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Molecular and phenotypic drug resistance pattern of common Mycobacterium tuberculosis complex species and associated mutation: Evidence from selected TB treatment initiating centers in Ethiopia.

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This is to certify that the Thesis prepared by Biniyam Dagne, entitled: Molecular and Phenotypic drug resistance pattern of common Mycobacterium tuberculosis complex species and associated mutation: Evidence from selected TB treatment initiating centers in Ethiopia and submitted in partial fulfillment of the requirements for the degree of Master of laboratory science in Diagnostic and Public Health Microbiology track. Complies with the regulations of the University and meets the accepted standard with respect to originality and quality.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

AFB	Acid Fast Bacilli
AM	Amikacin
BSC	Biosafety cabinet
KatG	Catalease Peroxidase G
CAP	Capreomycin
CFX	Ciprofloxacin
CI	Confidence intervals
DNA	Deoxy ribonucleic acid
DST	Drug susceptibility testing
EMB	Ethambutol
ETO	Ethionamide
EPHI	Ethiopian Public Health Institute
FLQ	Fluoroquinolones
DNA gyr.	DNA gyrase
HIV/AIDS	Human immunodeficiency virus
INH	Isoniazid
InhA	NADH enoyl ACP reductase
IR	Inactivation reagent
KAN	Kanamycin
LPA	Line probe assay
LJ	Lowenstein-Jensen
MDR TB	Multi-drug resistant TB
MGIT	Mycobacteria Growth Indicator Tube
MOX	Moxifloxacin
MTB	*Mycobacterium tuberculosis* complex
NALC	N-acetyl-L-cysteine
NTM	Non tuberculosis Mycobacteria
OFX	Ofloxacin
PCR	Polymerase chain reaction
Pre-XDR	Pre Extensive Drug Resistance

PTB	Pulmonary Tuberculosis
RIF	Rifampicin
RR-TB	Rifampicin-resistant TB
rpoB	RNA Polymerase Beta sub unit
SLID	Second line Injectable Drugs
STM	Streptomycin
SOP	Standard operational procedures
SPSS	Statistical Package for Social Sciences
TB	Tuberculosis
XDR-TB	Extensive Drug Resistance TB

Abstract

Background: Drug resistance in Mycobacterium tuberculosis complex remains major health burden in human history and still is a major leading cause of death in developing countries. Early detection of all forms of drug resistance tuberculosis is a key factor to reduce and contain the spread of these resistant strains. A better knowledge of the mechanisms of action of anti-tuberculosis drugs and the development of drug resistance will allow identifying new drug targets and better ways to detect drug resistance. Drug Susceptibility test of certain drugs can also be technically challenging, in resource limited areas and cost prohibitive.

Objective: The objective of this study was to asses molecular and phenotypic drug resistance Pattern of common Mycobacterium tuberculosis complex species and associated mutation.

Methods: A cross sectional study design was followed, the data was collected using standard check list and sputum samples from a total 204 study individuals with bacteriologically confirmed tuberculosis cases. Sputum samples was analyzed using conventional Tuberculosis culture and Identification followed by molecular and then phenotypic drug susptability test for both first line and second line drugs was done as a gold standard to see the drug susptability pattern and associated mutations. Data was entered in to SPSS version 20 and export to STATA version 12 for analysis. Descriptive analysis and frequencies was used for analysis.

Results: Out of 204 Clinical samples collected, Mycobacterium species were recovered from165 in which 160 were Mycobacterium tuberculosis complex and the remaining 5 isolates were non-tuberculosis Mycobacterium species. Differentiation of Mycobacterium tuberculosis complex isolates was conducted by using genotype MTBC assay and the result revealed that all were found out to be *M. tuberculosis*. Out of 5 Non Tuberculosis Mycobacterium species: 2 *M.fortuitum*, 2 *M.intracellulare* and 1 *M.gordonae* were identified. Among 160 species of *M. tuberculosis* isolates 110(68.8%) and 50(31.2%) were susceptible and resistance to one of all drugs tested, respectively. Ninety-nine isolates were Multi drug resistance while 8(5%) isolate was Pre-Extensive drug resistance, whereas 1(0.6%) isolate identified as Extensive drug resistance. Out of genotypic resistance isolates highest frequency of mutation detected for katG 86(92.5%) followed by rpoB (93(58.15%), 4(4.3%) inhA and4 (4.3%) gyrase genes. A total of 30(18.7%) isolates found to be discordant from this 12(7.5%) for Rifampicin, 16 (16%) for Isoniazid, 3(1.8%) for Fluoroquinolone and 3(1.8%) for Second line injectable drugs.

Conclusion: In conclusion, the present study findings in the phenotypic DST pattern showed that the magnitude of drug resistant TB in the previously treated TB cases was higher as compared to new cases. Highest proportion drug resistance detected for INH followed by RIF, resistance for EMB and STM all so higher. Determining the yield of drug resistance TB using both Phenotypic and Molecular DST methods were recommended for programmatic management of drug resistance Mycobacterium tuberculosis complex in Ethiopia.

Key words: Anti TB Drug Resistance, Gene mutation, Mycobacterium Tuberculosis Complex

1. Introduction

1.1 Background

Tuberculosis (TB) is a communicable infectious disease which has been a major health burden in human history and still is a major leading cause of death in developing countries. The causative agent is a complex of closely related organisms called Mycobacterium Tuberculosis complex mainly *M. tuberculosis, M. bovis, M. africanum, M. canettii, M. microti , M. caprae and M. pinnipedii* [1]. Its transmission is via air born in confined environment when a person with PTB expels the bacteria and also by close contact with infected cattle or by ingesting the organisms in raw untreated milk, a relatively small proportion of people infected with MTBC will develop TB disease. But peoples with HIV develop TB disease much higher than others [2].

M.tuberculosis is a slow-growing aerobe rod-shaped facultative intracellular bacterium characteristically acid-fast when microbiological staining techniques are used. The high lipid proportion cell wall makes the bacterium impervious to Gram staining, the most common method for bacterial staining, therefore the acid-fast stain is the preferred staining method for identifying MTBC [3]. In addition, the high lipid content in the cell wall provides M. tuberculosis the ability to persist in host cells by preventing attacks from oxygen radicals in phagolysosomes [4]. In extracellular environment, the cell wall of M. tuberculosis can also protect this bacterium from the activation of complement system in serum [5]. In contrast to other bacteria, the emergence of drug resistant strains of Mycobacterium tuberculosis is an increasing problem which adversely affects patient care and public health resistance of M. tuberculosis is exclusively associated with chromosomal mutation. Resistance of M.tuberculosis to anti-tuberculosis drugs is the result of a spontaneous genetic event and worse, a man-made amplification of the natural phenomenon [5, 6].

Multi drug resistance (MDR) TB is caused by strains of Mycobacterium tuberculosis that are resistant to at least rifampicin and isoniazid, two key drugs in the treatment of the disease. Resistant strains of M. tuberculosis labeled as Pre-extensively drug resistant TB (Pre XDR) were MDR cases are also resistant to any fluoroquinolone or one of the injectable Second-line drugs and Extensively drug resistant (XDR)-TB. In addition to being MDR are also resistant to any fluoroquinolone and to at least one of the injectable Second-line drugs: kanamycin, capreomycin

or amikacin. More recently, a more worrying situation has Emerged with the description of M. tuberculosis strains that have been found resistant to all antibiotics that were available for testing, a situation labelled as totally drug resistant (TDR)-TB[7].

Early detection of all forms of drug resistance in TB is a key factor to reduce and contain the spread of these resistant strains. A better knowledge of the mechanisms of action of anti-TB drugs and the development of drug resistance will allow identifying new drug targets and better ways to detect drug resistance [8].

Drug resistance in TB remains a man-made phenomenon. Among the reasons for this, the non-compliance with the treatment regimens is signaled as the first cause. The standard treatment of TB calls for a six-month regimen of four drugs that in the case of MDR-TB is extended to 18–24 months involving second-line drugs. This makes compliance with the treatment regimens very challenging and the rates of non-adherence could be high, resulting in poor outcomes and further dissemination of MDR strains a number of genes are clearly associated with drug resistance in M. tuberculosis, there are still many cases where resistant strains do not harbor any known mutation. More clarification is needed on the role of specific gene mutations and the development of MDR- or XDR-TB, or the relation between drug resistance and fitness of the bacteria [9].

First-Line Anti-TB Drugs: The mode of action of rifampicin in M. tuberculosis is by binding to the β-subunit of the RNA polymerase, inhibiting the elongation of messenger RNA. The majority of rifampicin-resistant clinical isolates of M. tuberculosis harbor mutations in the rpoBgene that codes for the β-subunit of the RNA polymerase. As a result of this, conformational changes occur that decrease the affinity for the drug and results in the development of resistance. In about 96% of M. tuberculosis isolates resistant to rifampicin, there are mutations in the so-called hot-spot region‖ of 81-bp spanning codons 507–533 of the rpoB gene. This region is also known as the rifampicin resistance-determining region. Mutations in codons 516, 526 and 531 are the most commonly associated mutations with rifampicin resistance in the majority of studies .Isoniazid is active against metabolically-active replicating bacilli. Also known as isonicotinic acid hydrazide, isoniazid is a pro-drug that requires activation by the catalase/peroxidase enzyme KatG, encoded by the katG gene, to exert its effect. [9] Isoniazid acts by inhibiting the synthesis of mycolic acids through the NADH-dependent Isoniazid and occurs more frequently in MDR strains. The

second most common mutation occurs in the promoter region of inhA causing an overexpression of InhA or less frequently, a mutation in its active site, which decreases its affinity for the isoniazid-NAD adduct. The most prevalent mutation found is at position −15C/T and is more commonly associated with low level resistance to mutations in inhA not only cause resistance to isoniazid but also to the structurally related drug ethionamide, which shares the same target . A recent study found that a mutation in the inhA regulatory region together with a mutation in the inhA coding region. Pyrazinamide is also a pro-drug that needs to be converted to its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase coded by the pncA gene. Mutations in the gene pncA remain as the most common finding in pyrazinamide resistant strains. Streptomycin consequently, mutations in rpsL and rrs are the major mechanisms of resistance to streptomycin but account for 60%–70% of the resistance found. Ethambutol is active against actively multiplying bacilli, disrupting the biosynthesis of the arabino galactan in the cell wall. The embCAB operon encodes the mycobacterial arabinosyl transferase enzyme.Resistance to ethambutol is mediated via mutations in the embBgene [9, 10].

Second-Line Anti-TB Drugs: Fluoroquinolones are currently in use as second-line drugs in the treatment of MDR-TB. Both ciprofloxacin and ofloxacin are synthetic derivatives of the parent compound nalidixic acid, discovered as a by-product of the antimalarial chloroquine. Newer-generation quinolones such as moxifloxacin and gatifloxacin are being evaluated in clinical trials and proposed as second-line antibiotics with the purpose of shortening the length of treatment in TB. The mode of action of fluoroquinolones is by inhibiting the topoisomerase II (DNA *gyrase*) and topoisomerase IV, two critical enzymes for bacterial viability. M. tuberculosis is by chromosomal mutations in the quinolone resistance-determining region of gyrA or gyrB. Injectable drugs: Kanamycin, Capreomycin, Amikacin, Viomycin .These four antibiotics have the same mechanism of action by inhibiting the protein synthesis but, while kanamycin and amikacin are aminoglycosides, capreomycin and viomycin are cyclic peptide antibiotics. All four are second-line drugs used in the management of MDR-TB [11].

Kanamycin and amikacin inhibit protein synthesis by alteration at the level of 16S rRNA. The most common mutations found in kanamycin-resistant strains are at position 1400 and 1401 of the rrs gene, conferring high-level resistance to kanamycin and amikacin. In concordance with this, a low-level resistance to kanamycin has been associated with mutations in the promoter region of the eis gene, encoding an aminoglycoside acetyltransferase. Mutations at position −10

and −35 of the eis promoter led to an overexpression of the protein and low-level resistance to kanamycin. Ethionamide is a derivative of isonicotinic acid structurally similar to isoniazid. It is also a pro-drug requiring activation by a monooxygenase encoded by the ethA gene. It interferes with the mycolic acid synthesis [12]

1.2 Statement of the Problem

The global burden of TB remains alarmingly high, according to 2019 WHO TB report, in 2018, an estimated 10 million people developed tuberculosis (TB) and 1.2 million died from the disease, 0.251 million of whom were HIV-positive. TB occurs in every part of the world, the largest number of new TB cases occurred in South-East Asia, with 44% of new cases, followed by the African region, with 24% of new cases. Overall treatment success rate was 75% and no data were reported by Angola, Chad and Ethiopia [13].

Globally in 2018 there were an estimated 484,000 incident cases of rifampicin resistance (RR-TB), with cases of MDR-TB accounting for 78% (377,520) of the total there were about 214,000 deaths from MDR/RR-TB. Despite the increase in testing, the number of MDR/RR-TB cases detected in 2018 only reached 38.6% (186,772) of the estimated one and 13,068 patients with XDR-TB was reported worldwide, 81 countries have reported. On average, an estimated 8.5% of people with MDR-TB have XDR-TB [13]. In 2018 about 156,071 (84%) patients were enrolled on MDR-TB treatment, equivalent to about 32% of the 484,000 incident MDR/RR-TB cases that year. Enrolments have increased over time and in several countries, the gap between detecting MDR/RR-TB cases and starting them on treatment has narrowed. In 2018, 11,403 patients with XDR-TB were enrolled in treatment, with 16% increase over 2017. However, unlike the drug sensitive TB, the treatment success in MDR/RR-TB and XDR-TB patients were only 56 % and 39% respectively, the outcome even more worsen than reported in some forms of PRE-XDR and XDR TB [13].

A World Health Organization (WHO) comprehensive report of 2019, involving 202 countries there were 30 TB high burden countries. Africa being represented by 17countries those countries are Angola, Central African Republic, Congo, Democratic republic of Congo, Ethiopia, Kenya, Lesotho, Liberia, Mozambique, Namibia, Nigeria, Papua New Guinea, Sierra Leone, South Africa, UR Tanzania, Zambia and Zimbabwe[13,14].

Ethiopia is home to over 100 million people and among the high Tuberculosis, MDR/RR-TB and TB/HIV burden countries in the world. It has an Annual Risk of TB Infection with estimated incidence of TB 151 per 100,000 populations in 2018. The TB related mortality rate 24 per 100,000 populations. The country in 2018 estimated incidence of MDR/ RR case 1.4 per 100,000 population. Among the notified TB cases 2700 (2.3%) of cases were estimated to harbor

MDR/RR-TB. According to National Tuberculosis program (NTP) report 635 DR TB cases detected in 2011 Ethiopian Calendar, 76% of them were only Rif resistance cases, 19 % of them MDR-TB cases, 5% of them were pre-XDR/XDR-TB [13,14,30].

Drug resistance complicates management of patients due to increased pressure on public health systems and cost of the treatment. It further aggravates the emergence of MDR-TB and XDR TB. The control of drug-resistant TB, in any form, requires accurate and prompt diagnosis of the type of resistance [14].

The timeliness of culture-based drug susceptibility testing (DST) is constrained by the slow growth characteristic of *Mycobacterium tuberculosis*, which can take 2 to 4 weeks after growth detected, depending on the method of testing. DST of certain drugs can also be technically challenging, in resource limited areas, cost prohibitive. The use of molecular methods to identify mutations associated with drug resistance can decrease diagnostic delay and in some cases, may prove to be more specific than phenotypic DST. The great potential of genetic testing to rapidly diagnose drug resistance has been the impetus behind a tremendous amount of basic and applied research. The result of this area of research has been used for the development of numerous commercial and laboratory designed diagnostic assays. These assays are increasingly being used in clinical and reference laboratories [15].

The emergence and transmission of drug-resistant TB is a major threat and challenge for TB control program particularly for MDR-TB and XDR-TB, representing a major public health problem globally but more specifically in resource-limited countries. In addition, a high prevalence of TB, poor treatment, limited access to health care and several other related factors make MDR-TB difficult to address in countries in the sub-Saharan region such as Ethiopia. In the region pattern of MDR-TB and XDR-TB is not well known and little information is available in high TB and HIV prevalence countries such as Ethiopia. Although drug-resistant TB development is a result of unsuccessful TB control programs characterized by inappropriate TB treatment, and poor diagnostic system, there are several risk factors related to drug-resistant TB in Ethiopia.

