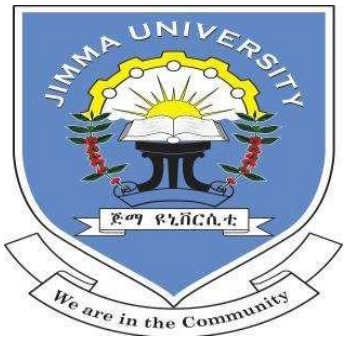
UTILITY OF MYCOBACTERIAL DNA EXTRACT FROM USED Xpert MTB/RIF CARTRIDGES FOR SECOND LINE GENOTYPIC DRUG SUSCEPTIBILITY TESTING AT EASTERN AND WESTERN OROMIA, ETHIOPIA



BY: TILAHUN KETEMA (BSc)

A RESEARCH THESIS SUBMITTED TO JIMMA UNIVERSITY INSTITUTE OF HEALTH, SCHOOL OF MEDICAL LABORATORY SCIENCE FOR THE PARTIAL FULFILMENT OF THE REQUIREMENT FOR MASTER OF SCIENCE DEGREE IN MEDICAL MICROBIOLOGY

NOVEMBER, 2021

JIMMA, ETHIOPIA

JIMMA UNIVERSITY

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BY: TILAHUN KETEMA (BSc)

ADVISORS: 1. MULUALEM TADESSE (PhD, Assistant Professer)
2. MEKDIM MEKONNEN (MSc, Lecturer)
3. WAKJIRA KEBEDE (MSc, Assistant Professor)
4. GEMEDA ABEBE (Professor)

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Abstract

Background: The emergence and spread of multidrug-resistant tuberculosis (MDR-TB) and extensive drug resistance (XDR-TB) is a threat to tuberculosis (TB) treatment, as only a few of these patients have access to drug susceptibility testing (DST). Xpert MTB/RIF is a test used for TB and rifampicin-resistance detection. Second-line genotypic DST, which is recommended by World Health Organization (WHO), requires additional specimen collection. This study aimed to determine the utility of mycobacterial genomic DNA extract from used Xpert cartridges for second-line DST, which may help patients to receive a diagnosis rapidly from a single specimen.

Objectives: To evaluate the Utility of Mycobacterial DNA extract from used Xpert MTB/RIF cartridges for second-line genotypic DST at eastern and western Oromia, Ethiopia

Methods: A cross-sectional study was conducted on 62 samples collected from two TB referral units in Ethiopia between June 2020 and May 2021. Collected sputum samples were allocated into two and processed by Xpert MTB/RIF and culture. Fifty used Xpert rifampicin-resistant (RR) cartridges were used for the mycobacterial genomic DNA extraction for second-line DST (SL-DST) by line probe assay. Descriptive statistics were performed using frequencies and percentages to describe the characteristics of the study population. The yield of a method was compared with DST performed on culture isolates.

Result: Of the 62 collected samples, Xpert detected *M. tuberculosis (MTB)* and RR-TB in 50 sputum samples. The sensitivity of MTBDR*plus* for rifampicin-resistant detection on cartridge extract (CE) was 22% and the assay was not feasible on CE. MTBDR*sl* had 100 % actionable results on CE for *MTB* detection. No resistance was found in any cartridge extract that was subjected to analysis. From CE and isolates, assay had a concordance of 100% and 90% for FLQ and SLID resistance detection respectively. All cartridge extract corresponding to C_T value ($C_T \leq 22$) had interpretable results.

Conclusion: MTBDR*sl* on CE has a high level of agreement with that from isolates. Our data showed that at the $C_T \leq 22$, CE from Xpert can be used for genotypic second-line DST. This demonstrates further testing of the second-line anti-TB drug resistance can be done directly on the used Xpert cartridge and minimize the time and resource needed for culture and mitigate additional sample collection. Further study with a large sample size is recommended.

Keywords: Genotypic Drug Susceptibility Testing, cartridge extract, Line Probe Assay

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Abbreviation/acronym

BSC	Biological Safety Cabinet
BSL	Biosafety Level
CAP	Capreomycin
CE	Cartridge Extract
DR-TB	Drug Resistant Tuberculosis
DST	Drug Susceptibility Testing
FLQ	Fluoroquinolones
INH	Isoniazid
JU-MRC	Jimma University Mycobacterial Research Center
KAN	Kanamycin
LPA	Line Probe Assay
MDR-TB	Multi Drug Resistant Tuberculosis
NALC	N-acetyl-L-cysteine
NaOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
RR	Rifampicin Resistant
SLID	Second Line Injectable Drugs
SOP	Standard Operating Procedures
SPSS	Statistical Package Software for Social Science
TB	Tuberculosis
WHO	World Health Organization

1. Introduction

1.1 Background

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* that largely affects the lungs. Mycobacteria are slender, curved rods that are aerobic no motile organisms. They are primarily slow-growing intracellular bacilli of the *Mycobacteriaceae* family of the order of *Actinomycetales* (1). Their main characteristic feature is their distinctive staining properties due to their unique and complex cell wall. The genus Mycobacterium is classified into members of the Mycobacterium tuberculosis complex, seven very closely related mycobacterial species. The classification is commonly depending on pigment production in light and/or dark, and different biochemical tests, such as catalase test and niacin production (2). The bacteria can also affect other sites than the pulmonary such as the brain, the kidneys, the spine, or bones (3).

Transmission of TB happens when a person inhales bacilli containing *M. tuberculosis*, which is responsible for the primary TB infection, and another strain of TB, *M. bovis,* can be transmitted by drinking raw milk (4). When person infected with pulmonary TB cough or sneeze, the infectious droplet nuclei are formed. These tubercle bacilli are suspended in the air for several hours and infection occurs when a healthy person inhales the bacilli and reaching to the alveoli of the lungs. They are ingested by alveolar macrophages; the majority of these bacilli are destroyed or inhibited. In other rare conditions, the bacilli may spread by way of lymphatic channels or through the bloodstream to more distant tissues and organs to cause extra-pulmonary tuberculosis (EPTB) (5).

The onset of active TB is gradual and insidious; the symptoms are unremarkable and nonspecific resulting in the delay of diagnosis, increase morbidity, and mortality as well as the continued spread of the disease. Manifestations often include progressive fatigue, malaise, unintentional weight loss, and a low-grade fever accompanied by chills and night sweats. Cough eventually develops and initially may not be productive. Upon progression of the disease, it becomes productive of purulent sputum (6).

TB remains one of the top ten leading causes of death from a single infectious agent. According to the Global TB Report of 2021, 10.0 million (8.9–11.0 million) people developed the disease, which is equivalent to 130 cases (116–143) per 100 000 population and 1.4 million died from the disease, including 208,000 deaths among people with Human Immunodeficiency Virus (HIV) (7)**.** The study also showed a TB case incidence of 226 (ranges 201–252) per 100,000 populations in Africa, but in the Central African Republic, Lesotho, and South Africa more than 500 cases per 100,000 populations. Another national TB prevalence survey conducted in twelve African countries found that the prevalence of bacteriologically confirmed pulmonary TB ranged from 119 (79–160) per 100,000 populations in Rwanda to 638 (502–774) in Zambia (8). Ethiopia sits 10th among the thirty high TB burden countries, with an estimated incidence of 157 (ranges 110–211) TB cases per 10^5 populations, and the mortality of 22 per 10^5 population (9).

The emergence of multidrug resistance TB (MDR-TB), TB with resistance to at least two main drugs isoniazid and rifampicin, and extensive drug-resistant TB (XDR-TB), TB caused by *MTB* strains that are resistant to isoniazid and rifampicin and which also resistant to any fluoroquinolones, and at least one additional Group A drug either bedaquiline or linezolid (or both) is a major public health security threat in many countries and could risk gains made in the fight against TB (10). Globally in 2020, there were an estimated 465 000 (400 000– 535 000) incident cases of rifampicin resistance. About 6.2% of MDR-TB cases had XDR-TB. The severity of national epidemics varies widely among nations with most cases occurring in India (27%) and China (14%) of the global burden (7).

According to a systematic review conducted in 2018, the total prevalence of MDR-TB in all TB forms was estimated to be 1.4 % in Ethiopia (11). As per a study conducted in Tigray, the overall percentage of MDR-TB was 16.7%. Pre-XDR-TB made for 5.3 % of all MDR-TB cases (12).The WHO End TB Strategy calls for early diagnosis and universal access to DST for diagnosis of DR-TB is a critical prerequisite in the management of TB. To satisfy this requirement several conventional culture and

molecular techniques are used to determine the DR patterns of MTB strain. Conventional DST is commonly performed either using a solid medium, Lowenstein - Jensen (LJ), and liquid medium-based methods, Mycobacterium Growth Indicator Tube (MGIT) System 960 (13,14). Phenotypic LJ-based uses proportion method to determine whether isolates of MTB are susceptible to anti-TB agents. The control medium without the anti-TB agent is inoculated, while media containing the critical concentration of the anti-TB drug is inoculated with a dilution of a culture suspension. Growth on the agent-containing medium is compared to growth on the agent-free control media, although results take at least four weeks to produce and are also time-consuming (15).

Drug susceptibility testing in MGIT 960 uses 7H9 liquid media which contains an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. During bacterial growth, the free oxygen is utilized and replaced with carbon dioxide. The depletion of free oxygen results in fluorescence of the sensor within the MGIT tube when visualized under ultraviolet light. The level of oxygen depletion is directly proportional to the intensity of fluorescence. The BACTEC MGIT 960 instrument automatically detects this fluorescence. Two MGIT tubes are inoculated with the test culture and a known concentration of a test agent is added to one of the MGIT tubes, and both tubes are incubated. Growth in the tube containing the agent is compared with that in the MGIT tube without the agent (16).

Xpert MTB/RIF is a breakthrough in TB diagnostics, a cartridge-based nucleic amplification assay that detects MTB and rifampicin (RIF) resistance in two hours. Using real-time PCR, the device combines automatic sample preparation, nucleic acid amplification, and detection of target sequences (17). But it has limitations, firstly it detects the genetic markers of RIF resistance only disregarding INH, an important component of *TB* treatment regimens; secondly, it uses a single gene copy (IS6110) for the detection of MTB missing the species which have zero or deleted copies of IS6110 (18)**.** LPA detects resistance to first-line drugs (INH and RIF) and for Fluoroquinolones and the second-line injectable drugs. This molecular assay is based on DNA strip technology and it includes DNA extraction from direct specimen or isolates, master mix

preparation, and addition, multiplex amplification with biotinylated primers, and detection with reverse hybridization (19).

The epidemiology of tuberculosis changed dramatically after the introduction of anti-TB drugs. WHO and national TB control program recommended standard first-line regimen for the treatment of drug susceptible TB comprises RIF, INH, PZA and EMB while the regimen construction for drug resistant TB, were depends on resistance obtained for the drugs, its effectiveness, implementation considerations and potential benefits when included in the treatment regimen. Fluoroquinolones (levofloxacin and moxifloxacin), bedaquiline and linezolid, were regarded highly effective and strongly recommended for inclusion in all regimens from group A, whereas clofazimine and cycloserine or terizidone were conditionally recommended as agents of second choice in a DR-TB regimen from group B. When drugs from Groups A and B can't be utilized, add-on agents (which not part of the core second line agents) like Pyrazinamide, high-dose isoniazid, and Delamanid is used to complete the regimen (20,21).

All confirmed RR/MDR-TB pulmonary TB patients must have baseline screening DST for the core-second lines medicines early before initiating the WHO or nationally recommended standard treatment, using the second line-Line Probe Assay (SL-LPA). These molecular methods were performed on the second sample collected. Therefore looking for the alternative from cartridge extracts would help the patient to receive the DST result early and in deciding best treatment options, but there is no adequate data on its feasibility for the methods.

