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Magnitude of *Mycobacterium tuberculosis*, drug resistance pattern, and associated factors among patients referred to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia.

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This is to certify that the thesis prepared by Melkayehu Kassa, entitled:

Magnitude of *Mycobacterium tuberculosis*, drug resistance pattern, and associated factors among patients referred to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia , and submitted in partial fulfillment of the requirements for Master of Science degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:

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Abbreviations

ATCC=American Type Culture Collection

DST= Drug Susceptibility Test

FMOH=Federal Ministry of Health

HAART=Highly Active Anti-Retroviral Therapy

HIV=Human Immunodeficiency Virus

INH=Isoniazid

IQC=Internal Quality Controls

LJ= Lowenstein-Jensen

LPA=line probe assay

MDR-TB=Multidrug-Resistant Tuberculosis

MOTT= Mycobacteria other than TB

MTB= Mycobacterium Tuberculosis

OADC = Oleic Acid Albumin Dextrose Complex

PPE=Personal protective equipment

PTB= Pulmonary Tuberculosis

RIF=Rifampicin

RMR= Rifampicin Mono-Resistant

SOP= Standard Operating Procedures

SPHMMC= Saint Paul's Hospital Millennium Medical College

SR=sample reagent

STM=Streptomycin

TB=Tuberculosis

TTD= Time to detection

WHO= World Health Organization

XDR-TB= Extensively Drug-Resistant Tuberculosis

Abstract

Background: Tuberculosis (TB) is an infectious disease caused by strains belonging to the Mycobacterium tuberculosis complex. *Mycobacterium tuberculosis* (M. *tuberculosis*) remains one of the most significant causes of death from an infectious agent and a major public health problem in the community. Drug development of drug resistance and its association with Human immunodeficiency virus (HIV) by far the major cause of the current increase in tuberculosis infection. Sub-Saharan Africa is endemic for both TB and HIV infection, and pulmonary tuberculosis (PTB) in the HIV-affected countries of eastern and southern Africa, such as Ethiopia, has increased rapidly in the past decades.

Objective:-To assess the magnitude of *Mycobacterium tuberculosis,* drug-resistance pattern ,and associated factors among patients referred to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia.

Methods: A cross-sectional study was conducted at SPHMMC, Addis Ababa, Ethiopia from Jan to July 2019. Socio-demographic data were collected by using structured questionnaire in face to face interview with patients. Sputum and non-sputum samples were also collected from 436 presumptive TB cases.

All sputum specimens collected underwent digestion and decontamination using the NaOH-NALC method, whereas and extra pulmonary clinical samples were used directly without decontamination. After centrifugation sputum samples, the sediments were examined using Ziehl-Neelsen technique, analyzed with Gene X-pert MTB/RIF assay, and culture using Lowenstein-Jensen (LJ) and positive culture results were tested for drug resistance pattern using Line probe Assay (LPA). The collected data was entered to EPI info 2002 version 3.32 after data editing and cleaning, exported to SPSS version 23 for analysis.

Results: The total of 436 respondents were included in the study, of this 223 (51%) were male. The mean ±SD age the participants was 38±17years. Regarding marital status, 238 (54.6%) participants were Julyried, and majority of the respondents 240(55%) urban resident, 214 (49 %) had monthly income 100-1000 Ethiopian Birr, 278 (63.8%) used carbohydrate as a usually monthly food item. Of the total, 374(85.8%) were diagnosed for pulmonary tuberculosis and 62(14.2%) were for extra-pulmonary tuberculosis, and from all 130 (30-%) were HIV positive individuals. Out of the total participants, the overall LJ culture confirmed *Mycobacterium tuberculosis* was, 27 (6.2%), and three isolates were resistant for either INH or RIF drug, while two of them were MDR-TB based on line probe assays method. In a bivariate logistic regression analysis, having a previous TB-contact history (COR=3.1; 95 % CI: 1.1, 8.7; P=0.03), patient weight loss (COR=3.6; 95 % CI: 1.5-8.8; P=0.004), having pneumonia with chest X-ray diagnosis (COR=3; 95%CI:3, 33; P=0.02), and having $CD4^+$ T-cells count 200-350/mm^3 of blood (COR=8.9; 95%CI:0.5, 0.9; p=0.049) were significantly associated.

