**ADDIS ABABA UNIVERSITY**

**COLLEGE OF HEALTH**

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**SCHOOL OF MEDICINE**

**DEPARTMENT OF MEDICAL MICROBIOLOGY, IMMUNOLOGY**

**AND PARASITOLOGY**

# Performance evaluation of the first-generation (1G) color plate drug susceptibility testing assay identifying drug- resistant *Mycobacterium tuberculosis* isolates for selected anti-tuberculosis drugs

By

**Binyam Mebrat (B.Sc.)**

**A Thesis Submitted to the Department of Microbiology, Immunology and Parasitology, College of Health Sciences, Addis Ababa University, In Partial Fulfillment of the Requirement for the Degree of Masters of Science in Medical Microbiology**

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**Addis Ababa, Ethiopia**

**Addis Ababa University**

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**Department of Microbiology, Immunology and Parasitology**

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## Abbreviations

| | |
|---|---|
| CC | Critical concentrations |
| CFU | Colony forming unit |
| CFX | Ciprofloxacin |
| CI | Confidence interval |
| DNA | Deoxyribonucleic acid |
| DR | Drug-resistant |
| DST | Drug susceptibility testing |
| EPTB | Extra pulmonary TB |
| FNA | Fine needle aspirate |
| FQ | Fluoroquinolone |
| 1G | First generation |
| GU | Growth Units |
| INH | Isoniazid |
| IPM | Indirect proportional method |
| KM | Kanamycin |
| LJ | Lowenstein–Jensen |
| LPA | Line Probe Assays |
| MDR-TB | Multi-drug resistant |
| MGIT | Mycobacteria growth indicator tubes |
| MTBC | *Mycobacterium tuberculosis* complex |
| MXF: | Moxifloxacin |
| NGS | Next generation sequencing |
| OADC | Oleic acid, albumin, dextrose, catalase |
| OFL | Ofloxacin |
| PTB | Pulmonary TB |
| PZA | Pyrazinamide |
| RIF | Rifampicin |
| R | Resistant |
| S | Susceptible |
| SPSS | Statistical package for social sciences |
| TB | Tuberculosis |
| TLA | Thin-layer agar |
| WHO | World Health Organization |
| XDR | Extensive drug- resistant |

# Abstract

**Background**: Tuberculosis is a major global public health concern and the emergence of drug-resistant (DR)-TB has been a major challenge to the control of the disease, especially in developing countries, including Ethiopia. Despite availability of appropriate diagnostic tools for *Mycobacterium tuberculosis* drug susceptibility testing, accessibility and timely diagnosis of DR-TB in resource-constrained settings, such as Ethiopia, is often a challenge. The first- generation color plate assay (1G) is ideally suited for low resource settings due to low cost, minimal technical expertise needed, and rapid provision of DST results.

**Objective**: The current study aimed to evaluate the performance of the 1st generation TB Color plate test for selected anti-TB drugs: Isoniazid (INH), Rifampicin (RIF), and Moxifloxacin (MXF) in *MTB* isolates collected from different clinical specimens, particularly sputum and fine needle aspirates (FNA).

**Materials and Methods:**

A retrospective, cross-sectional study design using *MTB* clinical isolates isolated from 2020 to 2022 were used to evaluate the 1G test for testing the drug susceptibility patterns, which were purposively selected (based on quality of the specimens and availability of linked *MTB* clinical data) from stored isolates at the Armauer Hansen Research Institute (AHRI) TB laboratory in Addis Ababa, Ethiopia. A total of 78 MTB isolates that were sub-cultured on LJ media were selected for this study.

Data were entered into an Excel spread sheet and then exported to be analyzed by SPSS version 27; kappa statistic was applied to test agreement between the results of the three assays, one independent reader were involved, and a p-value $<0.05$ was considered statistically significant.

**Result**: For detecting resistance to INH, RIF, MXF and MDR- TB, the sensitivity of the 1G test was 96.8%, 96.4%, 100% and 100%, respectively, and the specificity was 89.4%, 90%, 98.7% and 86% *vs.* IPM. The diagnostic accuracy of the 1G test for INH, RIF, MXF resistance and MDR-TB was 92%, 92%, 99% and 90%, respectively *vs.* IPM. There was strong agreement between the 1G test and IPM ($\kappa$-value = 0.84) for both INH and RIF. Further, INH and RIF susceptibility for 63 *MTB* isolates were also compared using the 1G test *vs.* Mycobacteria Growth Indicator Tube (MGIT) DST. The agreement between the 1G test and MGIT was 86% and 89% for INH and RIF,

respectively.

**Conclusion**: The phenotypic DST 1G test is considered inexpensive (~$1.75), easy to use, rapid for identifying DR-TB using archived pulmonary and extra pulmonary *MTB* isolates from sputum and FNA and thus, it may be a viable option for DST in high endemic and resource limited settings like Ethiopia. The 1G test concurrently performs TB growth detection and DST in stored isolate's, enabling timely treatment and preventing spread of DR-TB within the community. Additionally, the 1G test demonstrated good performance in detecting FNA (extra pulmonary) isolates, suggesting that it could be a useful alternative method for extra-pulmonary TB and DST diagnosis.

**Key Words**: 1G test, color plate test, indirect proportional DST Method, Multidrug-resistant tuberculosis, Pre-XDR Tuberculosis, Tuberculosis

# 1. Introduction

## 1.1. Background

Tuberculosis (TB), is the second leading cause of death by a single infectious agent, after the coronavirus disease 2019 (COVID-19)(WHO, 2023), is caused by *Mycobacterium tuberculosis* complex (MTBC) (Van Soolingen, 1997, WHO, 2023). The MTBC comprises closely related species responsible for strictly human and zoonotic TB. The complex consists of seven species including *M. bovis, M. africanum, M. microti, M. canetti,M. caprae and M. pinnipedi* (Van Soolingen, 1997). The disease is a global public health issue that affects approximately one fourth of the world's population (WHO, 2023) and often manifested either in pulmonary or extra pulmonary form. While the pulmonary form mainly involves the lung, the extra pulmonary TB form may involve organs other than the lung.

According to the World Health Organization (WHO), about 10.6 million people developed TB and 1.3 million died of TB in 2022(WHO,2023).In the WHO African Region, the reported number of people newly diagnosed with TB was limited. Ethiopia transitioned out of the 30 high multi-drug resistant (MDR)/ rifampicin resistant (RR)-tuberculosis (TB) burden countries. It may be due to under diagnosis as there is a limitation of diagnosis of MDR/extensive drug- resistant (XDR)TB detection which requires bacteriological confirmation of TB and testing for drug resistance using rapid molecular tests, culture methods or sequencing technologies(WHO, 2022). The emergence of drug resistance-tuberculosis (DR-TB) also threatens the national TB control program efforts in numerous low and middle-income countries. Furthermore, the incidence rate of RR/ MDR-TB was 1.6%, and eight pre-XDR/ XDR-TB cases were reported in Ethiopia (WHO, 2023)**.** The control and management of the rising burden of DR-TB requires universal access to drug susceptibility testing and individualized treatment approaches. Currently there are limited diagnostic options to test for drug-resistant (DR) -TB, in high TB burden countries. Tests such as the BACTEC™Mycobacteria Growth Indicator Tubes (MGIT), Gene X-pert and Line Probe Assays (LPA), although they provide results for DR-TB, they are costly and require complex equipment, laboratory infrastructures, biosafety needs, and training for lab technicians. These limits

their deployment in point of care settings in low resource communities, where DR-TB cases are rising, especially due to the impact of the COVID-19 pandemic limiting DR-TB testing and treatment (lack of drug supplies), and dedicated personnel (WHO, 2020).

In recent years, the development and evaluation of rapid and low-cost culture methods is considered a priority in the end TB strategy of TB. Several new methods have been developed to reduce the time to diagnosis of TB, such as the liquid media microscopic observation drug susceptibility assay, the nitrate reductase assay, or the colorimetric redox-indicator assay (Moore et al., 2006, Martin et al., 2008). One of the new, low-cost culture methods that reduce the time to diagnose TB is the MDR/XDR-TB color test (TB-CX), a thin-layer agar (TLA) method that allows the initial identification of *M. tuberculosis* complex based on its characteristic cording morphology under the microscope(Robledo et al., 2006). *M. tuberculosis* colonies show irregular wavy margins and appear like small spirals initially, but as they mature, these small colonies acquire cording with distinct irregular margins and ultimately spread over the entire area when viewed through the microscope (Satti et al., 2010, Welch et al., 1993).

TB-CX test has been shown as simple and rapid for the simultaneous detection of *M. tuberculosis* and isoniazid (INH), rifampicin (RIF), and ciprofloxacin (CFX). This method might be a priority for TB prevention and care by providing earlier results directly from specimens than conventional methods and reducing prolonged empirical treatment, facilitating early appropriate treatment of patients with MDR-TB in resource-constrained settings (Shibabaw et al., 2019).

## 1.2. Statement of the Problem

Drug-resistant tuberculosis diagnosis is established by laboratory methods requiring advanced laboratory capacity; however, these methods are not available in most resource-limited settings like Ethiopia. The first step for initiating effective treatment for TB and interrupting transmission is accurate and timely diagnosis. Worldwide, the majority of MDR-TB cases that occur globally are still undiagnosed and untreated. The emergence of extensively DR TB, in addition to challenges in detecting TB among children and people living with HIV has created an urgent need for better technologies. However, gaps remain. Currently, there is no replacement for sputum smear microscopy at the level of peripheral laboratories in resource-limited settings (Ghiasi et al., 2015).

In low-income countries, phenotypic DST is commonly performed with conventional methods such as the indirect proportion method on Lowenstein-Jensen (LJ) or Middlebrook 7H10 agar media owing to the relatively low cost. However, most of these conventional phenotypic methods require several weeks to months to obtain results. In contrast to phenotypic methods, the genotyping approaches of drug resistance detection are rapid but have limited implementation in daily routine, particularly in resource limited areas where the burden of TB is substantial, due to their high cost and need for continuous electric power supply (Palomino, 2009). Liquid medium-based methods such as MGIT can provide first and second-line DST results approximately 10 days after *M. tuberculosis* is isolated in sufficient concentrations for indirect DST but prohibitively expensive in resource-constrained settings (Martin et al., 2009d).

The emergence of strains resistant to the major anti-TB drugs speeds up the need for rapid methods for the identification of resistant *M. tuberculosis* strains to treat the disease effectively and, at the same time, to prevent the spread of resistant strains. Although molecular methods of TB diagnosis are effective, rapid, and detect a resistant strain of TB (Drobniewski et al., 2007), they are often costly, and require skilled personnel and also sophisticated equipment (Nahid et al., 2012).

In 2010, the WHO recommended GeneXpert MTB/RIF® as an initial TB diagnostic test, as it simultaneously detect *Mycobacterium tuberculosis* and RIF resistance, which is often considered as a surrogate marker for MDR-TB in the tested sample. Despite its sensitivity and utility, the

GeneXpert MTB/RIF® implementation in high TB burden areas is costly, requires expensive instrumentation with weekly maintenance and monthly calibration, a sustained power source, and laboratory technicians with specialized training (Evans, 2011).

Relatively the cost of TB-CX test per test is minimal with the advantage of its ability to detect two additional drugs in addition to RIF, such as INH and pyrazinamide (Mekonnen et al.,2019).