This study determines the drug susceptibility pattern of the isolates and its relationship to the bacteria species therefore it is important to detect if drug resistance is influenced by the genetic and evolutionary background of MTBC Species. Such studies are important in identifying the

anti TB drug resistant species and diversity of Mutations. Determining different mutation of genes for a single drug will have important role for effective treatment of the patient and indirectly will have benefit to decrease the spread of infection.

Understanding the correlation and discordant between different drug resistance TB detection techniques will all so have a role for increasing case detection, to control their distribution and also are helpful to provide information which will support modification of health policy and to develop recommendations which is important impute for the public health authorities to control drug resistance TB. Information generated during such type of study was publicized which is important to protect the inappropriate use of anti-TB drugs and it is useful to improve treatment quality. There-fore this study trys to fill the listed gap and can be used as a bench mark for further studies.

1.3 Rationale of the Study

This study will have important role in assessing drug resistance pattern of Mycobacterium tuberculosis to ensure that individuals with TB signs and symptoms are correctly diagnosed and get better treatment and also it is a key to control and prevent drug resistance based on identified risks associated with the emergence drug resistance tuberculosis.

Determining the yield of drug resistance TB using phenotypic and Genotypic DST methods will be advantageous for scaling up an efficient programmatic management and surveillance of drug-resistant TB. Drug resistance is mainly occurred as results of poor treatment outcomes, poor treatment adherence, poor quality of drugs and poor infection control practices.

Ethiopia is one of the high burden countries for MDR-TB. However, the extent and the magnitude of the problem are not well studied so it is important to study the assessment of drug resistance TB among tuberculosis patients to manage patients not to be transmitting the disease to others.

Laboratory Diagnosis of drug resistance TB is one of the interventions recommended by WHO in countries with high prevalence of TB to reduce the burden and this study will also assist the Patients, Clinicians, hospitals and health centers to be aware of the extent of TB infection and develop strategies for promoting and improving TB infection control practice.

2. Literature Review

The emergence of drug-resistant strains of Mycobacterium tuberculosis is a challenge to global tuberculosis (TB) control. Although culture-based methods have been regarded as the gold standard for drug susceptibility testing (DST), molecular methods provide rapid information on mutations in the M. tuberculosis genome associated with resistance to anti-tuberculosis drugs [15].

A systematic review on global Evolution of drug resistance in Mycobacterium tuberculosis on the molecular determinants of resistance and implications for personalized care indicated that DR-TB remains a key public health challenge of modern times. In light of recent reports of the dissemination of drug resistance beyond XDRTB to programmatically untreatable TB, there is an urgent need for personalized management for DR-TB through drug resistance screening. This review highlights the complexity of drug resistance in MTB, captured by the advances of technologies [16].

A Systematic Review and Meta-Analysis done in Iran to investigate Middle East Mycobacterium tuberculosis antibiotic resistance showed that resistance of M. tuberculosis to a single first-line drug was 14.7% of the new TB cases in the Middle East. High levels of drug-resistant TB, especially MDR-TB, which may be increasing in both groups of TB patients in the Middle East. Hence, in order to gain a more effective TB control, identifying individuals with TB signs and symptoms by new rapid diagnostic methods, performing drug susceptibility testing, detecting primary resistance to the first-line anti-TB drugs, providing effective treatment to prevent the emergence of other forms of drug resistance are needed [17].

Another study conducted in Pakistan to determine drug resistance pattern in Mycobacterium Tuberculosis to the first line drugs of pulmonary tuberculosis patients Drug susceptibility testing of M. tuberculosis isolates for 148 sputum samples were performed on first line anti tuberculosis drugs Prevalence of resistance to one drug was 3.4%. The highest proportion of mono-drug resistance was observed against E, 2%, followed by INH, 0.7%, and RIF, 0.7%. Pattern of resistant to two drugs was 9.5%. The proportion of poly resistant was 2%. 93.33% diagnose patients were MDR-TB. In this study the observed high rate of acquired drug resistance shows that National TB control program is not effective and during recent year's improper/irregular use of antituberculosis drugs have led to multiplication and accumulation of drug resistant strains of

M. tuberculosis. For the control of TB disease, a confounding factor is MDR-TB. MDR-TB is a man-made problem and efforts should be taken to prescribe the suitable regimen for the prescribed duration of the treatment. To formulate an effective regimen, it is important to know drug resistant pattern because drug resistant pattern varies from different period of time also from one place to another [18].

Similar study done in Bangladesh on Drug Resistance Pattern of *Mycobacterium Tuberculosis* Isolated from Patients Attending a Referral Hospital drug resistance of *M. tuberculosis* to single drug was found in 22.11 % cases, to two drugs in 15.79 %, to three drugs in 10.53 % and to four drugs in 4.21 % cases. One strain was resistant to all the five drugs. Creates the impression that fairly high rate of anti-tuberculosis drug resistance among the tuberculosis patients and also high MDR-TB. By taking history of previous treatment for tuberculosis, drug resistance patients group and new cases from same family should be identified. Susceptibility test of these groups should be performed with all commonly used first line anti-tuberculosis drugs, and then appropriate drugs should be selected for curative treatment to prevent spread of drug resistant tuberculosis. The priority is not the management, but the prevention of MDR tuberculosis [19].

Cross sectional study conducted to determine the frequency of drug resistance and the clonality of genotype patterns in M. tuberculosis clinical isolates from pediatric patients in Mexico. The greatest resistance was found against INH (23.3% of cases) followed by RMP (11.1% of cases). Resistance to RMP was always accompanied by INH-resistance leading to (11.1%) cases of MDR-TB. Of 71 patients from whom information was available, 14 patients had previous TB treatment, and secondary resistance was documented in 50% patients in contrast with primary resistance found in (24.5%) patients without previous TB treatment. study shows that TB among pediatric patients in Mexico is essentially caused by evolutionary recent genotypes characteristic of the Americas. However, the presence of the ancestral Manu lineage strains, supposed to be a missing link of the split between ancestral and modern tubercle bacilli during M. tuberculosis evolution [20].

A Phenotypic and Genotypic Analysis Study of Anti-Tuberculosis Drug Resistance in Mycobacterium tuberculosis Isolates in Myanmar. The overall concordance between phenotypic and genotypic DST was 99.5% (188/189) after the confirmation of these samples with additional phenotypic DST. The high MDR-TB rate, high accuracy of genotypic DST, and common drug

resistance-mutation pattern found in the this study suggested that genotypic DST should be more widely used as a routine test to determine drug susceptibility in newly diagnosed TB patients. Genotypic DST may also lead to the rapid detection of MDR-TB cases and be invaluable in preventing the transmission of drug-resistant strains and this study highlighted the high prevalence of drug resistance among new pulmonary TB cases and the usefulness of genotypic DST for determining drug susceptibility in TB patients and for the rapid and accurate diagnosis of TB in Myanmar [21].

A Comparative Study done in America on Genotypic and Phenotypic Second-Line Drug Resistance Testing of Mycobacterium tuberculosis Complex Isolates the results of second-line DST for M. tuberculosis complex bacteria obtained by the 7H10 agar dilution method and MGIT 960 method are largely similar the 7H10 agar dilution and MGIT960 phonotypical second-line DST methods for M. tuberculosis yielded largely identical results .The MGIT960 method has a much shorter turnaround time than that of the conventional 7H10 agar method, which is essential for the timely optimization of patient treatment regimens. Here, the MTBDRsl molecular assay proved to be a reliable method for predicting aminoglycoside and fluoroquinolone resistance and thus for the rapid screening of MDR-TB strains for possible extensive drug resistance [22].

Cross-sectional study was conducted from March 2013 to August 2013 at German-Nepal tuberculosis project laboratory. The prevalence of MDR-TB among all the cases of culture positive pulmonary tuberculosis was 15.6 %. The rate of MDR-TB among previously treated culture positive tuberculosis patients was 19.4 % and that among newly diagnosed culture positive pulmonary tuberculosis cases was 7.1 %. The highest rate of resistance of Mycobacterium tuberculosis, was toward streptomycin (24.4 %) followed by isoniazid (23 %), rifampicin (17.8 %) and ethambutol (15.6 %).The high prevalence of DR/MDR-TB in this study reflects poor implementation of tuberculosis control program. On the basis of the drug susceptibility patterns of M. tuberculosis it is recommend to include ethambutol instead of streptomycin in the multidrug therapy for the treatment of tuberculosis patients in Nepal. Further, due to high rate of MDR-TB among previously treated patients, the study do not recommend to use first line drugs for the treatment of pulmonary tuberculosis among previously treated patients [23].

In Cairo/Egypt the Patterns of drug resistance in cases of smear positive pulmonary tuberculosis showed that One hundred and nineteen sputum and culture positive patients underwent drug susceptibility to the first line drugs (Isoniazid, Rifampicin, Streptomycin and Ethambutol). 79/119 patients in addition underwent drug susceptibility to Pyrazinamide, Ofloxacin, Amikacin and Levofloxacin only 35 patients (29.4%) were sensitive to all the tested drugs. 84 of the included Patients (70.6%) showed drug resistance to at least one of the first line drugs by different patterns. was noticed that the most common drug resistant pattern among the newly diagnosed cases was mono-resistance with a significant statistical difference [24].

A study conducted in Zimbabwe to determine the drug resistance profiles in M. tuberculosis isolates that are phenotypically resistant but not detected by the GenXpert and MTBDRplus kit. The most frequent mutation responsible for rifampicin resistance was (25/92) S531L that was detected by using all molecular assays. Some inconsistencies were observed between phenotypic and genotypic assay results for both katG and rpoB genes in 30 strains. For these, eight codons; G507S, T508A, L511V, del513-526, P520P, L524L, R528H, R529Q and S531F were novel mutations These finding indicate that molecular assay kit diagnosis that is based on the rpoB and katG genes should be improved to cater for the genetic variations associated with the geographic specificity of the target genes and be able to detect most prevalent mutations in different areas [25].

A Rapid Molecular Screening Study for Multidrug-Resistant Tuberculosis in a High-Volume Public Health Laboratory in South Africa performed directly on 536 consecutive smear positive Sputum specimens from patients at increased risk of multidrug-resistant (MDR) TB 97% of smear-positive specimens gave interpretable results within 1–2 days using the molecular assay. Sensitivity, specificity, and positive and negative predictive values were 98.9, 99.4, 97.9, and 99.7%, respectively. This molecular assay is a highly accurate screening tool for MDR TB, which achieves a substantial reduction in diagnostic delay. With overall performance characteristics that are superior to conventional culture and drug susceptibility testing and the possibility for high throughput with substantial cost savings, molecular testing has the potential to revolutionize MDR TB diagnosis [26].

A cross sectional study done on analysis of gene mutations associated with isoniazid, rifampicin and ethambutol resistance among Mycobacterium tuberculosis isolates from Ethiopia. Of 260

isolates, mutations conferring resistance to INH, RMP, or EMB were detected in 35, 15, and 8 isolates, respectively, while multidrug resistance (MDR) was present in 13 of the isolates. Of 35 INH resistant strains, 33 had mutations in the katG gene at Ser315Thr 1 and two strains had mutation in the inhA gene at C15T. Among15 RMP resistant isolates, 11 had rpoB gene mutation at Ser531Leu, one at His526Asp, and three strains had mutations only at the wild type probes. The dominance of single gene mutations associated with the resistance to INH and RMP was observed in the codon 315 of the katG gene and codon 531 of the rpoB gene, respectively. According to this study the GenoType®MTBDRplus assay is a sensitive and specific tool for diagnosis of resistance to INH, RMP and MDR [27].

Another study done in south west Ethiopia on drug resistance-conferring mutations in Mycobacterium tuberculosis from pulmonary tuberculosis patients showed that high rate of drug resistance was commonly observed among failure cases. The most frequent gene mutations associated with the resistance to INH and RIF were observed in the codon 315 of the katG gene and codon 531 of the rpoB gene, respectively. Further studies on mutations in different geographic regions using DNA sequencing techniques are warranted to improve the kit by including more specific mutation probes in the kit [28].

Similar cross-sectional study was conducted on 413 TB-positive clinical specimens submitted between 2012 and 2014 to Bahirdar Regional Laboratory Center for confirmation of multidrug resistance. Resistance determining genes were analyzed using a line probe assay. 150 (36.3%) were multidrug-resistant, 19 (4.6%) were resistant only to rifampicin, and 26 (6.3%) were resistant to isoniazid. only eight (4.7%) showed rifampicin heteroresistance and only two (1.13%) showed isoniazid heteroresistance. Failing of the rpoB WT8 gene with corresponding hybridization of rpoB MUT3 (S531L substitution) accounted for 85 (50.3%) rifampicin-resistant mutations. Among 176 isoniazid-resistant isolates, 155 (88.1%) strains had the Ser315Thr1 substitution. The prevalence of multidrug-resistant M. tuberculosis was high in the study area. Ser531Leu and Ser315Thr1 substitutions were the highest gene mutations for rifampicin and isoniazid, respectively [29].

3. Objective of the study

3.1 General Objective

The objective of this study was to asses molecular and phenotypic drug resistance pattern of common Mycobacterium tuberculosis complex species and associated mutation: Evidence from selected TB treatment initiating center Health facilities in Ethiopia.

3.2 Specific Objective

1. To determine drug resistance patterns to first and second line anti TB drugs among new and retreatment cases.
2. To asses Correlation and Discordance between Genotypic and Phenotypic Testing for Resistance in Mycobacterium tuberculosis Clinical Isolates.
3. To assess the associated selected mutation in isolates with Rifampicin resistance, Multi drug Resistance, pre-Extensive drug resistance and Extensive drug resistance tuberculosis.
4. To describe the common Mycobacterium tuberculosis Complex species in association with DST pattern.

4. Materials and Methods

4.1 Study Area

This study was performed at selected treatment initiating centers in Ethiopia. These sites Were Addis Ababa, Bishoftu, Tulubolo, Fiche, Ambo, Weliso and Gambella. All Specimen analyses were performed at laboratory facilities of Ethiopia Public Health Institute (EPHI) National TB Reference Laboratory.

Addis Ababa is the capital city of Ethiopia. The estimated population size of Addis Ababa was more than 3.27 million. Administratively, the city is divided into 10 sub-cities and further classified into 99 Woreda (lowest government administrative unit). Health facilities which were providing laboratory services for MDR-TB diagnosis and health facilities with high TB patients load were selected from Addis Ababa.

Bishoftu is located in the East Shewa at a distance of 48 km at south east of Addis Ababa. The estimated population of 99,928, of whom 47,860 were men and 52,068 were women. Bishoftu, a rapidly growing town, has one Hospital (Bishoftu Hospital) and one Health center.

Third site was Fiche town and its surrounding in the northwest of Addis Ababa at 115km.The total population for Fiche of 27,493, of whom 12,933 were men and 14,560 were women. It is the administrative center of the North Shewa Zone of Oromiya Region and separate woreda. It is located about three km from the main Addis Ababa-Debre Markos road, Fiche has a latitude and longitude of 9°48′N 38°44′E and an elevation between 2,738 and 2,782 meters above sea level.

Tulu Bolo located in the south west Shewa Zone of the Oromiya Region, has an estimated total population of 14,307 of whom 6,837 are men and 7,470 are women it has a latitude and longitude of 8°40′N 38°13′ECoordinates: 8°40′N 38°13′E with an elevation of 2193 meters or 7195 feet above sea level. It is the largest settlement in Becho woreda. It is located around 80 Kilometers from Addis Ababa (the capital city of Ethiopia) on the way to Jimma.

Ambo Town is found at the West Shewa Zone of the Oromia Region, west of Addis Ababa, at a distance of 125 km from Addis Ababa, total population for Ambo of 48,171, of whom 24,634 were men and 23,537 were women this town has a latitude and longitude of 8°59′N 37°51′E and an elevation of 2101 meters.

