Correlation of Mutations with the Minimum Inhibitory Concentrations of Isoniazid Drug among Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates in Bangladesh

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Declaration

I hereby declare that the thesis entitled 'Correlation of Mutations with the Minimum Inhibitory Concentrations of Isoniazid Drug among Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates in Bangladesh' is my own work done under joint supervision of Dr. Syed Mohammad Mazidur Rahman, Associate Scientist, Programme on Emerging Infections, Infectious Diseases Division (IDD), International Centre for Diarrheal Disease Research, Bangladesh (icddr,b) and Dr. Iftekhar Bin Naser, Associate Professor, Department of Mathematics and Natural Sciences

It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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CERTIFICATE OF APPROVAL

We hereby declare that the thesis entitled **'Correlation of Mutations with the Minimum Inhibitory Concentrations of Isoniazid Drug among Isoniazid-Resistant *Mycobacterium tuberculosis* Isolates in Bangladesh'** is from the student's own work and effort, and all other sources of information used have been acknowledged. This Thesis has been submitted with our approval.

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Abstract:

Tuberculosis is still one of the major causes of mortality in the globe. Isoniazid (INH), a first-line medication used to treat tuberculosis is becoming increasingly resistant. With the rise of INH resistance we need more data on the genes responsible for the mutations and their correlated MIC values. In our study, we tested the MIC (Minimal inhibitory concentration) of 47 INH resistant Mycobacterium tuberculosis isolates that were obtained from Xpert MTB/RIF positive pulmonary tuberculosis patients diagnosed from TB Screening and Treatment Centers located at Dhaka, Sylhet and Chittagong divisions. Xpert XDR assay has been used in this study, which is a nested real time PCR application that can detect Mycobacterium tuberculosis as well as resistance to six leading antibiotics for TB treatment - INH (isoniazid), AMK (amikacin), KAN (Kanamycin), CAP (Capreomicin), FLQ (Fluroquinolone), and ETH (Ethionamide). The four most prevalent mutations that are responsible for INH resistance- KatG, inhA, fabG147 and ahpC can also be detected with the Xpert/XDR assay. About 485 of the isolates were tested by the Xpert/XDR assay. Isoniazid (INH) resistant samples were identified and were further tested for MIC (Minimal inhibitory concentration) on a 96-well plate ranging from 0.0156 ug/ml to 8 ug/ml. Among 485 isolates tested for Xpert/XDR assay, 47 were found to be INH resistant. The Xpert XDR assay results INH of mutation regions were then compared with the MIC value of the respective isolate. It was found that among the 47 INH Resistant samples examined for MIC, those with the KatG mutation had the highest MIC value - 8 ug/ml - and also the highest frequency among the isolates, accounting for about 61.7% (29 isolates). The mutation in the inhA promoter region was found in around 25.53% (12 isolates) of the isolates, having MIC values ranging from 0.03125 ug/ml to 2 ug/ml. The ahpC and FabG1 genes had the fewest cases, each accounting for one case with a modest MIC value of 1 ug/ml. In a few cases, double mutations have been identified, which are thought to lead to a higher MIC value. This study provides some insight into the frequency of INH resistance patterns in this country, as well as the related MIC values with the most prevalent mutations. More research and data are required in order to clearly anticipate the INH drug resistance mutation pattern and the drug concentration associated with the mutations in order to battle MDR-TB/XDR-TB and resist AMR (Antimicrobial resistance) in the field of tuberculosis.

SIGNIFICANCE OF THIS STUDY

1. Monitoring Resistance Trends:

Monitoring changes in the prevalence of resistance-associated genes and their minimal inhibitory concentrations over time provides valuable information about evolving resistance patterns

2. Treatment Selection with effective dose:

Knowledge of the genes associated with MIC values helps clinicians make informed decisions about antibiotic treatment.

Objectives

1. ldentify Mutations

   To Identify the Most Prevalent Mutations conferring INH resistance in Bangladeshi strains.

2. Determine MIC Values

   To Measure the MIC Value of Each Resistant Isolate and Find the Most Frequent Ones.

3. Correlate Mutations with MIC

   To Understand If Certain Mutations Are Associated with Certain Levels Of Drug Resistance

4. Classify Resistance Profiles
   To Group the Isolates Based on Their Resistance Profile with Associated Mutations

5. Evaluate Phenotypic and Genotypic Concordance
   To Find Out the Concordance Between Xpert MTB/XDR Assay With MIC And DST.

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Abbreviations

TB: Tuberculosis

MDR-TB: Multi-Drug Resistant Tuberculosis

XDR-TB: Extensively Drug Resistant Tuberculosis

pre-XDR-TB:pre Extensively Drug Resistant Tuberculosis

LTBI: Latent Tuberculosis Infection

DST: Drug Susceptibility Testing

PCR: Polymerase Chain Reaction

TB-HIV: Tuberculosis-Human Immunodeficiency Virus Co-infection

DOTS: Directly Observed Treatment, Short-Course

WHO: World Health Organization

M.tb: Mycobacterium tuberculosis (the bacterium causing TB)

RIF: Rifampicin (an antibiotic used in TB treatment)

INH: Isoniazid (an antibiotic used in TB treatment)

AMK: Amikacin (an antibiotic used in TB treatment)

KAN: Kanamycin (an antibiotic used in TB treatment)

CAP: Capreomycin (an antibiotic used in TB treatment)

FLQ: Fluoroquinolone (an antibiotic used in TB treatment)

ETH: Ethionamide (an antibiotic used in TB treatment)

SLID: second-line injectable drugs

PZA: Pyrazinamide (an antibiotic used in TB treatment)

EMB: Ethambutol (an antibiotic used in TB treatment)

IPT: Isoniazid Preventive Therapy

Chapter 1

Introduction:

Chapter 1

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1.1. Tuberculosis and Isoniazid Drug Medication

Tuberculosis is a global health issue accounting for hundreds of deaths each year. In 2021, 1.6 million people died from tuberculosis (including 187 000 HIV patients). TB is the 13th largest cause of mortality worldwide and the second leading infectious agent after COVID-19 (behind HIV and AIDS) .6.7% of all TB cases were among HIV-positive persons (WHO 2022). Geographically, the WHO areas with the highest number of TB cases in 2021 were South-East Asia (45%), Africa (23%), and the Western Pacific (18%), with lesser proportions in the Eastern Mediterranean (8.1%), the Americas (2.9%), and Europe (2.2%) (WHO, 2022). Globally, an estimated 10.6 million individuals contracted tuberculosis (TB) till 2021. Six million males, 3.4 million women, and 1.2 million children make up the population (WHO, 2021). Tuberculosis (TB) affects all nations and age groups. However, tuberculosis is both treatable and avoidable. MDR-TB is a public health concern as well as a health security hazard. In 2021, just around one-third of patients with drug-resistant tuberculosis received treatment. Between 2000 and 2021, TB detection and treatment saved an estimated 74 million lives (WHO, 2021).

Globally, the number of newly diagnosed and officially reported cases of tuberculosis dropped from 7.1 million in 2019 to 5.8 million in 2020 (-18%), with a partial rebound to 6.4 million in 2021 (WHO, 2022). Increases in the number of persons who aren't identified and consequently not treated for tuberculosis result in an increase in the number of TB fatalities (WHO, 2022) . Because there are more persons with untreated TB, the impact of lower case detection on TB mortality is severe and rapid (WHO, 2022). The cumulative drop in TB incidence rate between 2015 and 2020 was 13.5%, however the level in 2021 was only 10% lower than in 2015(WHO, 2022).

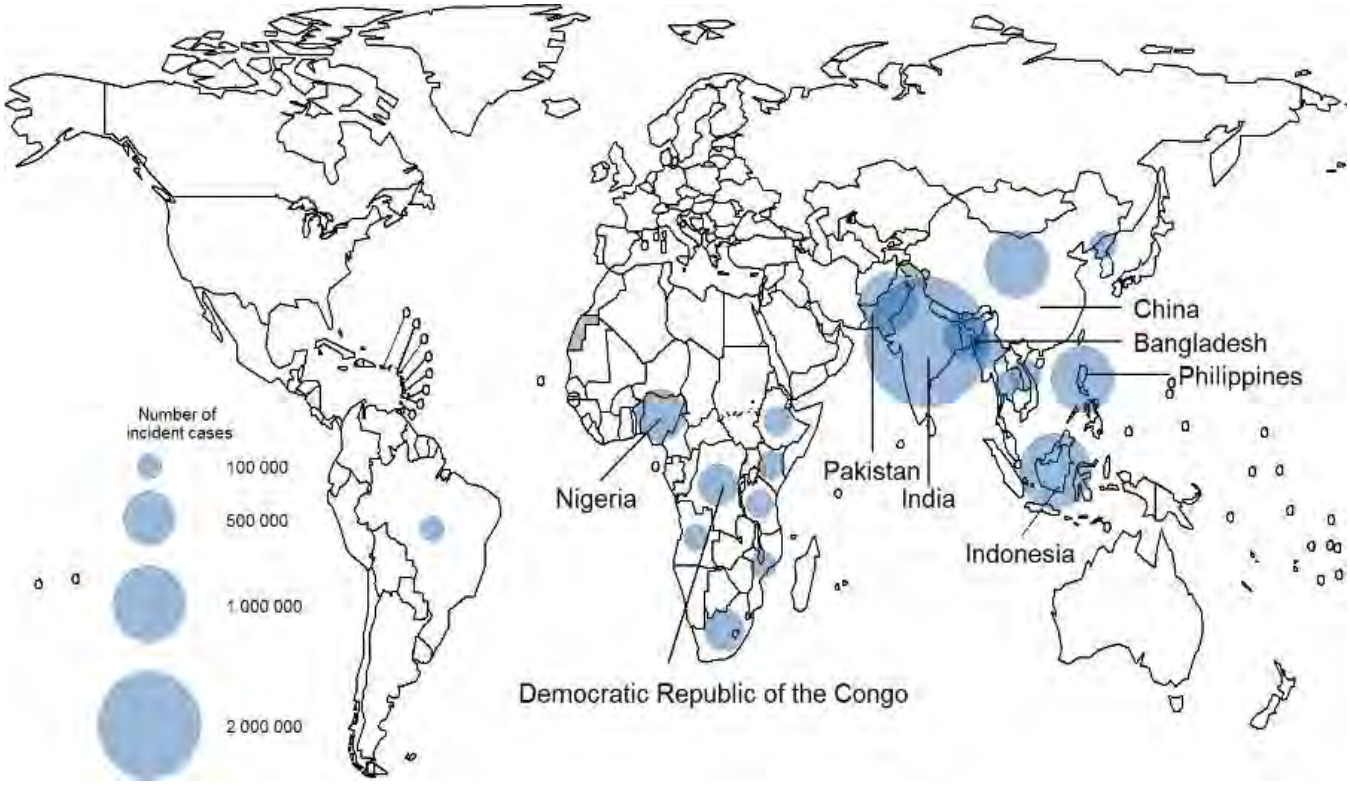

Figure 1: Estimated TB incidence in 2021, for countries with at least 100 000 incident cases (WHO, 2022)

In 2021, India (28%), Indonesia (9.2%), China (7.4%), the Philippines (7.0%), Pakistan (5.8%), Nigeria (4.4%), Bangladesh (3.6%), and the Democratic Republic of the Congo (2.9%) accounted for more than two-thirds of worldwide TB cases (WHO, 2022). The eight nations ranked first through eighth in terms of case count, accounting for two-thirds of global cases in 2021, are labeled in the figure 1. In terms of the number of incident TB cases per 100 000 population per year, the intensity of national TB epidemics varies greatly among nations (Fig. 2) (WHO, 2022). Countries having the highest rates in 2021 were largely in the WHO African Region (WHO, 2022). The

proportion of patients infected with HIV who had a new episode of tuberculosis (either new or relapse cases) was likewise greatest in WHO African Region nations, topping 50% in portions of southern Africa (WHO, 2022).