Conclusion: The magnitude of M. *tuberculosis* and MDR TB in this study highlights the need for further extended early case detection and managing MDR TB cases to minimize transmission and the suffering of patients.

Key words: *M. tuberculosis*, Resistance pattern, Lowenstein-Jensen and Line probe Assay.

1. Introduction

1.1.1 Background

Tuberculosis (TB) is an infectious disease caused by strains belonging to the *Mycobacterium tuberculosis* complex. *Mycobacterium tuberculosis* (*M. tuberculosis*) remains one of the most significant causes of death from an infectious agent and a major public health problem in the population. *M .tuberculosis* get in to the lungs leads to infection of the respiratory system; however, the organisms can spread to other organs, such as the lymphatics, pleura, bones/joints, or meninges, and cause extra pulmonary tuberculosis (1).

TB is transmitted by respiratory route when a patient is coughing or sneezing, and one strain of TB, Mycobacterium bovis, can be caused by drinking not boiled milk (2).

Airborne transmission of MTB is responsible for primary tuberculosis (TB) infection which can evolve in immune competent, but more frequently in immune compromised hosts into TB. The number of TB cases has increased dramatically worldwide, reflecting the susceptibility of HIV-infected patients to the M. *tuberculosis* complex. Because of this, pulmonary TB was considered in the Centers for Disease Control classification of AIDS as a true AIDS defining illness in HIV-infected patients, similar to Pneumocystis carinii pneumonia, cerebral toxoplasmosis (3).

1.1.2 Magnitude of *M*. *tuberculosis* Mortality and Morbidity

World Health Organization (WHO) estimated that 10 million people developed tuberculosis (TB) and 1.6 million died of TB globally in 2017 and one-fourth of people infected with latent *Mycobacterium tuberculosis* (4).

High mortality rate was observed in different health institution of the Northern Ethiopia; 87 (11.3%) patients died in Mekelle Hospital and Ayder Comprehensive Hospital (5) , 38 (14.02%) children from TB/HIV co-infected University of Gondar Comprehensive Specialized Hospital (6) and from multidrug resistant tuberculosis (MDR-TB) data showed that 61(29.47%) of the patients died in different hospitals of Amhara region, Northwest Ethiopia (7). Generally in Ethiopia, TB mortality rate declined from 393.8/100,000 to 100/100,000 between 1990 and 2016 (with a total decline of 75%), which indicates slow decline and resulted males had higher TB mortality rate than females (8).

1.1.3 Associated factors for *M. tuberculosis* infections

People at highest risk of progressing from latent to active TB disease are those who are immunosuppressed because of HIV or from treatment (e.g., tumour necrosis factor [TNF]-α inhibitors), who are preparing for organ or haematological transplant, who are on dialysis, who are household contacts of patients with pulmonary TB (particularly children <5 years of age) (4) and those exposed to infected animals in the case of *Mycobacterium bovis* (2).

Human immunodeficiency virus (HIV), multi-drug resistant tuberculosis (MDR) is emerging as major challenge facing tuberculosis control programs worldwide particularly in Asia and Africa (9–11).

Inadequate detection and cure rates have been identified as reasons for a mounting global tuberculosis burden. Human immunodeficiency virus (HIV) is by far the major cause of the current increase in tuberculosis infection. The presence of HIV increases the risk of reactivation of a latent *M. tuberculosis* infection and rapid thus progression of the infection (12,13); HIV also increases MTB transmission rates at the community level. Sub-Saharan Africa is endemic for both TB and HIV infection , and pulmonary tuberculosis (PTB) in the HIV-affected countries of eastern and southern Africa, such as Ethiopia, has increased rapidly in the past decades (14).

