Gln51Glu mutation disrupts the protein residue. Both residues are polar, but glutamic acid carries a negative charge. The minimal destabilization suggests that the substitution does not significantly affect overall protein stability but may slightly alter electrostatic interaction (Figure 4.26).

Our findings highlight the complexity of BDQ heteroresistance in addition to true resistance and the need for enhanced diagnostic and treatment strategies to manage drug-resistant TB effectively. Studies with larger sample size and regions with diverse genetic backgrounds and epidemiological patterns of TB might provide us more significant information about BDQ resistance and heteroresistance with the integration of multiple diagnostic approaches. The study primarily focused on the genetic and molecular aspects of BDQ resistance, with limited clinical correlation regarding patient outcomes and treatment efficacy. Further research should aim to link genetic findings with clinical data.

A central aim of this study was to explore both molecular and systemic contributors to Bedaquiline resistance in Mycobacterium tuberculosis. Although several mutations (e.g., *Rv0678*, *atpE*) linked to heteroresistance were found by the molecular data, the KAP survey exposed serious gaps in healthcare workers' practices and knowledge, especially with regard to infection control and second-line medication protocols. According to this dual analysis, deficiencies in clinical implementation could lead to selection settings that promote the evolution of resistance in addition to compromising patient outcomes. For instance, subpopulations of resistant bacilli may be able to endure and grow as a result of inadequate adherence monitoring or prolonged treatment instigation brought on by incorrect resistance classification, which would ultimately contribute to the genotypic patterns shown in this cohort. Therefore, operational gaps in TB control programs may contribute to the molecular level BDQ heteroresistance.

The KAP findings highlight the urgent need for structured, mandatory training programs focused on drug-resistant TB management, including the rational use

and monitoring of newer drugs such as Bedaquiline. Policies must go beyond passive dissemination of guidelines and incorporate hands-on clinical mentorship, periodic KAP assessments, and the integration of TB modules into continuous medical education frameworks. Moreover, monitoring and evaluation tools should be developed to regularly assess HCW adherence to treatment protocols, thereby limiting the unintended clinical behaviors that may promote resistance emergence.

Although exploratory, the GWAS analysis identified potentially significant genomic loci, including novel mutations in regulatory and efflux-related genes. These findings reinforce the need to extend resistance surveillance beyond recognized mutations, particularly in high-burden settings where drug pressure and treatment inconsistencies may accelerate the emergence of novel resistance pathways. Integrating these early signals into diagnostic platforms or resistance prediction models could dramatically improve early detection of heteroresistance and guide appropriate clinical responses before full resistance manifests. Moreover, protein crystallography of the novel marker such esXO will provide its insight to further decipher its contribution for BDQ resistance.

There are a number of limitations in the current study. First, the findings' generalizability may have been limited by the sample size's inability to fully reflect the variety of genetic variants across various geographical areas. Second, the KAP survey's cross-sectional methodology limited the capacity to draw conclusions about the causal relationship between the formation of resistance and deficits in knowledge or practice. Third, there are still questions regarding the specific involvement of the newly discovered loci (such as efflux and regulatory genes) in resistance because functional confirmation of these loci was outside the purview of this study, even if the molecular analysis revealed both known and novel alterations. Similarly, even though the GWAS offered valuable exploratory information, population structure or other variables might have an impact on the relationships found. Lastly, the KAP survey's self-reported data can be prone to social desirability bias and recall bias, which could result in an underestimation of clinical practice gaps. When combined, these limitations imply that in order to support and build upon

the current findings, longterm investigations incorporating functional tests, larger genomic datasets, and recurrent HCW practice assessments are required.

Chapter 6

Conclusion and Future Work

6.1 Conclusion

a) The TB KAP study among healthcare workers provides a valuable opportunity to identify knowledge gaps, assess TB-related attitudes such as stigmatization, and evaluate treatment adherence practices. These insights are essential for shaping effective policies, designing structured training programs, and strengthening healthcare systems to better support both HCWs and TB/DRTB patients.

b) Healthcare workers serve as role models within the medical field, and their improved knowledge, attitudes, and practices can positively influence patient behavior and treatment adherence. Enhancing HCW KAP scores not only improves treatment outcomes but also fosters a supportive environment for TB/DRTB patients, thereby reducing stigma and strengthening overall TB control efforts.

c) There are some gaps in healthcare worker (HCW) knowledge and adherence to TB management protocols were identified, particularly concerning BDQ resistance detection and treatment strategies as well as trengthening TB control efforts through targeted HCW training programs and improved molecular diagnostic tools is essential in combating drug-resistant TB.

d) Current dataset includes key mutations associated with BDQ resistance were identified in *Rv0678*, *pepQ*, *atpE* and *Rv1979c*, which may contribute to ATP synthase function alteration and efflux pump regulation.

e) The mutations found in non-heteroresistant isolates as well as in heteroresistant strains are providing the significance that, on acquiring possible true resistance in near future, these mutations would harbor as true resistant biomarkers.

f) All the novel mutations discovered in this study were present in single isolates for all three markers for Bedaquiline resistance (*Rv0678*, *Rv1979c* and *pepQ*) with a varied percentage of heteroresistance and need to be further investigated in diverse and larger DR-TB population for their fixation probability as a definite resistant markers.

g) The identification of resistance-associated mutations in *Rv0678*, *pepQ*, *atpE*, and *Rv1979c* has important clinical implications. These mutations, particularly when present in a heteroresistant state, might not be detected by conventional diagnostics, leading to true resistance and delayed or failed treatment. Recognizing heteroresistance as an early warning sign of true resistance highlights the need for more robust molecular diagnostics.

h) As a core part of DRTB treatment especially Bpal (bedaquiline, pretomanid, and linezolid), BDQ can improve the treatment outcomes for drug-resistant TB, but challenges in predicting BDQ heteroresistance and optimizing its use remain areas of concern.

i) Bedaquiline heteroresistance is an important indicator of emerging bedaquiline resistance, predominantly observed in previously treated cases without mixed infections, suggesting a higher likelihood of acquired resistance.

j) The study also focused on the genetic and molecular aspects of BDQ heteroresistance based on phenotypic DST and WGS, with no MIC testing and limited clinical correlation regarding patient outcomes and treatment efficacy. Further research should aim to link genetic findings with clinical data.

k) Phylogenetic analysis showed a higher prevalence of BDQ resistance in lineage 3 (CAS) in our population.

l) Our findings accentuate the complexity of bedaquiline heteroresistance and the need for better diagnostic and appropriate therapeutic treatment approaches for drug-resistant TB with bedaquiline-containing regimens.

m) A multifaceted approach integrating molecular surveillance, lineage-specific treatment adaptations, and healthcare capacity-building initiatives is crucial for managing BDQ - resistant TB effectively.

6.2 Future Directions

a) Implementation of targeted training programs to improve healthcare workers' knowledge and adherence to TB treatment protocols is necessary for the success of any TB control Programme to eradicate TB/DRTB from high burden countries like Pakistan.

b) For more significant mutational analysis and genetic insight of BDQ, larger-scale genomic wide association studies should be conducted to further elucidate emerging mutations and their impact on BDQ resistance.

c) Investigate the functional consequences of *Rv0678*, *atpE*, *pepQ* mutations and other novel biomarkers through in vitro and in vivo models and to explore alternative therapeutic strategies, including combination regimens, to counteract BDQ resistance.

d) Heteroresistance is an important yet an ignored phenomenon in the field of DRTB. To reduce the occurrence of DRTB especially resistance of new drugs including BDQ. It is mandatory to promptly detect heteroresistance in MTB isolates through innovating the advance detection techniques.

e) Develop new computational models to predict potential resistance mutations and their effects on protein targets so as to evaluate their efficacy effectively.

f) The resulting GWAS exploratory nature data emphasizes to validate it on a large cohort level to provide the definite and conclusive results.

g) It is required to strengthen molecular diagnostic tools for early detection of BDQ resistance in clinical settings and assess the transmission dynamics of BDQ-resistant TB to understand its epidemiological implications.

h) While acknowledging the significance of WGS, it would be worthwhile to use WGS for the prevalence and surveillance of DRTB so as to meet the goals of TB End strategy by 2035.

i) The study provides crucial pharmacogenomics insights by identifying genetic mutations and novel loci associated with bedaquiline resistance, enabling the development of personalized treatment strategies and early resistance prediction models.

Bibliography

[1] F. Rashid, S. Iqbal, S. Tahseen, and Y. Zhao, "Investigation of bedaquiline heteroresistance among *Mycobacterium tuberculosis* isolates from Pakistan," *Microbiol. Spectr.*, vol. 13, no. 1, p. e02181-24, 2025.

[2] R. M. Houben and P. J. Dodd, "The global burden of latent tuberculosis infection: A re-estimation using mathematical modelling," *PLoS Med.*, vol. 13, no. 10, p. e1002152, 2016.