Figure 2: Estimated TB incidence rates, 2021 (WHO, 2022)

Despite the prevalence and expansion of active TB in the public, modern medication is efficient in obtaining bacteriological cure. TB identification and treatment are expected to have saved 74 million lives between 2000 and 2021 (WHO, 2021). Patient noncompliance is the primary impediment to the widespread utilization of traditional TB management approaches. This is due to the current TB treatment's extended duration and use of toxic drugs, which frequently cause major

adverse effects in patients. Incomplete treatment and patient noncompliance are typical causes of drug-resistant MTB strains (Prasad el al., 2005). By WHO 2022, countries that had met the End TB Strategy's 2020 target for decreasing TB incidence rate by 2021 are 77 in number, but unfortunately Bangladesh is not one of them.

Figure 3: Countries which, by 2021, had reached the 2020 milestone of the End TB Strategy for reducing the TB incidence rate (WHO, 2022)

As a matter of fact, Bangladesh lies among the 30 High TB Burden countries (WHO, 2022). The severity of the situation has worsened with the emergence of multidrug-resistant (MDR) MTB strains that are resistant to INH and RIF and (Extensively Drug Resistant) XDR-TB disease caused by a strain of M. tuberculosis complex that is resistant to rifampicin (and may also be resistant to

isoniazid), and that is also resistant to at least one fluoroquinolone (levofloxacin or moxifloxacin) and to at least one other "Group A" drug (bedaquiline or linezolid) (WHO, 2022). According to the World Health Organization (WHO), 4.1% of new cases and 19% of previously treated cases in 2018 had rifampicin-resistant TB (RR-TB) or multidrug-resistant TB (MDR-TB), with up to 6.2% of MDR patients having extensively drug-resistant TB (XDR-TB) (WHO, 2019).

Despite the fact that MTB develops resistance to the drugs used to treat TB, isolates resistant to INH are found considerably more frequently than strains resistant to other treatments (1 in 106 bacilli) (Nachega and Chaisson, 2003). Furthermore, resistance to INH, either alone or in combination with other drugs, is the second most common kind of resistance reported in TB patients. INH resistance is discovered in 10.3% of new cases, 27.7% of treated patients, and 13.3% of combined cases worldwide (WHO, 2008). Globally, the rate of INH-resistant (INHR) TB infections is increasing, posing a severe challenge to the efficacy of TB therapy (Cattamanchi et al., 2009).

According to recent systematic reviews and meta-analyses, INH mono-resistance is associated with a lower likelihood of proper treatment outcomes for patients following conventional therapy (Menzies et al., 2009). INH resistance not only reduces the chance of effective treatment, but it may also increase the spread of MDR-TB and degrade the efficacy of IPT - Isoniazid Preventive Therapy (Jenkins et al., 2011). IPT is the use of INH to treat people who have been exposed to/infected with MTB but do not show clinical indications of current illness, a condition known as latent TB infection (LTBI) (Akolo et al., 2010). For these reasons, determining the scope of INHr TB is critical.

1.2: Isoniazid Drug Mechanism and Genes Associated with its Mutation

Isoniazid (INH) is one of the most effective medications used to treat tuberculosis (TB) over the world. INH has also been used to prevent the reactivation of latent Mycobacterium tuberculosis (MTB) infection in humans (Unissa et al., 2016). It is worth noting that MTB strain resistance to INH and rifampicin (RIF) serves as the foundation for the idea of multidrug resistance (MDR) in tuberculosis (Unissa et al., 2016). Despite its simple chemical makeup, INH has a complex and multidimensional method of action. Numerous macromolecular synthesis routes are affected, most notably mycolic acid production (Unissa et al., 2016).

Since its discovery as a strong antituberculosis medication in 1952, INH has also been a cornerstone of TB chemotherapy for over half a century (Pansy et al., 1952; Fox, 1952; Bernstein et al., 1952). INH is a prodrug, and its anti tuberculosis effect is dependent on in vivo activation by KatG, an enzyme having catalase and peroxidase activity. The role of KatG in INH action was originally suggested by an apparent link between KatG catalase activity reduction and INH resistance that was later verified by a genetic research (Middlebrook & Dressler, 1954; Zhang et al., 1992; Lei et al., 2000). Clinical *M. tuberculosis* isolates that were resistant to INH were later shown to have different mutations in the katG gene (Altamirano et al., 1994; Heym et al., 1995; Musser et al., 1996). INH activation inhibits the formation of mycolic acid, a fatty acid-containing long chain component of the mycobacterial cell wall (Winder & Collins, 1970; Takayama et al., 1972; Lei et al., 2000). The active inhibitor(s) target two enzymes involved in the elongation cycle of fatty acid biosynthesis, namely enoyl-acyl carrier protein reductase (InhA) (Banerjee et al.,1994; Quemard et al., 1995; Lei et al., 2000) and -ketoacyl-acyl carrier protein synthase (Mdluli et al., 1998; Lei et al., 2000).

In contrast, arylamine N-acetyltransferases (NATs) deactivate INH (Unissa et al., 2016). As a result, numerous genes are involved in different biosynthetic networks and pathways in the molecular processes of INH resistance (Unissa et al., 2016).

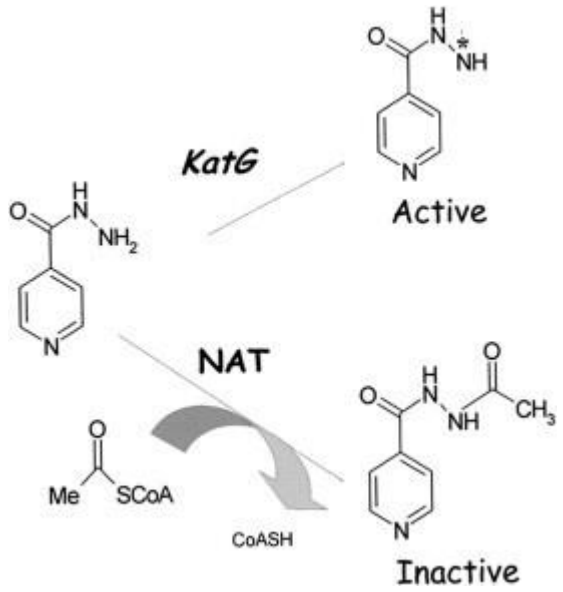

Figure 4: Schematic showing the activation and inactivation of INH (Jena et al., 2015).

Here, INH activation and inactivation are depicted schematically (Jena et al., 2015). INH is a prodrug that has to be activated by the catalase-peroxidase protein (KatG gene product) (Jena et al., 2015). The NAT enzymes have the ability to N-acetylate INH, rendering it therapeutically inactive(Jena et al., 2015). In the active form of INH, the asterisk adjacent to the terminal nitrogen suggests a kind of oxidized species (Jena et al., 2015). INH stands for isoniazid, while NAT is for N-acetyltransferase.

The most common source of INH resistance is a mutation in the katG gene, followed by fabG1 inhA, OxyR-ahpC intergenic region, kasA, ndh, fadE, furA, Rv1592c, and Rv1772 (Unissa et al., 2016). The recent link between efflux genes and INH resistance has also received a lot of attention.

Recently, mutations in nat, fabD, and accD have been reported in resistant isolates (Unissa et al., 2016). Another research suggests that furA and the iniBAC (iniA, iniB, and iniC) area are also linked to INH resistance, and that single nucleotide polymorphisms in efpA were found in both INH-resistant and -susceptible isolates (Ramaswamy et al., 2003). They also found 17 (44.7%) INH-resistant isolates carrying a single-locus, resistance-associated mutation in the katG, mabA, or Rv1772 genes (Ramaswamy et al., 2003). Seventeen (44.7%) INH-resistant isolates showed resistance-associated mutations in more than one gene, and the katG gene was mutated in 76% of all INH-resistant isolates (Ramaswamy et al., 2003). Mutations were also discovered in the genes fadE24, Rv1592c, Rv1772, Rv0340, and iniBAC (Ramaswamy et al., 2003).

In a study conducted in Brazil, katG mutations were detected in 83 (85.6%) of the 97 INH-resistant isolates, including codon 315 alterations in 60 (61.9%) of the INH-resistant isolates and 23 previously unreported katG mutations, mutations in the inhA promoter area were found in 25 (25.8%) of the INH-resistant isolates; 6.2% had inhA structural gene mutation, and 10.3% had oxyR-ahpC intergenic region mutations (one, nucleotide 48, previously unreported) (Cardoso et al., 2004). Besides, Two more novel mutations were identified in the KatG gene conferring resistance to INH - W341R KatG and L398P KatG (Hsu et al., 2020). A 2018 study also reported five novel mutations in the KatG region detected by recombineering method, not documented earlier - *katG* W161Q, W161R, E402stop, L415P, and A480del (Kandler et al., 2018). Apart from this the commonly found INH-associated genes noted in another study include *katG* (*Rv1908c*), *inhA* (*Rv1484*), *furA* (*Rv1909c*), *fabG1* (*Rv1483*), *ahpC* (*Rv2428*), and other INH-associated loci found in the literature are - *kasA* (*Rv2245*), *srmR* (*Rv2242*), *ndh* (*Rv1854c*), *iniB* (*Rv0341*), *iniA* (*Rv0342*),

iniC (*Rv0343*), *Rv0340*, *nat* (*Rv3566c*), *Rv1592c*, *fadE24* (*Rv3139*), *Rv1772*, *efpA* (*Rv2846c*), *fabD* (*Rv2243*), *accD6* (*Rv2247*), or *fbpC* (*Rv0129*) (Wilson et al., 1999).

1.3 Xpert MTB/XDR assay and MTB Detection

The Xpert MTB/XDR test is a 9-plex assay that uses 10 sloppy molecular beacon (SMB) probes (Chakravorty et al., 2011) to detect resistance to INH, ETH, FLQ, and SLID (second-line injectable drugs). Xpert XDR assay is a nested real time PCR application that can detect Mycobacterium tuberculosis as well as resistance to six leading antibiotics for TB treatment - INH (isoniazid), AMK (amikacin), KAN (Kanamycin), CAP (Capreomicin), FLQ (Fluroquinolone), and ETH (Ethionamide). Four probes target the inhA promoter (nucleotides 1 to 32), the katG (codons 311 to 319) and fabG1 (codons 199 to 210) genes, and the oxyR-ahpC (ahpC) intergenic region (nucleotides 5 to 50) to detect INH resistance. Because both resistance traits are encoded by mutations in the inhA promoter, detecting inhA promoter mutations in a particular optical channel also permits detection of ETH resistance and distinguishing of low-level INH resistance (Ghodousi et al., 2019; Seifert et al., 2015; Morlock et al., 2003). The Xpert MTB/XDR assay can also detect mutations associated with fluoroquinolone (FLQ) resistance in the gyrA and gyrB quinolone resistance determining regions (QRDR); and second line injectable drug (SLID) associated mutations in the rrs gene and the eis promoter region.(*Xpert® MTB/XDR*, n.d.) The extraordinary speed and simplicity of Xpert MTB/XDR assay are its distinguishing features. Unlike traditional procedures, which can take weeks or months to provide results, this technology can produce actionable insights in a matter of hours (*Xpert® MTB/XDR*, n.d.). This quick turnaround enables healthcare practitioners to quickly adapt treatment programs for patients, limiting the spread of drug-resistant strains and improving patient outcomes(*Xpert® MTB/XDR*, n.d.). The current gold

standard for finding drug resistance in M. tuberculosis, phenotypic drug susceptibility testing (pDST), takes 6 to 8 weeks to yield clear results and offers a biohazard risk to laboratory employees, especially when working with XDR strains (Cao et al., 2021).