Approximately 3 million people with TB were missed due to under-diagnosis as well as underreporting to national TB programs (WHO, 2019). Reductions in the reported number of people diagnosed with TB in 2020 and 2021 suggest that the number of people with undiagnosed and untreated TB has grown, resulting first in an increased number of TB deaths and more community transmission of infection and then, increased numbers of people developing TB (WHO, 2022). The WHO End TB Strategy calls for finding these missing millions in order to meet the sustainable development goal of ending TB by 2030.

New diagnostic tests and optimized test deployment strategies was critical for achieving this target. Detection time, sensitivity, and specificity of samples other than sputum were not assessed in the earlier studies conducted using TB color plate susceptibility test; this study evaluated the performance of the method for fine needle aspirate samples from lymph nodes. Group A fluroquinolone anti- TB drug,moxifloxacin (MXF) was assessed, which was not assessed in before.

## 1.3. Significance of the study

The findings of this study will contribute to the knowledge of the use of DR-TB diagnoses and its potential implication in the expansion and scale-up of the tool for use in resource poor settings, like Ethiopia. The present study will be one to determine the diagnostic accuracy, sensitivity, and specificity of first generation (1G) test (formerly TB-CX) for susceptibility testing of selected anti-TB drugs, compared to phenotypic DST. It enables patients to get their results in a few days than conventional DST methods. It also helps professionals to get results on time which plays an important role in decreasing of tuberculosis transmission. This study can be used as an input for TB control programs and base line data for further studies and investigation.

# 2. Literature review

## *2.1.* Biology of *Mycobacterium tuberculosis*

*M. tuberculosis* is a large non-motile and non-spore-forming, rod-shaped bacillus, grouped under the order Actinomycetales. It is known in the group for the high lipid content in their cell wall. The cell wall is the most distinctive anatomical feature of the bacteria. The bacteria cell wall is constituted by an inner peptidoglycan layer, which seems to be responsible for the shape-forming property and the structural integrity of the bacterium. The common characteristics of the group: acid fastness, extreme hydrophobicity, and resistance to weak disinfectants conferred by the lipid coat. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Murray et al., 2020). The mycobacterial cell envelope of MTBC comprised of four main layers: those are, the plasma membrane or inner membrane, the peptidoglycan can–arabinogalactan complex, an asymmetrical outer membrane, that is covalently linked to AGP via the mycolic acids, and the outermost capsule (Daffe and Reyrat, 2008). *Mycobacterium tuberculosis* is a facultative intracellular pathogen with the ability to infect and persist in humans for decades despite the presence of a completely functioning immune system. (Jankute et al., 2015, Dowdy et al., 2014, Turner and Bothamley, 2015).

## 2.2. Tuberculosis infection

A person can get *M. tuberculosis* through coughing, singing, shouting, sneezing, or any other ways that generate respiratory secretions from the airways, which is the most efficient ways of transmitting tuberculosis infection. TB infection occurs when the source of infection generates infectious particles that stay in the air and are inhaled by a susceptible individual who may become infected and who then may develop TB. Transmission can be interrupted through interventions that target either bacteria or host (Dowdy et al., 2014, Turner and Bothamley, 2015).

Organs other than the lung may be infected via the lymph vessels or bloodstream and produce dissemination foci there. Granulomas, foci fibrosis, scar, and calcification may be formed and the infection remains clinically silent. In about 10% of infected persons, the primary TB reactivates to become organ TB either within months or after several years. Cytokines like tumor necrosis factor alpha play an important role in tissue destruction. These cytokines are also responsible for

the cachexia associated with TB. The body's immune defenses have a hard time in containing necrotic tissue lesions in with large numbers of Mycobacterium cells occur (Kayser, 2005).

### 2.3. Anti-TB drugs and their mechanisms of action

Anti-TB drugs that are used for the treatment of all types of TB are classified as first- and second-line anti-TB drugs. First-line anti-TB drugs include isoniazid, rifampicin, pyrazinamide (PZA), ethambutol (Van Soolingen), and streptomycin. INH and RIF are the two most commonly used Anti-TB drugs. First-line anti-TB drugs are safe and effective if used correctly. The emergence of MDR/XDR-TB treatment makes DR -TB treatment complicated. Second-line anti-TB drugs that are used for treating DR-TB are listed as aminoglycosides; e.g., amikacin and Kanamycin (KM); polypeptides: e.g., capreomycin, viomycin and enviomycin; FQ; e.g., ciprofloxacin, levofloxacin, ofloxacin (OFL), MXF and gatifloxacin; and thioamides: e.g., ethionamide, prothionamide and cycloserine, and P-aminosalicylic acid (Biadglegne et al., 2014)

The mechanisms of action of INH are one of the most complexes of all antibiotics because they interfere with nearly every metabolic pathway in mycobacteria. The current model is: INH enters mycobacteria by passive diffusion and is activated by KatG to a range of reactive species or radicals and isonicotinic acid. These reactive species and radicals attack multiple targets, e.g., mycolic acid synthesis, deoxyribonucleic acid (DNA) damage, lipid peroxidation, and nicotinamide adenine dinucleotide metabolism in the cell (Shi et al., 2007). RIF is the most widely used drug in TB treatment, and resistance mechanisms are also paid more attention in research fields. RIF binds with RNA polymerase of *M. tuberculosis* interferes with protein synthesis, thereby a bactericidal effect will occur (Goldstein, 2014). Inhibiting DNA topoisomerase IV and DNA gyrase is the mechanisms of action for Fluoroquinolones, such as ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, and moxifloxacin. The main targets of fluoroquinolones are the GyrA and GyrB proteins (Mayer and Takiff, 2014).

## 2.4. Drug Susceptibility Test

Phenotypic drug susceptibility testing (DST) takes weeks to yield a result and is not widely available for newer drugs. Currently, molecular testing is increasingly the method of choice for the diagnosis and assessment of drug-resistant mutations for TB. Several molecular methods have been developed over the past decade. These include the Xpert® MTB/RIF, Ultra, MTB-XDR, LPA, and Loop-mediated isothermal amplification (Ciesielczuk et al., 2020).

### 2.4.1. Proportion method using Lowenstein– Jensen medium

Using the proportion method, the Lowenstein–Jensen (LJ medium can be used for susceptibility testing to anti-TB drugs. Media containing the critical concentration of the anti-TB drugs is inoculated with a dilution of a culture suspension and control media without the anti-TB drug is inoculated with a 1:100 dilution. The growth of the drug-containing media is compared with the growth of the drug-free control media. The ratio of the number of colonies on the medium containing the anti-TB drug to the number of colonies on the medium without the anti-TB drug is calculated, and the proportion is expressed as a percentage. It takes 3-4 weeks of incubation to get a result for susceptible isolates and 6 weeks of incubation for resistant isolates. It is used for 1$^{st}$ and 2$^{nd}$ line anti TB drug susceptibility testing (WHO, 1998).

### 2.4.2. Proportion method using Middle-brook 7H10 or 7H11 agar media

Middle-brook 7H10 or 7H11 agar media is used to determine whether isolates of *M. tuberculosis* are susceptible to anti-TB drugs. A standardized suspension of *M. tuberculosis* is prepared, and specified dilutions are made; these are then inoculated onto media containing anti-TB drugs and on to control media that do not contain an anti-TB drug. The ratio of the number of colonies on the medium containing the anti-TB agent to the number of colonies without the anti-TB agent is calculated (corrected for dilution), and the proportion is expressed as a percentage. An isolate is considered resistant if the proportion of the number of colonies on the medium containing the anti-TB agent to the number of colonies on the control medium is 1% or greater. DST using

Middle-brook media is often performed using plastic quadrant Petri dishes in which quadrants contain either medium without an anti-TB agent or medium with one of the anti-TB agents being tested (Kent, 1985).

### 2.4.3. MGIT DST

The automated MGIT system requires an instrument called the BACTEC 960 & MGIT tube with 7ml medium containing growth supplement and oxygen-quenched fluorochrome – tris (4,7-diphenyl1,10-phenonthroline) ruthenium chloride pentahydrate – embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced by CO2. The depletion of free oxygen results in the fluorescence of the sensor within the MGIT tube, which is directly proportional to the extent of oxygen depletion. If the test agent is active against the mycobacteria, it will inhibit the growth and, thus, there will be suppression of fluorescence while growth in the control tube will be uninhibited and fluorescence will increase. Growth is monitored by the MGIT 960 instrument, and is recorded in values known as Growth Units (GU). In the case of first-line agents, the instrument automatically interprets the difference in GU values between the control tubes and the tubes containing the agent; results are reported as susceptible or resistant. The principles of the proportion method have been used to establish procedures for DST for both first-line and second-line agents. The protocol for the BACTEC MGIT 960 susceptibility test usually takes for 4-13 days (less turnaround time than manual methods) for both first-line and second-line agents (Siddiqi et al., 2012)

### 2.4.4. Gene-Xpert

Xpert MTB/RIF (Cepheid, United States), since 2013, has been recommended also for the diagnosis of TB in children and of some specific extra-pulmonary forms. It is one of the most frequently used molecular tests for the quick diagnosis of tuberculosis and drug-resistant TB. It is an automated, real-time nucleic acid amplification technology. The assay is used to diagnose TB and MDR-TB by simultaneously detecting *M. tuberculosis* and mutations that confer rifampicin resistance in a closed system, suitable for use outside of traditional laboratory settings in 2 hours The Xpert MTB/RIF assay uses semi-quantitative nested real-time PCR to amplify a fragment containing the 81 bp hotspot region of the rpoB gene (codons 507–533) that is then hybridized to

five molecular beacon probes. However, some studies reported false-positive results with Xpert MTB/RIF due to silent mutations (Bunsow et al., 2014).

### 2.4.5. Sequencing

Sequencing is the best technology to rapidly analyze the genotype of an organism. Besides targeted gene sequencing, the development of Next Generation Sequencing (NGS) has been a major breakthrough in molecular biology, because it can rapidly provide whole genome data in a single run. This allows species identification, screening of all (synonymous and non-synonymous mutations, insertions and deletions) in a sample, detecting of drug resistance, and prediction of the organism's evolution. (Dheda et al., 2017, Manson et al., 2017). NGS which is faster than traditional phenotypic culture is increasingly considered a promising option for comprehensive DST for TB. Unlike probe-based assays where detection is limited to probe-specific targets, NGS-based assays can provide detailed and accurate sequence information for whole genomes, as with whole-genome sequencing, or multiple gene regions of interest, as with targeted NGS (WHO, 2018). The drawbacks of molecular tests are that they are unable to cover the entire genome, and numerous mutations causing resistance.

### 2.4.6. Thin Layer Agar

The TLA technique is also known as the micro-colony method which uses Middlebrook 7H11 agar for the detection of micro-colonies of mycobacteria. It was originally described for the rapid detection of mycobacterial growth and later applied as a rapid DST method (Welch et al., 1993). TLA uses quadrant agar plates with drug-free and drug-containing quadrants with critical concentrations. The quadrants are inoculated with the processed sample and incubated at 37 ºC with 5% CO2. The plate is then examined under a light microscope with 10x magnifications for cording characteristics of the colony growth (Martin et al., 2009d).

## 2.5. Management of drug resistance to tuberculosis

Combinations of first-line and second-line drugs are used for the treatment of MDR and XDR tuberculosis according to the results of DST. Long regimens for MDR and XDR TB are longer, less effective, less tolerable, and more expensive than standardized short-course chemotherapy. The percentage of patients who are cured from MDR-TB is estimated to be no more than 69 % on the basis of results from retrospective cohort studies, even if they are treated for more than 18 months (Orenstein et al., 2009).