Woliso is located in south west Shewa zone; the town has a population of 54,248 people (28,620 males and 25,628 females). Woliso, a rapidly growing town, has one hospital (St. Luke Hospital), one health center (Woliso Health Center). The height of Woliso above sea level is about 1900 meter with annual rain fall of 1200mL, has a latitude and longitude of 8°32′N 37°58′E and temperature of 18-27 0c. At the moment the town of Woliso has area coverage of 2,225.25 hectare. St. Lukas Hospital is found in this town which is the only Hospital which provides various health services for woliso society the surrounding areas or woredas.

The Gambella Region is located at the west of Ethiopia nearest to Sudan, at a distance of 714 km from Addis Ababa, has total population of 307,096, consisting of 159,787 men and 147,309 women; urban inhabitants number 77,925 or 25.37% of the population. With an estimated area of 29,782.82 square kilometers, this region has an estimated density of 10 people per square kilometer. Gambella Hospital is found in this region which is the only Hospital which provides various health services for Gambella Town society and neighboring woredas.

4.2 Study Design and Period

A Health facility based cross sectional study was conducted on New and Retreatment TB Cases from November 2019 to June 2020.

4.2.1 Source population

All TB patients attend the selected Treatment Initiating Center during the study period.

4.2.2 Study population

Consecutive adults presented to the study area during the study period with Bacteriological confirmed pulmonary TB cases with any technology available at study sites and cases that fulfilled the inclusion criteria were included.

4.3 Inclusion and Exclusion criteria

4.3.1 Inclusion criteria

Patients with age of ≥15 years

Confirmed pulmonary TB cases

New and re-treatment TB patients

4.3.2 Exclusion criteria

Those who are presumptive for extra pulmonary TB

Patients those unable to produce sputum

Patients on anti TB treatment for more than one month

4.4 Study variables

4.4.1 Dependent variables

Phenotypic and Genotypic Drug Resistance Pattern

4.4.2 Independent variables

Age, sex, New, contact history, HIV, Previous TB treatment, HIV and Any chronic illness.

4.5 Sample size and sampling method

4.5.1 Sample size Calculation

The sample size was determined using the formula for estimating a single Population proportion. Sample and a proportion, which could give maximum sample size was chosen.

The required sample size was determined by using single population proportion formula considering the following assumptions: at 95% confidence level (precision) to be 5%.

$$n = \frac{(Z\ \alpha/2)^2\ \ p\ (1-p)}{d^2}$$

Where:

- Level of significance = 0.05
- Marginal of error (**d**) = 5%
- Proportion/ prevalence = ***P***
- Z (α/2) = Z-score at 95% confidence interval = 1.96
- Sample size = **n**

The formula for calculating the sample size (n) will be:

According to the publication on molecular epidemiology of TB, the prevalence of drug-resistant TB among Isoniazid resistance cases was 14%. *P*= 0.14

$$\mathbf{n= (1.96)^2 x\ 0.14\ (1-0.14)/\ (0.05)^2 = 185,\ } \boldsymbol{n}\mathbf{=185}$$

By considering estimated 10% non-response rate, the overall sample size (N) was found to be as follows:

N = 185 + 19 (10 % non-response rate) = **204**

The final sample size for confirmed TB cases was **204**

4.5.2 Sampling method

Non-probability, convenience sampling technique was applied until the achievement of the expected sample size within the given study period. All eligible samples from bacteriologically confirmed TB patients visiting TIC then referred for culture was included in the study until the achievement of expected sample size.

4.6 Measurement and Data collection

4.6.1 Data collection procedure

A Data collection sheet was used to collect medical history and socio demographic information results of the participants after ethical clearance was obtained. Data log book was used to record all information of the patients enrolled in the study in a consecutive order.

4.6.2 Laboratory analyses

Specimen collection

All specimens were collected at TB treatment centers by trained TB focal person according to standard operating procedure. Morning sputum samples were collected from each participant in containers that are sterile, clear, plastic, leak-proof, screw cap and 50 ml capacity Falcon tube, transported to National Tuberculosis Reference Laboratory using triple packaging system with ice pack.

MTB culture and identification

All specimens were processed by standard N-acetyl-L-cysteine NaOH (NALC-NaOH) digestion decontamination technique. A final concentration of 1% NaOH was used for decontamination. Sputum samples were first decontaminated by addition of an equivalent volume of NaOH-NALC sodium citrate solution. Samples were then vortexed for 15-30 seconds and then left for 15 minutes to liquefy and add phosphate buffered saline up to 45ml. Specimen was then centrifuged at a speed of 3000 relative centrifugal force (G) or 3200 revolution per minute(RPM) for 15 minutes in a refrigerated centrifuge. After the centrifugation, the supernatant was decanted the sediment was resuspended by adding 2 ml of phosphate buffered saline (pH 6.8) to the sediment. The re-suspended pellet then used, for 0.8 ml of MGIT growth supplement/PANTA and 0.5 ml of a well-mixed processed/concentrated specimen was added to the appropriately labeled MGIT tube ,0.1 ml was added in to LJ Tube, 0.5 ml then used for GenXpert and 0.1 ml was used for smear microscopy. Then Leftover sediment was stored at 4 ^{0}c for 60 days until it was confirmed that the inoculated media were not contaminated. In case, there was contamination in the MGIT culture, the decontamination procedure was repeated with the remaining sediment following the same procedure and new culture inoculated. Quality control was assured by adding a negative tube in the middle of each batch of specimens processed in order to ensure that there was no contamination present in stock solutions and no carry-over of *M. tuberculosis* from one specimen to another. The negative control was treated the same as the patient samples. In case of contamination in the control tube, the results of specimens done in the same batch were checked to determine whether there was an influence from the contamination. If *M. tuberculosis* was present in the negative control tube, the results of specimens done in the same batch were checked to determine whether false positive culture were present, which might have indicated carry over from one specimen to another [34,36].

MGIT Tube

The BBL MGIT tube (from Becton Dickinson) containing 7 ml modified middle brook 7H9 broth was used, to which an enrichment supplement OADC (Oleic acid ,Albumin, Dextrose and Catalease) as well as a mixture of antibiotics consisting of PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) were added. After inoculation, the tubes were incubated at 37°C. Readings were taken daily for the first three weeks and once a week thereafter

for culture positivity until the end of six weeks using the BBL Micro MGIT system(Becton Dickinson Company). All the positive tube was further confirmed by ZN staining and a sub culturing on brain heart infusion agar plate. The time to detection (TTD) of *Mycobacterium* was based on the date of the earliest instrumental indication of positivity. The instrument reports a tube negative if it remains negative for six weeks (42 days) [34].

LJ Culture

LJ slant was inoculated with the 0.1 ml of the processed sample and incubated at 37°C for a maximum of 8 weeks. It was checked twice weekly for first two weeks and then once every week for maximum period of 8 weeks.

TB Ag rapid test

TB Ag rapid test and BHI culture were performed on MGIT positive samples for identification and differentiation between MTB and Other Mycobacterium (non-tuberculosis mycobacteria (NTM)) after the sputum culture but before DST was performed. The SD Bioline TB Ag MPT64 Rapid test is an immune chromatographic method which can detect MPT64 antigens produced by M. tuberculosis complex (ABBOT DIAGNOSTICS).

Zehel Nelsen AFB sputum Smear microscopy

The air dried smear was heat fixed then treated with carbolfuschin, acid alcohol and Methylene blue for 5, 3 and 1 minutes respectively, after complete slide wash with water in each step. Using with oil immersion, Acid-fast bacilli were seen as a bright red and rod shaped, slender rods, some time with one or more granules. The bacilli may occur singly, V-shaped forms, or as clumps, and fragments of bacilli often seen during treatment.

Xpert MTB/RIF testing

The Xpert MTB/RIF assay is almost fully automated cartridge-based system, utilizing real-time PCR technology to both diagnose TB and detect rifampicin resistance in less than 2 hours (Cepheid international). The assay uses molecular beacon technology. Xpert MTB/RIF testing was performed following Cepheid instructions. Three volumes of the Sample Reagent were added to one volume of processed specimen. The sample was shaken vigorously 10-20 times. After 10minutes of incubation at room temperature, the sample was shaken again 10-20 times

then left at room temperature for another 5 minutes. The sample was then transferred into the cartridge and ready to be loaded in the Xpert MTB/RIF module [31, 32].

Genotype MTBDRplus assay and MTBDR*sl*

This assay was performed as per standard protocol provided by the manufacturer (Hain Life science GmbH, Nehren, Germany). Briefly it was performed in three steps.

DNA extraction

DNA was extracted using the Genolyse kit which comprises of Lysis buffer (LB) and neutralization buffer (NB).For each isolate, a loopful of colonies was taken from the LJ culture and suspended in 100 µl of A-LYS in a sterile 2 ml cryotube and vortexed. The suspension was then incubated at 95°C for 5 min. After 5 min of incubation, 100 µl of A-NB was added to the mixture of A-LYS and isolate and vortexed for 5 s. The cryotubes were then centrifuged for 5 min at a speed of 25000 g. After centrifugation, the supernatant which is the DNA solution was transferred into another sterile 2 ml cryotube, stored at −20°C until ready for PCR.

Master Mix preparation

All the reagents needed for amplification are included in the Amplification Mix-A (AM-A) and Amplification Mix-B (AM-B). For each sample, 10 µl of AM-A which contains 10 × buffer, nucleotides, and DNA polymerase was mixed in a PCR tube with 35 µl of AM-B containing Magnesium Chloride (MgCl2), biotinylated primers, and dye [33,35].

DNA addition

A 5µl of the DNA solution was added to the master mix to give a total volume of 50 µl. For positive control, 5 µl of MTB strain H37Rv was added to the mixture, and for negative control, 5 µl of sterile distilled water was used [33].

Amplification

The PCRs consisted of 1 cycle of 15 min of denaturing at 95°C, followed by 10 cycles of 30 s at 95°C and 2 min at 65°C, followed by 20 more cycles of 25 s at 95°C, 40 s at 50°C, and 40 s at 70°C, with a final extension of 1 cycle at 70°C for 8 min.

Detection

Hybridization was performed using a TwinCubator prewarmed to 45°C. The hybridization buffer (HYB) and stringent wash solution (STR) were prewarmed to 37°C–45°C before use. For each strip, 10 µl of conjugate concentrate was diluted in a sterile tube with 990 µl of conjugate buffer (CON-D). In a separate tube, 10 µl of substrate concentrate (SUB-C) was diluted with 990 µl of substrates buffer (SUB-D) for each strip. In each of the wells of a 12 well tray, 20 µl of the PCR product was mixed with 20 µl of denaturation solution (DEN) and incubated for 5 min at room temperature. After incubation, 1 ml of prewarmed HYB was added to each well and mixed carefully until the solution was homogenous. A prelabeled strip was then placed in each well, completely covered by the solution, and allowed to incubate while on the TwinCubator for 30 min at 45°C and shake at 300RPM. At the end of the 30 min of hybridization, all the HYB solution was aspirated using a sterile pasture pipette. Then, 1 ml of STR was added to each strip and incubated for 15 min at 45°C on the TwinCubator. The STR was then removed, and 1 ml of Rinse solution (RIN) was added to each strip and incubated for 1 min. The RIN was removed; 1 ml of diluted conjugate was added to each strip and allowed to incubate for 30 min. The solution was removed, and the test strips were rinsed twice by using a RIN for 1 min at each wash, followed by sterile distilled water for 1 min. All solutions were removed between washes. One milliliter of diluted substrate was then added to each strip and incubated for up to 20 min. The diluted substrate was removed, and the reaction was stopped by rinsing twice with sterile distilled water. The test strips were then affixed to the Genotype MTBDR*plus* Ver 2.0 assay evaluation sheet provided in the kit, for interpretation of results. To determine the intra-assay precision (parallel testing), 10 isolates and 2 controls were set up in triplicates and tested under identical conditions on three different days. Interpretation of the assay results was performed according to the package insert. For accurate evaluation, a provided template was used to align the CC, AC, and the TUB with the respective bands on the sheet. The presence of WT bands with the absence of the MUT band was interpreted as sensitive. The absence of a WT band and the presence of a MUT band for a specific gene on the strip were interpreted as resistance. A strain that revealed the presence of reaction to both a MUT band and the corresponding WT band was considered heteroresistant [27, 35].

MGIT 1ST and 2ND LINE Antibiotic Susceptibility Test

Susceptibility testing in the MGIT 960 system is based on the same principles as isolation from sputum (detection of growth). DST is performed using an AST (antibiotic susceptibility testing) set or TB exit, which consists of a Growth Control tube and one tube for each drug. A known concentration of drug is added to a MGIT tube, along with the specimen, and growth is compared with a drug-free control of the same specimen. If the drug is active against the mycobacterial isolate (isolate susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube; meanwhile, the drug-free control will grow and show increasing fluorescence. If the isolate is resistant, growth and its corresponding increase in fluorescence was evident in both the drug-containing and the drug-free tube. The MGIT 960 system monitors these growth patterns and can automatically interprets results as susceptible or resistant. An isolate is defined as resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug [34, 36].

4.7 Quality assurance

All samples was collected, packaged, Transported according to standard operating procedure of the laboratory. All patient information was cheeked for the clarity and completeness in a regular basis. The sterility of the culture media was checked by incubating the whole media at 37 ^{0}C for 48 hours and performance of the media was cheeked by Known susceptible *M. tuberculosis* (H37Rv) and well characterized local laboratory resistant isolates for each drug were included as controls. The sterility of sample processing reagent cheeked by inoculating all reagents in a separate BHI. Start and end control were included in every run of MTB culturing. For Molecular operational activities Sterile molecular grade water and Reagent control used as negative control and H37Rv ATCC 25177 used as a positive control. Moreover to check the quality of PCR and Reverse Hybridization process we see the presence of the amplification control (AC) band indicates that the DNA extraction and PCR procedures were carried out successfully and Conjugate control (CC) documents two steps in the procedure: The efficiency of the conjugate (streptavidin-horseradish peroxidase) binding to the biotinylated primers (incorporated into the PCR products that have been hybridized to specific probes on the strip) and the efficiency of the substrate (hydrogen peroxide) reaction with the conjugate to detect the positive bands. All

laboratory results were recorded on a logbook during the study period. The collected data was analyzed and interpreted accordingly after it's checked for its completeness, accuracy and clarity.

4.8 Data Analysis and interpretation

Data was entered in to SPSS version 20 and export to STATA version 12 for analysis. Descriptive analysis, frequencies, and odds ratios (OR) with 95% confidence interval was calculated. In order to determine independent risk factors, OR and 95% confidence intervals (CI) was calculated using logistic regression analysis. In the logistic regression model demographic variables, previous treatment history, and drug resistance were include as variables. Results with p-values less than 0.05 was considered as being statistically significant.

4.9 Operational Definition

- **Bacteriologically confirmed TB case:** When a biological specimen (sputum sample) is positive by smear microscopy, culture, Xpert MTB/RIF or any other WHO Approved Rapid diagnostic method.
- **New patient:** a patient who has never had treatment for TB, or has taken anti-TB drugs for less than 1 month; the patient may have positive or negative bacteriology.
- **Previously treated patient:** A patient, who has received 1 month or more of anti-TB drugs in the past, may have positive or negative bacteriology.
- **Confirmed Case**: a patient with M. tuberculosis complex identified from a clinical specimen or one in which a health worker has diagnosed TB and has decided to treat the patient with a full course of TB treatment.
- **Rifampicin resistance TB (RR -TB) case:** resistance to rifampicin detected using phenotypic or genotypic methods, with or without resistance to other anti-TB drugs. It includes any resistance to rifampicin.
- **Multi-drug-resistant- tuberculosis (MDR-TB):** it is defined as M. tuberculosis that is resistance to both rifampicin and isoniazid.

- **Pre Extensively drug-resistant (Pre-XDR):** defined as M. tuberculosis that is MDR-TB with additional resistance to any fluoroquinolone but Susceptible for second-line injectable drugs or vice versa.
- **Extensively drug-resistant (XDR):** is defined as M. tuberculosis that is MDR-TB with additional resistance to any fluoroquinolone and at least one of the second-line injectable drugs.

4.10 Ethical considerations

The study was approved by Research and Ethics Review Committee of the Department of Medical Laboratory Sciences, College of Health Sciences; Addis Ababa University. As well as Ethiopian public Health Institute research ethics committee. All results were kept confidential; the participants were not identified by their name; instead appropriate coding system was used. The results were provided to the study participants and those in need of medical attention were communicated to respective physicians and laboratory personnel.