[3] N. A. Menzies *et al.*, "Progression from latent infection to active disease in dynamic tuberculosis transmission models: A systematic review of the validity of modelling assumptions," *Lancet Infect. Dis.*, vol. 18, no. 8, pp. e228–e238, 2018.

[4] J. C. Emery *et al.*, "Self-clearance of *Mycobacterium tuberculosis* infection: Implications for lifetime risk and population at-risk of tuberculosis disease," *Proc. Roy. Soc. B*, vol. 288, no. 1943, p. 20201635, 2021.

[5] M. A. Behr, P. H. Edelstein, and L. Ramakrishnan, "Is *Mycobacterium tuberculosis* infection life long?" *BMJ*, vol. 367, p. l5770, 2019.

[6] World Health Organization, *Global tuberculosis report 2024*, Geneva, Switzerland: WHO, 2024.

[7] K. Bi, Y. Zhao, S. Lu, K. Xu, and Y. Zhang, "Comment on WHO global tuberculosis report 2024: Progress, challenges and innovations for achieving end TB strategy by 2035," *J. Infect. Dis.*, vol. 228, no. 12, pp. 1839–1841, 2023.

[8] World Health Organization, *Global tuberculosis report 2023*, Geneva, Switzerland: WHO, 2023.

[9] Y. Zhang, "The magic bullets and tuberculosis drug targets," *Annu. Rev. Pharmacol. Toxicol.*, vol. 45, no. 1, pp. 529–564, 2005.

[10] World Health Organization, *Global tuberculosis report 2015*, Geneva, Switzerland: WHO, 2015.

[11] World Health Organization, *Global tuberculosis report 2022*, Geneva, Switzerland: WHO, 2022.

[12] C. C. Boehme *et al.*, "Rapid molecular detection of tuberculosis and rifampin resistance," *New England J. Med.*, vol. 363, no. 11, pp. 1005–1015, 2010.

[13] World Health Organization, "The use of molecular line probe assay for the detection of resistance to isoniazid and rifampicin: Policy update," WHO, Geneva, Switzerland, 2016.

[14] A. Dixit *et al.*, "Estimation of country-specific tuberculosis antibiograms using genomic data," *medRxiv*, 2021, doi: 10.1101/2021.09.23.21263991.

[15] M. Farhat *et al.*, "Drug-resistant tuberculosis: A persistent global health concern," *Nature Rev. Microbiol.*, vol. 22, no. 10, pp. 617–635, 2024.

[16] D. Yee *et al.*, "Incidence of serious side effects from first-line antituberculosis drugs among patients treated for active tuberculosis," *Amer. J. Respir. Crit. Care Med.*, vol. 167, no. 11, pp. 1472–1477, 2003.

[17] J. F. Murray, D. E. Schraufnagel, and P. C. Hopewell, "Treatment of tuberculosis: A historical perspective," *Ann. Amer. Thoracic Soc.*, vol. 12, no. 12, pp. 1749–1759, 2015.

[18] N. Ahmad *et al.*, "Treatment correlates of successful outcomes in pulmonary multidrug-resistant tuberculosis: An individual patient data meta-analysis," *Lancet*, vol. 392, no. 10150, pp. 821–834, 2018.

[19] K. R. Jacobson *et al.*, "Treatment outcomes of isoniazid-resistant tuberculosis patients, Western Cape Province, South Africa," *Clin. Infect. Dis.*, vol. 53, no. 4, pp. 369–372, 2011.

[20] World Health Organization, *WHO consolidated guidelines on tuberculosis. Module 4: Treatment-drug-resistant tuberculosis treatment, 2022 update*, Geneva, Switzerland: WHO, 2022.

[21] H. R. Stagg *et al.*, "Fluoroquinolones and isoniazid-resistant tuberculosis: Implications for the 2018 WHO guidance," *Eur. Respir. J.*, vol. 54, no. 4, p. 1900982, 2019.

[22] World Health Organization, *WHO treatment guidelines for isoniazid-resistant tuberculosis: Supplement to the WHO treatment guidelines for drug-resistant tuberculosis*, Geneva, Switzerland: WHO, 2018.

[23] C. J. Murray *et al.*, "Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis," *Lancet*, vol. 399, no. 10325, pp. 629–655, 2022.

[24] World Health Organization, *Global tuberculosis report 2024*, Geneva, Switzerland: WHO, 2024.

[25] L. Villegas *et al.*, "Prevalence, risk factors, and treatment outcomes of isoniazid- and rifampicin-mono-resistant pulmonary tuberculosis in Lima, Peru," *PLoS ONE*, vol. 11, no. 4, p. e0152933, 2016.

[26] L. Sharling *et al.*, "Rifampin-resistant tuberculosis in the United States, 1998–2014," *Clin. Infect. Dis.*, vol. 70, no. 8, pp. 1596–1605, 2020.

[27] A. S. Dean *et al.*, "Prevalence and genetic profiles of isoniazid resistance in tuberculosis patients: A multicountry analysis of cross-sectional data," *PLoS Med.*, vol. 17, no. 1, p. e1003008, 2020.

[28] World Health Organization, *Web Annex C. WHO operational handbook on tuberculosis*, Geneva, Switzerland: WHO, 2020.

[29] K. R. Jacobson *et al.*, "Implications of failure to routinely diagnose resistance to second-line drugs in patients with rifampicin-resistant tuberculosis

on Xpert MTB/RIF: A multisite observational study," *Clin. Infect. Dis.*, vol. 64, no. 11, pp. 1502–1508, 2017.

[30] C. Oga-Omenka *et al.*, "Factors influencing diagnosis and treatment initiation for multidrug-resistant/rifampicin-resistant tuberculosis in six sub-Saharan African countries: A mixed-methods systematic review," *BMJ Glob. Health*, vol. 5, no. 7, p. e002280, 2020.

[31] A. Svadzian *et al.*, "Differential yield of universal versus selective drug susceptibility testing of patients with tuberculosis in high-burden countries: A systematic review and meta-analysis," *BMJ Glob. Health*, vol. 5, no. 10, p. e003438, 2020.

[32] S. Kim, "Drug-susceptibility testing in tuberculosis: Methods and reliability of results," *Eur. Respir. J.*, vol. 25, no. 3, pp. 564–569, 2005.

[33] World Health Organization, *Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis*, Geneva, Switzerland: WHO, 2018.

[34] H.-J. Yu *et al.*, "Performance evaluation of the BACTEC MGIT 960 system for rifampin drug-susceptibility testing of *Mycobacterium tuberculosis* using the current WHO critical concentration," *J. Clin. Microbiol.*, vol. 61, no. 1, p. e01086-22, 2023.

[35] J. Shea *et al.*, "Low-level rifampin resistance and rpoB mutations in *Mycobacterium tuberculosis*: An analysis of whole-genome sequencing and drug susceptibility test data in New York," *J. Clin. Microbiol.*, vol. 59, no. 4, p. e01885-20, 2021.

[36] G. Torrea *et al.*, "Variable ability of rapid tests to detect *Mycobacterium tuberculosis* rpoB mutations conferring phenotypically occult rifampicin resistance," *Sci. Rep.*, vol. 9, no. 1, p. 11826, 2019.

[37] World Health Organization, *Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin*

resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children: Policy update, Geneva, Switzerland: WHO, 2013.

[38] World Health Organization, *Rapid implementation of the Xpert MTB/RIF diagnostic test: Technical and operational 'how-to'; practical considerations*, Geneva, Switzerland: WHO, 2011.

[39] S. E. Dorman *et al.*, "Xpert MTB/RIF Ultra for detection of *Mycobacterium tuberculosis* and rifampicin resistance: A prospective multicentre diagnostic accuracy study," *Lancet Infect. Dis.*, vol. 18, no. 1, pp. 76–84, 2018.

[40] J. S. Zifodya *et al.*, "Xpert Ultra versus Xpert MTB/RIF for pulmonary tuberculosis and rifampicin resistance in adults with presumptive pulmonary tuberculosis," *Cochrane Database Syst. Rev.*, no. 2, p. CD009593, 2021.

[41] A. Penn-Nicholson *et al.*, "Detection of isoniazid, fluoroquinolone, ethionamide, amikacin, kanamycin, and capreomycin resistance by the Xpert MTB/XDR assay: A cross-sectional multicentre diagnostic accuracy study," *Lancet Infect. Dis.*, vol. 22, no. 2, pp. 242–249, 2022.

[42] A. Meaza *et al.*, "Evaluation of genotype MTBDR plus VER 2.0 line probe assay for the detection of MDR-TB in smear positive and negative sputum samples," *BMC Infect. Dis.*, vol. 17, no. 1, p. 280, 2017.

[43] M. Driesen *et al.*, "Evaluation of a novel line probe assay to detect resistance to pyrazinamide, a key drug used for tuberculosis treatment," *Clin. Microbiol. Infect.*, vol. 24, no. 1, pp. 60–64, 2018.

[44] World Health Organization, *Catalogue of mutations in Mycobacterium tuberculosis complex and their association with drug resistance*, Geneva, Switzerland: WHO, 2023.