Drug susceptible TB trials are investigating the options to reduce the treatment duration down to 4 months, for MDR-TB the research efforts are devoted to the possibility to reduce it to 6-9 months. New WHO recommendations are bridging the implementation gap and facilitating access to treatment with all oral, shorter, less toxic treatment regimens. DR-TB patients with extensive pulmonary disease, severe forms of extra pulmonary TB, additional resistance to fluoroquinolones or those who have been exposed to treatment with second-line medicines for more than 1 month will require an individualized longer regimen, including at least three Group A agents (i.e. bedaquiline, levofloxacin,MXF and linezolid) and at least one Group B agent (cycloserine/terizidone and/or clofazimine) (Battista et al., 2020 ).Group C drugs may be added to the regimen through resistance or tolerability a regimen. WHO has updated recommendations and is now phasing out the shorter injectable-containing regimens and recommending a shorter all-oral bedaquiline-containing regimen for eligible MDR/RR-TB patients under specific conditions (WHO, 2019).

To combat the global emergence of drug resistance, beyond conventional approaches alternative approaches such as pathogen-centric (use of repurposed drugs, novel analogs of existing anti-TB drugs, novel and compounds with a different mechanism of action), host-centric (immune-modulatory agents, therapeutic vaccines, immune and cellular therapies) and nano- based drug/vaccine delivery should be used singly or in combination. Diverse types of nano- carriers have assessed as auspicious diagnostic and drug-delivery systems (Sharma et al., 2020).

## 2.6. Mechanisms of Drug Resistance

The mechanism involved in DR-TB is mainly due to primary or secondary drug resistance. Acquired/secondary drug resistance is due to incomplete or suboptimal treatment resulting in the selection of mutant resistant strain which is acquired during treatment in initial sensitive TB. In cases of primary resistant TB, the individual is infected with DR-TB strain. Worldwide including in India, secondary MDR-TB is more common than primary DR-TB infection.(Moore et al., 2006,Shah et al., 2020) .

Particularly, INH makes a covalent adduct with the nicotinamide adenine dinucleotide (NAD) co-factor and that adduct complex acts as a tight-binding competitive inhibitor of inhA. Resistance to INH is a complex process. Mutations in several genes, including katG, 82 ahpC, inhA, kasA and ndh, have all been associated with INH resistance.

Mutations in 83 katG are the major mechanism of INH resistance**.** Mutations in a 'hot-spot' region of the 81-bp region of rpoB are found in 101 about 96% of RIF-resistant *M.tuberculosis* isolates (Lohrasbi et al., 2018).

RIF inhibits RNA synthesis, while oxazolidinones (linezolid), aminoglycosides, macrolides, and cyclic peptides inhibit protein synthesis, among them linezolid is the potential orally existing drug targeting MTB protein synthesis. Mutated rpoB encourages a conformational change towards the binding affinity of RIF at β-subunit of the RNA polymerase, and the drug became inactive without proper binding to the exact target site (Campbell et al., 2001).

The only mutations proven to confer fluoroquinolones (FQ) resistance in *M. tuberculosis* occur in the FQ target, the DNA gyrase, at critical amino acids from both the gyrase A and B subunits that form the FQ binding pocket. Substitutions in GyrA are much more common and generally confer higherlevels of resistance than those in GyrB. Variable percentage of phenotypically resistant strains, which confirmed by phenotypic methods are missed by molecular techniques. The inability to find gyrase mutations may be explained by hetro-resistance: bacilli with a resistance- conferring mutation are present only in a minority of the bacterial population and are therefore detected by the proportion method, but not in a sufficient percentage to be reliably detected by molecular techniques. Moxifloxacin is currently the best anti-TB FQ and is being tested for use with other new drugs in shorter first-line regimens to cure drug-susceptible TB (Moore et al., 2006).

Poor regimen selection, inadequate drug supply, and poor adherence of patients to the 6-months therapy may lead to the development of drug-resistant *M.tuberculosis* strains, including (MDR: resistant at least to INH and RIF) and XDR strains (Nahid et al., 2019).

## 2.7. TB color test(1G) and its performance

An in-house technique for DST that enables concurrent early micro-colony detection and concurrent DST interpretation is the thin-layer agar MDR/XDR-TB 1G Test (CT) (WHO, 2011).The 1G medium includes a redox indicator 2,-3-diphenyl-5-(2-thienyl) tetrazolium chloride that stains the micro-colonies red and makes them visible to the naked eye when approximately 1 mm in diameter (Toit et al,2012).1G consists of a 4-quadrant Petri dish of which 1 quadrant serves as a growth control with no anti-TB drugs and 3 quadrants are supplemented with food colouring.1G used for DST to several drugs, e.g., INH,RIF and ciprofloxacin (CFX)(WHO,2011).

The predominant infectious disease that causes death in developing countries is due to TB than any other infectious disease. This is due to less health care access, as well as higher exposure to unhealthy and crowded living and working conditions, under-nutrition, HIV infection, diabetes mellitus, smoking, alcohol and drug abuse, and several other TB risk factors (Luciani et al., 2008).

Multidrug-resistant MDR -TB, defined as a disease caused by *Mycobacterium tuberculosis* strains with resistance to, at least, INH and RIF, is a growing public health and clinical problem worldwide (Zignol et al., 2006). The emergence of XDR-TB defined as MDR-TB strain that is resistant to either fluoroquinolones or second-line injectable drug but not both, has risen in every region of the world. TB drug resistance is caused by inadequate therapy enabling the selection and growth of resistant populations (i.e., acquired resistance) or by infection with a drug-resistant strain (Shah et al., 2007). The MDR/XDR- TB-CX-test is a non-commercial, thin-layer agar-based tuberculosis culture method capable of simultaneously detecting M. tuberculosis and tuberculosis drug susceptibility in approximately 14 days. The TB-CX contains four quadrants: one quadrant detects M. tuberculosis growth and the other three quadrants detect resistance to INH, RIF and a FQ (i.e., MXF). The effective control of TB is based on the immediate detection of *M. tuberculosis*, followed by the prompt implementation of adequate anti -TB therapy (Shibabaw et al., 2019 and Coll et al., 2005).

A study conducted in France compared the sensitivity and time to detection of growth of *M tuberculosis* in the TLA with BACTEC MGIT960. The average time for growth of *M. tuberculosis* in TLA and BACTEC MGIT960 was 10.6 and 9.6 days, respectively. The sensitivity of detection

of *M. tuberculosis* was 97.3% on TLA and 97% on BACTEC MGIT960 for smear positive samples. TLA showed comparable results to BACTEC MGIT960 and could be an alternative method for low-income countries (Martin et al., 2009a).

A prospective study conducted on a large number of sputum samples (1413) in people with suspected pulmonary TB in Indonesia evaluated the performance of TLA for diagnosing TB suggested as an equally sensitive and faster alternative for the diagnosis of TB. The sensitivity of TLA was 0.86 (95% CI 0.80–0.90), significantly higher than for LJ (0.76; 95% CI 0.69–0.81). The median time to detection in days was significantly shorter for TLA (12 days) than for LJ (44 days).Studies that should be implemented to evaluate the cost-effectiveness and impact of its introduction into programmatic settings are urgently needed (Battaglioli et al., 2013).

A study conducted in Georgia compared 279 TLA DST results with those of MGIT for INH and RIF, and 280 results for OFX and KM with those of the 7H11 agar proportion method, showed 94.7%, 98.2%, 100% and sensitivity, respectively, for INH, RIF and OFL, with 100% specificity. The average time to results was 7 days in TLA, 23 in MGIT and 49 for 7H11 agar. The culture positivity rate for TLA and MGIT was respectively 95.3% and 97.8% (Ardizzoni et al., 2015).

In a study conducted in Estonia using 201 archived *M. tuberculosis* clinical isolates archived during the years 2004–2010 setting, the TB-CX-test detected drug resistance, with 98% sensitivity for INH, RIF, and CPF and 99% for MDR-tuberculosis, compared to drug susceptibility testing results using BACTEC MGIT 960. The average time to positivity was 13 days, with interpretable results (Toit et al., 2012).

A study conducted in rural Malawi showed a high level of concordance between the AFB smear (12 positive) and TB CX-test with an average time of 10 days to a positive TB CX-test. The TB CX test detected no cases of DR from the positive sample, which was later confirmed by the GeneXpert. TB-CX-test accurately diagnoses active pulmonary tuberculosis and detects drug susceptible tuberculosis in low-income and high tuberculosis-burden settings (Zhang et al., 2018).

A prospective study performed in Homa Bay District Hospital in Kenya showed that TLA could become an affordable method for the diagnosis of smear-negative tuberculosis in resource- limited settings, with results available within 2 weeks. From a total of 1,584 smear-negative sputum samples, 212 (13.5%) were positive by culture in LJ medium and 220 (14%) were positive by the TLA method. The sensitivities of LJ and TLA were 71% and 74%, respectively.(Martin et al., 2009c) (Martin et al.,2009). A comparative study conducted in Gonder, Ethiopia, showed that the detection time for TB -CX was 12 days, which was significantly lower than LJ-MGIT (35 days). TB -CX test had: 59%, 96% and 95% sensitivity; 96%, 94% and 98% specificity; and 85%, 94% and 98% agreement for INH, RIF and MDR-TB, respectively. All ciprofloxacin DST results were susceptible to both methods (Shibabaw et al., 2019).

A cross-sectional hospital-based validation study conducted in Addis Ababa, Ethiopia, witha total of 137 participants showed that the average time that TB CX was detected TB was 13 days. The sensitivity and specificity of the TB-CX test for detecting *M. tuberculosis* were 94% and 98%, respectively. The sensitivity of the TB-CX test to detect drug resistance to INH, RIF, and MDR-TB was 91%, 100%, and 90% respectively. The specificity of the TB-CX test for detectingINH, RIF, and MDR-TB was 94%, 40%, and 94% respectively. Overall agreement between TB- CX test and LJ DST for detection of MDR-TB was 93 %.(Mekonnen et al., 2019).

# 3. Objective

## 3.1. General objective

To evaluate performance of 1st generation color susceptibility testing (1G test) with IPM reference standard for selected anti-TB drugs (INH, RIF, MXF) and MGIT DST in *M. tuberculosis* isolates collected from different specimens archived from 2020 to 2022.

## 3.2. Specific objectives

- To evaluate sensitivity, specificity, and positive and negative predictive values of 1G test in detecting resistance against RIF, INH & MXF in MTB isolates with IPM and MGIT
- To estimate the average time to positivity (turnaround time) of 1G test
- To evaluate performance of 1G test among sputum and fine needle aspirate (FNA) specimen- derived isolates.

# 4. Methods and materials

## 4.1. Study Setting

The AHRI is one of the biomedical research institutes in Ethiopia, under the Ministry of Health with a P-3 lab and the capacity to isolate, grow, characterize, and perform DST on *M. tuberculosis* isolates. The laboratory is based within one of the national MDR-TB inpatients hospitals, the All-Africa Leprosy Rehabilitation and Training Hospital (ALERT). As one of the national TB diagnostic and research laboratories, the AHRI laboratory receives specimens from patients being evaluated for MDR-TB for drug resistance testing and has a large repository of *M. tuberculosis* isolates from diverse pool of specimens. The source of archived isolates was from patients of the National TB control program of Ethiopia, those who are tested at AHRI.