4.11 Dissemination and Utilization of Results

The final result of the study was submitted to Department of Medical Laboratory Sciences, AAU, which could serve as a reference material to researchers and experts. In addition, a copy of this material was given to Ethiopian public Health Institute and respective health institutions. The result was also be disseminated through publication in peer reviewed local and international journals and through presenting it in relevant workshops and seminars.

5. Results

5.1 Socio-demographic Characteristics of the study participants

A total of 204 Sputum Samples were collected from New and Retreatment Cases attending eight treatment initiating centers. Among these participant 140 (68.6 %) were males and 64 (31.4%) were females with a minimum and maximum age of 15 and 85 years. The majority of the study participants had previous treatment for TB 116(56.9%) and Urban Residences 173 (84.8%). Thirty one (15.2%) of the participants was HIV Positive and 88 (43.1%) were New TB Cases (Table 1).

Table 1 Socio-demographic and clinical data of the study participants in selected drug resistant TB Treatment Centers, Ethiopia, May 2020

Variable		Confirmed TB Cases N=204(%)
Gender	Male	140 (68.6)
	Female	64 (31.4)
Age	15-24	61 (29.9)
	25-34	83 (40.7)
	35-44	33 (16.2)
	45-54	14 (6.8)
	≥ 55	13 (6.4)
Residence	Rural	31 (15.2)
	Urban	173 (84.8)
Treatment History	New	88 (43.1)
	First line	96 (47.1)
	Second line	20 (9.8)
TB Classification	New	85 (41.6)
	Relapse	66 (32.4)
	Failure	44 (21.6)
	Defaulter	9 (4.4)
HIV Status	Negative	173 (84.8)

	Positive	31 (15.2)
Smear Results	Negative	100 (49)
	Positive (Scanty)	23 (11.3)
	Positive (+1)	37 (18.1)
	Positive (+2)	21 (10.3)
	Positive (+3)	23 (11.3)
Living Region of Participant	Addisabeba	86 (42.2)
	Amhara	13 (6.4)
	Benishangul gumz	5 (2.4)
	Gambella	12 (5.9)
	Oromia	35 (17.1)
	SNNP	53 (26)

5.2 Drug resistance pattern to first line and second line anti-TB drugs

Among 204 bacteriologically confirmed sputum samples, 160 (77.3%) were found to be culture positive for Mycobacterium tuberculosis Complex and 5(2.4%) were identified to be Non tuberculosis mycobacterium whereas 4 (1.9%) were experienced contamination, 35(16.9%) were culture negative thus excluded from further analysis. Drug sensitivity tests to four first line and five second line anti-TB drugs were performed for all 160 M. tuberculosis isolates from this 64 were new cases and 96 were retreatment cases, 50 (31.2%) were susceptible to all drugs but 110 (68.9%) of them were developed resistant to one or more drugs. MDR were detected in 37/64(57.8%) of New cases and 62/96(64.5%) of retreatment cases. Pre-XDR were observed in 8(5%) of all case and XDR was observed in 1(0.6%) of all case (Figure 1).

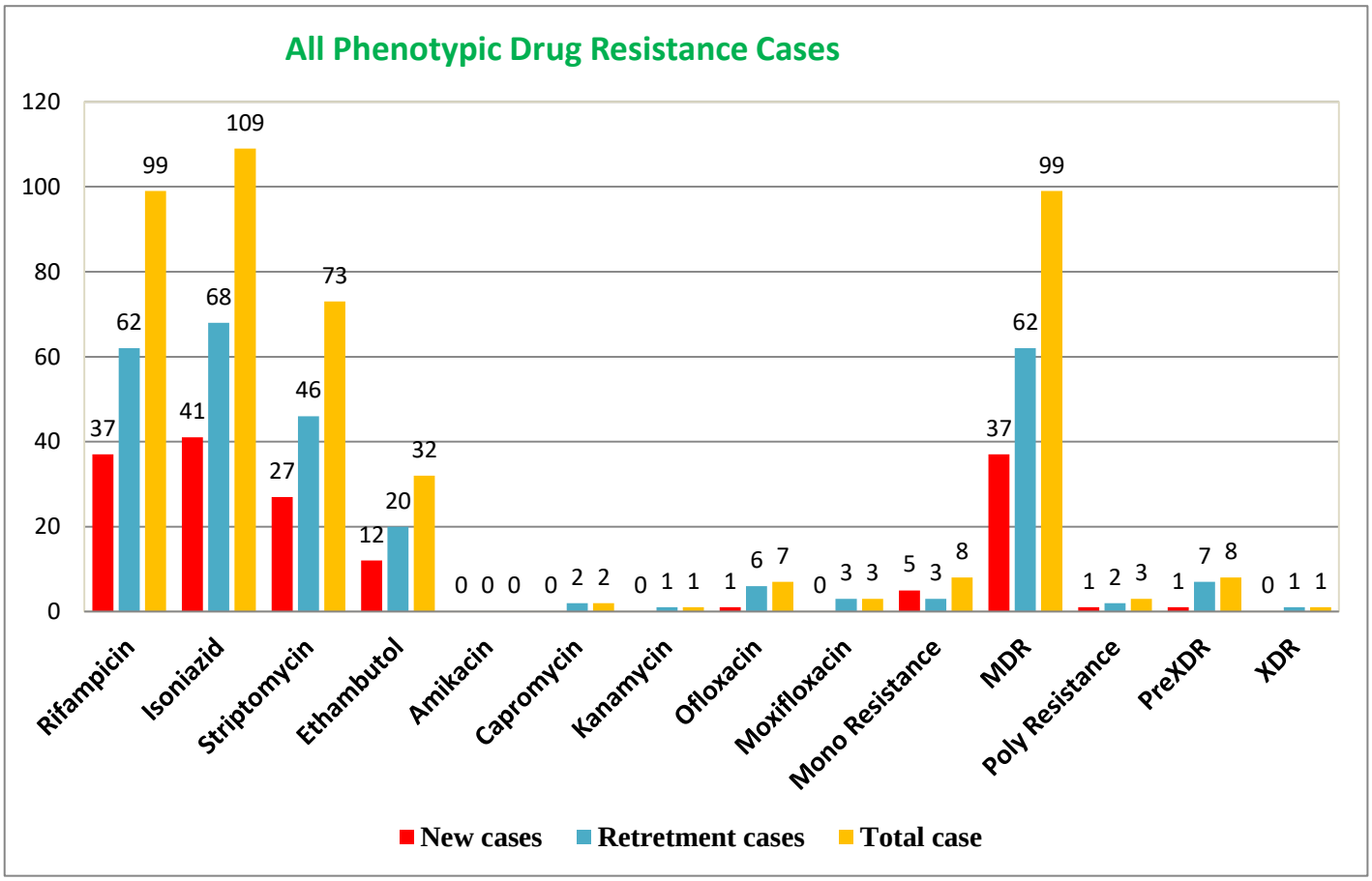

Figure1: Proportion of drug resistance for first and second line anti TB Drugs with respective frequencies, May2020

Table 2 Drug resistance pattern to first line and second line anti-TB drugs in selected drug resistant TB Treatment Centers, Ethiopia May2020

	New Cases	Retreatment Cases	All Cases
Resistance Pattern	Number (%)	Number (%)	Number (%)
Total	N=64	N=96	160
Susceptible	21 (32.8)	29 (30.2)	50 (31.2)
Any Resistance	43 (67.2)	67 (69.8)	110 (68.8)
All Resistance			
RMP	37 (57.8)	62 (64.5)	99 (61.9)
INH	41 (64)	68 (70.8)	109 (68.1)
STM	27 (42.2)	46 (47.9)	73 (45.6)
EMB	12 (18.8)	20 (20.8)	32 (20)
Mono Resistance			
RMP	0	0	0
INH	4 (6.2)	3 (3.1)	7 (4.3)
EMB	0	0	0
STM	1 (1.5)	0	1 (0.6)
All MDR	37 (57.8)	62 (64.5)	99 (61.9)
INH+RMP (Only)	10 (15.6)	15 (15.6)	25 (15.6)
INH+RMP+SM	15 (23.4)	28 (29.1)	43 (26.9)
INH+RMP+EMB	11(17.2)	17 (17.7)	28 (17.5)
INH+RMP+SM+EMB	1 (1.5)	2 (2.1)	3 (1.9)
Poly Resistance			
INH+STM	1(1.5)	1 (1)	2 (1.2)
INH+EMB	0	1 (1)	1 (0.6)
Pre XDR	1(1.5)	7 (7.3)	8 (5)
MDR+AMK	0	0	0
MDR+CAP	0	1 (1)	1 (0.6)
MDR+KAN	0	1 (1)	1 (0.6)

MDR+MOX	0	0	0
MDR+OFX	1(1.5)	2 (2.1)	3 (1.8)
MDR+MOX+OFX	0	3 (3.1)	3 (1.8)
XDR (MDR+CAP+OFX)	0	1 (1)	1 (0.6)

5.3 Comparison of drug susceptibility results determined by Genotypic (MTBDR Plus, MTBDRsl and X-Pert MTB Rif Assay) and phenotypic method (BACTEC MGIT 960)

Among 160 Clinical Isolates analyzed by phenotypic method, 99 were found to be resistant to Rifampicin and isoniazid, 99 were resistance to rifampicin, 106 to isoniazid. Comparison of MTBDR Plus assay with BACTEC MGIT 960 showed that out of 99 Rifampicin and isoniazid resistant isolates identified by conventional drug susceptibility testing by indirect proportion method, MTBDRplus assay detected 78 isolate as Rifampicin and isoniazid resistant, 9 as isoniazid resistant, 12 isolates as rifampicin resistant and 51 were Susceptible for both drugs (Table3).

Table 3 Comparison of frequency MDR and Mono resistance results determined by MTBDR Plus and Phenotypic method

		MGIT Phenotypic DST				
		RIF(R) INH(R)	RIF(S) INH(R)	RIF(R) INH(S)	RIF(S) INH(S)	Total
MTBDR Plus	RIF(R) INH(R)	78	3	0	0	81
	RIF(S) INH(R)	9	3	0	0	12
	RIF(R) INH(S)	12	0	0	0	12
	RIF(S) INH(S)	0	4	0	51	55
	Total	99	10	0	51	160

Out of 99 MDR Isolates identified by Conventional DST, 8 were pre XDR, 1 was XDR and 90 were susceptible for both fluoroquinolones and second line injectable drugs by both methods. MTBDR Plus detected 0nly 3 of pre XDR Cases and no XDR Case detected (Table 4).

Table 4 Comparative analysis of frequency of XDR and Pre XDR resistant results by the Conventional drug susceptibility test and MTBDRsl method

		MGIT Phenotypic DST				
		FLQ (R) SLI(R)	FLQ (S) SLI(R)	FLQ (R) SLI(S)	FLQ (S) SLI(S)	Total
MTBDRsl	FLQ (R) SLI(R)	0	0	0	0	0
	FLQ (S) SLI(R)	0	0	0	0	0
	FLQ (R) SLI(S)	1	0	3	0	4
	FLQ (S) SLI(S)	0	2	3	90	95
	Total	1	2	6	90	99

Among 160 drug susptability test analyzed by conventional method, GenXpert assay detected 95 as rifampicin resistance and 65 as Rifampicin Sensitive Mycobacterium tuberculosis. From 99 rifampicin resistance cases detected by phenotypic Method, GenXpert assay detected 91 cases as Rifampicin resistance and 8 as Rifampicin sensitive (Table5).

Table 5 Comparison of drug susceptibility results determined by phenotypic DST and GenXpert

		MGIT Phenotypic DST		
		Resistant	Sensitive	Total
GenXpert	Resistant	91	4	95
	Sensitive	8	57	65
	Negative	0	0	0
	Total	99	61	160

Comparative result of GeneXpert MTB/RIF with MTBDRplus assay showed that among 160 isolates 91 detected as Rifampicin resistance and 63 as Rifampicin sensitive by both methods. The rest 6 samples were discordant result among the two methods (Table 6).

Table 6 Comparative result of GeneXpert with Line Probe Assay for Rifampicin Resistance

		Line Probe Assay			
		Resistant	Sensitive	Negative	Total
GenXpert	Resistant	91	4	0	95
	Sensitive	2	63	0	65
	Negative	0	0	0	0
	Total	93	67	0	160

5.4 Summary of Discordant Result Determined by Phenotypic and Genotypic DST methods

The Genotype DST method detects 90 true resistances and 58 true Sensitive respectively to rifampicin and 93 true resistances and 51 true sensitive respectively for isoniazid. This assay identifies MDR-TB Correctly in 78 cases out of 99 Phenotypically Confirmed MDR Cases. Similarly these assays correctly identify 4 Pre XDR Cases out of 8 cases and miss an XDR Case out of 1 XDR case which was confirmed by the conventional method (Table 2).

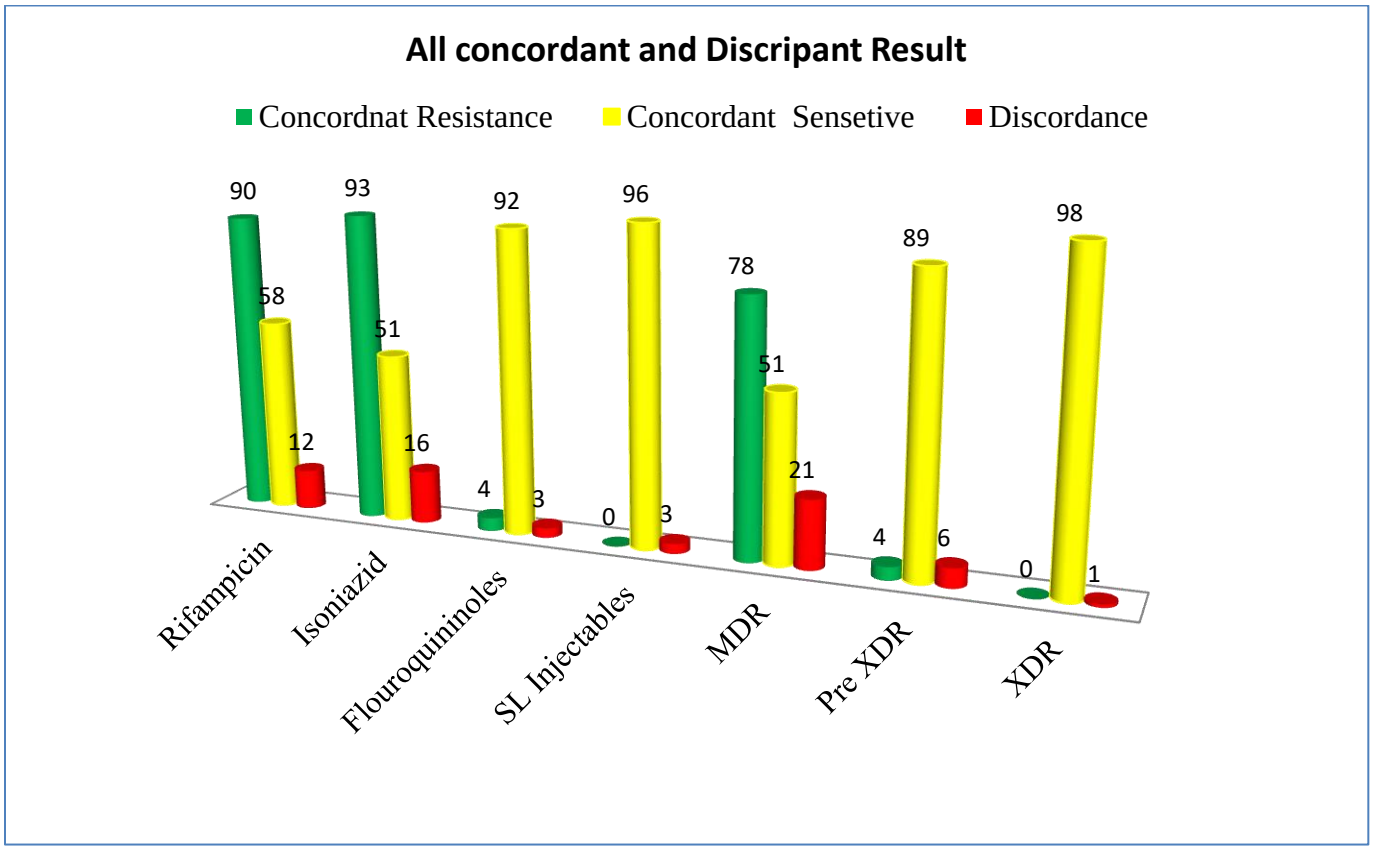

Figure2: Proportion of Concordant and Discordant results using Phenotypic and Genotypic Method

The sensitivity, specificity PPV and NPV of the GenoType MTBDRplus assay for the detection of RIF-resistant M tuberculosis isolates were 94.2,95,97 and 90.6 Percent respectively. Its sensitivity, specificity, PPV and NPV for detecting INH-resistant M. tuberculosis isolates were 87.2,100,100 and 76.1 respectively, whereas sensitivity, specificity, PPV and NPV Values of GenoType MTBDRplus assay were 82.5, 94.4,97 and 70.8 Percent respectively, for detecting MDR M. tuberculosis isolates.