1.2 Statement of the problem

Since 2007, tuberculosis has surpassed HIV/AIDS as the world's 10th biggest cause of death from a single infectious agent, as this curable disease becomes more difficult to treat due to drug resistance. Drug-resistant tuberculosis (DR-TB) continues to be a public health concern. Reduced access to diagnosis and treatment has resulted in a 1.3 million rise in mortality in 2020 compared to 1.2 million in 2019. Although 71 % of people with bacteriologically diagnosed tuberculosis were tested for rifampicin resistance, global coverage of fluoroquinolones resistance testing remains substantially lower, which was 50 % in 2020 (7). In the case of Africa, only 53% of MDR/RR-TB cases received second-line DST, and 47% of member nations lack a laboratory capable of performing second-line resistance testing (22).

Patients with rifampicin-resistant tuberculosis frequently experience treatment failure and resistance amplification remains challenging. To provide better patient care and reduce the rate of MDR-TB, which is a precursor of Pre-XDR and XDR, performing rapid DST with line probe assay (LPA) at the start of treatment would help identify many more cases of drug resistance common mutation pattern (23). Study conducted on the accessibility of second-line DST in Zimbabwe found that, of the 133 RR-TB cases identified by Xpert, sputum samples were not obtained from 12% of those who were eligible for second-line DST (24).

Many new cases of MDR-TB have emerged as a result of diagnostic errors in TB care, such as failing to detect preexisting resistance (25,26). Another problem for TB preventive and treatment efforts is the rise of pre-XDR-TB and XDR-TB which comprises 10% of MDR-TB cases, yet under diagnosed than MDR-TB, which is very costly to treat (27). WHO and national TB control program recommended all rifampicin resistant detected by Xpert to be further tested for second line DST, but only 64% were tested in 2017 in Ethiopia (28,29).

As the level of resistance is a key forecaster of treatment outcome and the toll of drug resistance is escalating timely, DST results for all patients are vital. This can only be achieved with genotypic DST assays, provided that phenotypic methods are costly, need specialized facilities, and take a long period because of the slow growth rate of the bacilli. Moreover, sub-culturing can lead to the loss of clinically-meaningful resistance (30,31). WHO endorsed molecular test Xpert MTB/RIF that detects MTB and RIF-resistance directly from sputum within hours and if resistance detected by Xpert, it recommended further DST using MTBDR*plus* and MTBDR*sl* by collecting additional sputum sample (32,33). DNA was taken from sputum samples or culture isolates to continue with the genotypic DST technique. Studies reported that MTBDR*sl* have suboptimal sensitivity on direct specimens, and culture is often required prior to DNA extraction for genotypic MTBDR*sl* testing (34,35). This causes diagnostic delay and also many high TB burden countries lack the necessary biosafety and laboratory infrastructure for mycobacterial culture.

As alternative, MTBDR*sl* on cartridge extract does not require extra patient visits and additional specimen collections. Testing second-line DST on the first available specimen could potentially reduce the number of patients lost during the diagnostic cascade and allow earlier diagnosis of XDR-TB leading to earlier effective treatment initiation, reducing health system cost, and better clinical outcomes. As a result, this study aims to evaluate for genotypic second line DST on used Xpert cartridge extract, which requires no culture, DNA purification or repeated sample requests.

1.3 Significance of the study

The MTBDR*sl* Line probe assays allow for rapid TB diagnosis as well as drug resistance target profiling. Currently, DR-TB patients are diagnosed either from direct clinical sample or culture isolate. This study is important to minimize the diagnostic delay that takes a long time with the conventional method by reducing turnaround time for DST result. This allows early initiation of treatment with the best treatment option/constructed regimen. It also reduces the patients' loss in DR-TB diagnostic cascade. The study is also important in reducing the cost needed for culture and DNA extraction/purification and mitigates additional sample collection.

This study's findings provide important and concrete information about the use of CE for second-line genotypic DST. Furthermore, the findings of this study can be used as a source of information and baseline data for researchers, policymakers, and other stakeholders involved in TB control programs and who want to conduct more research on the method and scale up the implementation of this protocol in the national TB control program.

2. Literature

Tuberculosis (TB) is a major worldwide health problem with a high prevalence in developing countries. At least 90% of patients must be diagnosed and offered treatment, and 90% must be cured if the global MDR-TB epidemic is to be controlled. All suspected TB cases must be tested using a WHO-approved fast molecular assay like Xpert MTB/RIF and LPA to increase detection and shorten the time to diagnosis. Patients with rifampicin-sensitive TB will be cured quickly with the first TB treatment regimen; however, patients with RR-TB will take longer to cure and will require molecular testing to discover resistance to INH, FLQ, and SLID (36).

Xpert MTB/RIF was used to quickly diagnose TB and RIF resistance; however second-line DST necessitates the collection of additional specimens. A total of 85 Xpert TB-positive CE (56 RIF-susceptible, 29 RIF-resistant) were examined for first and second-line molecular assays in a study conducted in South Africa. The MTBDR*sl* results from rifampicin susceptible CE were valid, with 95% for FLQ and 91% for SLID. The assay on RIF-resistant CE was 83% reliable results. The overall proportion of resistance detected by the assay from RIF-resistant cartridge extract compared to that detected from the isolate was 90% for FLQ and 84% for SLID (37).

Another study conducted in South Africa on used Xpert ultra cartridge also reported, 100% FLQ and 97% SLID results from ultra-cartridges extract were concordant with sputum results (38). In the report from Gabonese, there were 124 interpretable results out of 130 sputum samples examined with the Xpert assay; 21 (17%) were found to be RIF resistant. LPA on the Xpert remnants identified 18 of the 21 samples (86 %) as MDR(39).

All of the above findings, which were conducted at different times and locations, evaluated the assay on direct clinical specimens and cartridge extract. But to our knowledge, no study has been undertaken in this study site to evaluate SL- LPA on cartridge extract. As a result, the goal of this research is to evaluate assay on used Xpert cartridges that would otherwise be discarded.

3. Objective of the study

3.1 General Objective

- To evaluate the utility of Mycobacterial DNA extract from used Xpert MTB/RIF cartridges for second-line drug susceptibility testing using Line Probe Assay

3.2 Specific objective

- To determine the feasibility of cartridge extract for LPA for Mycobacterium tuberculosis complex and second line drug resistance detection
- To compare the proportion of second-line drug resistance determined by LPA from used cartridge extract and culture isolates
- To compare the LPA strip band quality (interpretability of the bands) of tests performed on used Xpert cartridge extracts and culture isolates

4. Materials and Methods

4.1 Study area

A study was conducted at Jimma University Mycobacteriology research center (JU-MRC) and Adama Regional Laboratory (ARL). These institutions were chosen purposefully for their high workload and the fact that they provide referral services for several treatments initiating centers and different levels of hospital in Oromia and neighboring regions, as well as being the region's only functional TB culture center. The JU-MRC at JU was established as part of an inter-university collaborative research project between Jimma University and a consortium of Flemish Universities from Belgium in 2010. The research center is equipped with culture, drug susceptibility testing (DST), Xpert MTB/RIF assay, and LPA, and it serves as both a research center and a patient care facility for the country's southwestern region. ARL is Ethiopia's first state-of-the-art regional laboratory, which established in 2003. Aside from patient care, the regional laboratory functions as a training center for laboratory personnel in HIV, TB, and malaria diagnosis, antiretroviral (ART) monitoring, and quality management system. The facility is Biosafety Level 2 (BSL2) certified and is equipped with Xpert assays, culture, phenotypic, and genotypic DST to serve Ethiopia's Southeastern Oromia region.

4.2 Study design and period

A cross-sectional diagnostic study was conducted at JU-MRC and ARL from June 2020 to May 2021.

4.3 Population

4.3.1 Source population

All pulmonary TB patients diagnosed with MDR/RR-TB and linked to treatment initiating centers, and whose samples were referred to JU-MRC and ARL during the study period.

4.3.2 Study population

A bacteriological confirmed RR-TB patient with Xpert MTB/RIF and who's their samples were presented to JU-MRC and ARL from fourteen treatment initiating centers for diagnosis of second line drug susceptibility testing were included in the study.

4.4 Inclusion and exclusion criteria

4.4.1 Inclusion criteria

MDR/RR-TB patients

4.4.2 Exclusion criteria

Patients who were receiving second line anti-TB drugs

Sample with insufficient volume

4.5 Sample size determination and sampling technique

Consecutive sampling technique was used. The rule of verification and validation studies was used to determine the sample size required for this study. The minimum sample size recommended for user-conducted verification and validation, according to the Clinical and Laboratory Standard Institute (CLSI EP09-A3) guideline, is forty (40). In this study, the estimated calculated sample size was 40.

4.6 Variables

4.6.1 Dependent variables

Utility of cartridge extract

4.6.2 Independent variables

C_T value, sputum quality

4.7 Study procedures

4.7.1 Sample collection and Laboratory tests

4.7.2.1 ***Sample collection***

A total of 62 sputum samples with a minimum volume of 5ml were collected. The samples were referred to JU-MRC and ARL from fourteen Treatment initiating centers

(TIC) for second-line DST. The collected sputum sample was allocated into two falcon tubes in the Biosafety Cabinet (BSC): one falcon tube was used for the Xpert MTB/RIF assay running and cartridge extract was obtained from used Xpert MTB/RIF cartridge, and the second falcon tube was used for the inoculation on the LJ culture media. The sputum samples were maintained in the refrigerator at 2-8°C until they were processed for the corresponding test (Xpert MTB/RIF and culture).

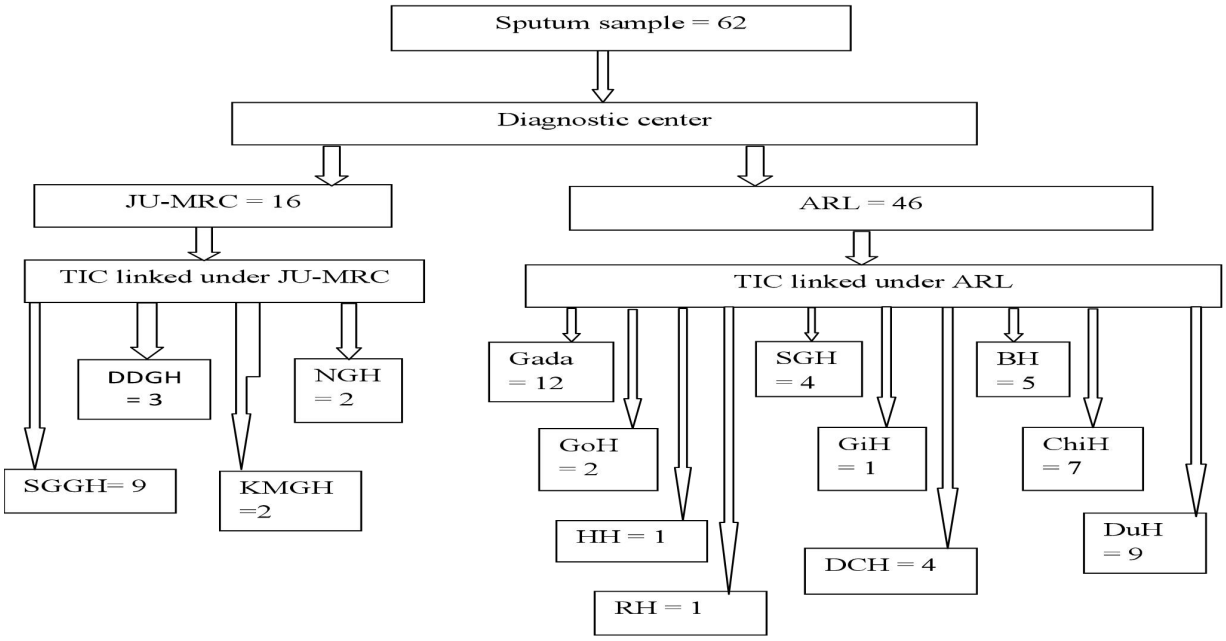


Figure 1: Flow of sample collection

Abbreviations: DDGH= DembiDollo General Hospital, NGH= Nekemte General Hospital, SGGH= ShenanGibe General Hospital, KMGH= Karl Mettu General Hospital, DCH= Dil chora Hospital, RH= Robe Hospital, SGH= Shashamene Hospital, BH= Bishoftu Hospital, GoH= Goba Hospital, GiH= Ginir Hospital, ChiH= Chiro Hospital, DuH= Dupti Hospital, HH= Harar hospital

4.7.2.2 ***Xpert MTB/RIF assay on clinical sample***

Xpert MTB/RIF assay is a nucleic acid amplification test that simultaneously detects DNA of MTB and resistance to RIF that is a mutation of the *rpoB* gene in less than 2 hrs.