[45] World Health Organization, *The use of next-generation sequencing for the surveillance of drug-resistant tuberculosis: An implementation manual*, Geneva, Switzerland: WHO, 2023.

[46] A. Dippenaar *et al.*, "Nanopore sequencing for *Mycobacterium tuberculosis*: A critical review of the literature, new developments, and future opportunities," *J. Clin. Microbiol.*, vol. 60, no. 1, p. e00646-21, 2022.

[47] E. Sanchez-Padilla *et al.*, "Detection of drug-resistant tuberculosis by Xpert MTB/RIF in Swaziland," *New England J. Med.*, vol. 372, no. 12, pp. 1181–1182, 2015.

[48] M. R. Farhat *et al.*, "Genetic determinants of drug resistance in *Mycobacterium tuberculosis* and their diagnostic value," *Amer. J. Respir. Crit. Care Med.*, vol. 194, no. 5, pp. 621–630, 2016.

[49] S. Mahomed *et al.*, "Discordant line probe genotypic testing vs culture-based drug susceptibility phenotypic testing in TB endemic KwaZulu-Natal: Impact on bedside clinical decision making," *J. Clin. Tuberc. Other Mycobact. Dis.*, vol. 20, p. 100176, 2020.

[50] L. J. Pankhurst *et al.*, "Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: A prospective study," *Lancet Respir. Med.*, vol. 4, no. 1, pp. 49–58, 2016.

[51] A. A. Votintseva *et al.*, "Same-day diagnostic and surveillance data for tuberculosis via whole-genome sequencing of direct respiratory samples," *J. Clin. Microbiol.*, vol. 55, no. 5, pp. 1285–1298, 2017.

[52] G. A. Goig *et al.*, "Whole-genome sequencing of *Mycobacterium tuberculosis* directly from clinical samples for high-resolution genomic epidemiology and drug resistance surveillance: An observational study," *Lancet Microbe*, vol. 1, no. 4, pp. e175–e183, 2020.

[53] A. Van Rie *et al.*, "Sequencing Mycobacteria and Algorithm-determined Resistant Tuberculosis Treatment (SMARTT): A study protocol for a phase IV pragmatic randomized controlled patient management strategy trial," *Trials*, vol. 23, no. 1, p. 864, 2022.

[54] M. Farhat *et al.*, "Drug-resistant tuberculosis: A persistent global health concern," *Nature Rev. Microbiol.*, pp. 1–19, 2024.

[55] World Health Organization, *WHO bacterial priority pathogens list, 2024: Bacterial pathogens of public health importance, to guide research, development, and strategies to prevent and control antimicrobial resistance*, Geneva, Switzerland: WHO, 2024.

[56] World Health Organization, *Rapid communication: Key changes to treatment of multidrug- and rifampicin-resistant tuberculosis (MDR/RR-TB)*, Geneva, Switzerland: WHO, 2018.

[57] World Health Organization, *Key updates to the treatment of drug-resistant tuberculosis: Rapid communication, June 2024*, Geneva, Switzerland: WHO, 2024.

[58] World Health Organization, *Global tuberculosis report 2013*, Geneva, Switzerland: WHO, 2013.

[59] M. Nadeem, "Factors influencing access to tuberculosis diagnostic services in Pakistan–An analysis," 2023, [Online]. Available: Not specified.

[60] G. N. Kazi, K. B. Mohamud, A. Quadir, S. K. Shah, and Z. ul Haq, "Tuberculosis control in Pakistan: A decade (2011-2020) in review," *Pakistan J. Public Health*, vol. 12, no. 1, pp. 17–22, 2022.

[61] Pakistan National Tuberculosis Control Program, [Online]. Available: https://ntp.gov.pk/about-us/.

[62] T. Gordon, F. Booysen, and J. Mbonigaba, "Socio-economic inequalities in the multiple dimensions of access to healthcare: The case of South Africa," *BMC Public Health*, vol. 20, no. 1, p. 289, 2020.

[63] H. Ullah, H. Ahmed, S. A. Salman, R. Iqbal, S. J. Hussaini, and A. Malikzai, "The rising tide of tuberculosis in Pakistan: Factors, impact, and multifaceted approaches for prevention and control," *Health Sci. Rep.*, vol. 7, no. 10, p. e70130, 2024.

[64] A. Ghodousi *et al.*, "Acquisition of cross-resistance to bedaquiline and clofazimine following treatment for tuberculosis in Pakistan," *Int. J. Mycobacteriol.*, vol. 10, no. Suppl 1, p. S6, 2021.

[65] E. B. Chahine, L. R. Karaoui, and H. Mansour, "Bedaquiline: A novel diarylquinoline for multidrug-resistant tuberculosis," *Ann. Pharmacother.*, vol. 48, no. 1, pp. 107–115, 2014.

[66] T. V. A. Nguyen *et al.*, "Bedaquiline resistance: Its emergence, mechanism, and prevention," *Clin. Infect. Dis.*, vol. 66, no. 10, pp. 1625–1630, 2018.

[67] B. Alotaibi *et al.*, "Tuberculosis knowledge, attitude and practice among healthcare workers during the 2016 Hajj," *PLoS ONE*, vol. 14, no. 1, p. e0210913, 2019.

[68] N. P. Hoa, V. K. Diwan, and A. E.-K. Thorson, "Diagnosis and treatment of pulmonary tuberculosis at basic health care facilities in rural Vietnam: A survey of knowledge and reported practices among health staff," *Health Policy*, vol. 72, no. 1, pp. 1–8, 2005.

[69] Z. Kanjee *et al.*, "Tuberculosis infection control in rural South Africa: Survey of knowledge, attitude and practice in hospital staff," *J. Hosp. Infect.*, vol. 79, no. 4, pp. 333–338, 2011.

[70] S. Naidoo *et al.*, "Changes in healthcare workers' knowledge about tuberculosis following a tuberculosis training programme," *Educ. Health*, vol. 24, no. 2, p. 514, 2011.

[71] A. Roy *et al.*, "A controlled trial of the knowledge impact of tuberculosis information leaflets among staff supporting substance misusers: Pilot study," *PLoS ONE*, vol. 6, no. 6, p. e20875, 2011.

[72] N. Awofeso, I. Schelokova, and A. Dalhatu, "Training of front-line health workers for tuberculosis control: Lessons from Nigeria and Kyrgyzstan," *Human Resources Health*, vol. 6, no. 1, p. 20, 2008.

[73] K. V. Driessche *et al.*, "Training health care workers to promote HIV services for patients with tuberculosis in the Democratic Republic of Congo," *Human Resources Health*, vol. 7, no. 1, p. 23, 2009.

[74] C. R. Iturregui Paucar *et al.*, "Factors associated with knowledge, attitudes, and practices about tuberculosis in Peruvians," 2023, [Online]. Available: Not specified.

[75] A. O. Bashorun *et al.*, "Knowledge, attitude and practice towards tuberculosis in Gambia: A nation-wide cross-sectional survey," *BMC Public Health*, vol. 20, no. 1, p. 1566, 2020.

[76] G. Du *et al.*, "Study on the influencing factors of knowledge, attitudes and practice about tuberculosis among freshmen in Jiangsu, China: A cross-sectional study," *Infect. Drug Resist.*, vol. 15, pp. 1235–1245, 2022.

[77] M. Minnery *et al.*, "A cross sectional study of knowledge and attitudes towards tuberculosis amongst front-line tuberculosis personnel in high burden areas of Lima, Peru," *PLoS ONE*, vol. 8, no. 9, p. e75698, 2013.

[78] P. J. Haasnoot *et al.*, "Knowledge, attitudes, and practice of tuberculosis among Maasai in Simanjiro district, Tanzania," *Am. J. Trop. Med. Hyg.*, vol. 83, no. 4, pp. 902–905, 2010.

[79] S. Arif *et al.*, "Knowledge, attitudes and practices (KAP) relating to brucellosis in smallholder dairy farmers in two provinces in Pakistan," *PLoS ONE*, vol. 12, no. 3, p. e0173365, 2017.

[80] R. A. Laar *et al.*, "Impact of physical activity on challenging obesity in Pakistan: A knowledge, attitude, and practice (KAP) study," *Int. J. Environ. Res. Public Health*, vol. 17, no. 21, p. 7802, 2020.

[81] T. Ahmed *et al.*, "Knowledge, attitude and practice (KAP) survey of canine rabies in Khyber Pakhtunkhwa and Punjab Province of Pakistan," *BMC Public Health*, vol. 20, no. 1, p. 1293, 2020.

[82] S. Faisal, J. Khotib, and E. Zairina, "Knowledge, attitudes, and practices (KAP) towards COVID-19 among university students in Pakistan: A cross-sectional study," *J. Basic Clin. Physiol. Pharmacol.*, vol. 32, no. 4, pp. 681–686, 2021.

[83] A. Shahzad *et al.*, "Knowledge, attitude and practices (KAP) of the families of β-thalassaemia children in thalassaemia centers of Rawalpindi and Islamabad, Pakistan," *Blood Transfus.*, vol. 15, no. Suppl 1, p. s22.4, 2017.