The discordance results were subsequently repeated to confirm the reproducibility of the results, finally a total of 30 isolates detected as discordant. From this 12 isolates were discordant to Rifampicin and Another 16 isolates to isoniazid was found to be discordant. On the other hand 21 MDR Cases, 3 isolates to Fluoroquinolone and 3 isolates to second line injectable drugs were found to be discordant (Table 7).

Table 7 Summary of Concordant, Discordant and Performance assay of Genotype MTBDRplus and MTBDRsl Method by comparing the result with that of MGIT 960 System.

	Rifampicin	**Isoniazid**	**MDR**	**Fluoroquinolones**	**Second line Injectable drugs**
True Resistance	90	93	78	4	0
True Sensitive	58	51	51	92	96
Discordant	12	16	21	3	3
Sensitivity	94.2 %	87.2%	82.5%	57.1%	50%
Specificity	95%	100%	94.4%	100%	100%
PPV (%)	97%	100%	97%	100%	100%
NPV (%)	90.6%	76.1%	70.8%	94.84%	95.8%

5.5 Molecular analysis of mutation in various drug resistance determining gene loci (rpoB, katG,inhA,gyrA,gyrB,rrs and eis)

All culture positive isolates subjected to first line and second line genotypic DST, 160 isolates abele to show interpretable results. Various controls were used before interpreting the result these are: Amplification control used to check the successfulness of amplification or PCR process. Conjugate Control check the detection process on enzyme substrate reaction step. Locus control check the sensitivity of reaction in each drug resistance determining gene loci in addition to this to check the whole process Negative control and positive control were used. TUB band zone is gene loci shared by all members of MTBC. If TUB band is missed the tested bacterium did not belongs to MTBC.

Since the Majority of detected cases are MDR a total of 93(58.15%) mutation were detected in rpoB gene, Of this cases 70(75.3%) had a mutation at WT8 MUT3 loci the amino acid Serine was Substituted to Leucine at 531 Codon, 6(6.4%) were found to have mutation within WT3 at Codon 513-517 , another 6 (6.4%) had a mutation within WT7 at a codon 526-529 this are the three most frequently detected mutation in rpoB gene for RIF resistance, another 7 rpoB mutations were identified at Codon 510-517,516,526 and 531 as shown in Table8.

Among mutation associated with the resistance to isoniazid the dominance mutation 86 (92.5%) occurred in the codon 315 of KatG result in Substitution Serine to Tyrosine1. Additionally another kat G wild type missing mutation without mutant band occurred in three of the isolates. Low level resistance mutation detected in 4(4.3%) of the isolate at -15 Promoter region Cytosine were changed to Thymine.

From a total of 99 isolates subjected for second line genotypic DST only 4 had a mutation in gyrase A gene loci responsible for fluoroquinolones resistance. Of this 1 isolate had a mutation with in WT2 MUT1 in the codon 89- 93 amino acid modification occurred due to substitution of Alanine to Valine, another 3 had a mutation in the codon of 90, 94 and 92-97 region (Figure3, Table 8).

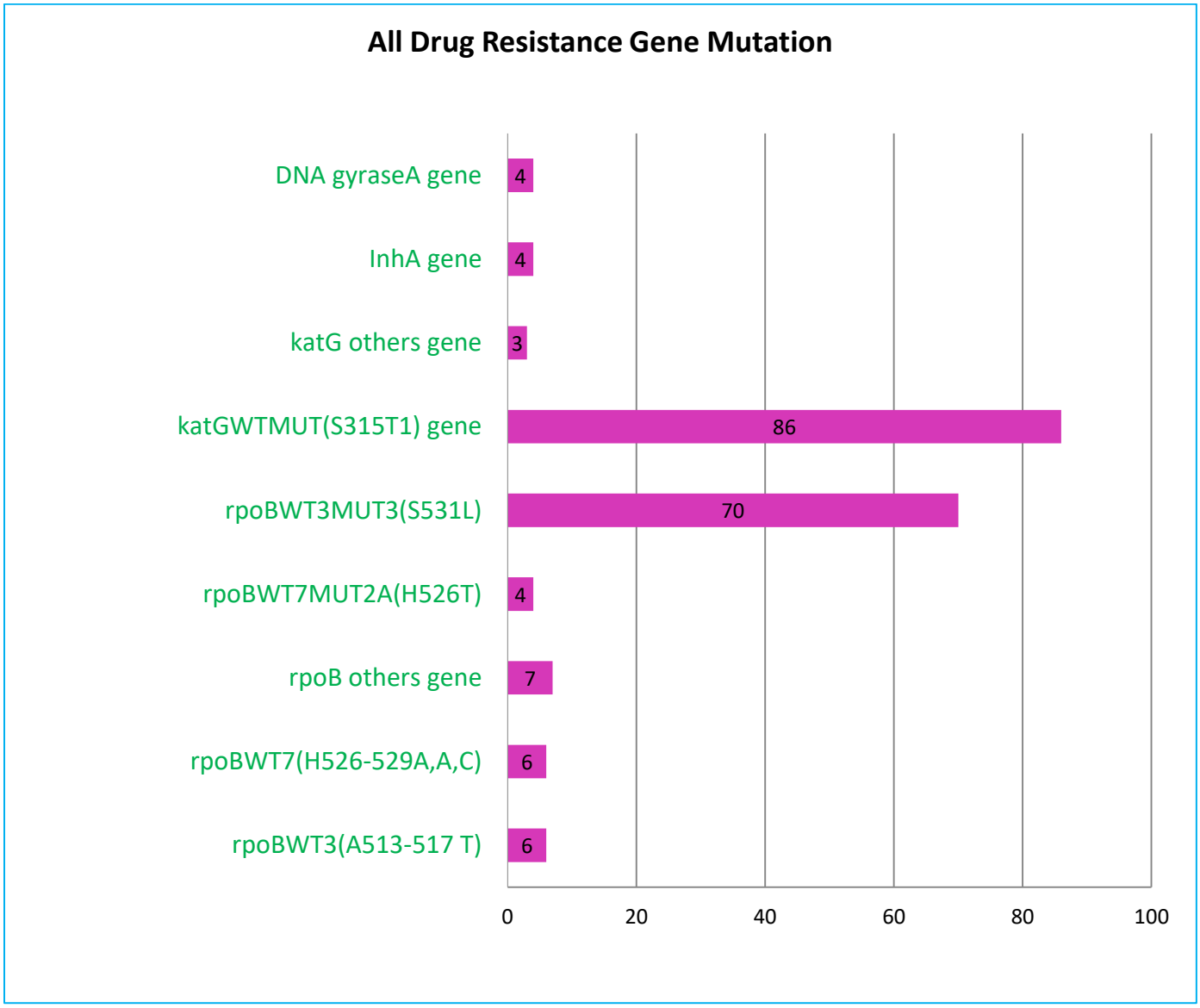

Figure3: The frequency of Mutations in various drug resistance determining gene loci

Table 8 Frequency of Mutations in various drug resistance determining gene loci

Gene	Wild type Band	Mutation Band	Mutated Codon	Specific Mutation	Aminoacid change	Number (%)
rpoB	3	-	513-517	GAC-TAC	Asp-Tyr	6 (6.4)
	7	-	526-529	CAC-CGC CAC-AAC CAC-TGC	His-Arg His-Asn His-Cys	6 (6.4)
	8	-	530-533	TCG-TGG CTG-CCG	Ser-Trp Leu-Pro	1 (1)
		3	531	TCG-TTG	Ser - Leu	2 (2.1)
	2 & 3	-	510-517	CAA-CCA	Gln-Pro	1 (1)
	3 & 4	1	516	GAC-GTC	Asp -Val	1 (1)
	4	1	516	GAC-GTC	Asp -Val	1 (1)
	7	2A	526	CAC-TAC	His- Tyr	4 (4.3)
	WT-8	Mut-3	531	TCG-TTG	Ser- Leu	70(75.3)
	-	2A & 3	526&531	CAC-TAC TCG-TTG	His-Tyr Ser-Leu	1 (1)
katG	WT	1	315	AGC-ACC	Ser-Thr1	86(92.5)
	WT	-	315	AGC-ACA	Ser-Thr2	2 (2.1)
	-	1	315	AGC-ACC	Ser-Thr1	1 (1)
inhA	1	1	-15	C-15T	Promoter	4 (4.3)
gyr A	2	1	89-93	GCG-GTG	Ala-Val	1 (1)
	3	3B	92-97	GAC-AAC GAC-TAC	Asp-Asn Asp -Tyr	1 (1)
	3	3B&3C	92-97	GAC-AAC GAC-TAC GAC-GGC	Asp-Asn Asp -Tyr Asp -Gly	1 (1)
	-	1&3B	90 94	GCG-GTG GAC-AAC GAC-TAC	Ala- Val Asp -Asn Asp -Tyr	1 (1)

Mutation in rifampicin resistance determining region of rpoB gene were identified in 95 of the160 total samples. The most common mutation were in Codon 529-533 of the Probe E Which were found in 72 (75.8%) of the 95 isolates. Mutation in Probe D (523-529) were detected in 9(9.5%) of the samples. Mutation in probe B (511-518) Occurred only in 8(8.4%) of RIF resistant isolates, whereas the remaining 6 (6.3%) of isolate detected as rifampicin resistance without the presence of missed probe (Table 9).

Table 9 Mutation patterns for the rpoB genes by Xpert MTB/RIF Assay found in 95 isolates

Probe failure	Codon	Amino acid	Number (%)
A	507-511	Gly,Thr,Ser,Gln & Leu	0
B	511-518	Leu,Ser,Gln,Phe,Met,Asp,Gln&Asn	8(8.4)
C	518-523	Asn,AsnPro,Leu,Ser & Gly,,	0
D	523-529	Gly,Leu,Thr,His,Lys,Arg&Arg	9 (9.5)
E	529-533	Arg,Leu,Ser,Ala,Leu	72(75.8)
None	-	-	6 (6.3)
	Total		95

0f 12 discrepant cases identified for RIF, 3 were resistant by both genotypic method but sensitive by phenotypic method and also had mutation in Probe D of GenXpert and missed WT7, MUT2A of MTBDRplus assay meanwhile 3 were resistant by phenotypic method but sensitive by both genotypic method .Three isolates had mutation without failed probe of GenXpert and resistant by Phenotypic method but sensitive by MTBDRplus assay. Another 2 were identified as a resistance by phenotypic and MTBDRplus assay at WT8, Mut3 band but sensitive by GeneXpert (Table 10).

Table 10 Rifampicin Resistance Discrepant cases between two molecular and phenotypic DST methods

Isolate No.	Genotypic DST				Phenotypic DST
	MTBDRplus		Xpert MTB RIF		BAC TECH MGIT960
	Mutation	Result	Failed Probe	Result	Result
GP-42	WT7, Mut2A	R	D	R	S
GP-46	WT8, Mut3	R	-	S	R
GP-48	WT7	R	D	R	S
GP-54	-	S	-	S	R
GP-55	-	S	-	S	R
GP-65	-	S	No	R	R
GP-92	WT7	R	D	R	S
GP-115	-	S	D	R	S
GP-121	Mut3	R	-	S	R
GP-146	-	S	No	R	R
GP-150	-	S	No	R	R
GP-156	-	S	-	S	R

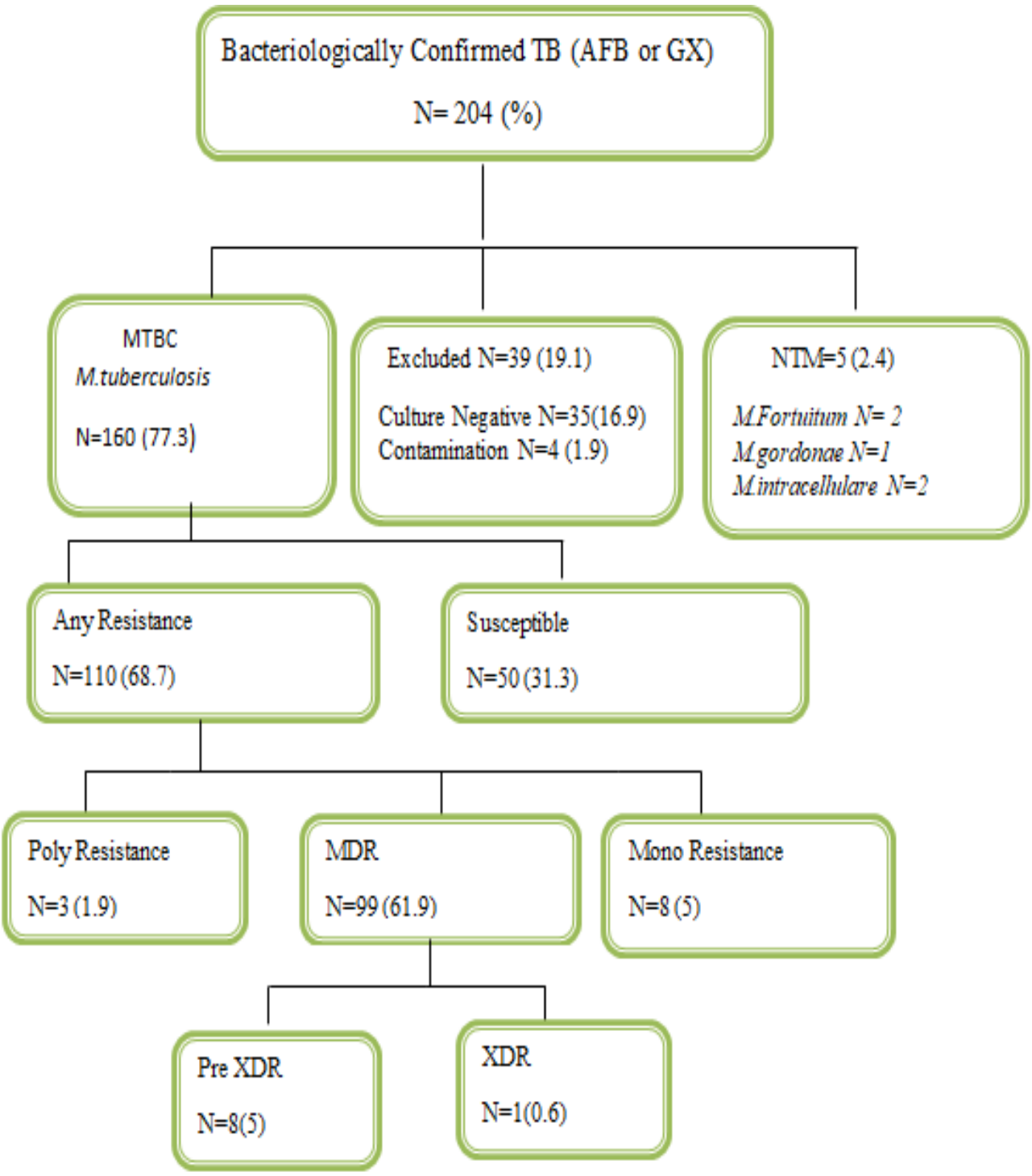

Figure4: Flowchart of Smear, GenXpert, Culture, MTBC assay, Genotypic and Phenotypic DST result

6. Discussion

Drug resistance strains of MTB transmitted in the community, replacing susceptible strains and consequently making first-line regimens inadequate for achieving high cure rates. The efficiency of current tuberculosis control program in any country is assayed by drug resistant pattern. The spread of drug resistance tuberculosis can only be prevented by rapid identification of these cases and treatment with combination of effective drugs. Meanwhile, in the present study, we analyzed resistance to 9 anti TB drugs (RIF,INH,STM,EMB,MOX,OFX,AMK,CAP and KAN) detected in 160 isolates, using both phenotypic and molecular methods.