Sputum liquefaction and inactivation with the reagent buffer were done by mixing in 1:2 ratios of sample to the reagent. Then, 2 ml of processed sample was carefully transferred into the test cartridge, and then inserted into the Xpert MTB/RIF automated test platform, after an average of 2 hours, the result was displayed. MTB was detected using five overlapping molecular beacon probes (probes A to E) that are complementary to the entire 81-bp rifampicin resistance determining the core region of the wild-type *rpoB* gene. The assay was done following the manufacturer's instruction (41).

4.7.2.3 ***Extraction of mycobacterial DNA from used Xpert MTB/RIF cartridges***

For the extraction of mycobacterial DNA extract, used Xpert MTB/RIF rifampicin-resistant cartridges were utilized, which were done on sputum samples. Used Xpert cartridges were collected and kept at 4 °C until DNA extracted from the cartridge within 5 days. Cartridge and the surroundings were cleaned carefully with 1% bleach and 70% alcohol before extraction. The transparent diamond-shaped reaction chamber on the back of the cartridge was punctured with a sterile fixed-needle insulin syringe in the biosafety level 2 Cabinet. The full cartridge extract volume, typically 15µl, was withdrawn and stored in sterile, safe-lock micro-centrifuge tubes at −20 °C until analyzed for further downstream Genotype MTBDR*sl* assay (37).

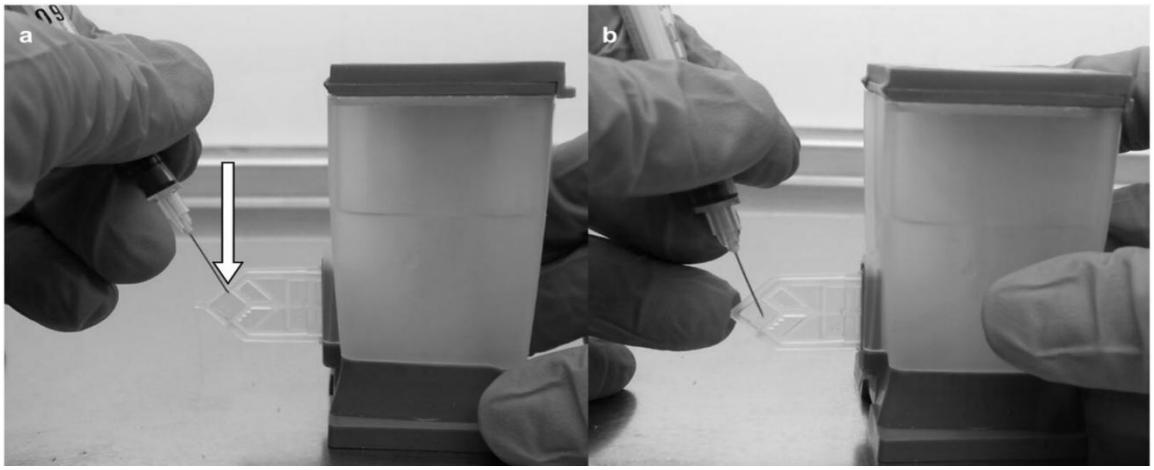


Figure 2: Picture of DNA extraction procedure from used Xpert cartridge

4.7.2.4 ***Genotype MTBDRsl assay on used Xpert MTB/RIF cartridge extract***

MTBDR*sl* was done on cartridge extract after validating the protocol in our lab, as indicated above in section 4.7.2.2. PCR was performed using pre-made amplification mixes that contained all the necessary components. Following amplification, DNA was hybridized with specific oligonucleotide probes immobilized on a strip which enables the detection of the presence of MTBc as well as simultaneously the presence of wild-type and mutation probes for resistance to FLQ and SLID. Finally, the result was interpreted based on the deletion of wild type and the addition of mutant bands following the protocol. The same protocol was followed as when processed from culture isolate or direct clinical sample. Without knowledge of the result indicated by MTBDR*sl* on culture isolates, the assay was performed and interpreted (42).

4.7.2.5 ***Culture and Identification***

Sputum samples were first decontaminated by addition of an equivalent volume of NaOH-NALC-sodium citrate solution. Then the solution was centrifuged for 15 minutes at speed of 3000rpm. The supernatant was decanted and the sediment was re-suspended with 1-2ml PBS. Approximately 100µl or 3-4 drops of the suspension were used to inoculate two LJ slants and the specimens inoculated into LJ media were incubated at $37^{\circ C}$ for a maximum of 8 weeks in a vertical position for the better development of individual colonies. MTBc isolates were identified using the time to visible growth at 37^{oC}, colony pigmentation and morphology, AFB smear and Capilia test (16).

4.7.2.6 ***Ziehl–Nielsen Smear microscopy***

Direct smears were prepared from the colony grown on LJ slant using saline, which were then air-dried, heat-fixed, and treated with carbolfuschin, acid alcohol, and Methylene blue for 5, 3, and one minute respectively, after complete slide wash with water in each step. The smears were graded according to WHO guidelines after being scanned with an oil immersion objective (16).

4.7.2.7 ***MPT 64 Rapid (Capilia)***

Following the bacilli growth on LJ culture media, the test method was carried out. The loop full colony was taken from the culture media and emulsified in 200µl MPT64 Ag buffer. The kit was then injected with 100 µl of the emulsified colony. According to the standard protocol, the chromatographic technology assay was interpreted as positive or negative after 15 minutes (43). These confirmed MTBc isolates were used for genotypic DST utilizing the LPA (Genotype MTBDRsl test) as described in section 4.7.2.9 below.

4.7.2.8 ***Genotype MTBDRsl assay on culture isolate***

The Genotype MTBDR*sl* is qualitative in vitro test for the identification of the MTBc and its resistance to second-line anti-TB drugs from smear-positive or -negative sputum specimens and cultivated samples. The test was performed according to the instructions supplied by the manufacturer. DNA was extracted from confirmed MTBc isolates and master mix was prepared in a clean room. 5µl of DNA extracts was added to the corresponding PCR tubes. After completion of the PCR process, the applicant was detected with a series of procedures. Finally, the result was interpreted based on the deletion of wild type and the addition of mutant bands following the protocol (42).

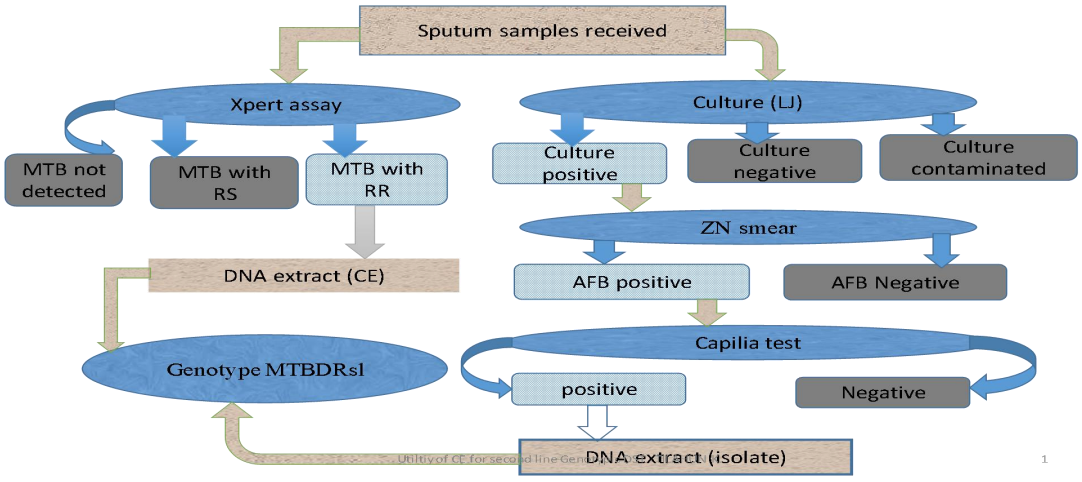


Figure 3: Flow diagram for laboratory procedure

4.8 Data analysis procedures

All laboratory and clinical data were recorded in a logbook during the study period. Each completed data extraction was properly coded, cleaned, verified and then transferred for statistical calculation. The results were entered into EpiData version 3.1 and double-checked for completeness. Statistical Package for Social Science (SPSS) version 23 was used for the statistical analysis. Descriptive statistics were performed using frequencies and percentages to describe the socio-demographic characteristics of the study population. The yield of a method was compared with DST performed on culture isolates. In light of the study's objective, the result was analyzed and interpreted.

4.9 Data quality management and Quality control

All samples were collected and processed following the laboratory's standard operating procedure. The performance of the culture media was tested by inoculating H37Rv and incubating at 37 ^{0}C for 48 hours. By inoculating all reagents in a separate Brain heart infusion (BHI), the sterility of sample processing reagents was checked. Start and end control were included in every run of sample inoculation. To avoid cross-contamination, DNA extraction was done in the BSC2, master mix preparation was done in a second room with a UV chamber hood, and PCR amplification and hybridization were done in a third room with a UV chamber hood for master mix and sample addition. Throughout the study, all laboratory results were recorded in a logbook

4.10 Ethical consideration

Ethical approval was received from Jimma University's Institute of Health Ethical Review Board (IRB000145/2020) prior to data collection. After obtaining approval, a letter of support (MELT46/2012) was written to JU-MRC and ARL from the school of Medical Laboratory Science. To that end, the study's objective was briefly explained, and an agreement was made with the study site that the data would be kept private and utilized solely for research purposes.

4.11 Report writing and dissemination of results

First, the study's final results were submitted to the Institute of Health Jimma University's School of Medical Laboratory Sciences, where they will be presented as a thesis for partial fulfillment of an MSc degree in Medical Microbiology. ARL and JU-MRC were also provided copies of this material. Finally, the findings of this study will be presented at various seminars and workshops, as well as published in peer-reviewed journals.

4.12 Operational definition

Rifampicin resistance: resistance to rifampicin detected using genotypic methods with or without resistance to INH.

Multi-drug-resistant- tuberculosis: *Mycobacterium tuberculosis* that is resistant to both rifampicin and isoniazid.

Pre-XDR-TB: TB caused by Mycobacterium tuberculosis (M. tuberculosis) strains that fulfill the definition of MDR/RR-TB and that are also resistant to any fluoroquinolones.