## 4.2. Study design, sampling method and period

This study was retrospective, cross-sectional by design and was conducted from February 2023 to July 2023 to evaluate the 1G test for testing the drug susceptibility of INH, RIF, and MXF of stored *M. tuberculosis* isolates at AHRI laboratory. A purposive sampling method was used to select *M. tuberculosis* stored isolates from the AHRI laboratory repository, depending on the availability and quality of isolates. Linked clinical and/or additional laboratory data (such as HIV status, treatment history, age, sex and origin of samples, either from FNA and sputum) were also collected. *M. tuberculosis* isolates archived from 2020 to 2022 were selected and retrieved for this project. Isolates collected from pulmonary TB (PTB) and extra pulmonary TB (EPTB) patients and that were grown on culture media (regardless of smear, gene-Xpert result or DST pattern) were considered for the study.

## 4.3. Sample source

The source of sample of this study was all *M. tuberculosis* isolates from 2020 to 2022 which were stored at AHRI TB Laboratory

## 4.4. Study material

*M. tuberculosis* isolates from sputum samples that were positive less than 1-month, from Fine needle aspiration of lymph nodes regardless of their resistance pattern were considered for this study.

## 4.5. Inclusion and exclusion criteria

### 4.5.1. Inclusion criteria

- Isolate from sputum which was MTBC positive and stored at AHRI TB laboratory, with clinical and laboratory information (Age, sex, HIV status, Treatment history, and MTBC result).
- All isolates from lymph node aspirate which were MTBC positive

### 4.5.2. Exclusion criteria

- Mislabeled & Non- *M. tuberculosis isolates*, volume less than 1 ml

## 4.6. Study variables

### 4.6.1. Dependent variables

Specificity and sensitivity, Accuracy, Positive and negative predictive value

### 4.6.2. Independent variables

Age, sex, treatment status, sample type &, TB Type,

## 4.7. Sample size

The sample size required for this study was calculated using the formula estimating sample sizes for evaluating the sensitivity and specificity of diagnostics test.

To determine the sample size, 95% anticipated sensitivity and specificity with absolute precision of less than 7% at 95% confidence interval (CI) was used (Baveja and Aggarwal, 2017, Abate et al., 2012). The prevalence of MDR-TB of 46.3% was used from a previous study in the TB referral hospital of Ethiopia (Abate et al., 2012).

Sensitivity 95%, specificity =95% (from previous research), level of precision (7%)

$$TP + FN = Z^2 \, x \, \frac{Sensitivity\ (1 - Sensitivity)}{W^2}$$

$$TN + FP = Z^2 \, x \, \frac{Specificity\ (1 - Specificity)}{W^2}$$

TP+FN=$1.96^2$ ×0.95(1-0.95)/$0.07^2$ =37.24

TN+FP=$1.96^2$ ×0.95(1-0.95)/$0.07^2$ =37.24

N required for sensitivity = $\frac{TP+FN}{P}$ = 37.24/0.46=81

N required for specificity= $\frac{TN+FP}{1-P}$ =37.24/1-0.46=69

Therefore, the larger one-81- was selected as sample size.

### 4.8. Data collection

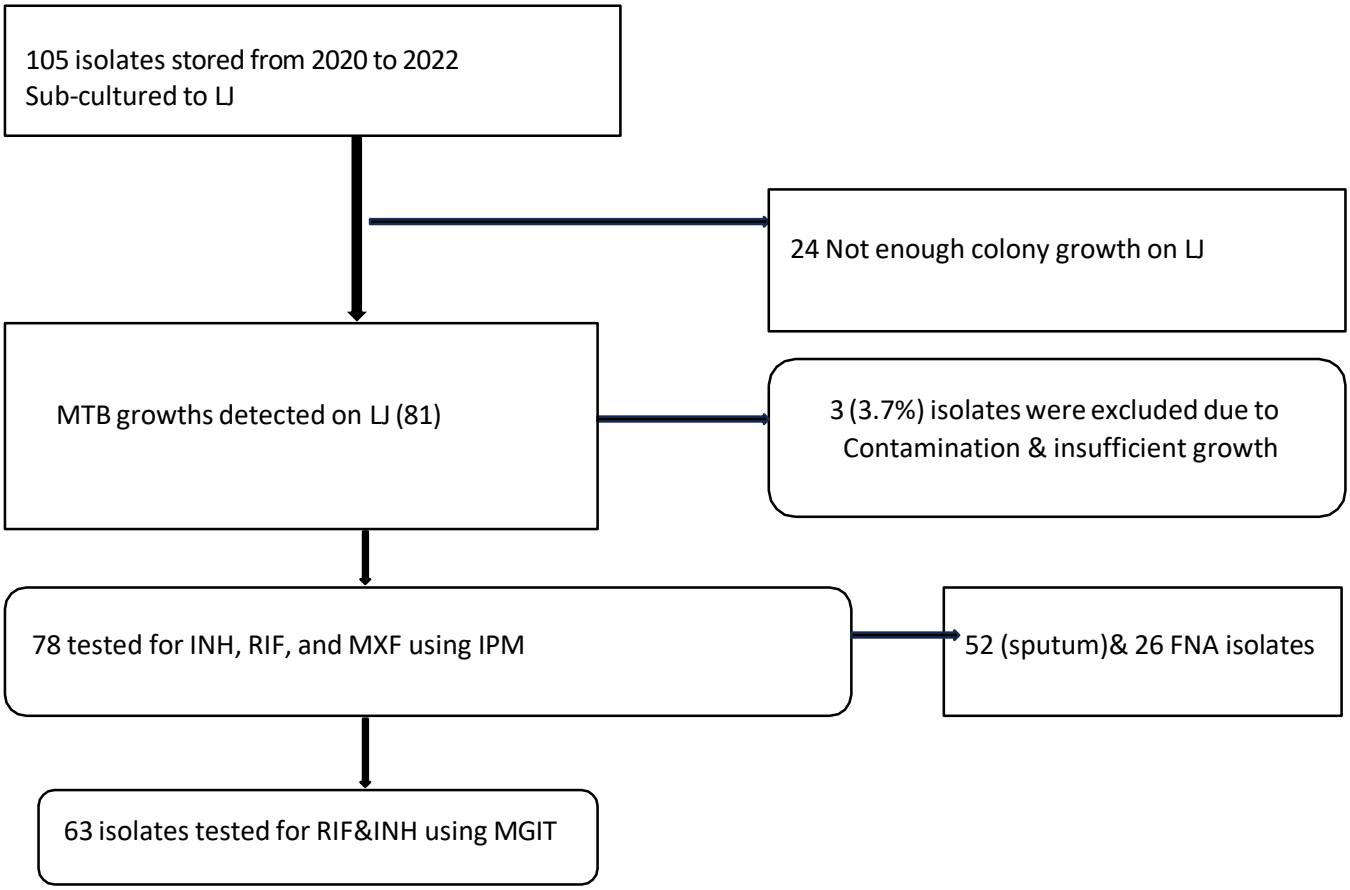
Demographic (age and sex), clinical (HIV status and treatment history) and laboratory information of isolates (MTB result and sample type) were collected from AHRI laboratory record based on the format (table 4) which is designed for this purpose.

### 4.9. Laboratory method

In this study, three laboratory tests were included namely 1G test (new), the IPM (reference method) and MGIT DST.

A total of 105 stored *M. tuberculosis* isolates were cultured to Lowenstein–Jensen (LJ) medium, 81 of them were included in this study; the remaining isolates were rejected, as the number and quality of colony growth did not pass the quality checks (fig 2). Following, the 81 *M. tuberculosis* isolates were inoculated to a 24 IPM and 1G plates to evaluate the performance of 1G test DST for selected anti-TB drugs (INH, RIF, and MXF), of which 3/81 (3.7%) were excluded because of contamination and insufficient growth and the majority, 78 (96.3%) produced findings that could be interpreted.

Figure 2: Diagrammatic presentation of how isolates were selected & tested with DST methods



### 4.9.1. Suspension preparation

All work manipulating *M. tuberculosis* isolates took place in a certified biosafety cabinet to protect the user from exposure to high concentrations of pathogens. Identify mycobacterial colonies and suspend the mycobacteria in 5 drops (~200 µl) of sterile distilled water in the base of a plastic universal container which is covered with 2-3 mm sterile glass beads. The plastic universal container was tightly closed and vortexes for 30 seconds. It was settled down for 20 minutes. A few ml of sterile water was added and checked for no macroscopic clumps. McFarland 1 Standardization was prepared and diluted accordingly for inoculation of IPM and 1G test.

### 4.9.2. A24-well plate assay susceptibility testing (Indirect proportion method)

The drug susceptibility patterns of the clinical isolates were determined using a modified indirect proportion method(IPM) adapted for 24-well agar plates containing 7H10 medium. Plates were prepared manually. Drug susceptibility was read by visual comparison of the drug- containing media (1:1 bacterial suspensions) with the drug-free control on which 1:100 bacterial suspensions was inoculated. The growth was evaluated according to the proportion method by comparing the 1:100 diluted controls to the drug containing wells. The test was repeated was if there was equal growth on the drug control and drug containing wells. The strain was reported susceptible (S) if there is clearly more growth in the 1:100 diluted control than in the drug containing well with the critical concentration and resistant (R) if there is more growth in the drug containing well than in the 1:100 control (Wedajo et al., 2014).

#### 4.9.2.1. Preparation of drug-containing agar series in 24-well plates

Two days before inoculation, each experimental batch consisted of twenty DST 24-well plates were prepared. To achieve the necessary antibiotic critical concentrations (cc) (INHcc=0.2ug/ml, RIFcc 1.0Ug/ml, MXFcc 0.5ug/ml (WHO, 2018), the antibiotics were diluted for each of three tested drugs (Table 1). A volume of each antibiotic (100µl for INH, RIF=500µl, MXF=250µl) was mixed with 7H10 medium in a 50 ml Falcon tube in a water bath to achieve the required drug concentration needed for 20 plates. After that, manual transfers of 2 ml of the media were performed into the appropriate wells of the 24-well DST plates, both drug containing and drug free

media. 2-3 DST plates were incubated in an incubator following preparation to ensure sterility before use.

*Table 1*: Schematic arrangement of the 24-well plate

| | | | | | |
|---|---|---|---|---|---|
| **INH**<br>0.064 µg/ml | | **INH**<br>0.125 µg/ml | | **INH**(CC)<br>0.2 µg/ml | |
| **INH**<br>1.0 µg/ml | | **RIF**(CC)<br>1.0µg/ml | | **MXF** (CC)<br>0.5 µg/ml | |
| | | | | | |
| | | | | **Control**<br>**(1:1)** | **Control**<br>**(1:100)** |

CC=Critical Concentration

#### 4.9.2.2. Inoculation

One McFarland Suspensions of *M. tuberculosis* complex isolates were prepared in biosafety cabinet, in bottle containing water and glass beads. Each and a bottle with glass beads were labeled with identification number.10 µl of the suspension was added to each well, using the micropipette, but was not inoculated in the second control well. 10 µl of bacterial suspension was added to the tube containing 1ml of H2O and swirled to prepare 1:100 suspension. Then 10 µl of this diluted suspension was added to the second control well. The plates were placed in a rack, in stacks of 3 pieces, in a 37 °C incubator for 48 hrs. Then the lid was covered with adhesive tape on the plates on two sites for reasons of safety and incubated at 37° C for about 21 days until there was growth. H37Rv strain, which is sensitive to all of the test drugs, and resistant strains were inoculated as controls for each batch of tests.