[84] R. Kumar, R. Somrongthong, J. Ahmed, and A. J. Almarabheh, "Correlates of knowledge, attitude and practices about health care waste management among hospital workers of Pakistan," *J. Liaquat Univ. Med. Health Sci.*, vol. 17, no. 1, pp. 1–7, 2018.

[85] M. Saqlain *et al.*, "Knowledge, attitude and practice among healthcare professionals regarding COVID-19: A cross-sectional survey from Pakistan," *J. Hosp. Infect.*, vol. 105, no. 3, pp. 441–451, 2020.

[86] M. A. Khan, M. F. Khilji, A. Rauf, M. I. Janjua, and K. H. Altintas, "Hepatitis B and C related knowledge, attitudes and practices of health care workers in Azad Kashmir, Pakistan–A potentially disastrous area," 2019, [Online]. Available: Not specified.

[87] Z. U. Mustafa *et al.*, "Knowledge, attitude, practices, and barriers of pharmacovigilance among healthcare workers: A cross-sectional survey from Lahore, Pakistan," *Bull. Faculty Pharm. Cairo Univ.*, vol. 59, no. 1, pp. 33–43, 2021.

[88] F. Habib, D. K. Khan, F. Bhatti, and A. Zafar, "Knowledge and beliefs among health care workers regarding hepatitis B infection and needle stick injuries at a tertiary care hospital, Karachi," *J. Coll. Physicians Surg. Pakistan*, vol. 21, no. 5, p. 317, 2011.

[89] M. Mushtaq *et al.*, "Knowledge, attitudes and practices regarding tuberculosis in two districts of Punjab, Pakistan," *Int. J. Tuberc. Lung Dis.*, vol. 14, no. 3, pp. 303–310, 2010.

[90] M. Z. I. Hydrie *et al.*, "Assessment of tuberculosis related knowledge, attitude and practices among final year medical students in Karachi," *Ann. Abbasi Shaheed Hosp. Karachi Med. Dent. Coll.*, vol. 25, no. 3, pp. 165–171, 2020.

[91] C. Bell, G. Duncan, and B. Saini, "Knowledge, attitudes and practices of private sector providers of tuberculosis care: A scoping review," *Int. J. Tuberc. Lung Dis.*, vol. 15, no. 8, pp. 1005–1017, 2011.

[92] S. Ahmad *et al.*, "Assessing the knowledge, attitude and practice (KAP) measures against tuberculosis in patients in the ambulatory department facilities in Pakistan: A cross-sectional analysis," *Monaldi Arch. Chest Dis.*, vol. 93, no. 2, 2023.

[93] M. T. Khan *et al.*, "Prevalence of pyrazinamide resistance in Khyber Pakhtunkhwa, Pakistan," *Microb. Drug Resist.*, vol. 24, no. 9, pp. 1417–1421, 2018.

[94] S. Tahseen, A. Van Deun, B. C. de Jong, and T. Decroo, "Second-line injectable drugs for rifampicin-resistant tuberculosis: Better the devil we know?" *J. Antimicrob. Chemother.*, vol. 76, no. 4, pp. 831–835, 2021.

[95] A. Jabbar *et al.*, "Whole genome sequencing of drug resistant *Mycobacterium tuberculosis* isolates from a high burden tuberculosis region of North West Pakistan," *Sci. Rep.*, vol. 9, no. 1, p. 14996, 2019.

[96] E. Terracciano, F. Amadori, L. Zaratti, and E. Franco, "Tuberculosis: An ever present disease but difficult to prevent," *Igiene Sanita Pubblica*, vol. 76, no. 1, pp. 59–66, 2020.

[97] P. K. Drain *et al.*, "Incipient and subclinical tuberculosis: A clinical review of early stages and progression of infection," *Clin. Microbiol. Rev.*, vol. 31, no. 4, p. e00021-18, 2018.

[98] P. Supply *et al.*, "Genomic analysis of smooth tubercle bacilli provides insights into ancestry and pathoadaptation of *Mycobacterium tuberculosis*," *Nature Genet.*, vol. 45, no. 2, pp. 172–179, 2013.

[99] Y. Blouin *et al.*, "Progenitor '*Mycobacterium canettii*' clone responsible for lymph node tuberculosis epidemic, Djibouti," *Emerg. Infect. Dis.*, vol. 20, no. 1, pp. 21–28, 2014.

[100] R. Brosch *et al.*, "A new evolutionary scenario for the *Mycobacterium tuberculosis* complex," *Proc. Nat. Acad. Sci.*, vol. 99, no. 6, pp. 3684–3689, 2002.

[101] M. B. Reed *et al.*, "Major *Mycobacterium tuberculosis* lineages associate with patient country of origin," *J. Clin. Microbiol.*, vol. 47, no. 4, pp. 1119–1128, 2009.

[102] B. C. De Jong, M. Antonio, and S. Gagneux, "*Mycobacterium africanum*—Review of an important cause of human tuberculosis in West Africa," *PLoS Neglect. Trop. Dis.*, vol. 4, no. 9, p. e744, 2010.

[103] K. A. Alexander *et al.*, "Novel *Mycobacterium tuberculosis* complex pathogen, *M. mungi*," *Emerg. Infect. Dis.*, vol. 16, no. 8, pp. 1296–1299, 2010.

[104] D. Brites and S. Gagneux, "Co-evolution of *Mycobacterium tuberculosis* and *Homo sapiens*," *Immunol. Rev.*, vol. 264, no. 1, pp. 6–24, 2015.

[105] T. Paulson, "Epidemiology: A mortal foe," *Nature*, vol. 502, no. 7470, pp. S2–S3, 2013.

[106] World Health Organization, *Global tuberculosis report, 2020*, Geneva, Switzerland: WHO, 2020.

[107] National Tuberculosis Control Program, *DRTB national guidelines*, Islamabad, Pakistan: NTCP, 2020.

[108] S. Gagneux *et al.*, "The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*," *Science*, vol. 312, no. 5782, pp. 1944–1946, 2006.

[109] S. Gagneux, *Strain variation in the Mycobacterium tuberculosis complex: Its role in biology, epidemiology and control*, Springer, 2017.

[110] M. Coscolla *et al.*, "Phylogenomics of *Mycobacterium africanum* reveals a new lineage and a complex evolutionary history," *Microb. Genom.*, vol. 7, no. 2, p. 000477, 2021.

[111] J. C. S. Ngabonziza *et al.*, "A sister lineage of the *Mycobacterium tuberculosis* complex discovered in the African Great Lakes region," *Nature Commun.*, vol. 11, no. 1, p. 2917, 2020.

[112] J. O'Neill, "Review on antimicrobial resistance. Antimicrobial resistance: Tackling a crisis for the health and wealth of nations," 2014, [Online]. Available: Not specified.

[113] A. P. MacGowan, "Clinical implications of antimicrobial resistance for therapy," *J. Antimicrob. Chemother.*, vol. 62, no. suppl_2, pp. ii105–ii114, 2008.

[114] R. Laxminarayan *et al.*, "Antibiotic resistance-the need for global solutions," *Lancet Infect. Dis.*, vol. 13, no. 12, pp. 1057–1098, 2013.

[115] K. T. Kibret *et al.*, "Treatment outcomes for multidrug-resistant tuberculosis under DOTS-Plus: A systematic review and meta-analysis of published studies," *Infect. Dis. Poverty*, vol. 6, no. 1, p. 7, 2017.

[116] L. Zhang *et al.*, "Treatment outcomes of multidrug-resistant tuberculosis patients in Zhejiang, China, 2009–2013," *Clin. Microbiol. Infect.*, vol. 24, no. 4, pp. 381–388, 2018.

[117] L. S. Roope *et al.*, "The challenge of antimicrobial resistance: What economics can contribute," *Science*, vol. 364, no. 6435, p. eaau4679, 2019.

[118] C. A. Winston and K. Mitruka, "Treatment duration for patients with drug-resistant tuberculosis, United States," *Emerg. Infect. Dis.*, vol. 18, no. 7, pp. 1201–1202, 2012.

[119] T. Dalton *et al.*, "Prevalence of and risk factors for resistance to second-line drugs in people with multidrug-resistant tuberculosis in eight countries: A prospective cohort study," *Lancet*, vol. 380, no. 9851, pp. 1406–1417, 2012.

[120] M. Merker *et al.*, "Evolutionary history and global spread of the *Mycobacterium tuberculosis* Beijing lineage," *Nature Genet.*, vol. 47, no. 3, pp. 242–249, 2015.

[121] G. Alvarez-Uria, S. Gandra, and R. Laxminarayan, "Poverty and prevalence of antimicrobial resistance in invasive isolates," *Int. J. Infect. Dis.*, vol. 52, pp. 59–61, 2016.