Patient ID: INH 425
Sample ID: Xpert M 021122153343
Test Type: Specimen
Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-XDR	1	In Vitro Diagnostic

Test Result:

MTB DETECTED;
INH Resistance NOT DETECTED;
FLQ Resistance NOT DETECTED;
AMK Resistance NOT DETECTED;
KAN Resistance NOT DETECTED;
CAP Resistance NOT DETECTED;
ETH Resistance NOT DETECTED

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
SPC-ahpC	27.8	284	NA	PASS
inhA	17.7	1324	POS	PASS
katG	26.8	502	POS	PASS
fabG1	16.5	975	POS	PASS
gyrA1	20.2	396	POS	PASS
gyrA2	28.1	185	POS	PASS
gyrA3	22.3	373	POS	PASS
gyrB2	19.7	283	POS	PASS
rrs	16.0	554	POS	PASS
eis	19.1	95	POS	PASS

Figure 5: Example of result of a sensitive strain by Xpert MTB/XDR assay

The Cepheid GeneXpert Instrument Systems with GeneXpert 10 color modules are used to carry out the Xpert MTB/XDR Assay. The GeneXpert Instrument System combines and automates

sample processing, nucleic acid amplification, and melt peak detection to identify target sequences in samples. A device, a computer, a barcode scanner, and preloaded software are all included in the GeneXpert Instrument Systems. These components executed tests on the samples and displayed the findings. The technology facilitates the polymerase chain reaction (PCR) procedure and melt peak detection and calls for the use of single-use disposable Xpert cartridges that contain target-specific PCR chemicals. Cross-contamination between samples is less likely since the Xpert cartridges are self-contained. The Xpert MTB/XDR Assay cartridge contains Sample Reagents for XDR MTB profile detection and sample processing control (SPC) to ensure that the target bacteria were properly processed and to check for the presence of any inhibitors during the PCR reaction. The Sample Reagent that is given contains Sodium Hydroxide with Isopropanol with 8 mL volume each cartridge which ranges from transparent, colorless to golden yellow. The Probe Check Control (PCC) checks the integrity of the probe, the stability of the dye, and the rehydration of the reagents. With the exception of the sample reagent (SR), which must be added to the specimen before the treated specimen is loaded into the cartridge, the Xpert MTB/XDR Assay cartridge contains all of the reagents needed for the test. The test is meant to be used as a follow-up for samples that tested positive for MTB.

A fluorescent dye or probe is used in a real-time PCR test to monitor the amount of amplified DNA in real time as the amplification process advances. At a high temperature, the DNA strands are first denatured (separated) into single strands during the PCR amplification process. The single strands are then bound by particular primers (short DNA sequences corresponding to the target area) during the annealing phase. The temperature is subsequently raised again for the extension stage, during which a DNA polymerase enzyme creates a complementary strand to each single-stranded DNA template, culminating in double-stranded DNA creation.As the PCR proceeds, more DNA copies of

the target sequence are produced. A fluorescent dye or probe attaches to the amplified DNA in a real-time PCR experiment, the Xpert MTB/XDR assay, resulting in a detectable fluorescence signal. The melt peak temperature is the temperature at which double-stranded DNA begins to disintegrate into single strands when hydrogen connections between complementary bases are broken. The fluorescence signal is reduced as a result of this dissociation.

Melt Peaks

Analyte Name	Melt Peak Temperature	Melt Peak Height
inhA-melt	76.2	249.1
katG-melt	73.6	62.0
fabG1-melt	71.5	222.2
ahpC-melt	68.6	40.5
gyrA1-melt	76.2	46.4
gyrA2-melt	70.3	57.4
gyrA3-melt	71.2	81.3
gyrB2-melt	69.5	72.2
rrs-melt	74.9	170.2
eis-melt	68.4	94.5
inhA-mut melt		
katG-mut melt		
fabG1-mut melt		
ahpC-mut melt		
gyrA1-mutA melt		
gyrA1-mutB melt		
gyrA1-mutC melt		
gyrA2-mutA melt		
gyrA2-mutB melt		
gyrA3-mutA melt		
gyrA3-mutB melt		
gyrA3-mutC melt		
gyrB2-mut melt		
rrs-mut melt		
eis-mutA melt		
eis-mutB melt		

Figure 6: The Melt Peak Temperature of a sensitive isolate by Xpert MTB/XDR assay.

The melt peak temperature in the Xpert MTB/XDR test is significant because it can offer information regarding the specificity of the amplification response. Each DNA sequence has a

distinct melt peak temperature, which may be used to validate the existence of the target sequence and grade the amplification reaction's correctness. Deviations from the predicted melt peak temperature may indicate the existence of non-specific amplification or other difficulties that may compromise the assay's credibility.

Figure 7: Xpert MTB/XDR cartridge (Cepheid, 2022)

However, because WHO has since downgraded the role of second-line injectable agents in treating drug-resistant tuberculosis and has revised case definitions of drug-resistant tuberculosis to include resistance to new drugs, the latest version of the Xpert MTB/XDR cartridge may have less of an effect than expected (Naidoo et al., 2022). Early trials of the Xpert MTB/XDR assay showed high estimated sensitivity (88.5-100%) and specificity (97.3%-100%) for resistance detection in a clinical analysis of 310 clinical specimens (Cao et al., 2021; Georghiou et al., 2021). In a clinical investigation using 100 sputum samples and 214 clinical isolates, the test demonstrated sensitivity of 94% to 100% and specificity of 100% for all medications except ETH when compared to sequencing (Cao et al., 2021). In another recent study, sensitivity and specificity (95% confidence interval (CI)) against pDST(phenotypic drug susceptibility testing) were reported to be 94.2% (87.5

to 97.4) and 98.5% (92.6 to 99.7), respectively, in the case of determining isoniazid resistance by Xpert MTB/XDR, (6 cohorts, 1083 participants, moderate-certainty evidence, sensitivity and specificity)(Pillay et al., 2022). In another trial with 710 patients, sensitivity of isoniazid for Xpert MTB/XDR detection of resistance against a composite reference standard that included phenotypic drug susceptibility testing and whole-genome sequencing was 94% (460 of 488, 95% CI 92-96) whereas specificity was 98-100% (Penn-Nicholson et al., 2022).

Figure 8: Workflow of a Xpert MTB/XDR cartridge (Cepheid, 2021)

The influence of Xpert MTB/XDR assay may be seen in its involvement in improving the management of drug-resistant tuberculosis(Xpert® MTB/XDR, n.d.). It helps greatly to worldwide efforts to combat TB and reduce the danger presented by drug-resistant strains by providing focused and effective treatment choices. The incorporation of this technology into healthcare systems

throughout the world represents a significant step forward in the fight against XDR-TB, highlighting its importance in the domain of diagnostic advances

1.4. MIC (Minimum Inhibitory Concentration) and Isoniazid Resistance

The measurement of MIC (Minimum Inhibitory Concentration) is an important component of antimicrobial susceptibility testing, notably for medications used to treat tuberculosis (TB). The MIC determination aids in determining the efficacy of medicines against M. tuberculosis. Clinicians can anticipate whether a given antibiotic will be useful in treating an illness by identifying the lowest dose of a medicine that suppresses bacterial growth. This information is critical for determining the correct antibiotic dosage and developing an effective treatment program. M. tuberculosis can develop antibiotic resistance through genetic alterations. Knowing the MIC value of the INH resistant strains with different mutations hence remains an imperative task.

In a study by Lempens et al., in 2018, they discovered primarily moderate-level resistance to INH, with a median of 6.4 mg/L for the fairly common katG Ser315Thr mutation, and invariably extremely high MICs (19.2 mg/L) for the combination of katG Ser315Thr and inhA c-15t. Another study reported some isolates harboring katG loss-of-function mutations MICs of more than 64 ug/ml (Ghodousi et al., 2019). They also reported that only an inhA promoter alteration or a mutation at codon 315 of katG resulted in nonoverlapping MIC distributions of 0.25 to 2 and 4 to 16 ug/ml, respectively, whereas both mutations resulted in MICs of 8 to 64 g/ml (Ghodousi et al., 2019). A C deletion 34 nucleotides upstream of inhA's primary transcriptional start site likely contributed for the katG S315N mutant's extremely high MIC of 64 g/ml (Karunaratne et al., 2018).

Strains with just inhA promoter mutations showed MIC distributions of 0.25 to 2 ug/ml, whereas those with additional inhA coding mutations at codon 21, 94, or 194 had MIC distributions of 0.5 to 2 ug/ml (Ghodousi et al., 2019).

Meanwhile, in another study in South Korea among the 71 INHr isolates mutations in the katG codon 315 were related with MICs greater than 1 g/ml, whereas mutations in the inhA promoter area were associated with low-level INH resistance MICs ranging from 0.2 to 1 ug/ml (Kim et al., 2003). According to research done in the Netherlands, a substantial proportion of INH-resistant M. tuberculosis isolates contain the amino acid 315 mutation in the katG gene (van Soolingen et al., 2000). This mutation was linked to reasonably high levels of drug resistance (MIC of 5-10 ug/mL) as well as resistance to more than one drug (van Soolingen et al., 2000).

In another research, the following genomic areas were included in the analysis: katG, inhA, ahpC, oxyR-ahpC, furA, fabG1, kasA, rv1592c, iniA, iniB, iniC, rv0340, rv2242, and nat (Rivière et al., 2020). MIC was used to assess the amount of INH resistance: low-level resistance was defined as 0.1-0.4 ug/mL on liquid and 0.2-1.0 ug/mL on solid media, while high-level resistance was defined as >0.4 ug/mL on liquid and >1.0 ug/mL on solid media (Rivière et al., 2020). They showed that 93% (n = 810) of the 867 isolates with a katG mutation at codon 315 showed high-level INH resistance. In comparison, only 50% (n = 72) of the 144 isolates with katG variants other than 315 demonstrated high-level resistance (Rivière et al., 2020). Hence pointed out that the presence of a variation in the katG gene is only a reliable indicator of high-level INH resistance if it is positioned in codon 315 (Rivière et al., 2020).

Moaddab and his team in their 2011 research found mutation in 56% and 20% in the KatG315 and KatG463 loci of 25 INH-resistant strains in Iran. There was no mutation in the investigated loci (KatG315 and KatG463) in 24% of INH-resistant strains. INH MIC was 0.2 ug/ml in all susceptible bacteria, but varied from 0.2 to 3.2 ug/ml in 25 INH-resistant isolates (Moaddab et al., 2011). Another investigation conducted in Ethiopia found that for 58% of MDR TB isolates, the MIC for isoniazid was 2-4 ug/ml (moderate resistance), and 95.6% (n = 25) of the isolates exhibited mutations in the katG gene (Getahun et al., 2022).

Furthermore, by using recombineering technology Kandler and his team found out about five new mutations in the KatG region that were not previously documented- katG W161Q, W161R, E402stop, L415P, and A480del (Kandler et al., 2018). All recombineered katG mutations provided resistance to INH at a MIC greater than 0.25 ug/ml (Kandler et al., 2018). In a study in Poland, the katG gene mutation was detected in 43 (93%) out 46 M. tuberculosis strains isolated from MDR-TB patients in 2004 (Jagielski et al., 2013). Mutations at codon 315 were the most common, occurring in 34 (74%) of the strains (Jagielski et al., 2013). Surprisingly, two strains were discovered in Poland, with no mutations at codon 315 of the katG gene exhibiting a unique translation termination mutation, resulting in polypeptide truncation and the production of defective catalase polypeptides (Jagielski et al., 2013). Both of these strains had the highest MIC values for INH (80 and 100 ug/mL) and had lost all catalase activity (Jagielski et al., 2013). The MICs of INH for the remaining 41 strains with katG mutations ranged from 0.2 to 10 ug/mL with thirty-six of the strains (88%) preserving the catalase activity (Jagielski et al., 2013).

In addition to this, to characterize mutations in the katG, ahpC, and inhA gene loci and correlate with minimal inhibitory concentrations (MIC) levels and spoligotype strain family, a group of researchers performed a comprehensive evaluation of INH resistant M. tuberculosis strains (n = 224) from three South American countries with a high burden of drug resistant TB (Dalla Costa et al., 2009). Mutations in katG were found in 181 (80.8%) of the isolates, with the katG S315T mutation accounting for 178 (98.3%)(Dalla Costa et al., 2009). Other alterations discovered included oxyR-ahpC, the inhA regulatory area, and the inhA structural gene (Dalla Costa et al., 2009). The S315T katG mutation was shown to be notably more likely to be related with an MIC greater than 2 ug/mL (Dalla Costa et al., 2009).