#### 4.9.2.3. Reading and interpretation of IPM DST

After 2 to 3 days, the plates were read to see if there was any contamination. The plates were checked for mycobacterial growth at 6, 12, and 19 days. The presence of growth on the 1:10 and 1:100 control wells was examined to ensure its validity. The result of the isolates was read by visual comparison of the drug-containing Media (1:1 bacterial suspension) with the drug-free control on which 1:100 bacterial suspensions were inoculated. The Critical concentration determines whether a strain is sensitive or resistant. The strain was reported susceptible if there was clearly more growth in the 1:100 diluted control than in the drug-containing well with the critical concentration of the drug and resistant if there was clearly more growth in the drug-containing well containing the critical concentration than in the 1:100 diluted control.

### 4.9.3. MGIT DST

The automated MGIT system requires an instrument called the BACTEC 960. The MGIT medium consists of modified Middlebrook 7H9 broth and an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced by $CO_2$. The depletion of free oxygen results in fluorescence of the sensor within the MGIT tube when visualized under ultraviolet light.

#### 4.9.3.1. Inoculation and Reporting

One ml of the supernatant from fresh-grown MGIT culture was transferred to a clean sterile container containing 4.0 ml of sterile saline and mixed well. A 1:5 dilution of the test culture was used to inoculate the test medium in tubes containing the anti-TB drug. Three tubes were labeled one tube as “growth control” and two tubes for INH and RIF. Aseptically 0.8 ml of BACTEC 960 SIRE supplement was added to each of the MGIT tubes and mixed well. Then 0.1 ml (100 ml) of the anti-TB agent was added to the appropriately labeled MGIT tube and mixed well. Finally, 0.5 ml of the well-mixed suspension (inoculum) was added to each of the tubes containing the anti-TB agent, but not added to the control tube. Loading of tubes to MGIT 960 was carried out by scanning the barcode and placed accordingly in the correct order in the carrier. Once the test had been

Once the test had been completed, the instrument indicated that the results were ready. The carrier was then removed, its barcode was scanned, and the report was printed.

### 4.9.4. TB Color plates(1G) susceptibility testing

#### 4.9.4.1. Preparation of TB color plate(1G)

The Ohio State University medical laboratory in the United States prepared a 1G test plate for the current study, which was then sent to Ethiopia. After that, until it was used, it was kept in a cold chamber (2–8 °C). This is a non-commercial direct method that allows MTB to be cultured and its DST tested simultaneously on a single agar plate. Based on color variations of the colony in the four color-coded quadrants of the plate, this particular color plate assay makes it easier to interpret DST results. One quadrant is used to detect growth (clear color), and the remaining three quadrants are used for DST. Figure 1 shows the quadrants for green-INH, yellow-RIF, and blue-MXF. Growth of MTB in drug-free media signifies a positive culture, whereas growth of MTB in media that contains both drugs and free agents suggests resistance.

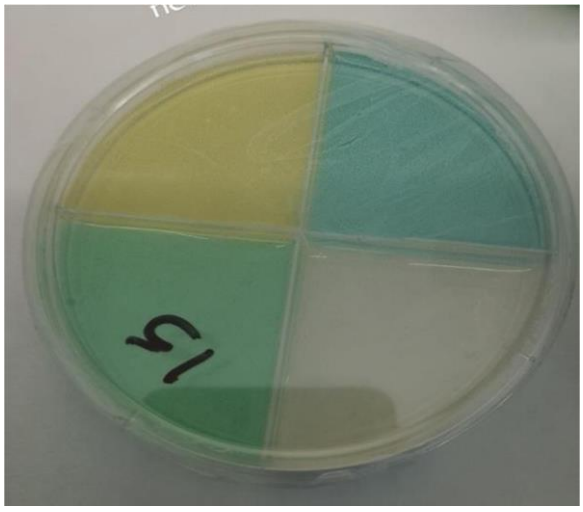


Figure 1**:** TB Color plate photography: growth detection quadrant (clear), INH (green), RIF (yellow) and MXF (blue)

#### 4.9.4.2. Reading and Interpretation

For a maximum of 42 days, the plates were visually examined three times per week until at least 11 colonies developed in the drug-free control quadrant. (Klaos et al., 2021).

For safety reasons, the reading of the plates was done without removing the plastic bag or opening the petri dish. The 1G test culture plate was read by holding it against a light background and checked for the growth of colonies that were visible to the naked eye as red spots.

The plate is identified as having TB if TB grows in the detection well, at which point the other 3 drug-containing quadrants are immediately inspected. There should be growth in the DS quadrant before proceeding to interpret the drug-containing quadrants. Once, you observe more than 10 colonies in the DS quadrant, the plate and the drug-containing quadrants are ready to interpret regardless of the days of incubation. Incubation of the plate was continued until >10 colonies grew in the detection quadrant and then reported drug-susceptibility for the drug-containing wells.

## 4.10. Laboratory quality control measures

All pre-analytical, analytical, and post-analytical steps were followed; briefly, the appropriate freezer temperature of the equipment and lab parameters were monitored during isolate storage and monitored regularly. Approved standard operating procedures were followed while sample preparation and handing; and growth of H37RV was run regularly before running the actual sample to check the performance of 1G & drug resistant strains (For RIF, INH&MXF) was used as a DR control.

## 4.11. Data analysis and interpretation

The data was entered into Excel, cleaned, and imported to SPSS (statistical package for social sciences) version 27 for further statistical analyses. Appropriate statistical analysis was used; sensitivity specificity, PPV, and NPV were calculated using SPSS. Statistical tool and agreements between the tests will be analyzed using kappa values. Data was summarized using descriptive statistics, frequency tables, and figures. A P-value less than 0.05 was considered statistically significant

## 4.12. Ethical considerations

Before the commencement of the study, ethical approval was sought from the Department of Research and Ethics Committee (DREC) of the Department of Microbiology, Immunology & Parasitology (DMIP), College of Health Sciences (CHS), and Addis Ababa University & AHRI/ALERT Ethics Review Committee (AAERC). Since the study used archived specimens and linked archived data, an institutional permit was sought from AHRI to access bio repository samples and linked archived data. To ensure the privacy and confidentiality of study participants, only coded samples were considered, while retrieving samples from AHRI bio repositories, and no personal identifiers were used during the communication of the research findings. A waiver of consent was requested and granted since it was not possible to contact the participants.

# 5. Result

## 5.1. Demographic and clinical characteristics of selected isolates

Of those 78 isolates, 26 (33.3%) were lymph node aspirates from extra pulmonary TB patients and 52/78 (66.7%) were sputum-derived from pulmonary TB patients. Most of these samples, 62/78 (79.5%) were collected from newly diagnosed TB patients who were also new to anti-TB drugs and about 5.1% (4/78) had a prior history of anti-TB treatment. The HIV positivity rate among the study population whose samples were extracted was 5/78 (6.4%) (Table 2).

*Table 2*: Demographic and clinical characteristics of participants whose isolates were included

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Treatment history | | Frequency | percent | | | | percent |
| | Failure | 6 | 7.7 | Age | Mean | 30.3 | |
| | New | 62 | 79.5 | | Median | 27 | |
| | Previously treated | 4 | 5.1 | | Minimum | 0.5 | |
| | Relapse | 3 | 3.8 | | Maximum | 70 | |
| | Unknown | 3 | 3.8 | | Range | 69.5 | |
| | Total | 78 | 100 | | Interquartile range | 11.5 | |
| HIV status | Positive | 5 | 6.4 | Sex | Female | 43 | 43.6 |
| | Negative | 44 | 56.4 | | Male | 34 | 55 |
| | Unknown | 29 | 37.2 | | Unknown | 1 | 1.3 |
| | Total | 78 | 100 | | Total | 78 | 100 |

## 5.2. Time to MTB Culture Growth Detection and DST Results

Time to culture growth (red colonies) as depicted in figure 2 was calculated in terms of days from the date of inoculation to the date of interpretable/valid positive results (Table 3). The date of positive-result growth of red colonies availability for each isolate was registered as a turn-around time (TAT). The TAT for 1G test plate test (concurrent MTB detection and susceptibility results) ranged from 5 to 18 days with a median and mean time of 9 days, while the range TAT for reference DST using IPM (Indirect Proportional Method) ranged from 14 to 21 days. The time for detection of resistance to INH, RIF, MXF and MDR-TB by 1G test was relatively faster (average of 9 days, range of 13 days) than IPM average of 15 days, and indirect MGIT DST with an average of 19 days. The average time of detection of resistance of 1G test for *MTBC* isolates from pulmonary (sputum) and extra pulmonary (FNA) was 9 days (8.5) and 9 days (8.9), respectively. 87.3% (24/26)of FNA isolates and 96% (50/52) of sputum isolates tested with 1G test had results within 12 days.

Table 3: 1G test DST Result reported in different days in FNA and sputum isolates

| | | | FNA | Sputum | Total |
|---|---|---|---|---|---|
| TAT days | 5 | Count | 1 | 0 | 1 |
| | | % Within Sample type | 3.8% | 0.0% | 1.3% |
| | 6 | Count | 8 | 19 | 27 |
| | | % Within Sample type | 30.8% | 36.5% | 34.6% |
| | 8 | Count | 0 | 1 | 1 |
| | | % Within Sample type | 0.0% | 1.9% | 1.3% |
| | 9 | Count | 12 | 23 | 35 |
| | | % Within Sample type | 46.2% | 44.2% | 44.9% |
| | 12 | Count | 3 | 7 | 10 |
| | | % Within Sample type | 11.5% | 13.5% | 12.8% |
| | 14 | Count | 0 | 1 | 1 |
| | | % Within Sample type | 0.0% | 1.9% | 1.3% |
| | 15 | Count | 1 | 1 | 2 |
| | | % Within Sample type | 3.8% | 1.9% | 2.6% |
| | 18 | Count | 1 | 0 | 1 |
| | | % Within Sample type | 3.8% | 0.0% | 1.3% |
| Total | | Count | 26 | 52 | 78 |
| | | % Within TTP days | 33.3% | 66.7% | 100.0% |
| | | % Within Sample type | 100.0% | 100.0% | 100.0% |

1G test plates are based on a thin layer of 7H11 agar media with OADC (oleic acid, albumin, dextrose, catalase), Glycerol and selective agents to inhibit bacterial growth. 1G test Plates have 4 quadrants, 1 quadrant without drug, and the remaining 3 quadrants with specific drug (fig 2). 1G test plates have an oxidation-reduction indicator that changes from colorless to red as the MTB grows. 1G test plates have food colorants to distinguish each well containing drug (green, yellow, and blue).