Extensively drug-resistant tuberculosis: TB caused by *Mycobacterium tuberculosis* strains that is MDR-TB, resistant to isoniazid and rifampicin and which are also resistant to any Fluoroquinolones and at least one additional group A drug i.e bedaquiline or linezolid.

Second-line drugs: Fluoroquinolones and the three second lines injectable drugs

Second-line injectable drugs: Amikacin, Kanamycin and Capreomycin

New: patients who were never treated for TB or took anti-TB drugs for < 1 month.

Previously treated patient: Patients who have received anti-TB drugs for one or more months in the past.

Treatment initiating centers (TIC): Health facilities selected by the TB program to provide patient care and treatment services right from the time of DR-TB diagnosis and throughout the course of treatment with SLD (44–46).

4.13 Validation experiment (Xpert MTB/RIF on dilution series)

In phosphate buffer solution known phenotypically confirmed (drug-susceptible and drug-resistant) culture isolates were prepared and sub-cultured to fresh LJ culture media. to ensure all our replicate strains were at the same (or as close as possible) OD600 (0.6-0.8) turbidity of the suspension equivalent to 0.5 McFarland were prepared, then a tenfold serial dilution on three separate culture isolate was prepared. This was done to ensure we had the correct colony-forming unit (CFUs) that correlated to the semi-quantitation values/$C_T$min values and we also measured each dilution by NanoPhotometer to ensure CFUs were correct for each replicate which correspond to biological range (from 1 x 10^6 to 1 x 10^0). Dilutions containing bacilli (1 ml aliquot) were tested by Xpert (18 totally, ranging from 10^1–10^3 CFU/ml in triplicate for two strains and hence 9 dilutions each for the DS and DR strains) as well as 0 CFU/ml controls in triplicate, according to the manufacturer's instructions The Xpert MTB/RIF assay was used to test bacilli-containing dilutions and negative control with 0 CFU/ml (DNA free biological grade water), in the same way that clinical samples were analyzed according to the manufacturer's instructions. Used Xpert MTB/RIF cartridges were collected and stored at 4 °C until DNA was extracted (37).

4.14 Validation result (Xpert MTB/RIF on dilution series)

4.14.1 Validation results of MTBDR*plus* on extract from used Xpert cartridges done on dilution series

The Xpert MTB/RIF assay detected *M. tuberculosis*-complex DNA in all dilutions series and identified RIF-susceptible and resistant *M. tuberculosis*. MTBDR*plus* had high rates of non-actionable results (TUB-band positive but indeterminate) across all dilutions series irrespective of the DNA extract concentration and strain types. At all dilution series from both strains (drug-susceptible and drug-resistant) MTB complex DNA was correctly detected. At all dilution series regardless of strain type and concentration, all were INH indeterminate 18/18 (100%). From DR-TB strain, the sensitivity for RIF resistance identification was 2/9 (22%). Collectively, MTBDR*plus* has low overall sensitivity for

RIF resistance detection and high rates of non-actionable results (TUB-band positive but indeterminate) in the identification of RIF and INH resistance (figure 4).

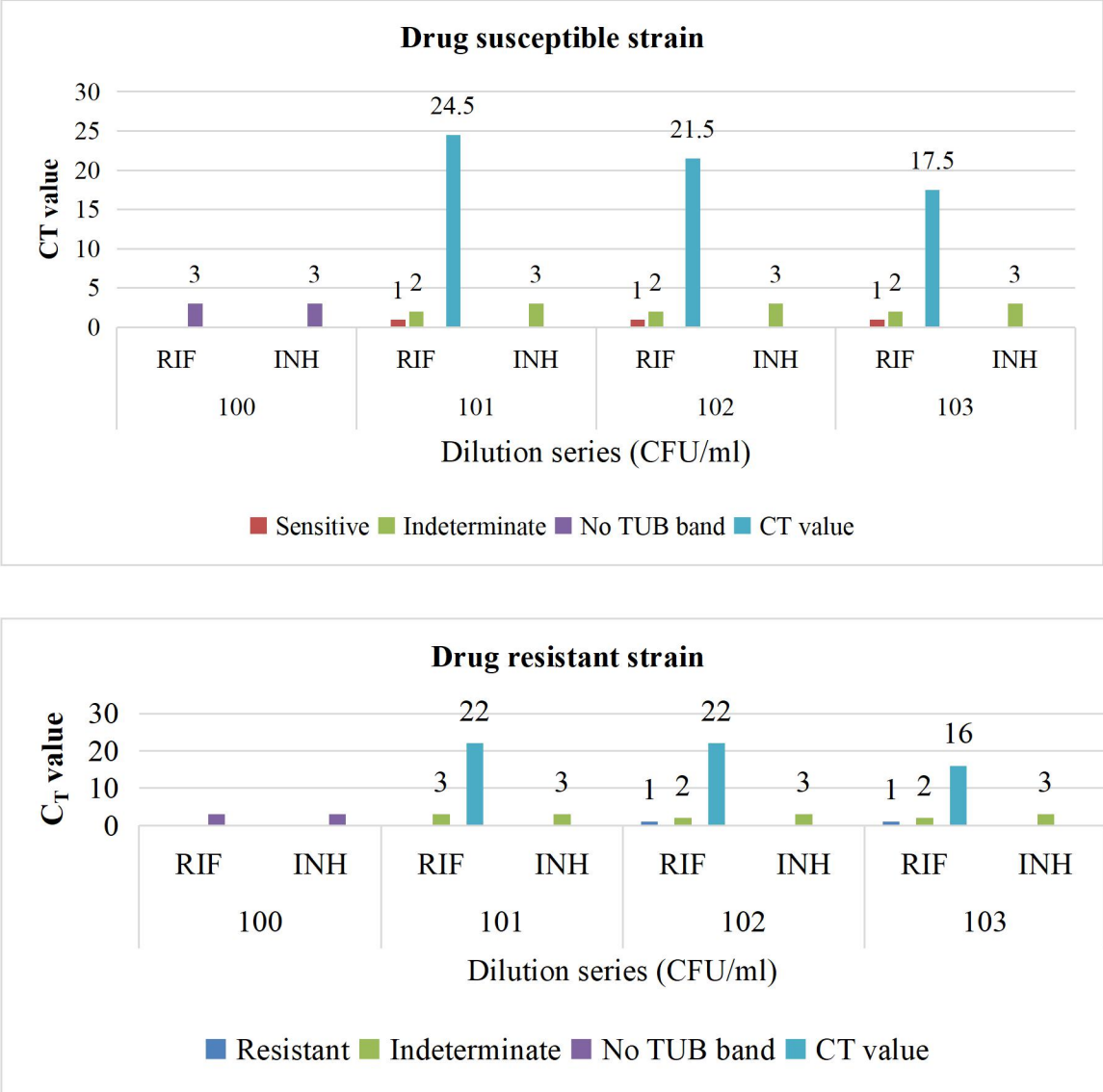


Figure 4: MTBDR*plus* done on CE from a dilution series of DS and DR- TB strains

4.14.2 Validation results of MTBDR*sl* on extract from used Xpert cartridges done on dilution series

Unlike MTBDR*plus*, MTBDR*sl* had actionable results for all cartridges extract at dilution series of $\geq 10^2$ CFU/ml. Regarding the susceptibility and resistance detection for FLQ and

SLID, MTBDR*sl* correctly identified on both strains (drug-susceptible and drug-resistant) at dilution series of $\geq 10^2$ CFU/ml, and no resistance was detected for both of the drugs. At 10^1 CFU/ml, 2/6 (33%) were interpretable and correctly identified as FLQ susceptible (four indeterminate) while all were indeterminate for SLID at this dilution series. Generally, MTBDR*sl* on CE demonstrated a high sensitivity [18/18 (100 %)] for *M. tuberculosis*-complex DNA detection. Susceptibility to FLQ and SLID were correctly identified for both strains at dilution series of $\geq 10^2$ CFU/ml, corresponding to $C_T \leq 22$ (the higher C_T range of the Xpert "medium" semi quantitation category). The $C_T$min threshold at which all MTBDR*sl* results were feasible on Xpert CE was $C_T \leq 22$, which was used for further experiments (Figure 5).

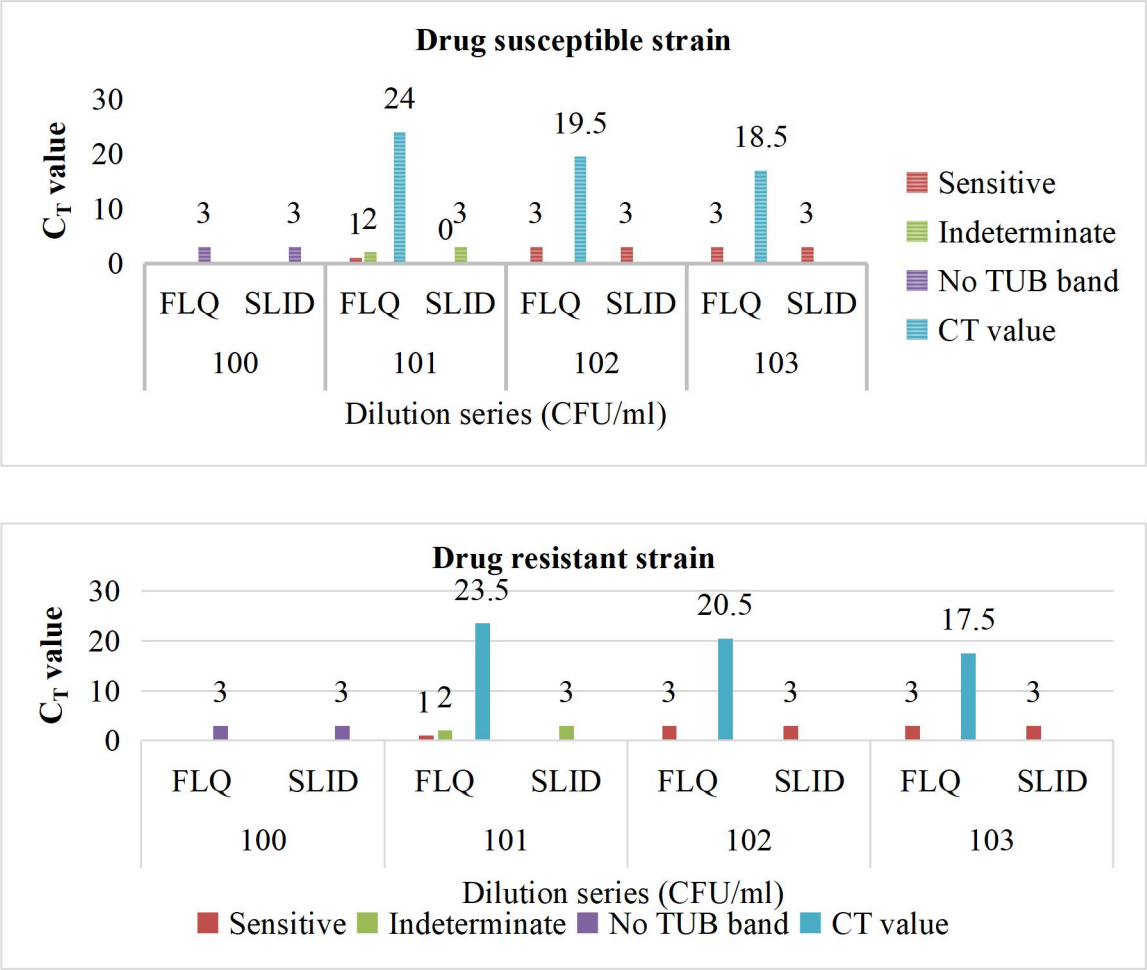


Figure 5: MTBDR*sl* done on CE from a dilution series of DS and DR- TB strains

The Mycobacterial DNA recovered from the used Xpert TB-positive cartridge was measured by Nano Photometer before and after the PCR procedure to determine the cause of non-actionable results in the identification of RIF and INH resistance. The result revealed no significant differences before and after the amplification technique (figure 6).