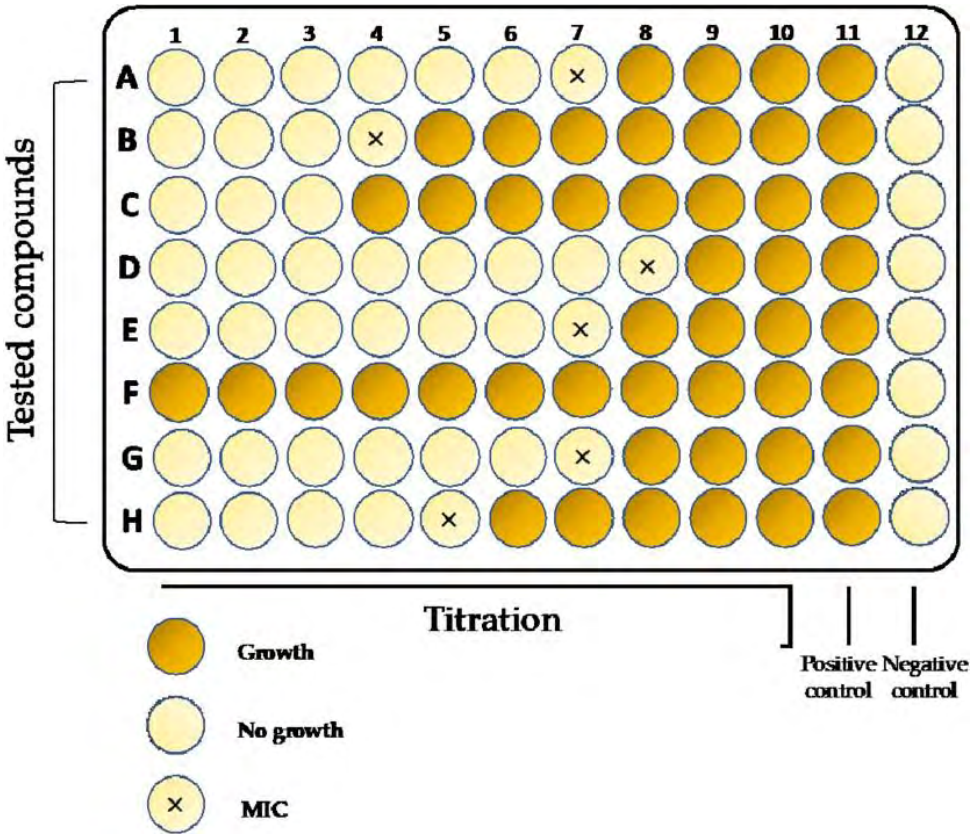

Figure 9: Example of a 96 well plate with bacterial culture (Jaśkiewicz et al., 2019).

Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains are more difficult to cure and can be fatal. MIC testing determines whether bacteria are sensitive or resistant to specific medications, hence assisting in the selection of appropriate treatment choices. In addition, various strains of M. tuberculosis may be more or less susceptible to particular medications. The susceptibility profile of the individual strain causing the illness is determined using MIC testing. This allows healthcare practitioners to adjust treatment regimens based on susceptibility patterns, boosting the chance of good treatment outcomes. Inadequate or insufficient therapy can lead to the emergence of drug-resistant TB strains. Knowing the MIC values allows healthcare practitioners to prevent overusing antibiotics that may be ineffective against a certain strain. This aids in the prevention of drug-resistant strains and the preservation of the effectiveness of current antibiotics. Furthermore, TB therapy frequently entails the use of numerous medicines in order to increase efficacy and avoid the development of resistance. MIC testing assists in the selection of the best medication combination that is likely to perform synergistically against the bacterium. MIC determination also helps with research and the development of novel anti-TB medications. Understanding the MIC values of novel drugs against Mycobacterium TB can aid researchers in assessing their potential efficacy and guiding the development of new treatment alternatives. To summarize, MIC determination is critical for directing successful and tailored TB treatment. It assists doctors in selecting suitable medicines, avoiding drug resistance, and optimizing treatment regimens, eventually enhancing patient outcomes and public health efforts to reduce tuberculosis.

Chapter 2

Methodology

Chapter 2

Methodology:

In this research, we obtained INH-resistant samples from patients, detected by means of GeneXpert -XDR cartridge and performed MIC on those selected INH -Resistant samples.

2.1. GeneXpert-XDR assay:

The GeneXpert Instrument Systems' Xpert MTB/XDR assay is a nested real-time PCR in vitro diagnostic test for the detection of DNA from the extensively drug-resistant (XDR) *Mycobacterium tuberculosis* (MTB) complex in unprocessed sputum samples, concentrated sediments made from sputum, or Mycobacterial Growth Indicator Tube.

Figure 10: Cartridge preparation and sample loading on Xpert MTB/RIF platform (Cepheid, 2022)

GeneXpert was performed on about 485 unprocessed sputum samples of patients infected with *Mycobacterium Tuberculosis* to find out INH-resistant strains. The experiment was carried out in a BSL2+ laboratory.

Requirements:

1. Unprocessed sputum samples
2. Sample Reagent (Sodium Hydroxide with Isopropanol, 8 mL)
3. XDR Cartridge
4. Dropper
5. Vortex machine
6. Droppers
7. 15 ml falcon tubes

Steps:

1. For the test around 1 ml of unprocessed sputum sample was required. The sputum sample was taken in a 15ml falcon.
2. Then the sputum sample was mixed with the sample reagent (SR) supplied along with the cartridge in the 1:2 ratio. So for example, if the sample volume was 1.5 ml, 3 ml of Sample Reagent was added.
3. The mixture was vortexed for at least 10 seconds and then was kept still for 10 minutes.
4. After 10 minutes, the mixture was vortexed again for the second time for 10 seconds.

5. Then after waiting for 5 minutes, 2 ml of the mixture was loaded into the XDR cartridge using a dropper.
6. The cartridge was then loaded in the module of the GeneXpert Instrument System using a barcode scanner. The test runtime is about 1 hour 30 minutes.

2.2. Microbial Inhibitory Concentration (MIC)

Minimal inhibitory concentration (MIC) defines in vitro levels of susceptibility or resistance of specific bacterial strains to an applied antibiotic (Kowalska-Krochmal B, 2021). In this research we followed the EUCAST protocol of AST (Antimicrobial Susceptibility Testing) for *Mycobacterium Tuberculosis* (Schön ET AL., 2020). The MIC was done in a 96-well plate and it was designed by having two samples for each plate. We observed resistance of Mycobacterium from 0.0156ug/ml upto 8ug/ml of the drug Isoniazid(INH). The critical concentration of Isoniazid is 0.2 ug/ml that lies in the 6th column. The critical concentration is defined as the lowest concentration of an anti-TB agent in vitro that will inhibit the growth of 99% of phenotypically wild type strains of *M. tuberculosis* complex (WHO, 2018).

For MIC, we needed culture of bacteria in the samples. Bacterial colonies were produced on egg based solid media - LJ media. Löwenstein-Jensen medium, better known as LJ medium, is a selective egg-based medium specifically used for the culture and isolation of Mycobacterium species, including *Mycobacterium tuberculosis*, from clinical specimens. It took about 21 days to 2 months for having mature colonies of the mycobacterial culture.

We designed our 96 well plate according to our needs. In each plate about two isolates can be tested with a duplicate row for each sample.

MIC PLATE DESIGN:

	1	2	3	4	5	6	7	8	9	10	11	12
A	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O
B	Negative Control	8 ug/ml Sample-1	4 ug/ml Sample-1	2 ug/ml Sample-1	1 ug/ml Sample-1	0.5 ug/ml Sample-1	0.25 ug/ml Sample-1	0.125 ug/ml Sample 1	0.0625 ug/ml Sample-1	0.03125 ug/ml Sample-1	0.0156 ug/ml Sample-1	200ul dH_2O
C	Negative Control	8 ug/ml Sample-1	4 ug/ml Sample-1	2 ug/ml Sample-1	1 ug/ml Sample-1	0.5 ug/ml Sample-1	0.25 ug/ml Sample-1	0.125 ug/ml Sample 1	0.0625 ug/ml Sample-1	0.03125 ug/ml Sample-1	0.0156 ug/ml Sample-1	200ul dH_2O
D	Negative Control	8 ug/ml	4 ug/ml	2 ug/ml	1 ug/ml	0.5 ug/ml	0.25 ug/ml	0.125 ug/ml	0.0625 ug/ml	0.03125 ug/ml	0.0156 ug/ml	200ul dH_2O
E	Negative Control	8 ug/ml Sample-2	4 ug/ml Sample-2	2 ug/ml Sample-2	1 ug/ml Sample-2	0.5 ug/ml Sample-2	0.25 ug/ml Sample-2	0.125 ug/ml Sample 2	0.0625 ug/ml Sample-2	00.3125 ug/ml Sample-2	0.0156 ug/ml Sample-2	200ul dH_2O
F	Negative Control	8 ug/ml Sample-2	4 ug/ml Sample-2	2 ug/ml Sample-2	1 ug/m; Sample-2	0.5 ug/ml Sample-2	0.25 ug/ml Sample-2	0.125 ug/ml Sample 2	0.0625 ug/ml Sample-2	0.03125 ug/ml Sample-2	0.0156 ug/ml Sample-2	200ul dH_2O
G	Negative Control	GC -100% Sample-1	GC -100% Sample-1	GC -1% Sample-1	GC -1% Sample-1	GC -1% Sample-1	GC -100% Sample-2	GC -100% Sample-2	GC -1% Sample-2	GC -1% Sample-2	GC -1% Sample-2	200ul dH_2O
H	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O	200ul dH_2O

Table no 1: MIC plate design for the Isoniazid Resistant strains

GC = Growth control

GC100% = Same inoculum as in the drug containing wells

GC1% = Hundredfold diluted inoculum compared to drug containing wells

Negative Ctrl = 200µl 7H9-OADC

dH20 = sterile distilled water

For use in the MIC plate, liquid 7H9 media has been used and distilled water has been used in all the peripheral wells except the first column. The peripheral wells were just supplied with sterile distilled water in order to prevent desiccation during the incubation time (Schön et al., 2020). Each well of the plate contains about 200 ul of volume. In the first column, only 7H9+ 10%OADC media has been used that serve as the negative control. The row D has been left without any sample to avoid any kind of contamination between the two samples. Meanwhile the row G serves as a growth control row without drugs for both the samples.

Steps:

The whole process for MIC can be majorly divided into 3 steps -

1. Decontamination and processing of the sample
2. Drug Preparation
3. Media Preparation
4. Microplate Preparation
5. Bacterial Inoculum Preparation

Step 1:

Decontamination and processing of the sample

Requirements:

1. 50 ml Falcon as per the number of samples
2. NALC powder
3. NaOH-Sodium citrate solution
4. Phosphate Buffer Saline (PBS; PH 6.8)
5. Lowenstein-Jensen (L-J) slants and loops

Procedure:

The N-Acetyl-L-Cysteine (NALC)-Sodium Hydroxide (NaOH) technique was employed to treat all specimens, which is a commonly used and approved procedure for MTB detection.

1. Unprocessed samples were taken in 50ml falcon and were decontaminated and digested in an equivalent amount of 2% NaOH, 1.45% Sodium citrate, and 0.5% NALC. The minimum volume of the sample was 1ml.
2. After 15 minutes, phosphate buffer saline (PBS; PH 6.8) is used upto 40 ml in the falcon.
3. After neutralization , the specimens were centrifuged at 3000 rpm for 15 minutes at 4°C.
4. The pellet was then resuspended in 1.5 mL of PBS
5. Then from the resuspended pellet, it was inoculated on Lowenstein-Jensen (L-J) slants using a loop. The L-J slants were incubated for six to eight weeks at 37°C and the culture growth was checked every 7 days.

Figure 11: LJ slants with positive growth of *Mycobacteria tuberculosis* (Singh et al., 2015).