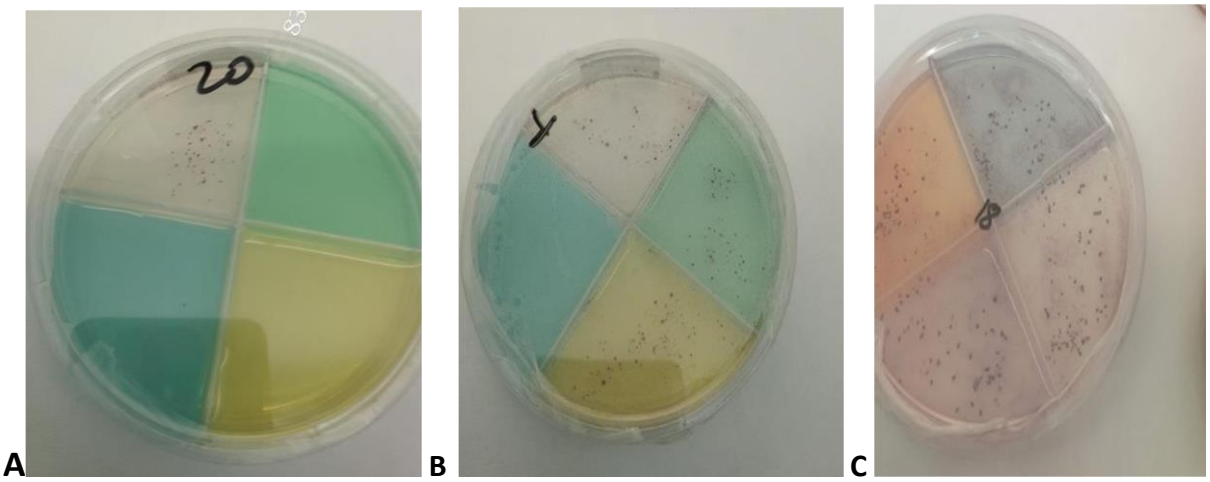


Figure 2; Photograph showing drug-resistant/susceptible TB detection on 1G test

**A. Susceptible**: MTB colony growth on drug-free quadrant (Top, clear) and susceptible to all three drugs.
**B. MDR-TB**: Concurrent MTB colony growth on drug-free quadrant (top, clear), green isoniazid-containing quadrant, and yellow RIF-containing quadrant.
**C. MDR-TB + MXF resistant**: Concurrent MTB colony growth on drug-free quadrant (top, clear), green INH-containing quadrant, yellow RIF-containing quadrant, and blue MXF-containing quadrant.

### 5.3. Diagnostic Accuracy of 1G test DST compared to IPM in all samples

For detecting INH, RIF, MXF resistance, and MDR- TB, the 1G test's sensitivity was 96.8%, 96.4%, 100%, and 100%, respectively, and its specificity was 89.4%, 90%, 98.7%, and 86%, respectively. 1G test diagnostic accuracy for INH, RIF, MXF, and MDR- TB was 92%, 92%, 99%, and 90%, respectively. Strong agreement was found between the 1G test and IPM, for INH and RIF with a kappa value of 0.838 (Table 4).

*Table 4*: Performance of the 1G test compared to IPM (*n* = 78 isolates)

| 2x2 Table | Reference test: IPM | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1G test **INH** | R | S | Sensitivity % (95% CI) | Specificity % (95% CI) | PPV % (95% CI) | NPV % (95% CI) | Kappa value | Level of significance | Accuracy (%) |
| R | 30 | 5 | 97 (87-100) | 89 (79-96) | 86 (72-95) | 98 (90-100) | 0.843 | <0.001 | 92 |
| S | 1 | 42 | | | | | | | |
| **RIF** | R | S | | | | | | | |
| R | 27 | 5 | 96.4 (85-100) | 90 (78-96) | 84 (69-94) | 98 (91-100) | 0.838 | <0.001 | 92 |
| S | 1 | 45 | | | | | | | |
| **MXF** | R | S | | | | | | | |
| R | 1 | 1 | 100 (2.5-100) | 99 (94-100) | 50 (38-96) | ----- | ---- | <0.001 | 90 |
| S | 0 | 76 | | | | | | | |
| **MDR** | R | S | | | | | | | |
| R | 21 | 8 | 100 | 86 (75-93) | 72 (55-86) | 100 | ---- | --- | 90 |
| S | 0 | 49 | | | | | | | |

**Note:** 1G test =Tuberculosis color test; IPM=Indirect Proportional Method; INH= isoniazid; RIF= rifampicin; PPV = Positive Predictive Value; NPV = Negative Predictive Value; R= resistant; S=susceptible; MDR=Multi Drug Resistant

## 5.4. Performance evaluation of 1G test Compared to IPM among isolates from different specimens

For the detection of INH, RIF, and MXF drug resistance, the performance of 1G test DST versus the reference IPM yielded the following results (Table 5). The sensitivity and specificity of 1G test among isolates from sputum were, 96% and 79% for INH, 77% and 76% for RIF, and 50% and 96% for MXF. Fifty-two sputum-derived isolates were evaluated for INH, RIF, and MXF; the agreement between 1G test and IPM was 88%, 77%, and 96%, respectively. INH (100%&100%), RIF (50%&83.3%), and MXF (100%&100%) were the isolates from FNA that 1G test was most sensitive to and specific to, respectively. The percentage of agreement between 1G test and IPM for the 26 isolates generated from FNA was 100%, 81%, and 100% for INH, RIF, and MXF, respectively. Kappa value & level of significance of TB CX for RIF in FNA isolates was relatively low in comparison with IPM, which is 0.250 and 0.198 respective.

*Table 5*-performance evaluation of 1G test Compared to IPM among different specimen-derived (FNA and sputum) isolates

| Reference test: 24well IPM | | | Specimen type | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1G test **INH** | R | S | | Sensitivity % (95% CI) | Specificity % (95% CI) | PPV % (95% CI) | NPV % (95% CI) | Kappa value | Level of significance | Accuracy (%) |
| R | 27 | 5 | sputum | 96 (85-100) | 79 (60-92) | 84 (69-94) | 95 (80-100) | 0.765 | <0.001 | 88 |
| S | 1 | 19 | | | | | | | | |
| **INH** | R | S | | | | | | | | |
| R | 3 | 0 | FNA | 100 | 100 | -------- | ------ | ------ | ---- | 100 |
| S | 0 | 23 | | | | | | | | |
| **RIF** | R | S | | | | | | | | |
| R | 21 | 6 | Sputum | 78 (60-91) | 76 (57-90) | 78 (60-91) | 76 (57-90) | 0.538 | <0.001 | 77 |
| S | 6 | 19 | | | | | | | | |
| **RIF** | R | S | | | | | | | | |
| R | 1 | 4 | FNA | 50 (38-96) | 83 (65-94) | 20 (13-63) | 95 (80-100) | 0.198 | <0.001 | 81 |
| S | 1 | 20 | | | | | | | | |
| **MXF** | R | S | | | | | | | | |
| R | 1 | 1 | Sputum | 50 (38-96) | 98 (92-100) | 50 (38-96) | 98 (92-100) | 0.480 | <0.001 | 96 |
| S | 1 | 49 | | | | | | | | |
| R | 0 | 0 | FNA | 100 | 100 | --- | ----- | | | 100 |
| S | 0 | 26 | | | | | | | | |

**Note:** 1G test = first generation test color test plate test; IPM=Indirect Proportional Method; INH= isoniazid; RIF= rifampicin=, MXF=moxifloxacin PPV = Positive Predictive Value; NPV = Negative Predictive Value; R= resistant*;* S=susceptible=Fine Needle Aspiration

## 5.5. Performance evaluation of 1G test DST Compared to MGIT

Sixty-three of the 81 isolates were evaluated for INH &RIF susceptibility using MGIT DST. The 1G test was in agreement by 86% (54 concordant, 9 discordant, kappa-value= 0.7, good agreement) and 89% (56 concordant, 7 discordant, kappa- value=0.742) for INH and RIF, respectively, between the 1G test and MGIT. For INH and RIF, the 1G test sensitivity and specificity were 89.5% 94.1%, and 84% and 87%, respectively (Table 6).

*Table 6*- performance of TB Color Plate DST Compared to MGIT

| | Reference test: MGIT | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 1G test **INH** | R | S | Sensitivity % (95% CI) | Specificity % (95% CI) | PPV % (95% CI) | NPV % (95% CI) | Kappa value | Level of significance | Accuracy | Discordance (%) |
| R | 17 | 7 | 90 (71-98) | 84 (72-93) | 71 (51-86) | 95 (85-99) | 0.684 | <0.001 | 86 | 14 |
| S | 2 | 37 | | | | | | | | |
| **RIF** | R | S | | | | | | | | |
| R | 16 | 1 | 94 (77-100) | 87 (75-95) | 73 (52-88) | 98 (90-100) | 0.742 | <0.001 | 89 | 11 |
| S | 6 | 40 | | | | | | | | |

**Note:** 1G test =1st generation Color plate test; IPM=Indirect Proportional Method; INH= isoniazid; RIF= rifampicin; PPV = Positive Predictive Value; NPV = Negative Predictive Value; R= resistant*;* S=susceptible

### 5.6. Diagnostic Accuracy of IPM compared to MGIT DST among isolates from different specimens

In sputum isolates, the sensitivity and specificity of IPM for detecting INH and RIF's resistance were 81% and 87%, respectively, with a moderate agreement of kappa value 0.62 when compared to MGIT DST (Table 7). In contrast, both the sensitivity and specificity of INH detection in FNA isolates were 100%. Additionally, the level of agreement between IPM and MGIT DST for determining INH (kappa value 0.74) and RIF resistance (kappa values =0.64) in combined sputum and FNA isolates was good.

*Table 7*: performance of IPM compared to MGIT DST

| IPM | MGIT DST | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 2 x 2 I N H | R | S | isolates derived | sensitivity % (95% CI) | specificity% (95% CI) | PPV% (95%CI) | NPV% (95%CI) | kappa value | level of significance | Accuracy in % |
| R | 13 | 4 | sputum | 81 (58-95) | 81 (61-94) | 77 (54-92) | 85 (66-96) | 0.62 | <0.001 | 81 |
| S | 3 | 17 | | | | | | | | |
| INH | R | S | | | | | | | | |
| R | 3 | 0 | FNA | 100 | 100 | 100 | 100 | 1 | <0.001 | 100 |
| S | 0 | 23 | | | | | | | | |
| INH | R | S | | | | | | | | |
| R | 16 | 4 | combined | 84% (64-96) | 91 (80-97) | 80 (60-93) | 93 (83-98) | 0.74 | <0.001 | 89 |
| S | 3 | 40 | | | | | | | | |
| RIF | R | S | | | | | | | | |
| R | 12 | 5 | sputum | 80 (56-95) | 77 (57-91) | 71 (47-88) | 85 (66-96) | 0.56 | <0.001 | 78 |
| S | 3 | 17 | | | | | | | | |
| RIF | R | S | | | | | | | | |
| R | 1 | 1 | FNA | 50 (38-96) | 96 (83-100) | 50 (38-96) | 96 (83-100) | 0.458 | <0.001 | 92 |
| S | 1 | 23 | | | | | | | | |
| | R | S | | | | | | | | |
| R | 13 | 6 | combined | 77 (54-92) | 87 (75-95) | 68 (46-86) | 91 (80-97) | 0.61 | <0.001 | 84 |
| S | 4 | 40 | | | | | | | | |

Note; MGIT=Mycobacteria Growth Indicator Tube; DST=Drug Susceptibility Test; INH= isoniazid; RIF= rifampicin; PPV = positive predictive value; NPV = negative predictive value; R= resistant; S=susceptible FNA=Fine Needle Aspiration

# 6. Discussion

This study evaluated the performance of the 1G test as an alternative testing method for the detection of MTBC, INH, RIF, and MXF resistance on stored clinical isolates from pulmonary and extra pulmonary sources. The goal of this study was to compare the performance characteristics of the 1G test with those of the MGIT and indirect proportional DST.