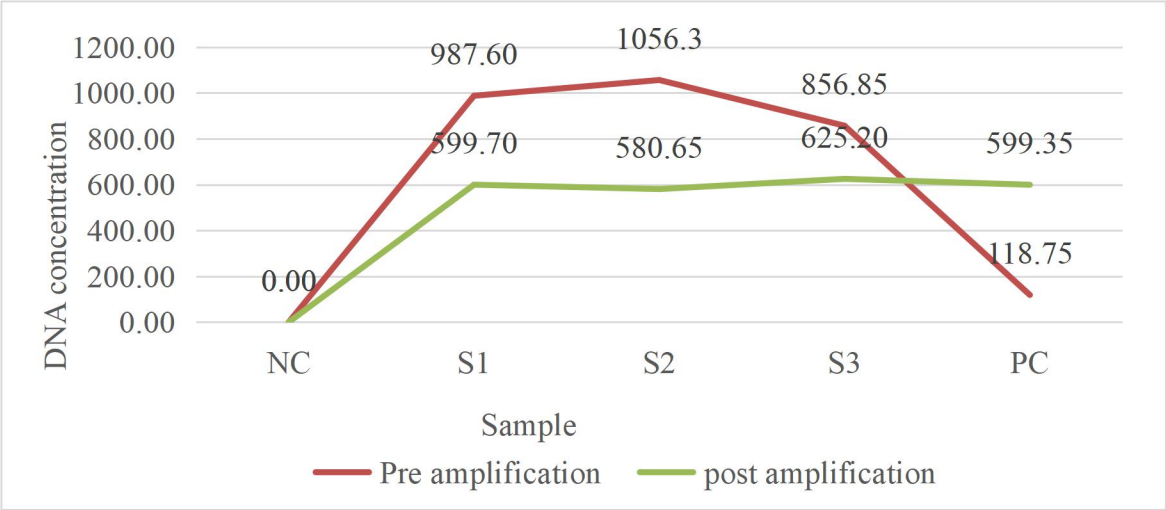


Figure 6: Pre and post amplification DNA quantification by NanoPhotometer

5 Result

5.1 Characteristics of study participants

A total of 62 sputum samples (17 new cases and 45 patients who failed to respond to first-line anti-TB drugs) referred to JU-MRC and ARL for further DST were tested for rifampicin- resistance by Xpert MTB/RIF. Out of 62 sputum samples tested by Xpert MTB/RIF, 50 were rifampicin-resistant. The remaining 12 (19%), were rifampicin-sensitive and were excluded from the further analysis. LJ culture was performed on the 50 rifampicin-resistant samples. Out of these, 40 were culture-positive. The left were culture-negative (six) and culture-contaminated (four) samples. As shown in Figure 7, a total of 40 rifampicin-resistant TB cases were included for the comparison in the final analysis, but the Mycobacterial DNA extracts collected from the ten Xpert TB-positive RIF resistant cartridge (with culture-negative and culture contaminated) were also subjected for the analysis to evaluate its utility for MTBDR*sl*.

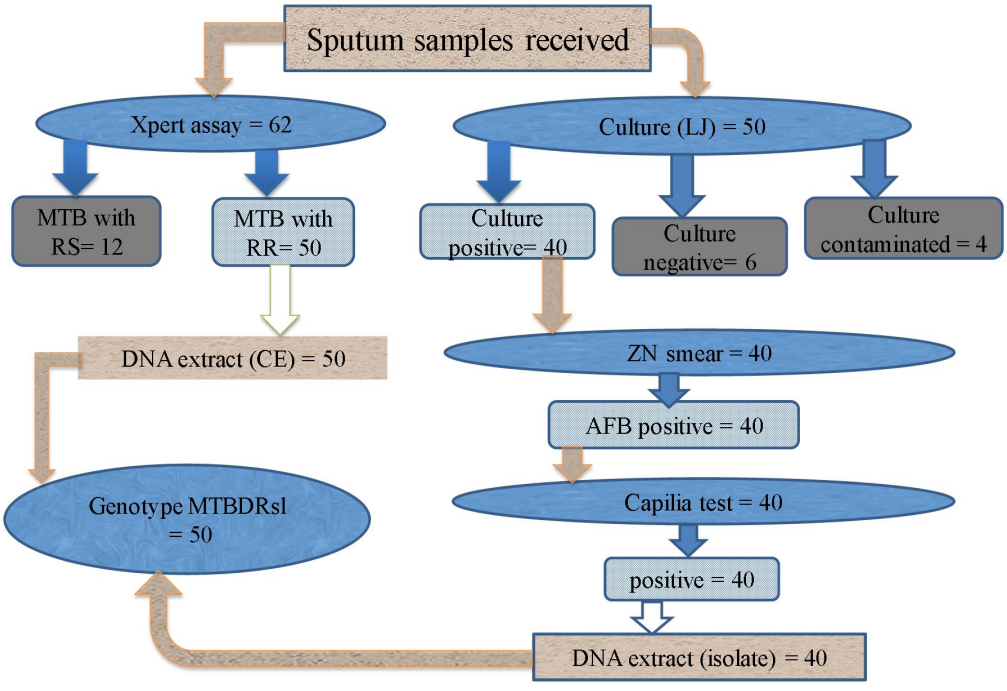


Figure 7: Flow chart for evaluation of MTBDR*sl* from cartridge extract

5.2 Utility of extract from used Xpert cartridge for Genotype MTBDR*sl*

For the reason that MTBDR*plus* was not practicable on the cartridge extract from the used Xpert cartridge done on dilution series during the validation assessment, the assay was not performed on cartridge extract obtained from used Xpert cartridge done on clinical specimens. MTBDR*sl* had 50/50 (100 %) actionable results on Mycobacterial DNA extracts from used Xpert rifampicin-resistant cartridge done on clinical sputa for MTB detection. No resistance was found in any cartridge extract that was subjected to FLQ susceptibility detection. Similarly, for the SLID, the assay gave actionable results in 43/50 (86 %) of the extracts, while 7/50 (14 %) of cartridge extract were indeterminate for SLID. MTBDR*sl* was feasible in 49/50 (98 %) of the extracts tested for low-level kanamycin resistance detection, while one sample was indeterminate (Figure 8).

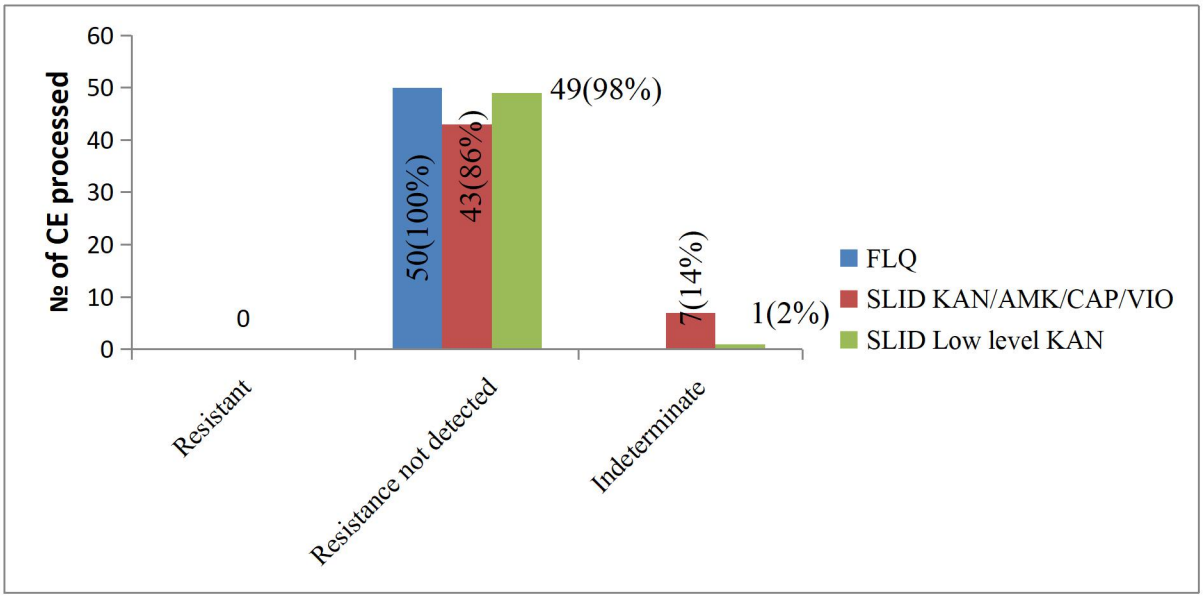


Figure 8: Results of MTBDR*sl* on Xpert cartridge extract

All CE corresponding to the higher C_T value ranges ($C_T \leq 22$) had interpretable results, whereas those that had indeterminate results corresponded to the Xpert semi quantitation levels of $C_T > 22.0$ (figure 9).

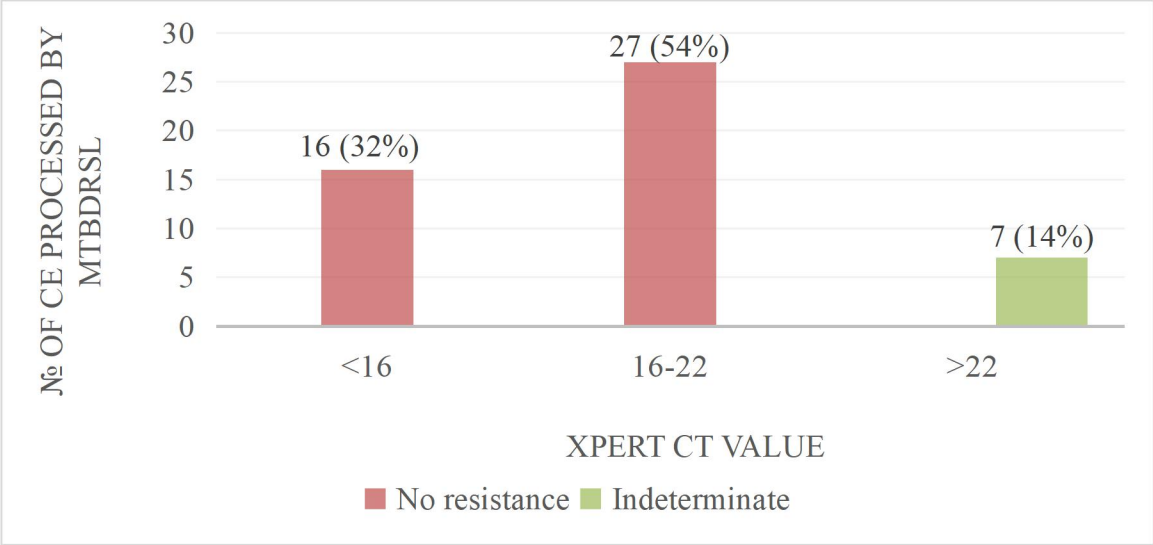
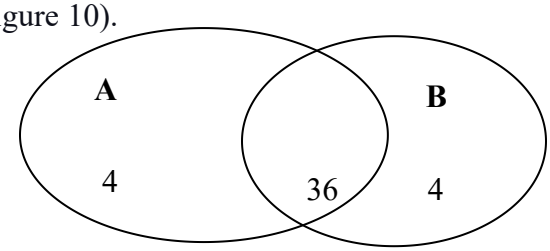


Figure 9: Genotype MTBDR*sl* on cartridge extract versus Xpert C_T value

5.3 Comparison of MTBDR*sl* results from Xpert MTB/RIF cartridge extract and culture isolates

Of 50 Xpert TB-positive rifampicin-resistant samples subjected for culture, 10 had negative or contaminated culture results and MTBDR*sl* was done on 40 culture-positive MTB isolates. Thus 40 (80%) of the 50 Xpert TB-positive rifampicin-resistant cartridge collected had matching culture isolates, for comparison of MTBDR*sl* on cartridge extract versus culture isolates. The cartridge extract and isolates had 40/40 (100%) matching MTBDR*sl* findings for FLQ resistance detection. Similarly, 36/40 (90%) of them demonstrated the concordant MTBDR*sl* results when it came to SLID resistance detection (Figure 10).