2. Drug preparation:

A. *Prepare Stock solution*

B. *Prepare working solution*

A. Stock solution preparation:

Requirements:

1. INH drug powder >99% potency
2. 15ml falcon -1
3. 50 ml falcon -2
4. 0.22 um filter paper
5. Autoclaved dH2O
6. 10ml syringe

Steps:

1. About 64mg of INH drug powder was measured using the measuring balance.
2. 50 ml of dH2O was taken and using the 0.22 um filter paper the water was filtered and transferred to another falcon tube of 50ml.
3. From the filtered sterile dH2O, 10 ml is taken to a 15ml falcon tube and 64mg INH powder is mixed by inverting up and down. Hence the concentration becomes 6400 ug/ml.
4. 250 ul of drug is aliquoted in eppendorfs with drug name, concentration, serial number and date of preparation properly labeled.
5. The aliquots were stored at -80C/20C in a DNA box and later was used from here while working.

B. Working solution:

The working solution needs to be 4X than the required highest concentration, which is 8ug/ml in this case. This is because with media and bacterial inoculum added the drug concentration decrease 4 times in the process. The drug then gets diluted in the progressing wells by repippeting. A 4X working solution from the stock aliquot was prepared in two dilution steps in 7H9/OADC from an aliquot of a stock solution.

Requirements - For each sample:

1. One stock aliquot solution -250 ul of INH -conc. 6400 microgram/ml
2. 3.8ml of 7H9 media is taken and 200ul drug from the aliquot is added. Concentration becomes 320 ug/ml.
3. Then 1 ml of this solution is added to the 9 ml of 7H9-10% OADC media.

Hence, we prepared 10ml of drug with 32ug/ml concentration in 7H9-10% media. T

Step 3:

Media Preparation:

A. *Stock media preparation*
B. *Working media preparation*

Middlebrook 7H9 medium (7H9) was prepared from the base , according to the manufacturer's instructions. After the medium is autoclaved, it is allowed to cool to 50°C in a pre-warmed water bath before adding 10% OADC (pre-warmed to room temperature (RT; 18-22°C)). Middlebrook

OADC Growth Supplement allows the isolation and cultivation of a wide variety of Mycobacteria. This contains oleic acid, bovine albumin, sodium chloride, dextrose, and catalase (Sigma Aldrich, 2023). For each 96-well plate, 10 mL of ready-made 7H9/OADC broth is needed.

A. *Stock media preparation (for bulk samples):*

7H9- Media prepared = 900 ml

OADC - 10% = 100 ml

Total media volume would be = 1000 ml

Requirements:

1. Middlebrook 7H9 Broth
2. Glycerol
3. Purified distilled water
4. Measuring cylinder
5. Media Jar

Steps (For 1000 ml media - 7H9-900ml, OADC-100ml) :

1. 4.7g of 7H9 media powder was measured.
2. 900 ml of purified distilled water using a measuring cylinder was measured.
3. The powder was added in the water and mixed by swirling.
4. 2 ml of glycerol was added.
5. The mixture was autoclaved at 121 C for 10 minutes.

Cooled to room temperature and stored at 4C for later use in the media jar with the proper labelling. OADC is added later while working.

B. Working media preparation (for each sample) :

Requirements:

1. 15ml falcon - 1
2. 1ml pipette and tips as required.

Steps:

1. Around 10 ml 7H9-10% OADC media is required for one 1 MIC plate (0.1ml * 96).

This is made by taking 9 ml of 7H9 media from the stock and 1ml of OADC.

Step 4:

Microplates preparation:

Requirements:

1. At Least 10 ml of 7H9-10% OADC media
2. Sterile dH20 water
3. Drug prepared at working concentration - 32ug/ml.
4. One reservoir.
5. One multichannel pipette , one 1ml pipette and one 200ul pipette and tips as required.
6. One MIC plate

Steps:

1. The MIC plate was labeled on the side with sample IDs and date.
2. The reservoir was filled with the 7H9-10% OADC.

3. Using the multipipette channel the wells of the MIC plate were filled with 100 ul of 7H9-10% OADC media except the peripheral walls, as outlined in the figure (B2 -G11).
4. 200 ul of 7H9-10% OADC media was added in the negative control wells of the first column (B1 - G1).
5. 200 ul of dH2O was added on the peripheral walls as outlined (A1-A12, B12, C12, D12, E12, F12, G12, H1-H12).
6. 100 ul of drug with working concentration (32 ug/ml) was added to all the second wells (B2-F2) of the second column using a multichannel pipette. It was mixed well by re-pipetting at least ten times.
7. 100 ul from these wells was taken and was diluted in the next column wells -(B3 -F3). Serial dilution like this was performed, by taking 100 ul till Column 11.
8. The last 100 ul of drug-media was discarded after reaching the 11th column. The total volume of liquid in the wells remains 100 ul.
9. The plate was covered with parafilm wax.

Step 5:

Bacterial inoculum preparation:

Requirements (for two samples):

1. 15 ml falcon - 8
2. 50 ml falcon tube -1
3. dH2O
4. MGIT tube - 2
5. Bijou bottle - 2

6. Bacterial culture from LJ.

7. Loops

8. One multichannel pipette , one 1ml pipette and one 200ul pipette and tips as required.

Steps:

1. In a 50ml falcon tube, 36 ml of 7H9 media aliquoted and 4ml of OADC is mixed. The media is mixed by inverting up and down. (Vortex not recommended as it may produce bubbles)
2. Around 4.5 ml of 7H9-OADC media is transferred to each of the 8 falcon tubes.

The next part is conducted in the BSL -2+ lab

3. All the materials were safely transferred and made sure that the plate didn't shake.
4. Bacterial colonies were sampled from several morphologically similar colonies (when possible to avoid selecting an atypical variant) at approximately 1 mg (4 loops of 1µl or a full 3 mm loop).
5. The colonies were emulsified using a loop in a bijou bottle containing beads . It is important to avoid scraping off medium.
6. For at least 2 minutes the bijou bottles were vortexed after careful closing of the cap. When clumps are well dispersed, 3-5 mL fresh sterile distilled water was added.
7. The cap was tightly closed and the tube's content was homogenized by vigorously vortexing the tube to swirl for at least 2 minutes.
8. 20-30 min waited for the remaining clumps to settle.

9. The turbidity of the supernatant was adjusted in a new MGIT tube to McFarland 0.5 by sterile dH2O by comparing with a model standard 0.5 McF.
10. It was vortexed for 30 seconds.
11. A 1:100 dilution of the bacterial suspension was prepared in 7H9/OADC broth by two steps of tenfold dilutions. A 10-1 suspension was prepared by adding 0.5 mL of the 0.5 McF bacterial suspension to 4.5 mL of 7H9/OADC and was vortexed for at least 30 seconds.
12. For the 10-2 inoculum, 0.5 mL of the 10-1 suspension to 4.5 mL of 7H9/OADC of another tube.
13. Additionally, from the 10-2 suspension (growth control (GC100%), a 10-4 (GC1%) suspension should be prepared in two dilution steps: 0.5+4.5 mL (10-3), then 0.5+4.5 mL (10- 4). This will be used as a GC for checking the inoculum and to assess the MIC values.
14. Then from the 10-2 inoculum of 0.5 McF, 100ul was added by using a 200ul pipette to antibiotic containing wells starting at the lowest concentration, where 100ul of antibiotic-media dilutions were pre-added.
15. Growth controls (GC100% and GC1%) was then inoculated as outlined in the table 1 - For sample 1, GC100% -G2, G3 and GC1% - G4, G5, G6 . For sample 2, GC100% - G7, G8 and GC1% - G9, G10, G11. The GCs consist of a 1:100 dilution of the 10-2 inoculum of 0.5 McF (i.e. 1% of the inoculum present in antibiotic containing wells; GC1%), and the same inoculum (10-2 suspension of 0.5 McF (i.e. 100% of the inoculum present in antibiotic containing wells; GC100%).

16. The MIC plate was wrapped on all the sides with parafilm wax very carefully so that the solution inside the well doesn't mix with one another or get on the lid.

By covering it with a zip lock bag with Study name, IDs and Inoculation Date written on it, keep it in the incubator. MIC culture reading was taken within an interval of 7 days upto 21 days and the result was recorded.

Chapter 3

Results

Chapter 3

Results:

3.1 Xpert MTB/XDR result:

Among the 485 samples that were tested, about 47 samples were found to be INH resistant. Those samples were given a new ID with "MIC".

Patient ID: INH 468
Sample ID: Xpert M 021122153039
Test Type: Specimen
Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-XDR	1	In Vitro Diagnostic

Test Result:

MTB DETECTED;
Low INH Resistance DETECTED;
FLQ Resistance NOT DETECTED;
AMK Resistance NOT DETECTED;
KAN Resistance NOT DETECTED;
CAP Resistance NOT DETECTED;
ETH Resistance DETECTED

Analyte Result

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
SPC-ahpC	26.3	296	NA	PASS
inhA	19.3	938	POS	PASS
katG	26.8	488	POS	PASS
fabG1	16.7	804	POS	PASS
gyrA1	19.4	407	POS	PASS
gyrA2	25.4	240	POS	PASS
gyrA3	22.9	329	POS	PASS
gyrB2	19.0	310	POS	PASS
rrs	15.9	613	POS	PASS
eis	18.4	130	POS	PASS

Figure 12: Example of a test report of an INH resistant isolate by Xpert MTB/XDR assay

Here in the report Low INH resistance and ETH resistance has been detected, where the strain is shown to be sensitive to the other four drugs (FLQ, AMK, KAN, CAP).

Melt Peaks

Analyte Name	Melt Peak Temperature	Melt Peak Height
inhA-melt		
katG-melt	73.7	79.8
fabG1-melt	71.5	207.6
ahpC-melt	68.7	51.9
gyrA1-melt	76.4	46.1
gyrA2-melt	70.2	49.5
gyrA3-melt	71.3	61.4
gyrB2-melt	69.4	72.6
rrs-melt	74.9	179.4
eis-melt	68.5	119.4
inhA-mut melt	70.9	235.0
katG-mut melt		
fabG1-mut melt		
ahpC-mut melt		
gyrA1-mutA melt		
gyrA1-mutB melt		
gyrA1-mutC melt		
gyrA2-mutA melt		
gyrA2-mutB melt		
gyrA3-mutA melt		
gyrA3-mutB melt		
gyrA3-mutC melt		
gyrB2-mut melt		
rrs-mut melt		
eis-mutA melt		
eis-mutB melt		

Figure 13: The Melt Peaks values from the INH resistant isolate (Figure 8) from Xpert MTB/XDR assay.

The melt peak temperature from the report gives the idea on which gene the mutation occurred. In the above mentioned figure we can understand that the probe specified for inhA region didn't bind

and hence there is no value there but it bound in the region with inhA mutation sequence, by giving it Melt Peak Temperature of about 70.9. Thus it can be said that the isolate in INH and ETH is resistant with InhA mutation.