[122] V. Eldholm *et al.*, "Armed conflict and population displacement as drivers of the evolution and dispersal of *Mycobacterium tuberculosis*," *Proc. Nat. Acad. Sci.*, vol. 113, no. 48, pp. 13881–13886, 2016.

[123] P. A. Zur Wiesch *et al.*, "Population biological principles of drug-resistance evolution in infectious diseases," *Lancet Infect. Dis.*, vol. 11, no. 3, pp. 236–247, 2011.

[124] D. Hughes and D. I. Andersson, "Evolutionary trajectories to antibiotic resistance," *Annu. Rev. Microbiol.*, vol. 71, pp. 579–596, 2017.

[125] J. Zhou *et al.*, "Selection of antibiotic-resistant bacterial mutants: Allelic diversity among fluoroquinolone-resistant mutations," *J. Infect. Dis.*, vol. 182, no. 2, pp. 517–525, 2000.

[126] C. B. Ford *et al.*, "*Mycobacterium tuberculosis* mutation rate estimates from different lineages predict substantial differences in the emergence of drug-resistant tuberculosis," *Nature Genet.*, vol. 45, no. 7, pp. 784–790, 2013.

[127] D. L. Huseby *et al.*, "Mutation supply and relative fitness shape the genotypes of ciprofloxacin-resistant *Escherichia coli*," *Mol. Biol. Evol.*, vol. 34, no. 5, pp. 1029–1039, 2017.

[128] L. Fenner *et al.*, "Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 56, no. 6, pp. 3047–3053, 2012.

[129] T. Vogwill, M. Kojadinovic, and R. MacLean, "Epistasis between antibiotic resistance mutations and genetic background shape the fitness effect of resistance across species of *Pseudomonas*," *Proc. Roy. Soc. B*, vol. 283, no. 1830, p. 20160151, 2016.

[130] K. A. Ängeby *et al.*, "Wild-type MIC distributions of four fluoroquinolones active against *Mycobacterium tuberculosis* in relation to current critical concentrations and available pharmacokinetic and pharmacodynamic data," *J. Antimicrob. Chemother.*, vol. 65, no. 5, pp. 946–952, 2010.

[131] N. Coeck *et al.*, "Correlation of different phenotypic drug susceptibility testing methods for four fluoroquinolones in *Mycobacterium tuberculosis*," *J. Antimicrob. Chemother.*, vol. 71, no. 5, pp. 1233–1240, 2016.

[132] R. Colangeli *et al.*, "Bacterial factors that predict relapse after tuberculosis therapy," *New England J. Med.*, vol. 379, no. 9, pp. 823–833, 2018.

[133] S. Borrell *et al.*, "Reference set of *Mycobacterium tuberculosis* clinical strains: A tool for research and product development," *PLoS ONE*, vol. 14, no. 3, p. e0214088, 2019.

[134] K. R. Wollenberg *et al.*, "Whole-genome sequencing of *Mycobacterium tuberculosis* provides insight into the evolution and genetic composition of drug-resistant tuberculosis in Belarus," *J. Clin. Microbiol.*, vol. 55, no. 2, pp. 457–469, 2017.

[135] S. Gagneux, "Ecology and evolution of *Mycobacterium tuberculosis*," *Nature Rev. Microbiol.*, vol. 16, no. 4, pp. 202–213, 2018.

[136] T. Vogwill, M. Kojadinovic, V. Furió, and R. C. MacLean, "Testing the role of genetic background in parallel evolution using the comparative experimental evolution of antibiotic resistance," *Mol. Biol. Evol.*, vol. 31, no. 12, pp. 3314–3323, 2014.

[137] National Tuberculosis Control Program, *National DRTB guidelines*, Islamabad, Pakistan: NTCP, 2020.

[138] M. Farhat *et al.*, "Drug-resistant tuberculosis: A persistent global health concern," *Nature Rev. Microbiol.*, pp. 1–19, 2024.

[139] D. I. Andersson, H. Nicoloff, and K. Hjort, "Mechanisms and clinical relevance of bacterial heteroresistance," *Nature Rev. Microbiol.*, vol. 17, no. 8, pp. 479–496, 2019.

[140] M. T. Zaw, N. A. Emran, and Z. Lin, "Mutations inside rifampicin-resistance determining region of rpoB gene associated with rifampicin-resistance in *Mycobacterium tuberculosis*," *J. Infect. Public Health*, vol. 11, no. 5, pp. 605–610, 2018.

[141] D. Mitchison, "Drug resistance in tuberculosis," *Eur. Respir. J.*, vol. 25, no. 2, pp. 376–379, 2005.

[142] C. P. Adikaram, J. Perera, and S. S. Wijesundera, "Geographical profile of rpoB gene mutations in rifampicin resistant *Mycobacterium tuberculosis* isolates in Sri Lanka," *Microb. Drug Resist.*, vol. 18, no. 5, pp. 525–530, 2012.

[143] A. R. Bahrmand *et al.*, "High-level rifampin resistance correlates with multiple mutations in the rpoB gene of pulmonary tuberculosis isolates from the Afghanistan border of Iran," *J. Clin. Microbiol.*, vol. 47, no. 9, pp. 2744–2750, 2009.

[144] O. Qazi *et al.*, "Mutation pattern in rifampicin resistance determining region of rpoB gene in multidrug-resistant *Mycobacterium tuberculosis* isolates from Pakistan," *Int. J. Mycobacteriol.*, vol. 3, no. 3, pp. 173–177, 2014.

[145] S. N. Khan *et al.*, "Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in Punjab, Pakistan," *Pakistan J. Zool.*, vol. 45, no. 1, pp. 93–100, 2013.

[146] A. Sajduda *et al.*, "Molecular characterization of rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland," *J. Clin. Microbiol.*, vol. 42, no. 6, pp. 2425–2431, 2004.

[147] Y. Tan *et al.*, "The beginning of the rpoB gene in addition to the rifampin resistance determination region might be needed for identifying rifampin/rifabutin cross-resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from Southern China," *J. Clin. Microbiol.*, vol. 50, no. 1, pp. 81–85, 2012.

[148] D. T. Hoagland *et al.*, "New agents for the treatment of drug-resistant *Mycobacterium tuberculosis*," *Adv. Drug Del. Rev.*, vol. 102, pp. 55–72, 2016.

[149] H. Blumberg *et al.*, "American Thoracic Society/Centers for Disease Control and Prevention/Infectious Diseases Society of America: Treatment of tuberculosis," *Am. J. Respir. Crit. Care Med.*, vol. 167, no. 4, pp. 603–662, 2003.

[150] M. A. Huaman and T. R. Sterling, "Treatment of latent tuberculosis infection—An update," *Clin. Chest Med.*, vol. 40, no. 4, pp. 839–848, 2019.

[151] S. Chakraborty and K. Y. Rhee, "Tuberculosis drug development: History and evolution of the mechanism-based paradigm," *Cold Spring Harbor Perspect. Med.*, vol. 5, no. 8, p. a021147, 2015.

[152] N. Lee and H. Nguyen, "Ethambutol," 2020, [Online]. Available: Not specified.

[153] K. Schubert *et al.*, "The antituberculosis drug ethambutol selectively blocks apical growth in CMN group bacteria," *MBio*, vol. 8, no. 1, p. e02213-16, 2017.

[154] C. Zhu *et al.*, "Molecular mechanism of the synergistic activity of ethambutol and isoniazid against *Mycobacterium tuberculosis*," *J. Biol. Chem.*, vol. 293, no. 43, pp. 16741–16750, 2018.

[155] M. S. Vasava *et al.*, "Drug development against tuberculosis: Past, present and future," *Indian J. Tuberc.*, vol. 64, no. 4, pp. 252–275, 2017.

[156] Y. Zhang, W. Shi, W. Zhang, and D. Mitchison, "Mechanisms of pyrazinamide action and resistance," *Microbiol. Spectr.*, vol. 2, no. 4, p. 2.4.03, 2014.

[157] D. Falzon *et al.*, "World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update," *Eur. Respir. J.*, vol. 49, no. 3, p. 1602308, 2017.

[158] A. D. Pranger, J. W. C. Alffenaar, and R. E. Aarnoutse, "Fluoroquinolones, the cornerstone of treatment of drug-resistant tuberculosis: A pharmacokinetic and pharmacodynamic approach," *Curr. Pharm. Des.*, vol. 17, no. 27, pp. 2900–2930, 2011.

[159] J. Garcia-Rodriguez and A. G. Garcia, "In-vitro activities of quinolones against mycobacteria," *J. Antimicrob. Chemother.*, vol. 32, no. 6, pp. 797–808, 1993.

[160] K. Drlica *et al.*, "Quinolones: Action and resistance updated," *Curr. Top. Med. Chem.*, vol. 9, no. 11, pp. 981–998, 2009.

[161] C. Xu *et al.*, "Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*," *J. Infect. Dis.*, vol. 174, no. 5, pp. 1127–1130, 1996.

[162] S. Kabir *et al.*, "Fluoroquinolone resistance and mutational profile of gyrA in pulmonary MDR tuberculosis patients," *BMC Pulm. Med.*, vol. 20, no. 1, p. 138, 2020.