Diagnosing drug-resistant TB with conventional methods (phenotypic and genotypic DST) is often challenging in resource-limited settings like Ethiopia since some tests require expensive equipment like MGIT, Gene-Xpert, and/or sequencing machines. The conventional LJ and agar DST methods also have drawbacks as they take a long time to get the results which impact patient diagnosis, treatment, and follow-up. Therefore inexpensive, easy-to-use, relatively quick, and accurate methods like 1G test would impact drug susceptible and drug resistant prevention, treatment, management and care.

One of the challenges with TB laboratories is culture contamination, which can result in misdiagnosis or missed diagnosis. To minimize misdiagnosis and the delay in patient results, a method that minimizes contamination is ideal. The contamination rate for the reference IPM DST method was compared to the 1G test. None of the 81 isolates tested by 1G test were contaminated, but two (2.4%) isolates were contaminated with the reference IPM DST method. Similar to the current study, no contamination was reported with other 1G test studies. (Mekonnen et al., 2019, Shibabaw et al., 2020, Toit et al., 2012).

When compared to IPM (15 days) and MGIT DST (19 days), 1G test was quicker at detecting MDR TB, INH, RIF, and MXF resistance which was an average of 9 days. This study's TAT was slightly faster than those of other studies (Martin et al., 2009c, Shibabaw et al., 2020), which may be due to the use of stored isolates rather than directly from clinical specimens. In this study *M. tuberculosis* isolates that had been stored and sub-cultured in LJ tubes were used for the 1G test and IPM.

The 1G test showed 97% sensitivity for INH resistance detection in combined isolates (FNA and sputum), which was comparable to other studies(Ardizzoni et al., 2015, Mekonnen et al., 2019, Toit et al., 2012), and higher than (Martin et al., 2009b) which was 59%. Similar to other studies (Ardizzoni et al., 2015, Shibabaw et al., 2020, Toit et al., 2012), the 1G test sensitivity (96%) and specificity (90%) for RIF resistance detection were both excellent.

Although no other studies evaluated MXF resistance, the 1G test had a sensitivity of 100% and a specificity of specificity of 86% in detecting MXF resistance which is comparable to a study that evaluated CFX (Shibabaw et al., 2020).

The 1G test had excellent sensitivity (100%) for MDR TB detection, comparable to other studies(Shibabaw et al., 2020) (95%), (Toit et al., 2012)(99%), and good specificity (86%), which was slightly lower than other similar studies (Shibabaw et al., 2020, Toit et al., 2012). It also had excellent sensitivity (97%) and specificity (90%) for detecting MDR+MXF resistant isolates, with a 93% agreement rate.

The sensitivity of 1G test for detecting INH resistance in sputum isolates were 96%, which was comparable with previous studies, (Ardizzoni et al., 2015, Martin et al., 2009b, Mekonnen et al., 2019, Toit et al., 2012) and the specificity was 79%, which was lower than studies (Ardizzoni et al., 2015, Martin et al., 2009b, Mekonnen et al., 2019, Toit et al., 2012) ,may be due to the use of stored isolates. Both sensitivity and specificity of 1G test for detecting INH resistance was 100% for FNA isolates, even though no previous study conducted with FNA isolates, but it was comparable with previous studies conducted with sputum isolates (Martin et al., 2009a, Mekonnen et al., 2019). The 1G sensitivity and specificity from pulmonary isolates (sputum) were 79% and 76%, respectively, while the sensitivity and specificity from extra pulmonary isolates (FNA) were 50% and 83%, respectively. The 1G test sensitivity for RIF from extra pulmonary sources was lower, which could be attributed to the small number of extra pulmonary isolates included in the study. The sensitivity and specificity for 1G test MXF resistance in sputum isolates were 96.4% and 79.2%, respectively, whereas it was 100% for both in FNA isolates**.**

Compared to the MGIT DST as a gold standard, the TB CX exhibited good sensitivity (90%)) for

detecting INH resistance which was comparable to a study conducted in Estonia using 201 archived M. tuberculosis clinical isolates archived during the years 2004–2010 setting (Toit et al., 2012). The current study's specificity for the detection of INH resistance was also excellent, which was comparable to the other similar study (Toit et al., 2012). Additionally, the 1G test has strong sensitivity and specificity for detecting RIF resistance, measuring 94% and 87%, respectively, which was comparable with similar studies that compared 1G test versus MGIT DST (Shibabaw et al., 2020),(Toit et al., 2012).

For both sputum and FNA isolates, the IPM demonstrated good sensitivity and specificity for identifying INH resistance as compared to MGIT DST.

Additionally, in sputum isolates there was good agreement between IPM's sensitivity and specificity in diagnosing RIF resistance compared to MGIT DST, which was comparable with previous IPM study (Wedajo et al., 2014). However, the sensitivity of 50% for identifying RIF in FNA isolates was rather poor; this could be because there weren't many FNA isolates utilized. There was no previous study that evaluated 1G test with FNA samples to compare. So, more research including higher extra pulmonary sample sizes ought to be carried out.

# 7. Limitations

- Other extra pulmonary specimens other than FNA (lymph node aspirate) were not evaluated, because of the absence of other types of stored extra pulmonary isolates
- 1G test for 2nd-line anti-drugs other than MXF, was not evaluated
- We ran 63/81 isolates in MGIT DST, the remaining 18 isolates were not tested using MGIT DST and MXF was not tested due to resource limitation and machine errors

# 8. Conclusion and recommendation

Because 1G test is generally inexpensive (~1.75$), easy to use, and does not require highly trained personnel, it may be a viable option for DST in high-endemic areas and resource-limited settings like Ethiopia. According to the current study, 1G test was quick and simple to use for identifying MTBC and resistance to INH, RIF, MXF, MDR TB and pre-XDR TB in isolates obtained from a variety of sources, particularly sputum and FNA. It demonstrated good performance in FNA (extra pulmonary) isolates in addition to pulmonary source isolates, suggesting that it could be a useful alternative method for extra pulmonary drug susceptibility testing.

Therefore, based on our findings we recommend:

- To include additional extra pulmonary samples from different body sites in future studies with a larger sample size.
- In addition, future research on 1G test should consider additional second-line anti-TB drugs
- Use of fresh specimen
- Making the plates in country for sustainability (Moving manufacturing to Africa)

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# *Appendix 1*: Laboratory Procedure

## 1.1. SOP For TB strain processing using 1G plate

### 1.2.1 Introduction:

This SOP describes the processing of the mycobacterial isolate / strain and the subsequent inoculation of the Color Test (1G) culture plates.

**Materials:**

Positive mycobacterial culture and Color Test plates(1G)

**Procedure:**

1. All work manipulating *M. tuberculosis* strains / isolates took place in a certified biosafety cabinet to protect the user from exposure to high concentrations of pathogens.
2. Suspend the mycobacteria in 5 drops (~200 µl) of sterile distilled water in the base of a plastic universal container. The base of the plastic universal container should be covered with 2-3 mm sterile glass beads.
3. Tightly close the plastic universal container and vortex for 30 seconds. Then wait approximately 20 minutes for most of the aerosol within the container to settle.
4. Open the plastic universal container within the biosafety cabinet, add a few ml of sterile water, (see below for volume), mix thoroughly by pipetting and check that no macroscopic clumps are visible within the suspension.

5. When a suspension free from macroscopically visible clumps has been obtained, the mycobacterial suspension should be diluted with a volume of sterile water sufficient to generate a suspension with optical density McFarland 1.

Inoculate the Color Test(1G) plates (see below)

### 1.2.2. Color Test plate(1G) inoculation and incubation

**Introduction:**

This SOP describes the inoculation of the Color Test culture plates with 8 drops of the sputum-disinfectant mixture from SOP 2.1 or the strain / isolates suspension from SOP 2.2.

**Materials:**

Color Test plates and sample for culture

**Procedure:**

1. To inoculate 1G plates with 8 drops of the sample, the following procedure was used: one drop of the sample should be dropped onto the left- hand side of each quadrant, inoculated first the detection quadrant, then isoniazidquadrant, then rifampicin, then Moxifloxacin quadrant in turn in a 'clockwise' order, without the drops coming into contact with the edge of the 1G plate. Second drops are then applied to the right-hand side of each quadrant. Inoculating in this manner, with one drop applied to all quadrants before the second drop is applied to any quadrants, reduces the variation in inoculum between the quadrants.
2. Close the lid of the culture plates, seal with Para film, then be double-sealed byenclosing in a Ziploc bag.
3. Transfer the plates to the incubator (37ºC).
4. Plates may be removed from the incubator typically 3x weekly for 42 day

**Notes:**

1. Standard biosafety precautions should be used and it is highly desirable that sputum containers should only be opened in a biosafety cabinet to protect the operator from TB exposure.
2. Use disposable plastic pipettes rather than reusable glass pipettes to reduce potential biohazard from broken glass.
3. The sample is dropped onto but is not 'spread' on the 1G plate. This simplifies the procedure and also reduces contamination, because non-viscous sputum-disinfectant mixtures are rapidly absorbed whereas viscous sputum- disinfectant mixtures continue to be liquefied and decontaminated during the 10-40 minutes whilst being absorbed into the 1G plate.
4. Because the sample contains colorant and because the drops of sample are not spread on the 1G plate, it is permanently visibly obvious when two drops have been applied to each quadrant.
5. For research purposes, the plates may be photographed and if this is done the plates should be photographed without being opened on the day that it is first read as positive.

### 1.2.3. 1G test reading

**Introduction:**

This SOP describes the process and timing of plate reading.

**Materials:**

- Inoculated 1G culture plates in incubator
- Microscope using approximately 40x magnification. The 4x objective of a standard microscope may be used. Adequate views may also be obtained with an inverted microscope or a pocket microscope. If a microscope is not available, then a hand-held magnifying ‘loop’

may be used, although this may delay definitive plate reading by a few days until the colonies are large enough for reliable interpretation with this level of magnification.

**Procedure:**

1. 1G plate is usually read three times weekly: Monday, Wednesday andFriday or Tuesday, Thursday and Saturday, for 6 weeks.
2. Check for growth visible to the naked eye as red spots.
3. The great majority of fungal and bacterial contamination is obviously non-mycobacterial from naked eye inspection either because no color change occurs in the surrounding culture medium or because of the rapid appearance of a large colony that on naked-eye inspection has the obvious morphological characteristics of fungal or bacterial contamination.
4. When mycobacterial growth is detected, the culture is declared positive.

**Notes:**

1. If the detection quadrant develops complete contamination, then the result is reported as a failed, contaminated culture and an additional sample is requested for repeat testing.
2. If partial contamination is present covering the minority of the detection quadrant and no mycobacterial growth is seen then the absence of mycobacterial growth by 6 weeks incubation is reported as a definitively negative culture.
3. The median time to positivity is 16 days. The great majority of positives occur within 4 weeks. We discard negative plates after 6 weeks incubation

**Thus, the result from the detection quadrant is interpreted as**

- **negative or**
- **uninterruptable (failed, needs to be repeated)**
- **Or positive for mycobacterial growth.**

### 1.2. Procedure for Indirect proportional Drug Susceptibility Testing (IPM)

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**Purpose**

**This procedure provides instructions for the preparation of 24 well drug-containing media.**

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**Principle**

Standard indirect drug susceptibility tests will be done to determine the susceptibility of isolates to anti-mycobacterial drugs. The mycobacterial cultures will be tested on their growth ability on Middlebrook 7H10 agar based on a modified proportion method using 24-well (IPM)DST plates (Van Soolingen, 2007). A suspension will be prepared from a bacterial culture. This suspension is distributedin10µl volumes on wells containing 7H10 medium with a precise concentration of the selected anti-Tuberculosis drugs, followed by incubation and reading of growth after 21days.