The result noted from all other INH resistant samples are presented in the table below-

SL.	Lab ID	MTB Detection	Summary (Resistant Drug)
1	MIC - 01	Detected	**INH (L) + ETH**
2	MIC - 02	Detected	**INH**
3	MIC - 03	Detected	**INH + ETH**
4	MIC - 04	Detected	**INH**
5	MIC - 05	Detected	**INH (L) + ETH**
6	MIC - 06	Detected	**INH + ETH**
7	MIC - 07	Detected	**INH**
8	MIC - 08	Detected	**INH**
9	MIC - 09	Detected	**INH (L) + ETH**
10	MIC - 10	Detected	**INH(L) +ETH**
11	MIC - 11	Detected	**INH(L) +ETH**
12	MIC - 12	Detected	**INH**

13	MIC - 13	Detected	**INH**
14	MIC - 14	Detected	**INH**
15	MIC - 15	Detected	**INH + ETH**
16	MIC - 16	Detected	**INH**
17	MIC - 17	Detected	**INH (L) + FLQ + ETH**
18	MIC - 18	Detected	**INH**
19	MIC - 19	Detected	**INH**
20	MIC - 20	Detected	**INH (L) + ETH**
21	MIC - 21	Detected	**INH**
22	MIC - 22	Detected	**INH**
23	MIC - 23	Detected	**INH**
24	MIC - 24	Detected	**INH**
25	MIC - 25	Detected	**INH (L) + ETH**
26	MIC - 26	Detected	**INH**
27	MIC - 27	Detected	**INH + ETH**
28	MIC - 28	Detected	**INH**

29	MIC - 29	Detected	**INH**
30	MIC - 30	Detected	**INH (L) + ETH**
31	MIC - 31	Detected	**INH**
32	MIC - 32	Detected	**INH (L) + ETH**
33	MIC - 33	Detected	**INH**
34	MIC - 34	Detected	**INH**
35	MIC - 35	Detected	**INH**
36	MIC - 36	Detected	**INH + FLQ**
37	MIC - 37	Detected	**INH**
38	MIC - 38	Detected	**INH**
39	MIC - 39	Detected	**INH**
40	MIC - 40	Detected	**INH + FLQ (L) + ETH**
41	MIC - 41	Detected	**INH + FLQ**
42	MIC - 42	Detected	**INH**
43	MIC - 43	Detected	**INH**
44	MIC - 44	Detected	**INH**

45	MIC - 45	Detected	**INH**
46	MIC - 46	Detected	**INH (L) + ETH**
47	MIC - 47	Detected	**INH**

Table 2: Xpert MTB/XDR assay result of the Resistant strains of INH

Here in table 2, we the Xpert MTB/XDR assay results of the 47 isolates. All the strains are all shown to be INH resistant and few along ETH or FLQ resistant. Two of the isolates are found to be INH, ETH and FLQ resistant. Whereas all of the isolates are found to be sensitive to AMK, KAN and CAP (all the second-line injectable drugs)

Mutation regions of the resistant INH strains detected by Xpert MTB/XDR:

Lab ID	***inhA***	***katG***	***fabG1***	***ahpC***	***inhA-mut***	***katG-mut***	***fabG1-mut***	***ahpC-mut***
MIC - 01	-	73.8	71.6	68.8	71	-	-	-
MIC - 02	76.3	-	71.5	68.7	-	68.2	-	-
MIC - 03	-	-	71.5	70	70.9	68.2	-	-
MIC - 04	76	-	71.3	68.4	-	68	-	-
MIC - 05	-	73.7	71.6	68.6	70.9	-	-	-
MIC - 06	-	-	71.3	68.4	70.6	68.0	-	-
MIC - 07	76.4	-	71.6	68.8	-	68.3	-	-
MIC - 08	76.2	-	71.4	68.6	-	68.1	-	-

MIC - 09	-	73.6	71.4	68.4	70.8	-	-	-
MIC - 10	-	73.5	71.2	68.4	70.6	-	-	-
MIC - 11	-	73.7	71.5	68.7	70.9	-	-	-
MIC - 12	76.1	-	71.4	68.5	-	68.1	-	-
MIC - 13	76.1	-	71.4	68.5	-	68.1	-	-
MIC - 14	76.3	73.7	71.5	68.7	-	68.2	-	-
MIC - 15	-	-	71.5	68.5	70.8	68.1	-	-
MIC - 16	76.1	-	71.4	68.5	-	68	-	-
MIC - 17	-	73.8	71.7	68.7	70.9	-	-	-
MIC - 18	76.4	-	71.6	70.1	-	68.3	-	-
MIC - 19	76.1	-	71.3	69.8	-	68.1	-	-
MIC - 20	-	73.8	71.6	68.8	71	-	-	-
MIC - 21	76.2	-	-	68.8	-	68.2	-	-
MIC - 22	76.1	-	71.3	68.4	-	68	-	-
MIC - 23	76.2	-	71.4	68.7	-	68.1	-	-
MIC - 24	76.2	-	71.4	69.9	-	68.1	-	-
MIC - 25	-	73.6	71.4	68.4	70.8	-	-	-
MIC - 26	76.3	-	71.6	68.7	-	68.2	-	-
MIC - 27	-	73.7	71.6	68.7	70.9	-	-	-

MIC - 28	76.1	-	71.3	68.5	-	68	-	-
MIC - 29	No			No				
MIC - 30	-	73.8	71.6	68.7	71	-	-	-
MIC - 31	76.2	-	71.4	68.6	-	68.1	-	-
MIC - 32	-	73.7	71.6	68.7	70.9	-	-	-
MIC - 33	76.2	-	71.4	69.9	-	68.1	-	-
MIC - 34	76.1	-	71.3	68.5	-	68	-	-
MIC - 35	76.4	-	71.6	68.8	-	68.3	-	-
MIC - 36	76.2	-	71.4	68.7	-	68.1	-	-
MIC - 37	76.2	-	71.4	68.6	-	68.1	-	-
MIC - 38	76.2	-	71.5	68.6	-	68.1	-	-
MIC - 39	76.2	-	71.4	68.6	-	68.1	-	-
MIC - 40	-	-	71.6	68.7	70.9	-	-	-
MIC - 41	76.3	-	71.5	68.7	-	68.2	-	-
MIC - 42	76.2	-	71.4	69.9	-	68.1	-	-
MIC - 43	76.1	-	71.3	68.5	-	68	-	-
MIC - 44	76.4	-	71.6	68.8	-	68.3	-	-
MIC - 45	76.3	-	71.5	68.7	-	68.2	-	-
MIC - 46	-	73.7	71.6	68.7	70.9	-	-	-

MIC - 47	76.4	-	71.6	-	-	68.3	-	-

Table 3: INH mutation regions information from the Xpert MTB/XDR assay -result

Table 3 contains records of the Melt Peak Temperatures of the INH resistant isolates indicating the mutated gene that is responsible for INH resistance. Blank value indicates that the probe didn't bind there.

3.2. MIC result

MIC readings were taken of the 47 isolates within a 7 days interval period till 21st day. The growth is observed using an inverted mirror. In case there was any kind of contamination the test was repeated on the sample. In two plates, mixed infection was also observed, that still remains to be identified. After 21 days the wells were found to be desiccated. All the growth results in the 3 weeks period were well documented. A picture of the MIC plate after 21 days of growth of an INH resistant isolate is given below.

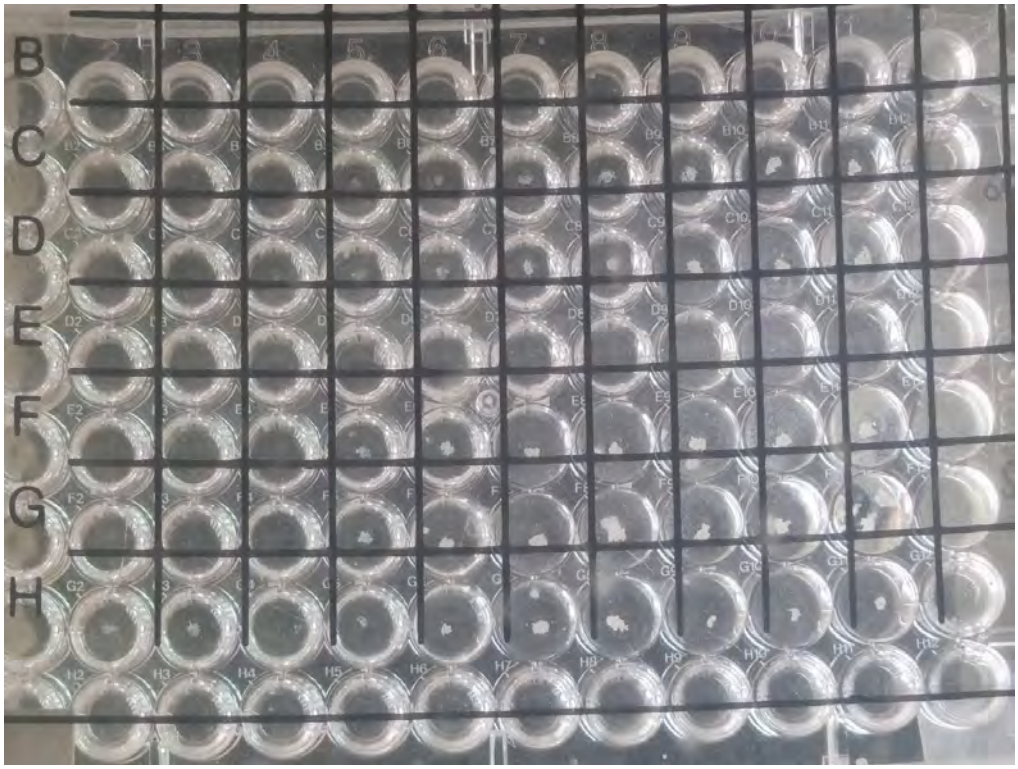

Figure 14: Result of MIC on a 96 well plate of two INH resistant isolates.

Here in the figure we can see that, in the first isolate growth stopped at column no 5 (c5, d5). Hence its MIC is calculated to be 1ug/ml which is the drug concentration of that specified column. The second isolate showed the same result by stopping growth at e5, f5. The growth control wells at row G gave positive results for both the isolates.

The MIC result of the 47 INH-resistant isolates are presented below:

MIC result of the resistant strains found from the GeneXpert- XDR

Lab ID	Week 1	Week 2	Week 3 (Final Growth)
MIC - 01	0.03125 ug/ml	0.25 ug/ml	0.25 ug/ml
MIC - 02	0.25 ug/ml	1 ug/ml	1 ug/ml
MIC - 03	2 ug/ml	4 ug/ml	8 ug/ml
MIC - 04	0.03125 ug/ml	1 ug/ml	2 ug/ml
MIC - 05	0.03125 ug/ml	0.25 ug/ml	0.25 ug/ml
MIC - 06	2 ug/ml	8 ug/ml	8 ug/ml
MIC - 07	0.25 ug/ml	2 ug/ml	4 ug/ml
MIC - 08	0.25 ug/ml	1 ug/ml	1 ug/ml
MIC - 09	0.25 ug/ml	0.25 ug/ml	0.25 ug/ml
MIC - 10	0.25 ug/ml	0.5 ug/ml	0.5 ug/ml

MIC - 11	0.0156 ug/ml	0.03125 ug/ml	0.03125 ug/ml
MIC - 12	2 ug/ml	2 ug/ml	4 ug/ml
MIC - 13	2 ug/ml	8 ug/ml	8 ug/ml
MIC - 14	**0.**25 ug/ml	4 ug/ml	8 ug/ml
MIC - 15	4 ug/ml	8 ug/ml	8 ug/ml
MIC - 16	8 ug/ml	8 ug/ml	8 ug/ml
MIC - 17	0.03125 ug/ml	2 ug/ml	2 ug/ml
MIC - 18	0.0156 ug/ml	1 ug/ml	2 ug/ml
MIC - 19	0.25 ug/ml	2 ug/ml	4 ug/ml
MIC - 20	0.03125 ug/ml	0.125 ug/ml	0.125 ug/ml
MIC - 21	0.03125 ug/ml	0.125 ug/ml	1 ug/ml
MIC - 22	0.25 ug/ml	0.5 ug/ml	1 ug/ml
MIC - 23	0.0156 ug/ml	2 ug/ml	2 ug/ml
MIC - 24	0.25 ug/ml	2 ug/ml	4 ug/ml
MIC - 25	0.03125 ug/ml	0.125 ug/ml	0.125 ug/ml
MIC - 26	2 ug/ml	2 ug/ml	2 ug/ml

MIC - 27	0.125 ug/ml	2 ug/ml	2 ug/ml
MIC - 28	2 ug/ml	2 ug/ml	8 ug/ml
MIC - 29	0.03125 ug/ml	0.5 ug/ml	2 ug/ml
MIC - 30	0.03125 ug/ml	0.125 ug/ml	0.125 ug/ml
MIC - 31	1 ug/ml	1 ug/ml	2 ug/ml
MIC - 32	0.5 ug/ml	2 ug/ml	2 ug/ml
MIC - 33	2 ug/ml	2 ug/ml	2 ug/ml
MIC - 34	1 ug/ml	1 ug/ml	2 ug/ml
MIC - 35	0.03125 ug/ml	0.5 ug/ml	1 ug/ml
MIC - 36	0.5 ug/ml	2 ug/ml	2 ug/ml
MIC - 37	0.5 ug/ml	1 ug/ml	1 ug/ml
MIC - 38	1 ug/ml	1 ug/ml	1 ug/ml
MIC - 39	0.5 ug/ml	2 ug/ml	2 ug/ml
MIC - 40	0.25 ug/ml	2 ug/ml	4 ug/ml
MIC - 41	1 ug/ml	2 ug/ml	2 ug/ml
MIC - 42	0.5 ug/ml	1 ug/ml	2 ug/ml

MIC - 43	4 ug/ml	8 ug/ml	8 ug/ml
MIC - 44	8 ug/ml	8 ug/ml	8 ug/ml
MIC - 45	0.03125 ug/ml	0.03125 ug/ml	0.0156 ug/ml
MIC - 46	0.5 ug/ml	0.5 ug/ml	0.5 ug/ml
MIC - 47	0.5 ug/ml	1 ug/ml	1 ug/ml

Table 4 : MIC results of the INH-resistant strains detected by Xpert MTB/XDR assay

Table 4 shows the MIC result of the 47 isolates recorded in the 7th day, 14th day and 21st day. The result ranges from 0.0156 ug/ml to 8ug/ml.