[163] J. Espinosa-Pereiro, A. Sánchez-Montalvá, and M. L. Aznar, "MDR tuberculosis treatment," *Medicina*, vol. 58, no. 2, p. 188, 2022.

[164] S. Kadura *et al.*, "Systematic review of mutations associated with resistance to the new and repurposed *Mycobacterium tuberculosis* drugs bedaquiline, clofazimine, linezolid, delamanid and pretomanid," *J. Antimicrob. Chemother.*, vol. 75, no. 8, pp. 2031–2043, 2020.

[165] H. L. Haver *et al.*, "Mutations in genes for the F420 biosynthetic pathway and a nitroreductase enzyme are the primary resistance determinants in spontaneous in vitro-selected PA-824-resistant mutants of *Mycobacterium tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 59, no. 9, pp. 5316–5323, 2015.

[166] G. V. Bloemberg *et al.*, "Acquired resistance to bedaquiline and delamanid in therapy for tuberculosis," *New England J. Med.*, vol. 373, no. 20, pp. 1986–1988, 2015.

[167] T. V. A. Nguyen *et al.*, "Delamanid resistance: Update and clinical management," *Clin. Infect. Dis.*, vol. 71, no. 12, pp. 3252–3259, 2020.

[168] J. S. Yang *et al.*, "Delamanid, bedaquiline, and linezolid minimum inhibitory concentration distributions and resistance-related gene mutations in multidrug-resistant and extensively drug-resistant tuberculosis in Korea," *Ann. Lab. Med.*, vol. 38, no. 6, pp. 563–568, 2018.

[169] A. Slee *et al.*, "Oxazolidinones, a new class of synthetic antibacterial agents: In vitro and in vivo activities of DuP 105 and DuP 721," *Antimicrob. Agents Chemother.*, vol. 31, no. 11, pp. 1791–1797, 1987.

[170] D. J. Diekema and R. N. Jones, "Oxazolidinone antibiotics," *Lancet*, vol. 358, no. 9297, pp. 1975–1982, 2001.

[171] K. Shaw *et al.*, "In vitro activity of TR-700, the antibacterial moiety of the prodrug TR-701, against linezolid-resistant strains," *Antimicrob. Agents Chemother.*, vol. 52, no. 12, pp. 4442–4447, 2008.

[172] S. Khoshnood *et al.*, "Bedaquiline: Current status and future perspectives," *J. Glob. Antimicrob. Resist.*, vol. 25, pp. 48–59, 2021.

[173] K. Andries *et al.*, "Acquired resistance of *Mycobacterium tuberculosis* to bedaquiline," *PLoS ONE*, vol. 9, no. 7, p. e102135, 2014.

[174] N. Dookie *et al.*, "Evolution of drug resistance in *Mycobacterium tuberculosis*: A review on the molecular determinants of resistance and implications for personalized care," *J. Antimicrob. Chemother.*, vol. 73, no. 5, pp. 1138–1151, 2018.

[175] S. Kundu *et al.*, "Bedaquiline targets the ϵ subunit of mycobacterial F-ATP synthase," *Antimicrob. Agents Chemother.*, vol. 60, no. 11, pp. 6977–6979, 2016.

[176] H. Hameed *et al.*, "Molecular targets related drug resistance mechanisms in MDR-, XDR-, and TDR-*Mycobacterium tuberculosis* strains," *Front. Cell. Infect. Microbiol.*, vol. 8, p. 114, 2018.

[177] D. Almeida *et al.*, "Mutations in pepQ confer low-level resistance to bedaquiline and clofazimine in *Mycobacterium tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 60, no. 8, pp. 4590–4599, 2016.

[178] H. Ghajavand *et al.*, "High prevalence of bedaquiline resistance in treatment-naive tuberculosis patients and verapamil effectiveness," *Antimicrob. Agents Chemother.*, vol. 63, no. 3, p. e02530-18, 2019.

[179] H. Hoffmann *et al.*, "Delamanid and bedaquiline resistance in *Mycobacterium tuberculosis* ancestral Beijing genotype causing extensively drug-resistant tuberculosis in a Tibetan refugee," *Amer. J. Respir. Crit. Care Med.*, vol. 193, no. 3, pp. 337–340, 2016.

[180] N. Riccardi *et al.*, "Clofazimine: An old drug for never-ending diseases," *Future Microbiol.*, vol. 15, no. 7, pp. 557–566, 2020.

[181] S. Polsfuss *et al.*, "Emergence of low-level delamanid and bedaquiline resistance during extremely drug-resistant tuberculosis treatment," *Clin. Infect. Dis.*, vol. 69, no. 7, pp. 1229–1231, 2019.

[182] A. Ghodousi *et al.*, "Acquisition of cross-resistance to bedaquiline and clofazimine following treatment for tuberculosis in Pakistan," *Antimicrob. Agents Chemother.*, vol. 63, no. 9, p. e00915-19, 2019.

[183] D. Liu *et al.*, "Whole-genome sequencing for surveillance of tuberculosis drug resistance and determination of resistance level in China," *Clin. Microbiol. Infect.*, vol. 28, no. 5, pp. 731.e9–731.e15, 2022.

[184] I. Baussano *et al.*, "Tuberculosis among health care workers," *Emerg. Infect. Dis.*, vol. 17, no. 3, pp. 488–494, 2011.

[185] R. Joshi *et al.*, "Tuberculosis among health-care workers in low- and middle-income countries: A systematic review," *PLoS Med.*, vol. 3, no. 12, p. e494, 2006.

[186] D. Menzies, R. Joshi, and M. Pai, "Risk of tuberculosis infection and disease associated with work in health care settings," *Int. J. Tuberc. Lung Dis.*, vol. 11, no. 6, pp. 593–605, 2007.

[187] L. Uden, E. Barber, N. Ford, and G. S. Cooke, "Risk of tuberculosis infection and disease for health care workers: An updated meta-analysis," in *Open Forum Infect. Dis.*, Oxford, UK: Oxford Univ. Press, 2017.

[188] A. Vigenschow *et al.*, "Knowledge, attitudes and practices regarding tuberculosis amongst healthcare workers in Moyen-Ogooué Province, Gabon," *BMC Infect. Dis.*, vol. 21, no. 1, p. 486, 2021.

[189] L. Uden, E. Barber, N. Ford, and G. S. Cooke, "Risk of tuberculosis infection and disease for health care workers: An updated meta-analysis," in *Open Forum Infect. Dis.*, Oxford, UK: Oxford Univ. Press, 2017.

[190] A. Vigenschow *et al.*, "Knowledge, attitudes and practices regarding tuberculosis amongst healthcare workers in Moyen-Ogooué Province, Gabon," *BMC Infect. Dis.*, vol. 21, no. 1, p. 486, 2021.

[191] G. Demissie Gizaw, Z. Aderaw Alemu, and K. T. Kibret, "Assessment of knowledge and practice of health workers towards tuberculosis infection control and associated factors in public health facilities of Addis Ababa, Ethiopia: A cross-sectional study," *Arch. Public Health*, vol. 73, no. 1, p. 15, 2015.

[192] S. J. Hoffman *et al.*, "Surveying the knowledge and practices of health professionals in China, India, Iran, and Mexico on treating tuberculosis," *Am. J. Trop. Med. Hyg.*, vol. 94, no. 5, pp. 959–970, 2016.

[193] M. A. Khan, S. Mirza, and E. Qadeer, "TB control in Pakistan," in *Handbook of Global Tuberculosis Control: Practices and Challenges*, Springer, 2017, pp. 15–25.

[194] L. Bradner *et al.*, "Chemical decontamination with N-acetyl-l-cysteine–sodium hydroxide improves recovery of viable *Mycobacterium avium* subsp. *paratuberculosis* organisms from cultured milk," *J. Clin. Microbiol.*, vol. 51, no. 7, pp. 2139–2146, 2013.

[195] B. van Klingeren *et al.*, "Drug susceptibility testing of *Mycobacterium tuberculosis* complex by use of a high-throughput, reproducible, absolute concentration method," *J. Clin. Microbiol.*, vol. 45, no. 8, pp. 2662–2668, 2007.

[196] A. N. Umubyeyi *et al.*, "Limited fluoroquinolone resistance among *Mycobacterium tuberculosis* isolates from Rwanda: Results of a national survey," *J. Antimicrob. Chemother.*, vol. 59, no. 5, pp. 1031–1033, 2007.

[197] D. Van Soolingen *et al.*, "Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: Evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis," *J. Clin. Microbiol.*, vol. 29, no. 11, pp. 2578–2586, 1991.

[198] A. M. Bolger, M. Lohse, and B. Usadel, "Trimmomatic: A flexible trimmer for Illumina sequence data," *Bioinformatics*, vol. 30, no. 15, pp. 2114–2120, 2014.

[199] H. Li, "Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM," *arXiv*, 2013, [Online]. Available: arXiv:1303.3997.