Key: A_ **Cartridge extract**
B_ culture isolate

Figure 10: Comparison of MTBDR*sl* from cartridge extracts and culture isolates for SLID resistance detection

The discordant MTBDR*sl* for SLID findings from cartridge extract versus isolates was found in 4/40 (10%) of the samples, with all discordant being indeterminate on cartridge extract but interpretable on culture isolate and no resistance were detected. The four discordant results obtained were determined to be interpretable for low-level kanamycin resistance on extract and similar to that of the culture isolate result, no resistance was detected. Each of the four contradictory outcomes was associated with a "low" Semi quantitation (C_T>22.0). When the four discordant results were excluded, all TUB-bands and susceptibility were consistent with culture isolate at $C_T \leq 22$, implying that the diagnostic utility of Genotype MTBDR*sl* on cartridge extract was likely possible at the above mentioned cut-off threshold.

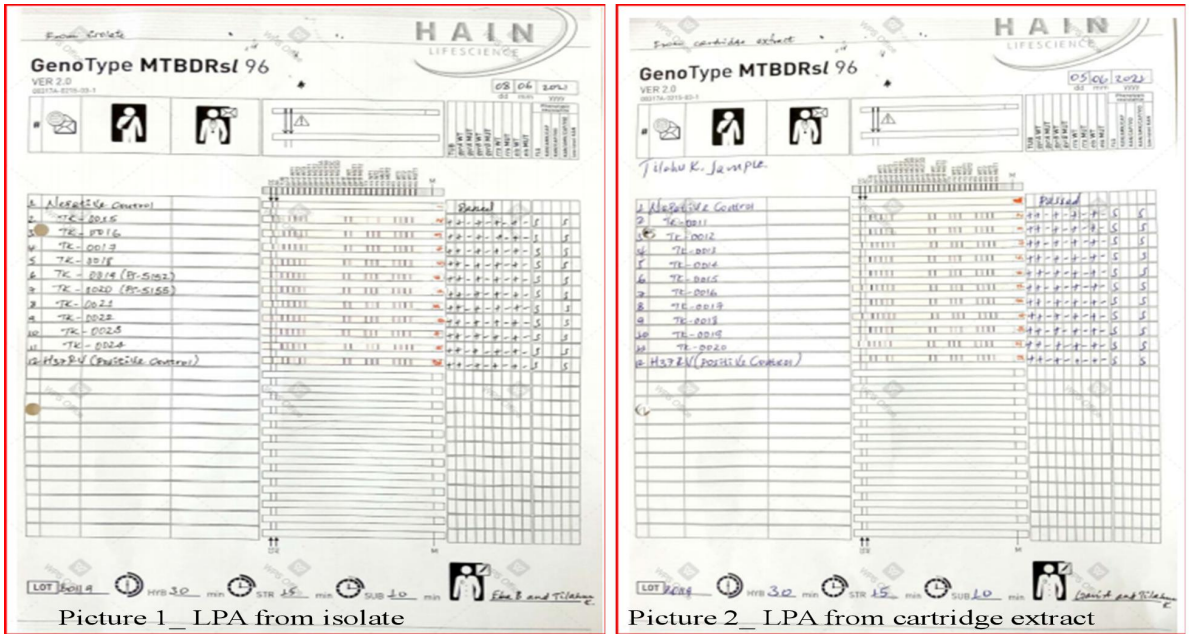
Table 1: Genotype MTBDR*sl* on cartridge extract with C_T value ≤22 of study participants data drown from JU-MRC and ARL Eastern and western Oromia, Ethiopia, October 2021 (N =36)

			Second line injectable drug (culture isolates)	Total
			Resistance not detected	
Second line injectable drug (cartridge extract)	Resistance not detected	Count	36	36
		% of Total	100.0%	100.0%
Total		Count	36	36
		% of Total	100.0%	100.0%

5.4 Description of band quality/interpretability of MTBDR*sl* performed on Xpert cartridge extracts and culture isolates

Various controls were considered while interpreting the LPA results. Amplification control was used to check the PCR process, Conjugate Control was used to check the

detection process on the enzyme-substrate reaction step, Locus control was used to check the reaction's sensitivity in each drug resistance gene locus, and negative and positive controls were also employed. The result is invalid when a complete gene locus (all bands including the Locus Control band) is missing, as determined by the test protocol. The visual quality and interpretability of MTBDR*sl* bands developed on cartridge extract and culture isolate were also assessed. The band strengths and quality of the MTBDR*sl* strip from the culture isolate and the cartridge extract were quite similar. The TUB band, which identified the amplicons as belonging to the *M. tuberculosis* complex, was found in all forty samples (culture isolate and cartridge extract), indicating that they were indeed *M. tuberculosis* complex. All of the control bands, as well as the wild type were visible and could be read with the guidance of the control bands and comparatively similar from both samples. Only the discordant result strip that performed on cartridge extract with C_T > 22 was being very faint (1) or missed/absent (3) for rrs band locus control (which was used to evaluate SLID resistance). Collectively with regards to the DNA concentration from the cartridges, this approach works best when done on cartridges with "medium" and "high" Xpert MTB/RIF results (figure 11).



Picture 1_ LPA from isolate

Picture 2_ LPA from cartridge extract

Figure 11: comparison of MTBDR*sl* strip done on culture isolate and CE

6 Discussion

Drug-resistant tuberculosis is a major challenge to global tuberculosis control efforts. A drug-resistant pattern evaluates the effectiveness of a country's current tuberculosis control program. Early detection of SLD resistance is critical for determining the best treatment regimen composition. Only by rapidly identifying drug-resistant tuberculosis cases and treating them with a combination of effective drugs can drug-resistant tuberculosis be prevented. The availability of low-cost, effective diagnostic tests that may be employed in resource-constrained settings is a critical gap in the fight against DR-TB (47). Nucleic acid-based diagnostics have provided a unique approach for developing very efficient point-of-care tests. Among these Xpert MTB/RIF has been widely used to diagnose DR-TB and brings significant improvement in time-to-treatment of MDR-TB. However, Xpert cannot detect resistance to second-line anti-TB drugs, and a follow-up DST test that requires an additional sample is recommended.

In Ethiopia, there is currently seven Line Probe Assay service providing site, which is well-established for the qualitative detection of MTB and resistance to FLQ and SLID from culture isolate samples (48). Because this LPA is already in use with a large number of qualified staff and quality procedures in place, utilizing it to detect second-line drug resistance from used Xpert MTB/RIF cartridge extract `could help with the TB control approach.

We have done verification on the performance of genotypic DST on cartridge extract and our main findings of MTBDR*plus* and MTBDR*sl* validation tests on cartridge extract (from used Xpert cartridge done on bacilli containing a dilution series) were: Genotype MTBDR*plus* had high numbers of non-actionable results (positive TUB-band but indeterminate) in all dilution series regardless of output. This might be due to the vast number of *rpoB* amplicons in the cartridge extract, which share binding sites with MTBDR*plus* probes and MTBDR*plus rpoB*, may drain reagents from the multiplex *inhA* and *katG* amplification reactions, resulting in incorrect results. MTBDR*sl* on cartridge extract has enabled genotypic drug-susceptibility testing of FLQ and SLID with high

accuracy and low indeterminate values when the Xpert semi quantification class was at least “medium” or $C_T \leq 22$ (corresponding to 10^2 CFU / ml). This data supports the use of used cartridge for second-line genotypic DST. We have defined the extent to which MTBDR*sl* is likely to feasible on Xpert cartridge extract, thus avoiding the time and resources spent on cartridge extract unlikely to provide a valid result. We therefore suggest that the results of MTBDR*sl* on CE be interpreted in the same way as the WHO recommended for MTBDR*sl* on clinical samples (42).

In the current study, we have evaluated MTBDR*sl* on cartridge extracts from used Xpert MTB/RIF cartridges for genotypic second-line DST. Despite the fact that only a small sample size was used in this investigation, we found that MTBDR*sl* on cartridge extracts enabled genotypic drug-susceptibility testing for second-line anti-TB drugs with the excellent concordance with MTBDR*sl* on culture isolates. MTBDR*sl* on used cartridge extract does not require extra patient visits and additional specimen collections. Testing second-line DST on the first available specimen could potentially reduce the number of patients lost during the diagnostic cascade and allow earlier diagnosis of XDR-TB leading to earlier effective treatment initiation, reducing health system cost, and better clinical outcomes.

A total of forty samples were included in the study to compare FLQ resistance determined by Genotype MTBDR*sl* from cartridge extract and culture isolate. The assay works well on cartridge extract and showed 100% concordance with that done on culture isolates for FLQ resistance detection. Similarly, our findings show that the assay was effective at detecting SLID resistance from cartridge extract (90%), even though there were four (10%) discrepant results obtained between the two samples (that from the cartridge extract and the culture isolate). The culture isolates of the four samples yielded interpretable results and were susceptible to the drugs, whereas on the cartridge extracts yielded indeterminate results. The overall observed rates of indeterminate results from the cartridge extract by the assay among the fifty samples, including ten culture excluded samples (culture contaminated and negative) in this study was 14%. This was due to a DNA concentration in the sample that is below the detection limit, which was low bacilli

load, corresponded to an Xpert semi quantitation category of at least "low." The rrs target region was associated with or encompassed the majority of the discrepancy (faint band staining, no locus control).

Overall agreement for SLID resistance detection from culture isolate versus cartridge extract of MTBDR*sl* in this study was comparable to a study conducted in South Africa (37). The proportion of the MTBDR*sl* assay for detecting low-level KAN resistance was 100% from both samples.

In this work, the band of the strip performed on cartridge extract and culture isolate was also evaluated visually. The band strength of the two strips, which were done from cartridge extract and isolate on forty samples, was comparable. All control bands, including TUB and wild types, as well as the mutant band, were visible and readable. Therefore, we recommend that Genotype MTBDR*sl* on used cartridge extract is interpreted in the same manner as recommended by the WHO for Genotype MTBDR*sl* on culture isolates.

Strength

- To the best of our knowledge, this is the first experimental study and goes through different procedures to evaluate the utility of extract from used Xpert cartridge for second-line genotypic DST in our country.
- Our findings provide key insight about the utility of cartridge extract for molecular DST in detecting susceptibility/resistance to second-line drugs.
- Validation experiment was done before conducting the study on clinical samples for both genotypic DST (MTBDR*plus* and MTBDR*sl).*

Limitation

- Due to COVID-19, a restriction was made on priority medical supplies to be imported and given for medical supplies used for COVID-19 protection. We were unable to get supplies and drugs for second-line DST. So phenotypic DST was not performed in this study which may be decisive to determining the correct resistance status of discordant findings between the cartridge extract and culture isolates by the assay and determining test performance/diagnostic accuracy.
- Only rifampicin resistant samples were analyzed in this study

7 Conclusion and Recommendations

Conclusion:

MTBDR*sl* performed on used cartridge extract has a high level of agreement with MTBDRsl performed on culture isolates. Our data showed that Genotype MTBDR*sl* is likely a useful tool to rapidly diagnose pre-XDR and XDR-TB in used cartridge extracts at C_T value $\leq$ 22 on a single specimen. Furthermore, our study demonstrates the feasibility of performing MTBDR*sl* on used cartridge extract dictating the material that would otherwise be discarded has a diagnostic utility.