3.3: Comparison of the Xpert MTB/XDR assay result with MIC value

The INH mutations associated genes detected from Xpert MTB/XDR assay of the 47 isolates and the MIC value found associated to those genes are presented below in different tables.

Strains with only InhA mutations and their MIC value

Sl.	**Lab ID**	**MIC value**	**XDR Summary (Resistant Drug)**
1.	MIC - 01	0.25 ug/ml	**INH (L) + ETH**
2.	MIC - 05	0.25 ug/ml	**INH (L) + ETH**
3.	MIC - 09	0.25 ug/ml	**INH (L) + ETH**
4.	MIC - 10	0.5 ug/ml	**INH(L) +ETH**

5.	MIC - 11	0.03125 ug/ml	**INH(L) +ETH**
6.	MIC - 17	2 ug/ml	**INH (L) + FLQ + ETH**
7.	MIC - 20	0.125 ug/ml	**INH (L) + ETH**
8.	MIC - 25	0.125 ug/ml	**INH (L) + ETH**
9.	MIC - 27	2 ug/ml	**INH + ETH**
10.	MIC - 30	0.125 ug/ml	**INH (L) + ETH**
11.	MIC - 32	2 ug/ml	**INH (L) + ETH**
12.	MIC - 46	0.5 ug/ml	**INH (L) + ETH**

Table 5 : MIC results of the INH-resistant strains with InhA mutations detected by Xpert MTB/XDR assay

Around 12 isolates were detected that contain inhA mutations. The MIC value of these isolates ranges from 0.125 ug/ml upto 2 ug/ml indicating low INH resistance. Double mutations have also been observed in these isolates with ETH and FLQ.

Strains with only KatG mutations and their MIC value

Sl.	**Lab ID**	**MIC value**	**XDR Summary (Resistant Drug)**
1.	MIC - 02	1 ug/ml	**INH**
2.	MIC - 04	2 ug/ml	**INH**
3.	MIC - 07	4 ug/ml	**INH**
4.	MIC - 08	1 ug/ml	**INH**
5.	MIC - 12	4 ug/ml	**INH**

6.	MIC - 13	8 ug/ml	**INH**
7.	MIC - 14	8 ug/ml	**INH**
8.	MIC - 16	8 ug/ml	**INH**
9.	MIC - 18	2 ug/ml	**INH**
10.	MIC - 19	4 ug/ml	**INH**
11.	MIC - 22	1 ug/ml	**INH**
12.	MIC - 23	2 ug/ml	**INH**
13.	MIC - 24	4 ug/ml	**INH**
14.	MIC - 26	2 ug/ml	**INH**
15.	MIC - 28	8 ug/ml	**INH**
16.	MIC - 29	2 ug/ml	**INH**
17.	MIC - 31	2 ug/ml	**INH**
18.	MIC - 33	2 ug/ml	**INH**
19.	MIC - 34	2 ug/ml	**INH**
20.	MIC - 35	1 ug/ml	**INH**
21.	MIC - 36	2 ug/ml	**INH + FLQ**
22.	MIC - 37	1 ug/ml	**INH**
23.	MIC - 38	1 ug/ml	**INH**
24.	MIC - 39	2 ug/ml	**INH**

25.	MIC - 41	2 ug/ml	**INH + FLQ**
26.	MIC - 42	2 ug/ml	**INH**
27.	MIC - 43	8 ug/ml	**INH**
28.	MIC - 44	8 ug/ml	**INH**
29.	MIC - 45	0.0156 ug/ml	**INH**

Table 6 : MIC results of the INH-resistant strains with KatGA mutations detected by Xpert MTB/XDR assay

Around 29 isolates were detected that contain KatG mutations. The MIC value of these isolates ranges from 0.0156 ug/ml upto 8 ug/ml indicating high INH resistance. Double mutations have also been observed in two of these isolates with FLQ.

Strains with Both KatG and InhA mutations and their MIC value

Sl	**Lab ID**	**MIC value**	**XDR Summary (Resistant Drug)**
1.	MIC - 03	8 ug/ml	**INH + ETH**
2.	MIC - 06	8 ug/ml	**INH + ETH**
3.	MIC - 15	8 ug/ml	**INH + ETH**
4.	MIC - 40	4 ug/ml	**INH + FLQ (L) + ETH**

Table 7 : MIC results of the INH-resistant strains with InhA + KatG mutations detected by Xpert MTB/XDR assay

Four isolates were detected with both KatG and InhA mutation and they show moderate high to very high INH resistance with their MIC values ranging from 4ug/ml to 8 ug/ml.

Strains with fabG1 and KatG mutations and their MIC value

Sl.	Lab ID	MIC value	XDR Summary (Resistant Drug)
1.	MIC - 21	1 ug/ml	**INH**

Table 8 : MIC results of the INH-resistant strains with FabG1 + KatG mutations detected by Xpert MTB/XDR assay

Only one isolate has been detected with fabG1 and KatG mutation with MIC value of 1ug/ml showing moderate resistance.

Strains with ahpC and KatG mutations and their MIC value

Sl.	Lab ID	MIC value	XDR Summary (Resistant Drug)
1.	MIC - 47	1 ug/ml	**INH**

Table 9 : MIC results of the INH-resistant strains with ahpC + KatG mutations detected by Xpert MTB/XDR assay

Only one isolate has been detected with ahpC and KatG mutation with MIC value of 1ug/ml showing moderate resistance.

Overall comparison of the INH mutation regions and their MIC values

Mutation regions	MIC value range	No. of samples	Final comment
KatG mutations	**0.0156 ug/ml - 8 ug/ml**	**29**	**Highly Resistant strains and most frequent number of resistance.**
InhA mutations	**0.03125 ug/ml - 2 ug/ml**	**12**	**Low Resistant strains and less frequent number of resistance.**
KatG + InhA mutations	**4 ug/ml - 8 ug/ml**	**4**	**Highly Resistant strains and less frequent resistance.**
FabG1 + KatG mutations	**1 ug/ml**	**1**	**Moderate Resistant strain and very less frequency.**
ahpC + KatG mutations	**1 ug/ml**	**1**	**Moderate Resistant strain and very less frequency.**

Table 10 : Overall comparison of the INH mutation regions and their MIC values

So overall, we identified 29 isolates with KatG mutations showing very high MIC values and also the most frequent mutation. 12 isolates with inhA mutations were identified showing lower range of

MIC value. And 6 isolates are identified that contain double mutations with MIC value ranging from moderate to high.

Strains with MIC value below critical concentration of INH but with INH- Resistant by GeneXpert -XDR assay

SL.	Lab ID	MIC value	Mutation region	XDR Summary (Resistant Drug)
1.	MIC - 11	0.03125 ug/ml	***inhA***	**INH + ETH**
2.	MIC - 20	0.125 ug/ml	***inhA***	**INH + ETH**
3.	MIC - 25	0.125 ug/ml	***inhA***	**INH + ETH**
4.	MIC - 30	0.125 ug/ml	***inhA***	**INH + ETH**
5,	MIC - 45	0.0156 ug/ml	**KatG**	**INH**

Table 11: Strains with MIC value below critical concentration of INH but with INH- Resistant by GeneXpert -XDR assay

This table shows 5 isolates whose MIC value was detected to be less than the critical concentration of INH which is 0.2 ug/ml.

Strains with MIC value at critical concentration of INH but with INH- Resistant by GeneXpert -XDR assay

SL.	Lab ID	MIC value	Mutation region	XDR Summary (Resistant Drug)
1.	MIC - 01	0.25 ug/ml	***inhA***	**INH (L) + ETH**

2.	MIC - 05	0.25 ug/ml	***inhA***	**INH (L) + ETH**
3.	MIC - 09	0.25 ug/ml	***inhA***	**INH (L) + ETH**

Table 12: Strains with MIC value at critical concentration of INH but with INH- Resistant by GeneXpert -XDR assay

This table shows 12 isolates whose MIC value was detected to be almost equal to the criticalconcentration of INH which is 0.2 ug/ml.

Chapter 5

Discussion

Chapter 5

Discussion:

In this research we found about 47 INH resistant strains with mutations in KatG, InhA, FabG1 and ahpC regions. There were few cases of double mutations as shown in the abovementioned tables. Now diving deep into it, from the XDR results it has been found the highest number of INH mutations reside in the region KatG (Table no. 6). The most frequent mutations, around 29 samples, that conferred resistance to INH were found in the katG1 gene, a pattern that has also been noted by several other investigations (Dean et al., 2020; Valafar et al., 2021). This region also gives the highest frequency of the highest concentration of the MIC which is 8 ug/ml. There has been a number of researches supporting the prevalence of KatG to be the number one cause of INH resistance. Out of the 47 INH resistant samples - 9 were found to be highly resistant with MIC value upto 8ug/ml, among which 8 of the strain contains mutation in the KatG region (Table no. 6). Among the strains with KatG mutations, only one had a MIC value as low as 0.0156 ug/ml below the critical concentration 0.2 ug/ml. This strain needs further investigation. This can be also because of the False Positive result of the Xpert MTB/XDR assay. In our analysis, we discovered isolates with mutations in the katG gene, inhA promoter, or both, a pattern that is typical of INH-resistant isolates in Bangladesh (Lempens et al., 2018). Another isolate with a fabG1 gene mutation was discovered but it also has mutation in the KatG region (Table no.8).

Mokrousov I, in their research discovered that the KatG gene mutation is the main reason for INH-specific resistance (Mokrousov et al., 2002). The most frequent mutation, which happens at

codon S315, is present in up to 94% of clinical isolates that are resistant to INH (Mokrousov et al., 2002). Approximately 300 mutations can be found in the katG gene's ORF in addition to the gene's complete deletion (Vilchèze et al., 2002). MIC greater than 256 ug/ml has been reported for mutations in the KatG and InhA genes, respectively (Ramaswamy et al., 2003). It's intriguing to notice that isolates with the Q434STOP mutation, which causes the protein to prematurely terminate, or those without the KatG gene both exhibit the same level of resistance (Vilchèze et al., 2014; Ramaswamy et al., 2003). In another study with 52 INH resistant isolates, at codon 315 in katG, 34 (65.4%) INHr isolates contained mutations (Caroline et al., 2005). In 31 strains, the wild-type codon, AGC (Ser), was changed to ACC (Thr), and in three strains, ACA (Thr) (Caroline et al., 2005). In a systematic review published in 2015, it was reported that South East Asia had thehighest prevalence of the katG315 mutation (78.4%) while the Western Pacific Region had the lowest frequency (55.5%) among isolates that were phenotypically INH resistant (Seifertet et al., 2015).