[200] H. Li, "A statistical framework for SNP calling, mutation discovery, association mapping and population genetical parameter estimation from sequencing data," *Bioinformatics*, vol. 27, no. 21, pp. 2987–2993, 2011.

[201] J. Xie *et al.*, "Tree Visualization By One Table (tvBOT): A web application for visualizing, modifying and annotating phylogenetic trees," *Nucleic Acids Res.*, vol. 51, no. W1, pp. W587–W592, 2023.

[202] A. Noé *et al.*, "Knowledge, attitudes and practices regarding tuberculosis care among health workers in Southern Mozambique," *BMC Pulm. Med.*, vol. 17, no. 1, p. 72, 2017.

[203] World Health Organization, *Global tuberculosis report*, Geneva, Switzerland: WHO, 2023.

[204] C. H. Rodrigues, D. E. Pires, and D. B. Ascher, "DynaMut: Predicting the impact of mutations on protein conformation, flexibility and stability," *Nucleic Acids Res.*, vol. 46, no. W1, pp. W350–W355, 2018.

[205] R. Salomon-Ferrer, D. A. Case, and R. C. Walker, "An overview of the Amber biomolecular simulation package," *Wiley Interdiscip. Rev. Comput. Mol. Sci.*, vol. 3, no. 2, pp. 198–210, 2013.

[206] C. H. Rodrigues, D. E. Pires, and D. B. Ascher, "DynaMut: Predicting the impact of mutations on protein conformation, flexibility and stability," *Nucleic Acids Res.*, vol. 46, no. W1, pp. W350–W355, 2018.

[207] F. Iqbal *et al.*, "Understanding how geographic, demographic and treatment history impact health outcomes of patients with multi-drug-resistant tuberculosis in Pakistan, 2014-2017," *Epidemiol. Infect.*, vol. 148, p. e253, 2020.

[208] L. Wang *et al.*, "Mixed infections in genotypic drug-resistant *Mycobacterium tuberculosis*," *Sci. Rep.*, vol. 13, no. 1, p. 17100, 2023.

[209] N. Madadi-Goli *et al.*, "The importance of heteroresistance and efflux pumps in bedaquiline-resistant *Mycobacterium tuberculosis* isolates from Iran," *Ann. Clin. Microbiol. Antimicrob.*, vol. 23, no. 1, p. 36, 2024.

[210] Y. Zhao *et al.*, "National survey of drug-resistant tuberculosis in China," *New England J. Med.*, vol. 366, no. 23, pp. 2161–2170, 2012.

[211] M. Ejaz *et al.*, "Prevalence of multi-drug resistant tuberculosis in Karachi, Pakistan: Identification of at risk groups," *Trans. Roy. Soc. Trop. Med. Hyg.*, vol. 104, no. 8, pp. 511–517, 2010.

[212] Z. Mor *et al.*, "Drug-resistant tuberculosis in Israel: Risk factors and treatment outcomes," *Int. J. Tuberc. Lung Dis.*, vol. 18, no. 10, pp. 1195–1201, 2014.

[213] A. M. Ahmad *et al.*, "Risk factors for multidrug-resistant tuberculosis in urban Pakistan: A multicenter case–control study," *Int. J. Mycobacteriol.*, vol. 1, no. 3, pp. 137–142, 2012.

[214] I. Ullah *et al.*, "Pattern of drug resistance and risk factors associated with development of drug resistant *Mycobacterium tuberculosis* in Pakistan," *PLoS ONE*, vol. 11, no. 1, p. e0147529, 2016.

[215] S. Hameed *et al.*, "Drug resistance profile of *Mycobacterium tuberculosis* and predictors associated with the development of drug resistance," *J. Glob. Antimicrob. Resist.*, vol. 18, pp. 155–159, 2019.

[216] A. Javaid *et al.*, "Prevalence of primary multidrug resistance to anti-tuberculosis drugs in Pakistan," *Int. J. Tuberc. Lung Dis.*, vol. 12, no. 3, pp. 326–331, 2008.

[217] H. D. Meriki *et al.*, "Drug resistance profiles of *Mycobacterium tuberculosis* complex and factors associated with drug resistance in the Northwest and Southwest Regions of Cameroon," *PLoS ONE*, vol. 8, no. 10, p. e77410, 2013.

[218] O. Daniel and E. Osman, "Prevalence and risk factors associated with drug resistant TB in South West, Nigeria," *Asian Pac. J. Trop. Med.*, vol. 4, no. 2, pp. 148–151, 2011.

[219] A. Rukmana *et al.*, "*Mycobacterium tuberculosis* lineage distribution using whole-genome sequencing and bedaquiline, clofazimine, and linezolid phenotypic profiles among rifampicin-resistant isolates from West Java, Indonesia," *Int. J. Microbiol.*, vol. 2024, no. 1, p. 2037961, 2024.

[220] N. Maghradze, "Molecular epidemiology of '*Mycobacterium tuberculosis*' in the country of Georgia," Ph.D. dissertation, Univ. Basel, Basel, Switzerland, 2024.

[221] J. Xu *et al.*, "Bedaquiline resistance mutations: Correlations with drug exposures and impact on the proteome in *M. tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 67, no. 7, p. e01532-22, 2023.

[222] F. Rashid *et al.*, "Investigation of bedaquiline heteroresistance among *Mycobacterium tuberculosis* isolates from Pakistan," *Microbiol. Spectr.*, vol. 13, no. 1, p. e02181-24, 2025.

[223] C. Nimmo *et al.*, "Dynamics of within-host *Mycobacterium tuberculosis* diversity and heteroresistance during treatment," *EBioMedicine*, vol. 55, p. 102747, 2020.

[224] A. M. Akhtar *et al.*, "Prevalence and drug resistance pattern of MDR TB in retreatment cases of Punjab, Pakistan," *J. Pak. Med. Assoc.*, vol. 66, no. 8, pp. 989–993, 2016.

[225] C. Zheng *et al.*, "Mixed infections and rifampin heteroresistance among *Mycobacterium tuberculosis* clinical isolates," *J. Clin. Microbiol.*, vol. 53, no. 7, pp. 2138–2147, 2015.

[226] G. Napier *et al.*, "Robust barcoding and identification of *Mycobacterium tuberculosis* lineages for epidemiological and clinical studies," *Genome Med.*, vol. 12, no. 1, p. 114, 2020.

[227] F. Coll *et al.*, "Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*," *Nature Genet.*, vol. 50, no. 2, pp. 307–316, 2018.

[228] A. S. Khan *et al.*, "Characterization of rifampicin-resistant *Mycobacterium tuberculosis* in Khyber Pakhtunkhwa, Pakistan," *Sci. Rep.*, vol. 11, no. 1, p. 14194, 2021.

[229] G. Napier *et al.*, "Characterisation of drug-resistant *Mycobacterium tuberculosis* mutations and transmission in Pakistan," *Sci. Rep.*, vol. 12, no. 1, p. 7703, 2022.

[230] D. K. Saeed *et al.*, "Variants associated with bedaquiline (BDQ) resistance identified in Rv0678 and efflux pump genes in *Mycobacterium tuberculosis* isolates from BDQ naïve TB patients in Pakistan," *BMC Microbiol.*, vol. 22, no. 1, p. 62, 2022.

[231] A. Ali *et al.*, "*Mycobacterium tuberculosis* Central Asian Strain (CAS) lineage strains in Pakistan reveal lower diversity of MIRU loci than other strains," *Int. J. Mycobacteriol.*, vol. 3, no. 2, pp. 108–116, 2014.

[232] A. Jabbar *et al.*, "Whole genome sequencing of drug resistant *Mycobacterium tuberculosis* isolates from a high burden tuberculosis region of North West Pakistan," *Sci. Rep.*, vol. 9, no. 1, p. 14996, 2019.

[233] A. Kanji *et al.*, "Alternate efflux pump mechanism may contribute to drug resistance in extensively drug-resistant isolates of *Mycobacterium tuberculosis*," *Int. J. Mycobacteriol.*, vol. 5, no. Suppl 1, pp. S97–S98, 2016.

[234] A. Ali *et al.*, "Whole genome sequencing based characterization of extensively drug-resistant *Mycobacterium tuberculosis* isolates from Pakistan," *PLoS ONE*, vol. 10, no. 2, p. e0117771, 2015.

[235] C. Consortium *et al.*, "Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing," *New England J. Med.*, vol. 379, no. 15, pp. 1403–1415, 2018.

[236] J. Werngren *et al.*, "Detection of pyrazinamide heteroresistance in *Mycobacterium tuberculosis*," *Antimicrob. Agents Chemother.*, vol. 65, no.

Appendix - I

TB KAP Questionnaire

Tuberculosis is the second leading cause of infectious disease killers in the world, and in order to popularize and strengthen the knowledge of tuberculosis prevention and control, we invite you to participate in this questionnaire on "Tuberculosis knowledge, attitudes and practices". The study is an anonymous online questionnaire and does not ask for any of your personal information. Your participation in this survey is completely voluntary and free, and you have the right not to participate or to withdraw from the survey at any time without any discrimination or unfair treatment. The results of the questionnaire are summarized statistically, and all information you fill in will be treated with the strictest confidentiality, and we will only explore the results of the survey in groups for academic purposes.