Time to detection of resistance to second-line anti-TB drugs was significantly reduced when MTBDR*sl* was performed on cartridge extract compared to MTBDR*sl* perfumed on culture isolates leading to rapid detection of XDR-TB. Despite the launch and recommendation of genotypic DST, RR-TB diagnosed cases still waiting for the culture to proceed downstream molecular assay, which takes a long time and is difficult in resource-constrained nations like ours, and this alternative is the best in terms of cost-cutting by employing materials that would otherwise be discarded and minimizes the need for mycobacterial culture.

Recommendation:

- Utilization of Mycobacterial DNA extract from used Xpert cartridge for MTBDR*sl* needs to be considered in Ethiopia for FLQ and SLID resistance detection.
- Further investigation using a more accurate Phenotypic DST technique that could discriminate indeterminate results is warranted to determine the diagnostic accuracy of the assay from the cartridge extract.
- We have not tested with a specific experiment for cartridges that stood at room temperature, and the impact of storing cartridges for a prolonged time demands a more thorough investigation and optimization of the method.
- Investigation on extra pulmonary samples, which is one third of tuberculosis cases
- The number of isolates obtained for this study was small, as samples were collected only from two TB culture referral centers, thus further study with large scale is recommended.

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Annex-I Data extraction sheet

Jimma University

Institute Of Health

School of Medical Laboratory

Data extraction sheet that was used to collect clinical and laboratory information to Evaluate Utility of Mycobacterial DNA extract from used Xpert MTB/RIF cartridges for second line Genotypic Drug Susceptibility Testing at Eastern and Western Oromia, Ethiopia

Medical record No._____ TB lab Ser No. _____ Date: _____ Contact address _____		
Part one: Socio demographic characteristics		
1.1	sex	Male ☐ Female ☐
1.2	Age in years	_____
1.3	Residence	Urban ☐ Rural ☐
Part two: Patients clinical data		
2.1	Previous TB treatment history	yes ☐ No ☐
2.2	HIV status	Positive ☐ Negative ☐
Part three: Laboratory aspect		
3.1	Reason for request	Diagnosis ☐ Follow up ☐
3.2	Gene Xpert result	MTB detected ☐ Not detected ☐ Invalid/errors ☐
3.3	Rifampicin status	Resistant ☐ Resistant not detected ☐ Indeterminate ☐
3.4	Semi-quantitative grades	High ☐ Medium ☐ Low ☐
3.5	C_T value:	Probe A___ Probe B___ Probe C___ Probe D__ Probe E___
3.6	Culture	MTB +ve ☐ MTB –ve ☐ contaminated ☐

		NTM suspected ☐
3.7	Second line LPA result:	FLQ: Resistant ☐ Resistant not detected ☐ Indeterminate ☐ SLID: Resistant ☐ Resistant not detected ☐ Indeterminate ☐
3.8	Second line LPA result from CE	FLQ: Resistant ☐ Resistant not detected ☐ Indeterminate ☐ SLID: Resistant ☐ Resistant not detected ☐ Indeterminate ☐

Laboratory Requesting and Reporting Form

1. Patient Identification:

Patient MRN _____ Age (Yrs.): _____Sex (M/F): _____

2. TB Disease Type & Treatment History:

Site: Pulmonary_____ Extra pulmonary (specify):_____

Registration Group: New_____ Relapse_____ Treatment after loss to follow-up_____

failure after retreatment_____

Previous TB drug use: First line_____ Second line _____ MDR-TB contact_____

3. Request for Testing at TB Laboratory:

Reason: Diagnosis_____ Follow up _____

If diagnosis: presumptive TB_____ Presumptive DR_____

Specimen: Sputum _____ Gastric lavage _____ Other (Specify): _____

Date specimen collected: ____/____/____ (Ethiopian Calendar)

Requested tests: Xpert MTB/RIF__ 1st line LPA__ 2nd line LPA___ Phenotypic DST ___

Person requesting examination: Name: _____ Date____/_____/_____

4. Laboratory Result:

TB Lab ID: _____ Date specimen received: ____/___/___

Xpert MTB/RIF test result

Result: M. tuberculosis: Detected____ Not detected____ Invalid / No result / Error _____

Rifampicin resistance: Detected _____ not detected _____ indeterminate _____

2nd line LPA

FLQ resistance: Detected _____ not detected _____ indeterminate _____

SLID resistance: Detected _____ not detected _____ indeterminate _____

Date of reporting _____Reported by----------

Laboratory data

1. Gene Xpert result collection form

SN	Lab code	M. tuberculosis status					Rifampicin resistance status		
		Detected	Not detected	Invalid	No result	Error	Detected	Not detected	Indeterminate

2. Microscopic Examination Result collection sheet

SN	Lab code	AFB result				
		Negative	scanty	1+	2+	3+

3. Culture follow up sheet

SN	Sample ID	Date Collected	Date processed	Primary smear	Culture	Growth detection								BHI	Media smear	Capilia	Culture Result	Report date	Initial
						Week													
						1	2	3	4	5	6	7	8						
					LJ														
					MGIT														
					LJ														
					MGIT														

4. TB culture result collection sheet

SN	Lab. code	Media used (LJ or MGIT)	Result						
			Negative (0 colonies)	1-9 (<10 colonies)	+ (10-100 colonies)	++ (>100 colonies)	+++ (Innumerable/confluent growth)	NTM	Contaminated

5. LPA from used Xpert MTB/RIF cartridges extract, result collection sheet

SN	Lab code	SL-LPA					Remark (Date)
		FLQ	SLID				
			KAN/AMK/CAP	KAN/CAP/VIO	KAN/AMK/CAP/VIO	Low level KAN	

6. LPA from culture isolate, result collection sheet

SN	Lab code	SL-LPA					Remark (Date)
		FLQ	SLID				
			KAN/AMK/CAP	KAN/CAP/VIO	KAN/AMK/CAP/VIO	Low level KAN	

Annex-II: Standard Operating Procedures

1. SOP for Xpert MTB/RIF

Principle of the Test

The Xpert MTB/RIF system consists of the instrument, a computer, a bar code scanner and requires single-use disposable Xpert MTB/RIF cartridges that contain assay reagents. Following a 3-step sample preparation in the laboratory, the specimen is transferred into the MTB/RIF cartridge and inserted into the Xpert instrument. By starting the test of the system software, the Xpert automates all following steps, including sample work-up, nucleic acid amplification, detection of the target sequence and result interpretation. The primers in the Xpert MTB/RIF assay amplify a portion of the *rpoB* gene containing the 81 base pair "core" region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with resistance to RIF.

Procedures

1. Label each Xpert MTB/RIF cartridge with the corresponding specimen ID.
2. Transfer 1.0 ml of expectorated sputum to a conical, screw-capped tube using a sterile transfer pipette.
3. Add 2.0 ml Xpert MTB/RIF Sample Reagent to the expectorated sputum using a sterile transfer pipette
4. Replace the lid, and shake the tube vigorously 10-20 times.
5. Allow the tube to stand upright for 10 min at room temperature.
6. Shake the tube vigorously 10-20 times.
7. Allow the tube to stand upright for another 5 min at room temperature.
8. Inspect the specimens: samples should be liquefied with no visible clumps of sputum.
9. Open the labeled Xpert MTB/RIF cartridge lid.
10. Using the sterile transfer pipette provided, aspirate 2 ml of the liquefied specimen and transfer the sample into the open port of the Xpert MTB/RIF cartridge.
11. Close the cartridge lid.
12. Turn on the computer, and then turn on the Xpert MTB/RIF diagnostic instrument.
13. On the Windows desktop, double-click the Xpert MTB/RIF diagnostic shortcut icon.

14. Log in to the Xpert MTB/RIF diagnostic System software using your user name and password.

15. In the Xpert MTB/RIF diagnostic System window, click Create Test. The Scan Cartridge bar code dialog box appears.

16. In the Sample ID box, scan or type the sample ID.

17. Scan the bar code on the Xpert MTB/RIF cartridge. The Create Test window appears.

18. Click Start Test, and enter your password in the dialog box that appears.

19. Open the instrument module door with the blinking green light, and load the cartridge.

20. Close the door. The test starts and the light stops blinking and remains constantly green. When the test is finished, the light turns off.

21. Wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.

22. Store used cartridges in the refrigerator at a temperature of 2-8^{o}C for further evaluation.

Result Interpretation

Xpert MTB/RIF result can indicate that *M. tuberculosis* was not detected, MTB was detected and was not resistant to rifampicin (that is, it is rifampicin susceptible), or that MTB was detected and it was resistant to rifampicin. A small proportion of tests may result in an error or invalid result; these tests need to be repeated.

Extraction of mycobacterial DNA from used Xpert MTB/RIF cartridges

Extracting liquid from diamond reaction chamber

Used Xpert MTB/RIF TB-positive cartridge performed on sputum from people with resistant to rifampicin was collected. A cartridge was stored at 4 °C prior to CE extraction within 5 days. The transparent diamond-shaped reaction chamber on the back of the cartridge was punctured with a sterile fixed-needle insulin syringe.

Note: The following steps are to be performed in a BSL2 cabinet. Ensure the surface is decontaminated with UV for 5min prior to start and properly wipe down all surfaces (including each cartridge) with 1% sodium hypochlorite and 70% alcohol before and

especially after performing experiment, including gloves and lab-coat sleeves that are in the cabinet.

1. Remove sterile insulin syringe from packaging and pull syringe up slightly to allow some air to enter the syringe (this makes the extraction of the solution easier).
2. Pierce the film in the top corner of the diamond shaped chamber with the syringe
3. Slowly move syringe deeper into the diamond chamber and pull back on the syringe drawing the solution into the syringe, taking care not to pierce the film anywhere else.
4. Eject the solution slowly into a pre-labeled Eppendorf tube, close the lid tightly.
5. Store at -20°C or place on ice if used immediately.

2. SOP Ziehl Neelson Staining

Procedures

1. Prepare smear and allow to air dry.
2. Heat fix smear, or pass slide through Bunsen burner flame. Don't over heat.
3. Flood the stain with carbolfuchsin.
4. Heat the slides to steaming with Bunsen burner, for 5 minutes.
5. Wash slides with water (use tap water or water from reservoir bottles)
6. Flood smear with acid alcohol, allow to stain for 3 minutes
7. Wash smear again with water and drain
8. Floods slides with methylene blue as a counter stain for 1 to 2 minutes
9. Rinse with water, drain and air dry .do not blot
10. Examine smears under oil immersion objectives lens of the microscope

Reporting of smear results

The smear should be searched in an orderly manner

- None……..….. No AFB seen
- 1-9/100 fields……. ……..…Exact number/scanty/
- 1-9 /10 fields……..…1+
- 1-9 /fields…….....2+
- >9 /fields……..…….....3

3. Sop for LJ culture

Principles

All sputum samples were homogenized and decontaminated by the N-acetyl L-cysteine-sodium hydroxide method (NALC-NaOH) methods. Equal amount of NALC-NaOH solution were added in to the sputum specimens. Specimen -NALC-NaOH solution were filled by Phosphate- buffer solution (PBS) of 6.8 PH units, to the level of 45ml. Then the solution was centrifuged for 15 minute at speed of 3000rpm.The supernatant was decanted and the sediment was re-suspended with 1-2ml PBS. Approximately 100µl or 3-4 drops of the suspension was used to inoculate two LJ slants and the specimens inoculated into L-J media are incubated at $37^{\circ C}$ for 8 weeks in a vertical position for the better development of individual colonies. MTB complex isolates are identified using time to visible growth, growth at 37^{oC}, colony pigmentation and morphology, AFB smear and Capilia assay.