In the present study, all culture positive MTBC isolates were identified as *M.tuberculosis* whereas five NTM isolates identified as *M.gordonae, M.fortuitum* and *M.intracellulare*. This study coincides with previous study conducted in Southern part of Ethiopia and Bangladesh [37,38].This result was dissimilar with the studies conducted in North West Ethiopia, Turkey, Djibouti and Ghana (97.6% *M.tuberculosis and* 2.4 % *M.africanum*)[39-42].The difference among species between different study settings may be due to difference in diagnostic methods, geographic area and study participants.

In this study the demographic data of participants from drug resistance TB treatment center indicated that bacteriologically confirmed cases were higher in previously treated cases 96(60%) than new cases 64(40%). Drug resistance to at least one of the anti TB drugs was 110/160 (68.8%) from this, 43/160 (26.8%) was newly diagnosed TB cases and 67/160 (37.5%) was from previously treated TB cases. which is consistent with other report in Addis Ababa resistance to any drug was (54%) [43].Similarly a report by Nafees etal. , from Pakistan, reported any drug resistance in (62.6%) cases [44]. Some countries like Germany and US has a very low drug resistance cases (12.7%) and (1%) respectively [45, 46].

The results of our study showed that highest proportion of drug resistance identified for INH (68.1 %), followed by RIF 61.9% cases. Resistance to STM were found in (45.6 %) and to EMB (20 %).Comparable proportion were reported in other areas of Ethiopia resistance to INH was (51.4 %), to RIF (32.9 %), STM (42.9 %) and to EMB (28.6 %) cases of tuberculosis [47]. However, lower level of resistance reported in Iran to INH (10 %), RIF (11.8 %), to STM (10.7 %) and to EMB (3.2 %) cases of tuberculosis [48]. The difference in findings may be due to the

nature of study participant because the participant in this study was from drug resistance TB treatment initiating center.

In the present study, indicated that high rate (68.1 %) of INH resistance is alarming since it is a potent first line drug used though out the course of treatment. INH also used as a chemoprophylaxis of TB especially in immune compromised Patients, TB High risk group Population and it acts on dormant stage of TB therefore used as monotherapy for treating latent TB infection. Any resistance to Rifampicin was found in (61.9%) of cases in this study. RIF is al so front line anti TB drug. The reason high resistance may be due to conditions that alter metabolism of the drug, treatment interruption and using in treatment of other bacterial diseases. Any resistance to Streptomycin was found in (45.6%) of cases. STM is the first anti tuberculosis drug. The reason high resistance due to previously used as monotherapy for treating TB for a long period of time, using for treatment of endocarditis, Plague and Brucellosis. Drug resistance against Ethambutol was found in (28.6%) in this study. It is a bacteriostatic drug but in large dose can act as bactericidal.

In this study 99/160 (61.9%) were MDR case. The emergence of drug resistance is great public health threat across the globe and access to universal DST is also another concern. Higher rate of Multidrug resistance also reported in Ethiopia (87%), Pakistan (75.7%) and India (69.4%)[49-51].Lower rate reported in Central Ethiopia (39.4), Sudan (51.8%) and Germany (16.7%)[43,45,52].The variation in proportion among different study setting could be due to study population because hospital based studies particularly in drug resistance TB referral centers increases the finding of MDR-TB cases.

In present study Out of 99 MDR-TB cases, 8(8.1%) and 1(1%) were found to be PreXDR and XDR case respectively. Similar rate of PreXDR was reported from Northern Ethiopia (5.7%) and Brazil (9.19%)[49,53].Higher rate was reported from China (30.9%) and Zimbabwe (27%)[54,55].The difference in finding may be due to different levels of health care delivery system in various countries.

In this study gene mutation associated with drug resistance was determined. The molecular mechanism of resistance to rifampicin determined by rpoB gene mutation on 81bp region. In this study 93 of 160(58.15%) isolates tested were resistance to rifampicin. The frequency of Serine to leucine substitution occurred in (75.3%) of the total rpoB mutation so it is the most common,

followed by missing of WT7 codon 526-529 and missing WT3 codon 513-517 without mutant band. However, other mutations at (codon 516-517, codon 16-26 and codon 531) are less frequently encountered. This finding was comparable with other studies from Ethiopia (73%), Cameron (71.4%) and Spain (72.3%) [27, 56, 57].

Isoniazid resistance determined by mutation on katG and inhA gene. In present study we found that (92.5%) mutation occurred at codon 315 an amino acid serine substituted to Threonine. The remaining occurred on the remaining katG codon 315 and Promoter region inhA gene. Our finding showed that most of resistance mutation to INH occurred at codon 315 this was in agreement with several studies from Ethiopia (87.8%), Georgia (95.1%) and Vietnam (76.83%) [28, 58, 59].

Fluoroquinolone resistance mutation detected by probes of two gene loci these are DNA gyrase A and DNA gyrase B also called DNA Topoisomerase II. In this study we found 4 fluoroquinolone resistance isolates, all had mutation to different codons of gyrase A. In gyraseA gene Mutation Occurred between codon 90-97.The findings was comparable with study done in Pakistan and China all mutations Occurred in gyraseA gene of fluoroquinolone resistance determining region [60, 61].Furthermore determining individual drug resistance mutation is important to determine low level resistance which can be treated with high dose of fluoroquinolones.

In the present study 95(59.4%) isolates found to be RIF resistance by GenXpert MTB/RIF method. The assay detect mutation in rpoB gene by using five overlapping probe called probe A(codon 507-511), probe B(codon 511-518),probe C(codon 518-523), probe D(codon523-529) and E(codon 529-533).The most frequent mutation occur in the region of probe E(75.8%),followed by Probe D(9.5%) and Probe B(8.4%). Moreover the rest (6.3%) RIF resistant detected without failed probe. No mutation detected on probe A and which is consistent with studies done in Ethiopia, Malawi [62, 63].

Phenotypic testing is currently the gold standard for DST of MTB. It is performed to test the in vitro capacity of a selected drug to inhibit the bacterial growth and is used as a surrogate to predict success of the therapy. The reliability of the results varies according to the drug tested, the concentration of bacteria, the media used and the incubation time. For these reasons, phenotypic is generally considered technically demanding; in addition, proper biosafety

precautions should be taken for manipulating live MTB isolate. By considering this, the finding of this study was compared with BACTEC MGIT 960 system.

In this study we analyzed the correlation between gene mutation pattern and conventional DST. Among 99 phenotypically RIF resistance isolates detected in the present study, 90 (90.1%) were found to possess resistance conferring mutation to RIF in the rpoBgene upon genotyping. The remaining 9 phenotypically resistance had no known mutation in the rpoB gene. Among the isolates with rpoB mutation, WT8Mut3 (S531L) was the most common mutation. This is a point mutation TCG-TTG, result in the substitution of serine to Leucine in the amino acid chain. The S531L mutation has been found in 75.3% of RIF resistance. Of the isolates with rpoB mutation WT3 (513-517) and WT7 (526-529) was the second most common mutations. This is found in up to 6.4% respectively. Furthermore, similar proportion reported in studies conducted in different country [56,57,64].

Our data showed that out of 109 Phenotypically INH resistance isolates detected in this study,93 (85.3%) were found to possess resistance mutation to INH in the katG and inhA gene up on genotyping. Whereas the remaining 16 phenotypically resistance with no detected mutation in the katG and inhA gene. Of the 93 isolates with KatG mutation, WTMUT1 (S315T) was found in 92.5% of INH resistance. Furthermore the S315T was the most common mutation. This is a point mutation AGC-ACC, result in the substitution of Serine to Threonine in the amino acid chain. Among the isolate with inhA mutation WT1MUT1(C-15T) was found the second most common mutation. This is a transitional mutation at -15 positions C-T, result in the substitution of Cytosine to Thymine in the promoter region of inhAgene. The C-15T mutation has been found in 4.3% of INH resistance. This finding was in consistence with studies done in different countries [42, 58,59]. However a similar study done in the Northern part of Ethiopia by Belay etal. , reported INH resistance in their isolates with all mutations at codon 315 of the *kat*G gene [27].

The relation between gene mutation patterns with phenotypic resistance for second line FLQ indicated that out of 7 phenotypically resistances isolates detected in present study,4 (57.1%) were found to possess resistance mutation to FLQ in the DNA gyraseA gene up on genotyping. However the remaining 3 phenotypically resistance isolates had no known mutation in the DNA

gyrase gene. This finding has been described previously by studies done in Vietnam, Pakistan and Morocco [59, 60, 65].

The proportion of discordance between several genotypic methods as compared to phenotypic method was analyzed. Most isolates yields completely concordant result. However there were some differences across the method. Among the RIF tested isolates the comparison of the phenotypic and genotypic resistance profiles were identified 12 (7.5 %) discordant results out of a possible 160 isolate. From this 3 discrepant results were isolates predicted to be genotypically resistant to RIF but found to be phenotypically susceptible. The rest 9 mismatched results were for isolates predicted to be phenotypically resistant to RIF but found to be genotypically susceptible, indicating that the determinant was present but not expressed or expressed poorly. The possible reason for this discrepancy could be mutation in region other than RR DR, The MTBDRplus and GenXpert MTB/RIF may detect Silent or Nonsense mutation which is a Cause for false positive results [66, 67].

This study showed that out of 109 phenotypically resistance isolates for INH a total of 16 (10%) yield discrepant result from a total tested. All discrepant results were phenotypically resistance but genotypically susceptible. The nonsense mutation, Mutation other than the hot spot region and Non chromosomal mutation could be a possible reason [25, 68].

In this study, comprehensive resistance Phenotypes of 160 *MTB* isolates along with their correlations with genotypic resistance were determined. To accomplish this, resistance for each of nine antimicrobials tested were defined. Six resistance genes along with a number of resistance-associated mutations were identified. There was a high correlation between phenotypic resistance to a given drug tested and the presence of one or more resistance genes expected to confer resistance to that drug. For second line drugs Out of 9 phenotypically resistance isolates a total of 6 (3.7%) isolates displayed discrepancies between resistance phenotypes and genotypes for fluoroquinolones and injectable drugs. Of the six discrepant results 2 isolate for Ofloxacin, 1 isolate for Moxifloxacin, 2 isolate for capreomycin and 1 isolate for kanamycin yield discordance results. All discrepant results were phenotypically resistance but genotypically susceptible. The possible reason could be the presence of Heteroresistance, lower sensitivity of the method and mutation other than drug resistance determining region [69-71].This assay was widely used in high TB burden countries like Ethiopia. However according to

the finding this assay wrongly classified four Pre-XDR cases as MDR and one XDR case as PreXDR case.

7. Conclusion

In this study we conclude that all detected isolates were belongs to species of *M.tuberculosis* circulating in the study area. No other Mycobacterium Tuberculosis complex species was detected. However five Non tuberculosis Mycobacterium species were identified namely *M.gordonae, M.intracellulare and M.fortuitum.*

The findings in the phenotypic DST pattern showed that the magnitude of drug resistant TB in the previously treated TB cases was higher as compared to new cases. Highest proportion drug resistance detected for INH followed by RIF, resistance for EMB and STM all so higher. Furthermore, for all RIF and MDR TB cases second line DST also conducted and the finding indicated that higher frequency of OFX resistance identified followed by MXF, few CAP,KAN and No AMK resistance Cases detected. Finally eight Pre-XDR cases and one XDR Cases were identified.

Our findings highlight high frequencies of drug resistance conferring mutations to first and second-line anti-TB drugs in culture positive isolates specially to INH, RIF and FLQ. The high rate of resistance conferring mutations in katG and rpoB was high relative to global report. Moreover, the high proportion of INH and RIF resistance is a significant risk for the potential emergence of MDR-TB in the study area because is the initial step towards anti-TB drug resistance and a common pathway to the development of MDR-TB.

Our finding indicated that Most of the isolate yields concordant results. However a high discordance cases detected as a result of this four PreXDR cases wrongly classified as MDR cases and One XDR case wrongly classified as PreXDR Case. Moreover discrepancy rate of routinely used molecular method will have direct clinical consequences especially in high TB burden countries like Ethiopia. Furthermore determining the yield of drug resistance TB using Phenotypic and Molecular DST methods were crucial for programmatic management of TB.

8. Strength and Limitation of the study

Limitations

The study had certain limitations. First, study participants were all patients visiting selected drug resistance TB treatment Centers in Ethiopia. Findings from such a selected population may not indicate the true burden of the problem at community level.

Total number of isolates that met this criteria n =204 was small relative to the Other high TB burden countries due to this factor true frequencies of drug resistance conferring mutations to second-line anti-TB drugs could be higher than we have reported if a larger sample size is used.

Strength

The strength of this study was First, the sample was analyzed by various laboratory assays such as Smear Microscopy, liquid culture, Solid culture, Brain Heart infusion agar, MPT64Antigen test, GeneXpert MTB/RIF, MTBDRplus, MTBDRsl, MTBC, CM assay, AS assay, SIRE/MGIT960 and Second line phenotypic DST.

Our findings provide key insight about the performance of molecular DST methods in detecting resistance to first line and second line drugs.

In Ethiopia the second line phenotypic DST was not be performed throughout the country therefore limited information was known about PreXDR and XDR Cases.so that such studies provide a great benefit for our community and country.

9. Recommendations

Universal access for DST is one of the strategies laid by WHO STOP TB Partnership for TB Control Program. However in High TB burden countries access for DST is a great problem. In Ethiopia we have ten TB Culture laboratories across the country. Seven in regions (two in Amahara, two in Oromia, One SNNPR, One Hareri and One Tigray) and Three in Addis Ababa (EPHI, AHRI and St.peter TB specialized Hospital) in all laboratories Genotypic DST was conducted but Phenotypic DST is not routinely practiced in any of the Laboratory. Moreover Genotypic DST method has high rate of discrepancy could lead to wrong diagnosis if utilized in isolation by a laboratory, lead to significantly different drug resistance TB regimens, it remains harder to prove that such a regimen would lead to a worse outcome.DR TB is a special scenario, in that it is treated with multiple drugs for several months and the drugs have several side effect. So we recommend MOH and EPHI to build the testing capacity of referral laboratories for second line Phenotypic DST test across the country.

TB laboratory personnel and clinicians should be aware of the selected method they use for DST and be mindful of possible discrepancies with other methods.

Finally large scale study should be done on Nation Wide Drug resistance TB prevalence, Molecular Epidemiology of DRTB and Whole genome sequencing was required in order to identify the molecular epidemiology and phylogenic strain diversity of drug resistance TB.