Tips: This questionnaire is expected to take 5-10 minutes, please answer carefully. Your valuable insights will inform important research on TB prevention and control.

1. How old are you?
2. What is your gender?
    A. male
    B. female
3. What is your academic background?
    A. elementary school

B. secondary school

C. Technical secondary school or junior college

D. undergraduate

E. Graduate student or above

4. What is your current work/study unit:

A. Tuberculosis Specialist Hospital

B. General Hospital

C. Cdc

D. Medical

E. other

5. Your job title is:

A. doctor

B. nurse

C. Medical technicians

D. Hospital infection control personnel

E. Laboratory staff

F. Students/interns

G. Researchers

H. other

6. Have you received any training for TB in the past 6 months?

A. yes

B. no

7. Have you ever had tuberculosis?

A. yes

B. no

8. Have people you had close contact with have had TB (e.g., family member, spouse, etc.)?

   A. yes

   B. no

Knowledge

Tuberculosis basics

1. What is the causative agent of tuberculosis?

   A. *Mycobacterium tuberculosis*

   B. *Mycobacterium avium*

   C. *Mycobacterium pneumoniae*

   D. *Mycobacterium leprae*

2. How is tuberculosis transmitted?

   A. Sexual transmission

   B. Airborne droplet transmission

   C. Direct contact transmission

   D. By sharing needles

3. Who is at higher risk of developing TB?

   A. Patients with chronic obstructive pulmonary disease

   B. People with HIV

   C. male

   D. People who live in the tropics

4. What are Preventive measures for tuberculosis?

   A. Use a mask

   B. Avoid people who are coughing

C. Avoid close contact

D. All of the above

Tuberculosis diagnosis

1. What are the most common symptoms of tuberculosis?

   A. hemoptysis

   B. Persistent high fever

   C. Weight loss

   D. Persistent cough

2. What is the best diagnostic tool for tuberculosis?

   A. Blood cultures

   B. Tuberculin skin test

   C. Tuberculosis culture

   D. Microscopic examination of sputum

3. Is tuberculosis in children harder to diagnose than in adults?

   A. yes

   B. no

4. Do you know what Gene Xpert is?

   A. Know

   B. I don't know

Tuberculosis treatment

1. Is tuberculosis a curable disease?

   A. yes

B. no

2. At least how long is first-line drug therapy for treatment-naïve non-resistant tuberculosis?

   A. 2 months

   B. 6 months

   C. 9 months

   D. 12 months

3. How many drugs are included in the first-line drug regimen for tuberculosis?

   A. 1

   B. 2

   C. 3

   D. 4

4. Did you know the direct-to-face short-course supervised chemotherapy (DOTS) strategy in the initial stages of TB treatment?

   A. yes

   B. no

5. What is MDR-TB?

   A. Resistant to both isoniazid and rifampicin

   B. Resistance to pyrazinamide and ethambutol

   C. Resistant to any anti-tuberculosis drug

   D. Resistant to all anti-tuberculosis drugs

6. In which group of people is MDR-TB most likely to occur?

   A. People with HIV

   B. People who have been in close contact with tuberculosis patients

   C. People who have been previously treated for tuberculosis

D. People who have never had tuberculosis

7. What are the consequences of non-standard anti-TB treatment?

   A. Development of drug-resistant tuberculosis

   B. Not completely cured

   C. Further spread of tuberculosis

   D. All of the above

8. What is the best time to get BCG vaccination?

   A. At birth

   B. 2 months after birth

   C. 6 months after birth

Attitude

1. Timely detection of new cases is essential to control TB

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

2. Public awareness of tuberculosis is adequate

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

3. Direct face-to-face short-course supervised chemotherapy (DOTS) is used to increase the success rate of treatment

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

4. Do TB patients feel discriminated against?

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

5. In tuberculosis prevention and control, the investment in education is more worthwhile than the investment in short-course supervised chemotherapy (DOTS) under direct observation.

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

6. Multidrug-resistant tuberculosis (MDR-TB) is a problem in our country

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

E. Strongly opposed

7. There are many barriers to TB treatment

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

8. Infection control is an important means of preventing tuberculosis

   A. Couldn't agree more

   B. agree

   C. neutrality

   D. oppose

   E. Strongly opposed

9. Do you often worry that you have tuberculosis?

   A. Always worried

   B. Constant worry

   C. Occasionally worried

   D. Never worried

   E. Didn't think about it

10. If I contracted tuberculosis, the unit allowed me to continue working

    A. Couldn't agree more

    B. agree

    C. neutrality

    D. oppose

    E. Strongly opposed

Practice

1. When should the first sputum sample be taken?

   A. Collected immediately at presentation

   B. 2-3 hours after presentation

   C. Deep sputum is collected the next morning

2. How should sputum samples be stored before they are sent to the laboratory?

   A. in culture medium

   B. It cannot be stored and should be sent to the laboratory urgently

   C. Store in the refrigerator at 4 ℃

3. When should a sputum sample be collected for the first follow-up visit after antituberculosis therapy is initiated?

   A. 1 month post-treatment

   B. 2 months post-treatment

   C. 3 months post-treatment

4. What are the main ways to monitor the effectiveness of anti-TB treatment?

   A. Chest X-ray

   B. Examination of a sample of sputum

5. The patient is a young male with persistent cough for half a month, night sweats, fatigue, and no shortness of breath, and his younger brother, who lives with him, has had the same symptoms since a month ago. What is the most likely diagnosis for this patient, and what method is used to assist in the diagnosis?

   A. Asthma, CRP

   B. Cough, blood clotting test

   C. Tuberculosis, sputum for tuberculosis bacteria test

6. What medications will you use in the initial stages of TB treatment, and for how long?

   A. Pyrazinamide, isoniazid, rifampicin, and ethambutol, 2 months

   B. bedaquiline, rifampicin, 6 months

   C. Ethambutol, bedaquiline, 4 months

7. What medications will you use during the continuation phase of your TB treatment and for how long?

   A. Isoniazid and rifampicin, 4 months

   B. Isoniazid, rifampicin, and ethambutol, 5 months

   C. Fluoroquinolones, pyrazinamide, and bedaquiline, 8 months

8. What do you do if a patient completes an initial course of treatment and the sputum sample is positive for TB bacteria?

   A. Assess for drug resistance and adjust treatment regimens

   B. Continue with the previous medication regimen

   C. I do not know

9. What should I do if my skin turns yellow after three weeks of anti-TB treatment?

   A. No special handling is required

   B. Discontinue treatment

Appendix - II

LOWENSTEIN-JENSEN (LJ) MEDIUM

Principle

Lowenstein-Jensen (LJ) medium is most widely used for tuberculosis culture. LJ medium containing glycerol favors the growth of *M. tuberculosis* while LJ medium without glycerol but containing Pyruvate encourages the growth of *M. bovis* as well as drug resistant strains of *M. tuberculosis*. The malachite green suppresses the growth of non-acid fast organisms. (L- Asparagine for nitrogen source).

Homogenization of egg

- Select eggs not older than 7 days for the preparation of egg fluid. (Note: Hens should be fed on food without antibiotics)
- Check Fresh eggs for minimum air space are checked for viability and is done by candling method.
- Clean eggs with soap water; Place in a basin and wash in running water until the water is clear, then rinse in distilled water and then again immerse finally in 70% alcohol for 5 minutes; Place the eggs on a clean towel to dry.
- Break the eggs individually and transfer into a stainless steel beaker and transfer the egg fluid into a 2 liters round flat bottomed flask.
- Homogenize the egg fluid using a mechanical egg churner.
- Filter the egg fluid using a sterile gauze and funnel.

- Measure one litre of egg fluid using a sterile measuring cylinder and transfer into a 3 or 5 litres conical flask.
- Transfer 600 ml of the sterilized mineral salt malachite green solution to the egg fluid.
- Gently shake to mix thoroughly.
- Fix the pourer to the mouth of the conical flask and distribute approximately 6 ml of medium in Universal container (McCartney bottle).

Coagulation of media

- Pour distilled water into the Inspissator tank through the side opening up to the mark
- Place the bottles in the Inspissator to coagulate the media for 60 minutes at 85^{o}C -90 ^{o}C
- Remove after 60 minutes from the Inspissator and leave at room temperature.
- Record the Inspissator temperature periodically in a note book (every 15 minutes)
- Re Inspissator the bottles at 85^{o}C - 90^{o}C C for 30 minutes on the consecutive day after overnight storage at room temperature.
- Label the media tray with batch number and date of preparation. The same should be recorded in the Media Preparation Register.

Sterility check

After inspissation randomly the whole media batch should be incubated at 37^o C for 24 hours select 2 bottles of plain LJ for sterility check and record in the Media Sterility register.