Preparing the medium

LJ medium is prepared without potato starch following the procedure described below.

• Use fresh eggs that are not more than 1 week old from chickens that have not been fed antibiotics.

• Scrub the eggs with a soap solution, and leave them in the solution for 30 minutes.

• Rinse the eggs thoroughly under running water, and then soak them in 70% ethyl alcohol for 15 minutes.

• Place eggs on a sheet of clean paper towel and allow to air dry

• Break the eggs into a sterile flask, and then shake the flask by hand to homogenize the eggs.

• Filter the egg suspension through four layers of sterile gauze, and collect the filtered suspension in a sterile measuring cylinder so that it can be measured.

• Prepare 600 ml of the recommended salt solution as described below and autoclave at 121 ^{O}C for 15 minutes. Cool to room temperature.

To prepare the salt solution dissolve the ingredients in the following order. (Alternatively, LJ media salt solution can be prepared from a commercial base following the manufacturer's instructions)

1. Monopotassium phosphate (anhydrous).....2.4 g
2. Magnesium sulphate 7H2O.....0.24 g
3. Magnesium citrate.....0.6 g
4. Asparagine.....3.6 g
5. Glycerol (reagent grade).....12.0 ml
6. Distilled or deionized water.....600.0 ml

• Add 20.0 ml malachite green solution to the cooled salt solution; use freshly prepared 2% malachite green solution in water.

• Add 1000 ml of the homogenized eggs.

• Mix thoroughly, and dispense 6-8 ml into each screw-capped sterile tube.

• Place the tubes in an inspissator on an angle.

• Inspissate the tubes for 45 minutes once the temperature has stabilized at 85 ^{O}C ± 1 ^{O}C.

• To prepare the medium containing the agent to be tested, the agents are incorporated into the liquid mixture before it is dispensed into tubes and inspissated. All tubes should be labeled with the name of the agent and the concentration.

• Inspissated LJ medium with and without anti-TB agents incorporated can be stored at 6^{O}C ± 2 ^{O}C for 1 month.

• After inspissation, randomly select about 5% of the tubes for a sterility test. These tubes should be incubated at 35-37 ^{OC} for 48 hours as an initial sterility check, and then at least 5 tubes should be incubated at 35-37 ^{OC} for 4 weeks to test for slow-growing bacteria and fungi. Tubes incubated for 48 hours and found to be sterile may be used for routine culture work.

• A quality control test should be run on each batch of freshly prepared medium

Specimen processing

Sputum specimens are homogenized and decontaminated by N-acetyl L-cysteine- sodium hydroxide method (NALC-NaOH) methods. Equal amount of NALC-NaOH solution will be added in to the sputum specimens. Specimen - NALC-NaOH solution will be filled by Phosphate- buffer solution (PBS) of 6.8 PH units, to the level of 45ml. Then the solution will be centrifuged for 15 minute at speed of 3000 rpm. The supernatant will be decanted and the sediment will be re-suspended with 1-2ml PBS

Inoculation and Incubation of Solid Cultures

1. Label LJ tube, using the study-specific labels that contain identifying information
2. Remove any excess water in the slant using a sterile transfer pipette.
3. Inoculate the tube with 200 µl (3-4 drop) of the sample using a sterile graduated disposable pipette. Spread inoculum evenly over entire surface of medium.
4. Replace cap and ensure there are no droplets around the rim of the tube. Wipe off the outside of the tube with a paper towel soaked in tuberculocidal disinfectant.
5. Leave tube in slanted position with cap loosened until inoculum is absorbed (about a week), then tighten cap securely and incubate in upright position at 37°C (±1°C). Alternatively, the tube can be incubated immediately in an upright position with cap loose for the first week of incubation.
6. Examine and record results for the cultures weekly, for 8 intervals. Cultures can be read on the bench, as long as the caps are NOT loosened.
7. To observe fine growth, a strong direct light from the angle poise lamp must be shone onto the slant surface. *M. tuberculosis* usually grows as a buff-colored, dry colony, which is very distinctive.

4. SOP for Second Line-Line Probe Assay

Principles of the Procedure

The Genotype MTBDRsl test is based on the DNA STRIP technology. The whole procedure is divided into three steps: (i) DNA extraction from decontaminated sputum specimens or cultured material (solid/liquid medium) – the necessary reagents are not included in the kit, (ii) a multiplex amplification with biotinylated primers, and (iii) a reverse hybridization. All reagents needed for amplification, such as polymerase and primers, are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. The membrane strips are coated with specific probes complementary to the amplified nucleic acids. After chemical denaturation, the single-stranded amplicons bind to the probes (hybridization). Highly specific binding of complementary DNA strands is ensured by stringent conditions which result from the combination of buffer composition and a certain temperature. Thus the probes reliably discriminate several sequence variations in the gene regions examined. The streptavidin-conjugated alkaline phosphatase binds to the amplicons' biotin via the streptavidin moiety. Finally, the alkaline phosphatase transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate. A template ensures the easy and fast interpretation of the banding pattern obtained.

Specimen Requirements and processing

Decontaminated smear-positive or -negative sputum samples as well as cultivated samples (solid/liquid medium) can be used as starting material for DNA extraction.

Preparation

Clinical specimens must be processed using the NALC/NaOH method. After decontamination, the cell pellet should be re-suspended in a maximum of 1 to 1.5 ml of phosphate buffer. When the sample is to be cultivated, cultivation can be performed either on solid medium (LJ) or in liquid medium (MGIT)

DNA Extraction

Decontaminated smear-positive or -negative sputum samples as well as mycobacteria grown on solid medium or in liquid medium can be used as starting material for DNA extraction. The working area must be free from contaminating DNA. For DNA extraction from decontaminated clinical specimens or cultured material, the Genolyse kit is used according to protocol A.

Amplification

All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B (AM-A and AM-B) and are optimized for this test. After thawing, spin down AM-A and AM-B briefly and mix carefully by pipetting up and down. Pipette AM-A and AM-B only in a room free from contaminating DNA. The DNA solution should be added in a separate working area.

Prepare for each sample:

– 10 µl AM-A

– 35 µl AM-B

– 5 µl DNA solution

Final volume: 50 µl

Determine the number of samples (number of samples to be analyzed plus control samples). Prepare the number of tubes needed. Prepare a master mix containing AM-A and AM-B and mix carefully but thoroughly (do not vortex). Please note that the master mix needs to be prepared freshly each time. Aliquot 45 µl into each of the prepared PCR tubes and add 5 or 10 µl water (see above) to one aliquot (negative control). In a separate working area, add 5 or 10 µl DNA solution to each aliquot (except for negative control).

Hybridization

Preparation

Prewarm shaking water bath to 45°C (the maximum tolerated deviation from the target temperature is +/–1°C) or switch on Tw incubator. Prewarm solutions HYB and STR to 37-45°C before use. The reagents must be free from precipitates (note, however, that solution CON-D is opaque).Warm the remaining reagents with the exception of CON-C

and SUB-C to room temperature. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 µl concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

1. Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.
2. Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for 5 minutes.
3. Carefully add to each well 1 ml of prewarmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color.
4. Place a strip in each well.
5. Place tray in shaking water bath/Tw incubator and incubate for 30 minutes at 45°C.
6. Completely aspirate Hybridization Buffer.
7. Add 1 ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in shaking water bath/Tw incubator.
8. Work at room temperature from this step forward.
9. Wash each strip once with 1 ml of Rinse Solution (RIN) for 1 minute on shaking platform/TwinCubator (pour out RIN after incubation).
10. Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on shaking platform/TwinCubator.
11. Remove solution and wash each strip twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on shaking platform/TwinCubator (pour out solution each time).
12. Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking.
13. Stop reaction as soon as bands are clearly visible by briefly rinsing twice with distilled water.

14. Using tweezers remove strips from the tray and dry them between two layers of absorbent paper.

Evaluation and Interpretation of Results

Paste strips and store protected from light. An evaluation sheet is included in the kit. When using this evaluation sheet, paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the sheet. For technical reasons the distances between single probes on the strips may vary slightly. For an accurate evaluation therefore please use the provided template and align it – separately for each locus – with the respective Locus Control band. Determine the resistance status and note down in the respective column.

Conjugate Control (CC)

A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.

Amplification Control (AC)

When the test is performed correctly, a control amplicon will bind to the Amplification Control zone. In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This might be due to competition of the single reactions during amplification. In this case the test was performed correctly and does not have to be repeated. When only the CC and AC bands are developed, this represents a valid negative result. A missing AC band in case of a negative test result indicates mistakes during amplification setup, or presence of amplification inhibitors. In this case, the test result is not valid and the respective sample has to be repeated.

M. tuberculosis complex (TUB)

This zone hybridizes, as far as is known, with amplicons generated from all members of the Mycobacterium tuberculosis complex. If the TUB zone is negative while no evaluable resistance pattern is developed, the tested specimen does not contain bacteria belonging to the M. tuberculosis complex and cannot be evaluated by this test system.

Locus Controls (*gyrA, gyrB, rrs, eis*)

The Locus Control zones detect gene regions specific for the respective locus. In case of a positive test result (evaluable wild type and mutation banding pattern), the signals of the Locus Control bands may be weak.

gyrA

Both the gyrA and gyrB genes are examined for detection of resistance to FLQ (e.g., Ofloxacin or Moxifloxacin). The wild type probes comprise the most important resistance regions of the gyrA gene. When all wild type probes stain positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal.

gyrB

Both the gyrA and gyrB genes are examined for detection of resistance to FLQ (e.g., Ofloxacin or Moxifloxacin). The wild type probe comprises the most important resistance region of the gyrB gene. When the wild type probe stains positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the wild type probe resulting in the absence of the wild type probe signal.

rrs

The rrs gene is examined for detection of cross-resistance to AG/CP antibiotics such as Kanamycin (KAN) and Amikacin (AMK), both AG, or Capreomycin (CAP) and Viomycin (VIO), both CP. The wild type probes comprise the most important resistance regions of the rrs gene. When both wild type probes stain positive, there is no detectable mutation within the examined region. In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal.

eis

The eis gene is examined for detection of a low-level KAN-resistance. The wild type probes comprise the most important resistance regions of the eis gene. When all wild type probes stain positive, there is no detectable mutation within the examined region. In case

of a mutation, the respective amplicon cannot bind to the corresponding wild type probe resulting in the absence of the wild type probe signal.

Quality Control

In order to control the correct performance of the test and the proper functioning of kit constituents, each strip includes 6 control zones:

– A Conjugate Control zone (CC) to check the binding of the conjugate on the strip and a correct chromogenic reaction

– An Amplification Control zone (AC) to check for a successful amplification reaction

– Four Locus Control zones (*gyrA*, *gyrB*, *rrs*, and *eis*) checking the optimal sensitivity of the reaction for each of the tested gene loci

Observe the usual precautions for amplification setup. It is essential that all materials (such as pipette tips) coming in contact with the reagents are free from DNases. Do not interchange or pool Amplification Mixes or membrane strips from different kits unless the lots are identical. A negative control sample for detection of possible contamination events containing water (molecular biology grade) instead of DNA should be part of each test run; the respective test strip should show the CC and AC bands only.