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11. Annex

Annex I: Data Collection form

Data Collection form		
USIN ☐☐☐☐☐☐		
Name of Diagnostic Center: _____		
Section 1 :		
1.1	Date of collection	____/____/_____ DD MM YYYY
1.2	Name of Clinician	_____
1.3	Patient. (Medical Record No)	_____
1.4	Age in Years	_____
1.5	Sex	☐ Male ☐ Female
1.6	Previous History of TB	☐ Yes ☐ No
1.7	Have you ever taken anti -TB drugs (>1 month)	☐ Yes ☐ No
1.8	Residence	☐ Urban ☐ Rural
1.9	Living Region of participant	_____
1.10	If yes to the question number 1.6	☐ Relapse ☐ Treatment failure ☐ Loss to follow up ☐other_____
1.11	Co-infections	☐ Diabetes mellitus ☐ Cancer ☐ HIV ☐ Other_____
1.12	Patient classification	☐Presumptive TB patient ☐Presumptive MDR-TB patient ☐MDR-TB follow up patient at

		month_____	
1.13	Sputum sample collection date and time Circle	____/____/ _____ DD MM YYYY	____:_____ AM/PM Hour Min
1.14	Volume of Sputum	_____ml	
1.15	Nature of sputum sample: **Circle one**	☐ Mucoid ☐ Muco-purulent ☐ Purulent ☐Saliva ☐ Bloody	
Sample Reception and processing :To be filled by professional at EPHI/NTRL			
1.16	Date Sputum received by EPHI/NTRL	____/____/_____ DD MM YYYY	
1.17	Time Sputum received by EPHI/NTRL	_____:_____ AM/PM Hour Min	
Ziehl Nelson Smear-From direct sample			
2.1	Date ZN test done	____/____/_____ DD MM YYYY	
2.2	Ziehl-Nelson Smear result	☐ Negative ☐ Scanty ☐ 1+ ☐ 2+ ☐ 3+	
GenXpert Testing			
2.3	Date of GenXpert testing	____/____/_____ DD MM YYYY	
2.4	GenXpert result (check box)	☐ MTB Detected RIF Resistant Detected ☐ MTB Detected RIF Resistant Not Detected ☐ MTB Detected RIF Resistant Indeterminate ☐ MTB not detected ☐ Invalid ☐ Error	
Solid media LJ results			
2.5	Date sputum sample processed	____/____/_____	

		DD MM YYYY
2.6	Date of LJ tube Inoculation	____/____/_____ DD MM YYYY
2.7	LJ result	☐MTBC Positive ☐MTBC Negative ☐Contaminated ☐MTBC positive with contamination
2.8	Date LJ result reported	____/____/_____ DD MM YYYY
BACTEC MGIT Culture		
2.9	Date of MGIT tube Inoculation	____/____/_____ DD MM YYYY
2.10	MGIT tube result	☐MTBC Positive ☐MTBC Negative ☐Contaminated ☐MTBC positive with contamination
2.11	Date BACTEC MGIT Culture result reported	____/____/_____ DD MM YYYY
Data Collection Form Reviewed by: _____ (for study staff only) Name _____ Signature _____ Date		

Annex II. Procedure for Ziehl-Neelsen staining technique

Principle	The property of acid-fastness is based on the presence of mycolic acids in the cell wall of mycobacterium. Primary stain (fuchsin) binds to cell-wall mycolic acids. Intense decolourization (strong acid or acid/alcohol) does not release the primary stain from the cell

wall and the mycobacterium retain the red colour of fuchsin hence acid-fastness. Counterstaining (with Methylene blue) provides a contrasting background.

While mycobacteria are AFB, very few other bacteria possess the property of acid-fastness, and then only weakly (e.g. *Nocardia*). AFB found in extra pulmonary specimens, particularly gastric washings, stool or urine, should never be automatically assumed to represent TB bacilli

Materials

Reagents
1%Carbolfuchsin staining solution.
3% Acid- alcohol.
0.1% Methylene blue counterstaining solution.
Supplies
•

Quality Control

Control	**Level**	**Stability**	**Frequency**	**Preparation (y/n)**
Unstained positive(1+)	positive	6 month	At least for every newly prepared staining solution	As need arise
Unstained negative	Negative			

Note: Positive and negative control slide should have expected result. If it hasn't this could be problem associated with staining solution preparation, staining procedure or reagent used for staining solution preparation. In this case root cause analysis should be done. The sample of that batch should be retested and patient result is not released.

Procedure

Step	**Action**
1.	Place the slides with smear upwards on the staining rack over a sink about

	finger-width apart and heat fix.
2.	Add carbol-fuchsin staining solutions over the smears
3.	Prepare the torch by dipping its cotton wool end in burning spirit and light it
4.	Heat all slides keeping the torch a little below them until steam arises
5.	do not let staining solution dry on the slides
6.	Leave the heated stain on the slide a minimum of 5 minute.
7.	Tilt each slide using forceps to drain off the staining solution
8.	Rinse the slides well with clean water from a beaker.
9.	Pour decolourising solution over the smears covering them completely
10.	Allow to act for 3 minutes
11.	Tilt each slide with forceps to drain off the acid
12.	Gently rinse each slide again with clean water. Do not splash adjacent slides.
13.	If needed, repeat until all macroscopically visible stain has been washed away.
14.	Flood smear with methylene blue solution for 1 minute.
15.	Gently rinse each slide with water. Do not splash adjacent slides
16.	Tilt each slide with forceps to drain off excess water
17.	Clean back of slide with moist paper
18.	Using forceps place it on draining rack. Always keep smears out of direct sunlight.
19.	Examine slides after it has dried

Appearance of AFB

Viewed with oil immersion , AFB are red , slender rods, some time with one or more granules. the bacilli may occur singly, V-shaped forms, cords, or as clumps. Fragments of bacilli often seen during treatment.

Result Interpretation

Recording

Observation	**Reporting**
No AFB found in at least 100 fields	**Negative**
1-9 AFB in100 fields	**Record exact number of bacilli**
10 - 99 AFB per 100 fields	**1+**
1 to 10 AFB per field (check 50 fields)	**2+**
More than 10 AFB per field (check 20 fields)	**3+**

Annex III. Procedure for Detection of TB and rifampicin resistance using Xpert MTB/RIF test from sputum sample

Principle

The GenXpert (Cepheid) is a closed, self-contained platform for the extraction, amplification and detection of *Mycobacterium tuberculosis* (*Mtb)* complex from unprocessed samples. The GenXpert system is able to generate a result within 2 hours.

The Xpert MTB/RIF assay allows for the rapid detection of *Mtb* and rifampicin (RIF) resistance by combining automated extraction, amplification and detection on a single system. RIF is one of the first line anti-TB drugs and is also a surrogate marker for multi-drug resistant TB. The assay amplifies a portion of the "rifampicin resistance determining region" of the *rpoB* gene, the most common site for RIF mutations, in real-time, using two sets of primers. Fluorescent probes

are then used to differentiate between wild-type and mutant strains so that if one or more probes do not bind, this indicates the presence of a mutation and therefore RIF resistance.

A sample processing control (SPC) consisting of spores from *Bacillus globigii*, is included in the assay as an internal control to ensure adequate processing of the sample as well as to monitor the presence of PCR inhibitors. A probe check control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity and dye stability.

Sample

Sample type	Amount required	Transport and Storage	Stability
Sputum sample	more than 1.0 ml	Triple packaging, cold chain	2-8°C for up to 7 days
Resuspended sputum sediment	More than 0.5ml		2-8°C for up to 7 days

Limitations:

Xpert MTB/RIF test has not been validated for blood samples

Environmental and safety controls

Refer to Health and Safety Manual for safety considerations

- The GenXpert system should be installed on a solid, even base
- Always wear a lab coat and disposable gloves when working with clinical samples.
- Treat all samples as potentially infectious
- If liquid containing potentially infectious agent is spilled, clean affected area with concentrate bleach
- Dispose of all waste in a biohazard medical waste bin

Quality Control	Control preparation: Lot verification for new arrived lots .

Result Interpretation	• **MTB DETECTED** – MTB target DNA is present hence the patient is TB positive • **MTB NOT DETECTED** – MTB target DNA is was not detected hence the test is negative for TB • **RIF Resistance DETECTED -** MTB present is resistant to rifampicin • **RIF Resistance NOT DETECTED** – MTB present is sensitive to rifampicin • **INVALID** – repeat test • **ERROR** – repeat test • **NO RESULT** – repeat test

Annex IV. Procedure for Sample Processing and inoculation using NALC-NaOH Method

Principle	Specimens for tubercle bacilli isolation do usually contain associated flora which has to be eliminated before inoculation of the specimen onto culture media. Culture examination detects fewer bacilli than microscopy and increases the number of tuberculosis cases found, in the range of 20-50%, subject to the local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and enable the detection of drug resistance.
	Reconstitution of PANTA solution Dissolve PANTA in 15 ml MGIT Growth Supplement. Label the date and initial of reconstitution **NALC-NaOH solution**, freshly prepared for daily use only Add 0.5 g NALC to 100ml of the mixture of sodium hydroxide-Trisodium citrate just before use **Reagents stability and storage:** **Reconstitution of PANTA: store at 2-8^{o}C and stable for 5 days.** NALC-NaOH solution is stable only for one day. Sore 2 to 25^{o}C

Quality Control

Control	Level	Stability	Frequency	Preparation (y/n)
Sterile distilled water	Start	Store at 18 to 25 ^{o}C for 3 months	For each test run	No
Sterile distilled water	End	Store at 18 to 25 ^{o}C for 3 months	For each test run	No

Note: Start and End control shouldn't show any growth. If it has growth it could be cross-contamination or any contamination from reagent or material. In this case cause analysis will be done and patient result is not issued. The sample of that run should be retested.

Procedure

Pre-processing preparation:

Label the sample and request form with culture number

Check all the material required for sample processing is in place

Arrange sample, and required material in the biological safety cabinet

Preparation for process using NALC-NaOH

Step	Action
	Check the volume of the sputum (at least 2 ml, not more than 5 ml) (if the volume of the sample is above 5ml, transfer 5ml of the purulent part to another falcon tube and proceed this allocate for the subsequent steps)
	Add equal volumes of NALC-NaOH solution. Use aliquots of NALC-NAOH(1 vial of NALC-NAOH per one specimen)
	Tighten cap of container and vortex slowly
	Shake intermittently to aid homogenization and decontamination
	Invert each bottle to ensure that NALC-NaOH solution contacts all the sides and

	inner portion of caps.
	Keep at 20°C – 25°C for 15 min for decontamination
	Fill the tube with phosphate buffer up to 45 ml mark on the tube. . Use aliquots of phosphate buffer(1 vial of of PBS per one specimen)
	Mix-well or vortex
	Centrifuge at 3,000 ×g for 15 minutes
	Carefully pour off the supernatant into a discard container containing 5% sodium hypochlorite or other germicide. Make sure the final concentration of bleach is 1% after pouring off the supernatant.
	Prepare smear on slide labelled with culture number for microscope
	Re suspend the deposit with 2ml PBS.

Inoculation

MGIT Media

Step	Action
	Mark each MGIT tube with laboratory number.
	Using repeater pipette Add 0.8 ml of the PANTA solution to each MGIT tube just prior to inoculation.
	with disposable Pasteur pipette, Add 0.5 ml of a well-mixed processed and/or direct specimen to the appropriately labelled MGIT tube
	Tightly recap the tube and mix by inverting the tube several times.
	Wipe tubes and caps with a mycobactericidal disinfectant.
	Leave inoculated MGIT tubes at room temperature for 30 min.

LJ Media

Step	Action
	Mark each LJ tube with laboratory number
	Decant excess water from the media

	Inoculate 2 to 3 drops of sediment and/or direct preparation in to two LJ media
	Wipe tubes and caps with a myco-bactericidal disinfectant.

Incubation and follow up of the culture tube

MGIT tube

Step	Action
	Open the desired MGIT 960 drawer and press the “tube enters” key
	The barcode scanner will light up
	Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960
	Check MGIT 960 daily for indicator lights flagging positive and negative cultures
	Incubate MGIT tubes until the instrument flags them as positive (red flag) or negative (green flag)
	Positive and negative tubes will be issued by pull the respective drawer and press “positive or negative button as needed”, the machine displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer
	Remove the tube and scan
	Continue MGIT culture work up
	Register the result in the work sheet if it is final result register in the final registration book (Annex Work sheet for culture reading)

LJ tube

Step	Action
	Loose the cap slightly and put the LJ tube in slant position facing upward in the incubator
	Make sure the fluid cover the surface
	Keep in a slant position for one week

	Check LJ two times a week for any contamination and fast grower Mycobacterium /NTM
	Read LJ tubes weekly until a positive growth obtained (Refer Annex characteristic of reference strain)
	If the LJ tube have growth and enough (>50 matured colony) for subsequent work , quantify the colony (Annex quantification scheme) and issue
	If the LJ tube have growth and not enough for subsequent work write "P" on the work sheet and wait 1 to 3 week till enough growth obtained
	Positive tube will be kept in the incubator till (maximum of 1 week) the subsequent work is done
	If the LJ tube have no growth in 8th week issued as negative
	Negative tube will be safely discarded from the laboratory
	Register the result in the work sheet weekly if it is final result register in the final registration book (Annex work sheet for culture reading)

For LJ: Growth on culture media shall be recorded in accordance with the following schema:

Description	Result/Grade	
Confluent growth	3+	
More than 100 colonies	2+	
20-200 colonies	1+	
< 20 colonies	Exact count	
No growth	Negative	

Annex V. Procedure for MPT 64 Antigen Test

Test procedure

Step	Action

1	Remove the test cartridge from the foil pouch and place it on the BSC work area
2	Avoid touching the specimen placing area on the cartridge with hands
3	Label each cartridge with the sample identification number
4	Place 100µL of the prepared bacterial culture on the pecimen placing area of the test cartridge. ipette tips should be changed between samples
5	Examine the reading area of the test plate after 15 minutes

Interpretation of the test

Step	Action
1	A colour band will appear at the left section of the result window to show that the test is working properly. This band is the Control Line
2	The right section of the result window indicates the test results. If another colour band appears at the right section of the result window, this band is the Test Line
3	The formation of a purple to red line on the reading areas labelled [T] and [C] of the cartridge indicates a POSITIVE result
4	The formation of a purple to red line on the reading area labelled [C] of the cartridge but not [T] indicates a NEGATIVE result
5	If no line is observed on the reading area [C], technical errors or product damage has occurred. In this case, the test should be considered invalid and repeated using a new cartridge

Annex VI. Procedure for Drug Susceptibility testing in the MGIT 960 System

Principle

Susceptibility testing in the MGIT 960 system is based on the same principles as isolation from sputum (detection of growth). DST is performed using an AST (antibiotic susceptibility testing) set or TB exit, which consists of a Growth Control tube and one tube for each drug. A known concentration of drug is added to a MGIT tube, along with the specimen, and growth is compared with a drug-free control of the same specimen. If the drug is active against the mycobacterial isolate (isolate susceptible), growth will be inhibited and fluorescence will be suppressed in the drug-containing tube; meanwhile, the drug-free control will grow and show increasing fluorescence. If the isolate is resistant, growth and its corresponding increase in fluorescence will be evident in both the drug-containing and the drug-free tube. The MGIT 960 system monitors these growth patterns and can automatically interprets results as susceptible or resistant. An isolate is defined as resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug.

Sample

Sample type	Amount required	Transport and Storage	Stability
MGIT positive tube 1 to5 days old	0.5 to 2.5mlof growth	Stored at -20^{0}C or -80^{0}C till tested	NA

Limitations: The test has to be carried out on pure cultures and 1 to5 days old otherwise it will yield invalid results

Sample retention: All positive culture should be stored in storage media at -20^{0}C for three years or -80^{0}C indefinitely

-

Quality Control

Control	Level	Stability	Frequency	Preparation (y/n)
$H_{37}Rv$	Susceptible	3 year when stored at -20°C	Each batch	Yes
MDR	Resistant	3 year when stored at -20°C	Each batch	Yes

Control preparation: Same as drug test for samples

Note: Sterility and performance is carried out for each shipment before used for patient test

If any control in a run gives an unexpected result, none of the results from the test run should be reported to the clinician. The whole batch must be re-examined and cause analysis is initiated.

Procedure

Dehydration of Lyophilized SIRE and PZA Drugs

Step	Action
1.	Using a sterile pipette or transfer pipette, reconstitute each of the SIRE (Streptomycin, Isoniazid, Rifampicin and Ethambutol) lyophilized drug vials with 4 ml of sterile distilled water.
2.	Reconstitute PZA drug vial with 2.5 ml of sterile distilled water. Use separate pipette for each drug.
3.	Aliquot in sterile cryovial tube by considering the average test done at each run
4.	Store aliquoted drugs at -20 ^{0}C up to 6 months or up to the date of original

	expiry, whichever comes first.
5.	Once thawed, discard any leftover drug and do not store or refreeze.