This demonstrates that the KatG gene may be the only factor causing the significant resistance to INH. Additionally, it has been discovered in several investigations that furA and sigI also control KatG (Vilchèze et al., 2014; Pym et al., 2001; Zahrtet al., 2001). Deletion of furA gene resulted in INH hypersusceptibility whereas mutations in FurA in regions (a-10c, g-7a) resulted in 80% reduction in katG expression, leading to 2 to 4 fold of INH resistance (Ando et al., 2011). Isolates with deleted sigI genes and reduced catalase activity produced INH resistance both in vitro and in vivo, whereas overexpression of sigI led to hypersusceptibility to INH (Vilchèze et al., 2014; Lee JH et al., 2012).

The second most common mutation, around 12 samples, has been found in the InhA region which is also a region for determining resistance to Ethionamide (Table no. 5). Both simple and complex processes underlie *M. tuberculosis*'s resistance to INH and ETH. Patients with TB who had undergone INH therapy but never ETH medication have occasionally developed clinical isolates that are co-resistant to both medicines (Hok TT et al., 1964; Stewart et al., 1962; Lefford et al., 1966). This contradiction led to the hypothesis that INH and ETH shared a common resistance mechanism, and it took decades to determine that INH and ETH both target the enoyl-ACP reductase InhA in M. tuberculosis (Banerjee et al., 1994). The medication can be titrated by overexpressing InhA, the target of both INH and ETH, or by reducing the binding of the INH-NAD or ETH-NAD adduct to InhA by having mutations (Vilcheze et al., 2006). Hence in the Xpert MTB/XDR assay table results (Table no. 2) We found a number of double mutations of INH and ETH where the mutated gene was InhA. Mutations in the mshA (Rv0486) gene, which codes for a glycosyl transferase involved in the production of mycothiol, are also being investigated for their potential role in the co-occurrence of INH and ETH resistance (Vilcheze et al., 2008). Along with the KatG mutation, a double mutation in mshA (V171G, A187V) was discovered in a clinical isolate that was extremely INH- and ETH-resistant (Brossier et al., 2011).

I21T, S94A, and I95P are the variants reported in both INH- and ETH-resistant clinical isolates among the 15 mutations inhA discovered in INH-resistant clinical isolates (Hazbon et al., 2006; Ramaswamy et al., 2003; Zhang et al., 2005; Cardoso et al., 2004; Basso et al., 1998; Ristow et al., 1995; Silva et al., 2003; Brossier et al., 2006). Besides all these there are ongoing investigations regarding the gene NudC and ndhA that can be faintly related to the INH and ETH co-resistance

(Wang et al., 2011; Vilchèze et al., 2014; Vilcheze et al., 2005; Miesel et al., 1998; Hazbon et al., 2006).

The oxyR-ahpC mutation is one example of a compensating mutation, in addition to others. Several deletions and frameshifts in the coding regions of the mycobacterial OxyR gene have rendered it inactive, however the ahpC gene, which codes for alkyl hydroperoxide reductase, is found downstream of OxyR (Vilchèze et al., 2014). Mutations in the ahpC region caused the overexpression of ahpC in INH-resistant isolates, which is likely to compensate for the loss of the KatG gene (Sherman et al., 1996; Ng et al., 2004). About 29% of clinical isolates that are resistant to INH have been shown to contain mutations in the oxyR-ahpC regions (Sreevastan et al., 1997). In our study only one of the isolates was found to have ahpC mutation, which surprisingly also has a KatG mutation. But the isolate is shown to have moderate value for MIC of about ug/ml . The purpose of ahpC is still unclear given that these mutations are seen in around 8% of isolates that are sensitive to INH (Baker et al., 2005). Additionally, Colangeli and his collaborators noted that the iniA operon deletion enhanced susceptibility to INH (Colangeli et al., 2005). Mutations in the iniBac operon are also considered to play a minor impact in INH resistance (Vilchèze et al., 2014; Colangeli et al., 2005).

Besides ahpC, the least number of mutations is also found in the FabG1 region among the INH resistant strains. We found only one isolate with FabG1 mutation that along with KatG mutation counting MIC value upto 1ug/ml. FabG1 and inhA both encode mycolic acid biosynthetic enzymes and are co transcribed (Banerjee et al., 1998). Lavender and Caroline in their 2005 research found 13 isolates (25%) with *fabG1-inhA* mutations among 52 INH resistant isolates. Besides, in a

systematic review on genetic mutations associated with Isoniazid Resistance in *Mycobacterium tuberculosis* in 2015, it has been reported that around 72 publications found mutations in fabG1-inhA regions (Seifert et al., 2015). They reported the most commonly reported gene areas for INH resistance mutations were furA-katG, reaching about 115 publications while ahpC-oxyR mutations were recorded in about 24 publications (Seifert et al., 2015). Additionally, the genes efpA, fadE24, iniA, iniB, iniC, kasA, nat, ndh, Rv1772, Rv1592c, Rv0340, and srmR were reported to have mutations; however, because the data retrieved for these genes were so sparse, they were disregarded for further study (Seifert et al., 2015).

With all these support from the other investigations, it can be claimed that our result is congruent with all these previous researches. KatG reported the highest resistance with the most number of resistant isolates, followed by InhA. Double mutations in KatG and InhA also resulted in very high resistance. In a study while discussing the combinatorial effect of mutations, a high level of MIC value above 6.4 ug/ml was observed among isolates with combined gene mutations in 79 (47% of 168) MDR-TB isolates and in 6 (30% of 20) INH mono-resistance isolates (Liu et al., 2018). Except for five isolates, the mutations were caused by a combination of katG mutations and some other gene (Liu et al., 2018). Based on information from all their investigations, we may infer that 4 isolates from our study have significant levels of INH resistance because they had double mutations in the katG gene and the inhA promoter. FabG1 and ahpC reported the lowest number of mutations and moderate resistance. The pattern of INH mutation resistance in Bangladesh is congruent with the global scenario.

In addition to the majority of mutations that have been studied, there may be many more variables that contribute to INH resistance. The detoxification of INH, increased expression of efflux pumps, and NAD+/NADP+ binding enzymes are all included in this (Vilchèze et al., 2014). These factors have been connected to several genes, all of which have been shown to have a minor part in conferring resistance to INH. These points contribute to the fact that other than genetic mutations there can be many other reasons for resistance to INH that cannot be detected by technology such as GeneXpert. To identify strain like these we need phenotypic testing like DST (Drug Susceptibility Testing). Besides, the Xpert MTB/XDR Cartridge can identify only four mutation regions responsible for INH resistance ignoring the many other mutation regions reported that are mentioned above. It is imperative to perform MIC tests on the sensitive isolates also from the Xpert MTB/XDR results to identify the isolates like these that contain mutations in other regions that are less frequent. DST (Drug Susceptibility Testing) and WGS (Whole Genome Sequencing) can aid in identifying many other mutation regions responsible for INH resistance.

Moreover, there lies also the possibility of False Positive result in this Xpert MTB/XDR assay testing due to the presence of silent mutation. A silent mutation is a type of substitution, or point, mutation, wherein the change in the DNA sequence of the gene has no effect on the amino acid sequence. Since the GeneXpert Instrument Systems' Xpert MTB/XDR assay is a nested real-time PCR, there remains a possibility that the probe designed for a certain region may not bind due to having silent mutation. This results in genotypically resistant but phenotypically sensitive ultimately giving rise to false positive results. In our study, we found about 5 isolates with MIC value lower than the critical concentration of INH (0.2 ug/ml). Four of these were found to have mutations in the inhA region and only one has mutation in the KatG region. These shouldn’t be the

case as all of the 47 isolates were found to be resistant by Xpert MTB/XDR assay. These indicate the above mentioned possibility for silent mutation. Besides, provided by the fact that GeneXpert/XDR assay is still a new technology (launched in 2020) there is scarcity of research and data that would predict its specificity and sensitivity accurately. More data regarding XDR assay results need to be evaluated before we start considering Xpert MTB/XDR assay result as a standard.

For having significant changes on the regulation of the INH drug regimen for the TB patients more data is needed. According to WHO recommendations, therapy with rifampicin, ethambutol, pyrazinamide, and levofloxacin is advised for a period of six months in patients with proven rifampicin-susceptible and isoniazid-resistant TB while it is not advised to include streptomycin or other injectable medications in the treatment plan (WHO, 2022). For the treatment of drug-susceptible TB, a usual dose of isoniazid is between 4-6 mg/kg/day, whereas a "high" dose, often 10-15 mg/kg/day, is used for individuals with a particular kind of isoniazid-resistant TB (limited to those with the inhA mutation) whereas, when INH resistance is caused by katG mutations, alternative medications and treatment regimens are considered. Typically, the standard dose for adults is 5 mg/kg to 10 mg/kg of body weight, up to a maximum of 300 mg daily (WHO, 2022). To predict whether the recommended dose can be altered on the basis of the MIC values generated from the INH resistant isolates we need extensive research. If Isoniazid (INH) can be used on the patients who are INH resistant but lies in the lower spectrum of MIC values, these can save the trouble from the intake of other antibiotics. If an INH resistant patient can be cured with the INH drug, which is a first- line drug, by adjusting its dose on the basis of its MIC value and mutations, the use of second line drugs can be avoided. This would be highly beneficial as it would resist antimicrobial resistance (AMR) to a great extent, which is a global cause nowadays. But to

conform this we still need a huge amount of investigations regarding the MIC values of the INH resistant strains of tuberculosis patients globally. A bulk of research and resources in this area would make it possible to fight against resistant strains of tuberculosis without having to contribute to drug resistance cases in the future .

Conclusion and Perspective:

TB remains a significant global concern, especially in congested countries like Bangladesh. Bangladesh is one of the 30 high TB-burden countries and accounts for 3.6% of the global total. There's a lot of scope of research here due to the high number of patients contributing to the higher sample size. Recently MDR-TB (Multidrug Resistant TB), pre-XDR TB (Extensively drug resistant) and XDR-TB (Extensively drug resistant - TB) is on the rise. To combat this grave situation we need more research on the resistant strains of tuberculosis.

In our study, we performed MIC on the INH resistant isolates till 8ug/ml concentration. But the isolates that showed MIC value at 8ug/ml, bear the possibility to be resistant at much higher concentration, as so many previous relevant researches mentioned above shows that there are chances. Even though this remains as one of the limitations of this research, this also provides a scope of designing another MIC experiment with the highly resistant strains detected from here at a much higher MIC value range to detect the possibly most resistant strain and its MIC value. Besides, since Xpert MTB/XDR assay also has some limitations, there remains a chance of false negative cases. The sensitive strains detected by the GeneXpert assay need to be tested on by MIC

also to find out if there are other phenotypically resistant isolates. There can be other mutation regions that might go undetected.

Mycobacterium tuberculosis, the TB-causing pathogen, frequently develops drug resistance as a result of a point mutation in a particular gene. This information has been utilized in the creation of molecular diagnostics as a quick replacement for culture-based techniques. However, a significant drawback of molecular testing is that it can only identify resistant mutations that the platform was intended to identify. Therefore, bacteria with unusual resistance mechanisms will evade identification. As a result, with an increase in the incidence of unusual mechanisms of resistance, the sensitivity in identifying resistance may decline. It is widely known that bacteria that are isoniazid-monoresistant (INHr) can include these mechanisms, avoid identification, and transform into MDR-TB (Torres et al., 2015; Vilchèze et al., 2019). Therefore, integration of all mutations imparting INH resistance is essential for thorough molecular detection.

A good undertaking could be to review the early studies on the mechanisms of action of INH in order to identify more factors of INH resistance. More mechanisms of resistance are likely still to be discovered. It may be possible to create new medications that avoid the known causes of drug resistance by using techniques based on knowledge of resistance genes and mutations. For instance, the natural substance pyridomycin inhibits InhA without necessitating KatG activation and is thus effective against clinical isolates o*f M. tuberculosis* that have a katG mutation and are highly INH-resistant (Hartkoorn et al., 2012). Last but not least, in addition to unique mutations that impart resistance to every cell in a population, new research that shows how a cell might temporarily gain

phenotypical resistance to INH will be crucial in creating more effective approaches to eradicate *M. tuberculosis.*

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