**Materials**

**Reagent's preparation: for drug stock refer in the procedure part below and for LJ solution refer AHRI/TBL/SOP5.5-003**

**Reagent's stability and storage: Stock solution of the drug should be stored at -20°C for further use and can be utilized up to 6 months.**

| **Supplies** |
|---|
| ✓ **DMSO**<br>✓ **Ethanol**<br>✓ **Glycerol**<br>✓ **OADC enrichment**<br>✓ **Middlebrook 7H10agar**<br>✓ **LJ media** |

| **Equipment and Device** |
|---|

| | |
|---|---|
| | ✓ **24-well plates, (transparent polystyrene), size 100x100x18mm**<br>✓ **Plastic tubes (Falcontubes)**<br>✓ **100ml bottles**<br>✓ **Pipetman**<br>✓ **Pipettes tips**<br>✓ **Pipetteman and plastic pipettes (10ml)**<br>✓ **Glass beads (diamerterca2mm)**<br>✓ **Autoclave**<br>✓ **Biosafety cabinet or hood**<br>✓ **Carbon dioxide incubator (with a water reservoir and 5%CO2 at 35°C).** |
| | • ✓ **Water bath**<br>• ✓ **Balance (sensitivity 0.01 g)**<br>• ✓ **Spoons or spatulas**<br>• ✓ **Weighing paper**<br>• ✓ **Vortex mixer**<br>• ✓ **Refrigerator** |

**Procedure**

**1.2.1. Preparation of the antibiotic concentration agar series in 24-well plates**

**1.2.1.1. Preparation of the Middlebrook 7H10media**

For all test drugs, the Middlebrook7H10 medium is used.

- Calculate how much media is needed and prepare one spare bottle. Sixty ml of 7H10 agar media is needed for each DST plate.
- Weigh an amount of Middlebrook7H10 agar need for one batch test and dissolve it with the required amount of distilled water containing glycerol. According to the supplier,
- 19.17g of 7H10 agar is dissolved in 900ml distilled water containing 5ml of glycerol.
- Place the bottles in a water bath of 100°C until the agar dissolves**.**

**After the agar is completely dissolved, sterilize the solution in the autoclave for 10 minutes at 121°Celsius.**

•Cool down the media to a temperature of 50°C in water bath and add warmed OADC enrichment to the bottle with7H10agar.

•Check the pH as soon as the agar is solidified (one ml of agar will be solidified to check the final pH), pH must be 6.6+/-0.2

#### 1.2.1.2. Preparation of a stock solution of the antibiotics

**The following first-line and second-line anti-tuberculosis drugs will be used for test.**

| Anti TB drugs | Abbreviations | Product number (Sigma) | Solvent(s) used | Stock soln (µg/ml) |
|---|---|---|---|---|
| **Isoniazid** | **INH** | **I-3377** | **dH2O** | **1000** |
| **Rifampcin** | **RIF** | **R-3501** | **DMSO** | **1000** |
| **Moxifloxacin** | **MOX** | **32477** | **NaOH** | **1000** |

**• Weigh the correct amount of the antibiotic.**

### 1.3. Storage of a stock solution

**After the stock solution is prepared, it will be stored with the following information.**

- Label one: Name, solvent, amount, the concentration of the stock solution, and the date. This label is for the bottle with the antibiotic.
- Label two: All the information on label one and the name of the person who weighed the antibiotic powder, this label is meant for the laboratory journal (log book).
- All the drugs will expire in 2days when kept at 4°C.
- If kept at minus 70°C the expiration date is one year.
- Once the is unfrozen, you cannot freeze it again. Hence an aliquot for each stock solution is necessary.
- When there is no solution fluid indicated at the label, distilled water is used.

### 1.4. Preparation of the 24 well plates

- Mark the bottles with the correct code.
- Add to the bottles the correct amount of antibiotics

**Table 8: Pippet schedule for 2 plates**

| Bottle code | Test Drugs | Conc. (µg/ml | µl/5ml agar | Worksol (µg/ml) |
|---|---|---|---|---|
| 0-1 | Control (1:1) | )No) drug | | |
| 0-2 | Control (1:100) | No drug | | |
| 1-1 | INH | 0.064 | 32 | 10 |
| 1-2 | INH | 0.125 | 62.5 | 10 |
| 1-3 | INH | 0.2 | 100 | 10 |
| 1-4 | INH | 1.0 | 50 | 100 |
| 2-1 | RIF | 1.0 | 50 | 100 |
| 1-1 | MOX | 0.5 | 25 | 100 |

**Table 9 pippet schedule for 10 plates**

| Bottle code | Test Drugs | Conc. (µg/ml) | µl/25ml agar | Worksol (µg/ml) |
|---|---|---|---|---|
| 0-1 | Control (1:1) | No drug | ═══ | ═══ |
| 0-2 | Control (1:100) | No drug | ___ | ___ |
| 1-1 | INH | 0.064 | 160 | 10 |
| 1-2 | INH | 0.125 | 312.5 | 10 |
| 1-3 | INH | 0.2 | 50 | 100 |
| 1-4 | INH | 1.0 | 250 | 100 |
| 2-1 | RIF | 1.0 | 250 | 100 |
| 11-1 | MOX | 0.5 | 125 | 100 |

**NB: highlighted in black are used for comparison (critical concentration)**

Table 3 Schematic arrangement of the 24- well plate: The green wells are critical concentrations of the drugs (WHO), which are used in our study to compare with the control.

Table 1: schematic arrangement of 24 well

| | | | | | |
|---|---|---|---|---|---|
| **INH**<br>0.064 | | **INH**<br>0.125 | | **INH**<br>0.2 | |
| **INH**<br>1.0 | | **RIF**<br>1.0 | | MXF<br>0.5 | |
| | | | | | |
| | | | | **Control**<br>**(1:1)** | **Control**<br>**(1:100)** |

**Inoculation**

**Preparation of the inoculum**

- Prepare in a Laminar Flow safety cabinet a suspension of 2-1x$10^5$ CFU/ml in bottle containing water and glass beads. Suspensions of *M. tuberculosis* complex isolates should have a density of one McFarland.

- Place the bottle into a special safety box on the orbital shaking platform and shake the bottles for 20 minutes at 350 r.p.m. When the cultures are not properly suspended within this period, shake for another 20 mins

**Preparation of the plates for inoculation**

- Collect all the culture tubes, which should be tested.
- Write the correct strain number on the 24 well plate
- Write for each strain also a bottle with glass beads.
- Fill short laboratory tubes with 1 ml of distilled water, needed for 1/100 dilution.
- Sterilize the pipette tips for 2 minutes

**Inoculation of the 24 well plates**

- Bring 10 µl of the suspension on each well, using the micropipette, but do not inoculate the second control well.
- The 1/100 suspension: bring 10 µl in the tube containing 1ml of H2O and swirl.
- Inoculate 10 µl of the diluted suspension on the second control well.
- Place the plates in a rack, in stacks of 3 pieces, in a 35°C or 37 °C incubator for 48 hrs.
- Fixate the lid with adhesive tape on the plates on two sites for reasons of safety, and incubate at 35° C in the presence of sufficient humidity
- Put the plates in the special's racks. Because there is condense at the lid the plate will be turned upside down and be read backward.

**N.B** As far as control strains are concerned, for each batch of sensitivity test H37Rv strain that is susceptible to all of the test drugs will be inoculated on to all drugs. Whereas control strains resistant to the different anti-TB drugs will be inoculated to the wells where the respective anti-TB drugs are mixed.

**Reading and interpretation of sensitivity**

After 2 to 3 days, the plates will be read to see if there are any contamination and mixtures with other bacteria. Besides checking the plates on day 2-3, all inoculated bacterial suspensions will also be inoculated on blood agar which is a better test for contamination. The plates will be checked for mycobacterial growth at 6 (used as early check as well as the plate can sometimes be read already here), 12 and 19 days. The control wells must be grown then. If there is no growth on either of these control wells the experiment will be repeated. The sensitivity result of the isolates will be read by visual comparison of the drug containing Media (1:1 bacterial suspension) with the drug-free control on which 1:100 bacterial suspensions will be inoculated. Equal growth on the two media, in principle, would mean 1% of the isolates are resistant to that particular drug. However**,** for reason of accuracy, the test will be repeated if the growth is equal or nearly equal for both.

## Appendix 2: Drug susceptibility result registration form

| S/N | Anti-TB drug | DST methods | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *24 well IPM* | | | *TB color plate* | | *MGIT DST* | | |
| | | ID NO | Result (S, R, I) | Date | ID NO | Result (S, R, I) | ID NO | Result (S, R, I) | Date |
| 1 | INH | | | | | | | | |
| 2 | RIF | | | | | | | | |
| 3 | MXF | | | | | | | | |
| 4 | Total | | | | | | | | |

Note; INH=Isoniazid, RIF=Rifampicin, MXF=Moxifloxacin, S=Susceptible=resistant, I,Invalid,ID No=identification number

# Appendix 3: Operational Definition

**Sensitivity**: the probability (percentage) of the screening test correctly identifying diseased subjects (determined by the result of the reference or 'gold standard' test)

**Specificity**: the probability of the screening test correctly identifying non-diseased subjects (determined by the result of the reference or 'gold standard' test)

**Positive predictive value (PPV):** is the probability that subjects with a positive screening test truly have the disease or the probability that a positive result accurately indicates the presence of infection.

**Negative predictive value (NPV):** is the probability that subjects with a negative screening test truly don't have the disease or the probability that a negative result accurately indicates the absence of infection.

**Bio-safety** is defined as the discipline addressing the safe handling and containment of infectiousmicroorganisms and hazardous biological materials

**New patients**: have never been treated for TB or have taken anti-TB drugs for less than 1 month.

**Previously treated patients**: A patient who has received 1 month or more of anti-TB (first line or second line) drugs in the past. They are further classified by the outcome of their most recent course of treatment as follows:

**Drug-susceptible isolate:** an organism's ability to be killed by a particular drug; the wild type

bacteria, not previously exposed to a drug.

**Drug-resistant isolate**: an organism's ability to grow despite the presence of a particular drug, if

more than 1% of the bacteria exhibit resistance to that drug.

**Mono-resistant TB**: TB that is caused by strains of *M. tuberculosis* (MTB) that is resistant to one first-line anti-TB drug only.

**MDR-TB**: TB caused by strains of MTB that are resistant to at least INH and RMP.

**XDR-TB**: resistance to any fluoroquinolone, and at least one second-line injectable agent (amikacin, kanamycin, and/or capreomycin), in addition to MDR-TB.

**Confidence interval**: the confidence interval quantifies the uncertainty in measurement; usually reported as the 95% confidence interval, the range that we can be 95% certain covers the true value.

**Accuracy**: the percentage of correct results obtained by the test under evaluation compared with the results of a reference or 'gold standard' test. Usually, it expressed as the number

## Declaration

I, the under signed, declare that this thesis is my original work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc. degree at Addis Ababa University.

Name of the student: _Binyam Mebrat_____

Date: _____

Signature: _____

**Approval of academic advisors**

Dr Yimitubezinash W/amanuel (MD, M.Sc., Ph.D.) signature_____

Dr Kelemework Adane (Ph.D.) signature_____