Inoculums preparation for MGIT DST

Age of MGIT	Activities
1-2 days old	1. Positive MGIT cultures must have pure growth of *M. tuberculosis* (ZN positive, BAP negative, MPT/MTB 64 antigen test positive; in order to be tested for drug susceptibility.
	2. DST must not be set up on the same day a MGIT tube signals positive. The day a MGIT tube is positive by the instrument is considered **Day 0.**
	3. If MGIT tube**1-2 days old** after signaling positive, Vortex each seed tube for 1 minute to break up clumps
	4. Leave the tube undisturbed for about 20 minutes to allow large clumps to settle.
3-5 days old	1. If growth is on Day 3, 4, or 5, Vortex each seed tube for 1 minute to break up clumps
	2. Let the large clumps settle for 20 minutes.
	3. Dilute 1.0 ml of the positive broth in 4.0 ml of sterile saline (1:5 dilutions).
	4. Use this well–mixed, diluted culture for inoculation of the drug set.
longer	1. Vortex MGIT broth for 1 min. Leave 20 minutes to allow any large clumpsto settle.
	2. Supplement a new MGIT tube with 0.8 ml Growth Supplement *without* PANTA.
	3. Remove inoculum from the supernatant broth and make a 1:100 dilution of the positiveMGIT tube into sterile saline

than five days	4. Mix tube well by inverting gently several times.
	5. Inoculate new MGIT tube with 0.5 ml of the 1:100 diluted specimen.
	6. Cap tube tightly and mix well by inverting gently several times.
	7. Enter tube into MGIT 960 instrument and monitor until it becomes positive.
	8. Use new tube for DST within one to five days of positivity as described above.

Preparation of inoculums from LJ Media

Step	Action
1.	Colonies from solid media may be used if they are no more than 15 days from the first appearance of positive growth.
2.	Using a sterile loop scrape as many colonies as possible trying not to remove any of the solid medium.
3.	Transfer the growth into a sterile tube containing 2-3drops of saline or distilled water and 8-10 sterile glass beads.
4.	Tighten the cap and vortex the tube for 2-3 minutes to break up any large clumps.
5.	The turbidity of the suspension should be greater than the McFarland number 1 standard.
6.	Let the suspension stand for 30 minutes undisturbed.
7.	Using a sterile pipette, carefully transfer the supernatant suspension into sterile tube containing 2-3drops of saline or distilled water
8.	The turbidity of this suspension should be greater than McFarland 0.5 standard.
9.	Adjust the turbidity of this suspension to McFarland 0.5 standard by adding sterile saline and adjusting visual comparison.

10.	Dilute 1.0 ml of this suspension in 4.0 ml of sterile saline and mix well. This 1:5 dilution will be used as the inoculum for DST

Preparation of 1:100 and 1:10 dilution (for SIRE and PZA growth control)

Step	Action
11.	Prepare the 1:100 by adding 100 µl of the seed (1-2 days old) or 1:5 dilution of 3-5 days old tube to 9.9 ml of saline
12.	Prepare the 1:10 dilution by adding 500 µL of the seed or 1:5 to 4.5 ml saline
13.	Repeat for all MGIT seed tubes or 1:5 dilutions.

Inoculation into SIRE set

Step	Action
1.	Label five MGIT tubes for each test isolate with GC (Growth Control), STR (streptomycin), INH (isoniazid), RIF (rifampicin), and EMB (ethambutol) and laboratory number.
2.	Place the tubes in the following sequence in the 5 tube AST set carrier, from left to right: GC, STR, INH, RIF, and EMB. N.B Epicenter label can be used for DST entry if AST carrier is not used
3.	Add 0.8 ml MGIT SIRE Supplement to each SIRE tube including growth control tube.
4.	Add 0.1 ml (100 µl) of reconstituted drug solutions to the corresponding labelled MGIT tubes.
5.	Do not add drugs to the MGIT GC tube.
6.	Using a sterile transfer pipette, dispense 0.5 ml from the top 1/3 of the 1:100 dilution tube in to the SIRE GC tube .

7.	Inoculate each labelled drug-containing tube with 0.5 ml of the inoculum prepared just inside the mouth of the tube so the inoculum runs down the inside of the tube.
8.	Immediately recap the tube tightly and mix by inverting the tube twice.
9.	Wipe all tubes and caps with a mycobactericidal disinfectant.

Inoculation into PZA set

Step	Action
1.	Label two PZA tubes for each test isolate with GC (Growth Control), or PZA (pyrazinamide).
2.	Place the tubes in the following sequence in the 2 tube AST set carrier, from left to right: GC, PZA. **N.B Epicenter label can be used for DST entry if AST carrier is not used**
3.	Add 0.8mL of PZA supplement to each PZA tube
4.	Add 0.1 ml (100 µl) of reconstituted PZA drug solutions to the corresponding labelled MGIT tubes.
5.	Do not add drugs to the PZA GC tube.
6.	Using a sterile transfer pipette dispense 0.5 ml from the top 1/3 of the **1:10** dilution tube in to the PZA GC tube
7.	Inoculate PZA drug-containing tube with 0.5 ml of the Inoculum prepared just inside the mouth of the tube so the inoculum runs down the inside of the tube
8.	Immediately recap the tube tightly and mix by inverting the tube twice.
9.	Wipe all tubes and caps with a mycobactericidal disinfectant.

Incubation

Step	Action
1.	Enter the inoculated set of DST specimens into the BACTEC 960 instrument using the AST set entry feature.
2.	Be sure that the tubes are loaded according to the order specified above

3.	Be sure that the caps are tightly closed
4.	Open the desired MGIT 960 drawer and press the "tube enter" key.
5.	The barcode scanner will light up.
6.	Scan the inoculated MGIT tube and load into the slot identified by the MGIT 960.
7.	Incubate MGIT tubes until the instrument flags them as positive
8.	Check MGIT 960 daily for indicator lights flagging positive
9.	Positive tubes will be displayed by the indicator light changing to green at the exact location of the tube in the instrument drawer.

Procedural Notes

- If the GC tubes become positive in less than 4 days or remain negative up to 21 days or some other conditions occur which may affect the test results, the instrument report will be as an Error ("**X**"). In such situations, the test needs to be repeated.
- If a laboratory routinely experiences x200 errors when performing DST in the local patient population, the 3-5 day culture can be used undiluted as a first step. Monitor this technique very closely to ensure that an excess of x400 errors is not subsequently produced.
- The turbidity should not be less than McFarland 0.5.
- The tube cap should be tightly closed.

Annex-VII: Procedure for Line probe Assay

Principle

Genotypic DSTs were designed as alternative methods to improve the speed of diagnosis of drug resistant-TB, especially MDR/XDR-TB. Resistance to first line drugs develops through sequential accumulation of mutations in genes targeted by the respective drugs. Several genes were linked to resistance to TB drugs, the most known and used are *KatG* and promoter region of *InhA* for INH, *rpoB* for rifampicin resistance. Mutations in specific codons were identified and used for detecting resistance to specific drugs. Genotype MTBDRplus is based on the DNA-strip technology and consist of three steps: DNA extraction from cultures or clinical. specimens, amplification of the target gene with biotinylated primers and a reverse hybridization. All

reagents needed for amplification are included in the master mix and are optimized for the PCR step of MTBDR*plus* test. The AM-A contains Taq polymerase, PCR buffer and nucleotides. The nucleotides acts as DNA precursors (the four deoxy nucleoside triphosphates, dATP, dCTP, dGTP and dTTP) which will be used as building blocks during the elongation of the single stranded DNA. DNA polymerase (Hot Start *Taq*) is required to elongate the DNA molecule by facilitating the incorporation of the free nucleotides onto the end of the primer, according to the complementary base on the single stranded target DNA. The AM-B contains biotinylated primers for the amplification of specific regions of the mycobacterial chromosome. The Mg2+ in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognise them as substrates during the amplification procedure. The membrane strips used in the hybridization or detection step are pre-coated with specific probes complementary to the amplified nucleic acids. After chemical denaturing, the single amplicons bind to the probes. 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 discriminates 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 coloured precipitate.

Procedure

Transfer 0.5 ml of sediment suspended in phosphate buffer into sterile 1.5 – 2.0 ml conical Eppendorf tubes properly labelled with the laboratory number and date

• Open one tube and one specimen at a time to prevent cross contamination

• Load the rotor inside BSC

• Gently wipe the tubes with 1% solution of sodium hypochlorite before removal from BSC and placing into the rotor

• Place the tube into the rotor in such a position so that to make location of the pellet position easily notable

• Secure the rotor lid

• Centrifuge the tubes at 10 000 g for 15 min

• Remove the tubes inside BSC in 2-5 min after the centrifugation stopped

• Let the tubes stand for 1-2 min in a rack before you proceed for decanting

• Gently aspirate and discard the supernatant using automated pipette and 1.0 ml sterile filtered tips

• Use new tip for each sample

• If the pellet is disrupted sediment it down again

• After all samples were decanted add 100 µl of molecular grade water, vigorously pipette the pellet and then vortex for 1 min to ensure homogeneity of the suspension to facilitate the heat-kill

Preparation of liquid cultures isolates for decontamination

• Work in a dedicated room/area (refer to SOP "Prevention of contamination in line probe assay laboratory")

• Work in BSC

• To prepare liquid culture isolates vortex MGIT tubes for 2 min to break the clumps

• Transfer 1.0 ml of culture to a sterile conical Eppendorf tube 1.5 – 2.0 ml

• Vortex the tubes again for 1 min to make sure the clumps completely by Preparation of solid culture isolates for decontamination

• Work in a dedicated room/area

• Work in BSC

• Re-suspend representative colonies in 300 µl of molecular grade water using sterile loops

• Make sure to avoid pick up of Lowenstein-Jensen media to prevent inhibition of PCR reaction

• Vortex the tubes for 1 min break the clumps

• Refer to the section 4.5.1 for the next steps

Decontamination (heat kill)

• Work in a dedicated room/area (refer to SOP "Prevention of contamination in line probe assay laboratory")

• Inactivate live bacilli in the suspensions by heating in pre-warmed heat block or hot oven at 950C for 20 min

• Check the temperature right before placing the tubes

• If you use a hot oven for decontamination keep an appropriate rack inside to make sure it is always pre-warmed

Preparation of master mix

• Work in a dedicated room/area.

• Clean the room/area

• Label PCR tubes with laboratory numbers quality control identifications

• Label the last 3 tubes as follows: PC (positive control), EC (extraction control) and MC (master mix control)

• Estimate amount of reagents to be used based on the number of samples and quality controls to be tested plus one or two to account for loss on pipettes tips

• Remove the needed amount of reagents from fridge/freezer and thoroughly thaw and mix

• Spin down the drops from the walls and the caps if necessary

• Keep the DNA polymerase and the reagents on the bench in a mini-cooler during the procedure

• Combine ingredients of the PCR reaction in the order indicated in the Table 1 in an Eppendorf tube of an appropriate size

• Open one reagent tube at a time only to prevent contamination and close the reagent tube as soon it is added

• Visually observe the addition of DNA polymerase the tube to make sure it is added

• Gently vortex the tube and spin down for 10 – 15 sec after all reagents added

• Distribute the master mix to labeled with laboratory numbers PCR tubes by 45 µl

• Cover the lid of the PCR rack with the master mix tubes and take the rack to the room/area for addition of DNA templates

• If the tubes are not used the same day keep them at – 20^{o}C

Detection

Step Action

1 Pre-warm HYB and STR solutions (green and red) to 45°C in water bath (15minutes total). Pre-warm RIN (rinse solution) and sterile distilled water to room temperature

2. Pre-warm TwinCubator to 45°C

3 Remove DNA strips from tube (shake strips down to end of tube then remove carefully holding the end of the strip with forceps) and mark them with provided pen according to detection worksheet.

4 Pipette 20µl DEN (denaturing solution) to one end of each well of a clean tray to be used

5 Add 20µl of corresponding amplified DNA sample to the denaturing solution in each well, and mix well by pipetting up and down 5 times.

6 Incubate for 5 minutes at room temperature

7. Carefully add 1 ml HYB (hybridization solution) to each well and in the opposite DEN/DNA mixture. Use single tip for each well. Do not splash mixture or contaminate neigh bouring well.

8 Gently tilt to shake and homogenize solution. Do not splash mixtures

9 Add each labelled strip to each well with coloured marker facing up. If strips turnover, re-position them with a fresh pipette tip. Strips must be completely covered by hybridization solution

10 Place tray on Twincubator and press “START” to incubate for 30 minutes at 45°C.

From this point, press right arrow on Twincubator once to advance steps in Protocol.

11 When alarm goes off, press right arrow key to stop

12 Remove HYB carefully by pipetting with individual sterile pipette tip or sterile disposable Pasteur pipette into a small plastic discard container containing undiluted bleach solution. Change tips or Pasteur pipettes between wells

13 Wipe off condensation that forms on Twincubator lid before every incubation step.

14 Add 1 ml STR (stringent buffer) per well and incubate for 15 minutes in Twincubator at 45°C. Press right arrow key to start.

15 When alarm goes off, press right arrow key. Completely remove STR as previously described for HYB removal.

16 Add 1 ml rinse solution (RIN) to each well. Press right arrow key to rinse the strips

For 1 minute. When alarm goes off, press right arrow key. Completely remove RIN.

17 Add 1 ml of diluted Conjugate per well. Press right arrow to incubate at 37°C for 30 minutes on Twincubator When alarm goes off, press right arrow to stop

19 Completely aspirate CON-D solution using Pasteur pipette

20 Add 1ml RIN per well. Press right arrow and incubate for 1 minute on Twincubator

21 When alarm goes off, press right arrow key to stop. Remove RIN completely and repeat Step 20.

22 Remove RIN completely and wash with 1 ml sterile distilled water per well on TwinCubator. Press right arrow key and incubate for 1 minute on TwinCubator

23 When alarm goes off, press right arrow key to stop. Remove water and add 1 ml of diluted substrate per well.

24 Place on TwinCubator under aluminum foil for a maximum of 10 minutes. Look for color reaction to indicate reaction completion after 4-5 minutes. If color reaction is too weak, replace the foil and re-incubate for several more minutes, up to a maximum of 10 minutes

25 Wash twice for 1 minute with distilled water. Remove distilled water after each wash

26 Use forceps to transfer membrane strips to an absorbent paper and allow to air dry

27 Soak trays in 0.5% bleach for 15 minutes and rinse with distilled water

28 Clean pipettes, instruments and work area with freshly diluted 0.5% bleach, followed by 70% alcohol

29 Switch off the TwinCubator after use Interpretation of the Result.

Annex-VIII: Laboratory Requesting and Reporting Form

1. PATIENT IDENTIFICATION:

 Patient code no _____Age (Yrs.): _____Sex (M/F): _____

 Referring Health Facility: _____ Co-infection: _____

2. TB DISEASE TYPE & TREATMENT HISTORY:

 Site: Pulmonary_____ Extra pulmonary (specify):_____

 Registration Group: New___ Relapse____ Treatment after loss to follow-up___

Treatment after failure of first treatment__ Treatment after failure of retreatment__

 Previous TB drug use: New___ First line___ Second line___ MDR-TB contact___

3. REQUEST FOR TESTING AT TB LABORATORY:

 Reason: Diagnosis__ If diagnosis, presumptive TB___DR___ Follow up___

If follow up, at ___ months during treatment, Follow up at ___ months after treatment

 Specimen: Sputum ____Other (Specify): _____

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

 Requested tests: Microscopy__ Xpert MTB/RIF test__ Culture Phenotypic DST __

 Person requesting examination: Name:_____ Date_____

4. LABORATORY RESULT: Xpert MTB/RIF test result

 Sample Number: _____ Date specimen collected: ____/___/___ (Ethiopian Calendar)

 Date of result: ____/___/__

 Result

M. tuberculosis: Detected_____ Not detected_____ Invalid / No result / Error (Repeat Test) __

Rifampicin resistance: Detected____ Not detected____ Indeterminate result___

Examined by (name and signature): _____

Declaration

I, the undersigned, declare that this M.Sc. Thesis is my original work, has not been presented for a MSc degree in this or any other university and that all sources of materials used for the Thesis have been duly acknowledged.

By: Biniyam Dagne (BSc)

Signature:_____

Date of submission:_____

This Thesis has been submitted with our approval as advisors:

Advisor: Kassu Desta (MSc, PhD fellow, Associate Professor)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.

Advisor: Misikir Amare (MPH, Associate Researcher)

Signature: _____

Date: _____

Place: Addis Ababa, Ethiopia.