Some of the risk factors may be malnutrition, tobacco smoke, air pollution, and lack of respiratory infection–control programs and persons who are socioeconomically poor populations, often live in crowded settings and work in poorly ventilated areas (15).

1.1.4 Drug Resistance

Drug-resistant TB, including multidrug-resistant TB (MDR-TB, defined as resistance to at least isoniazid and rifampicin, the two most important first-line anti-TB drugs) and extensively drug-resistant TB (XDR-TB, defined as MDR-TB plus resistance to any fluoroquinolone (such as ofloxacin or moxifloxacin) and to at least one of the three injectable second-line drugs (amikacin, capreomycin, or kanamycin) has emerged as a serious threat to global health (16).

Worldwide, a substantial percentage (~35%) of patients with drug-susceptible TB remains undiagnosed and a staggering percentage (~85%) of patients with MDR-TB remain undiagnosed (17). Out of the people diagnosed with TB, less than 3% are tested to determine the pattern of drug resistance. In addition to drug resistance, another major challenge is the accurate detection of smear-negative disease which disproportionately occurs in HIV-positive people with TB (18).

Immunosuppression induced by HIV modifies the clinical presentation of MTB and its management, while immune restoration induced by highly active anti-retroviral therapy (HAART) may be associated with paradoxical manifestations related to immune reconstitution. On the other hand, MTB influences the prognosis of HIV infection, and anti-tuberculosis drugs interfere with anti-retroviral drugs, including protease inhibitors and non-nucleoside reverse-transcriptase inhibitors (NNRTIs) (19,20).

1.1.5 Stages and diagnosis of tuberculosis

Stages: The stage of tuberculosis is depending on: features associated with duration of the disease, severity and its complications of the disease.

Early Primary Progressive (active) Stage: Immune system does not control initial infection. Patients often have nonspecific signs or symptoms (e.g. fatigue, weight loss, fever). Diagnosis can be difficult: findings on chest radiography may be normal and sputum smear may be negative.

Late Primary Progressive (active) Stage: Patients have more signs and symptom as disease progresses e.g. weight loss, anemia and findings on chest radiograph are normal but diagnosis is via culture of sputum.

Latent Stage: Mycobacteria persists in the body having no signs or symptom occur, do not feel sick.

Diagnosis of tuberculosis: The diagnosis of tuberculosis depends on signs and symptoms of the disease. By using Sputum smear, Polymerase chain reaction, Chest radiography , Sputum culture, drug susceptibility test (1, 14).

Sputum smear microscopy remains the most common way to diagnose pulmonary TB. Depending on the report and method used, smear microscopy can accurately detect TB in 20% to 80% (using fluorescence microscopy methods) of TB cases. However, it could be used to diagnose TB when sputum has sufficient bacillary load, and it cannot detect drug resistance. Thus, HIV-associated TB often goes undetected because people living with HIV (PLHIV), especially those with severe immunosuppression, generally have very low numbers of bacilli (22). Hence, accurate and rapid detection of tuberculosis (TB) and drug resistance are critical for improving patient care and decreasing the spread of TB especially in HIV infected patient and in the case of children (23). In people with HIV living in settings where mycobacterial culture is not routinely available to all patients, a third sputum smear adds little to the diagnosis of TB (24).

Xpert used as an initial diagnostic test for TB detection and rifampicin resistance detection in patients suspected of having TB, MDR-TB, or HIV-associated TB is sensitive and specific(23).

The line probe assay (LPA) method is based on the reverse hybridization principle, in which the mycobacterial 16S-23S ribosomal RNA (rRNA) spacer region is amplified by polymerase chain reaction (PCR) (25).

Rapid differentiation of the Mycobacterium tuberculosis complex (MTBC) and mycobacteria other than tuberculosis (MOTT) is crucial to facilitate early and effective treatment of the patients. Clinical presentation of MTBC and MOTT is not always very clear and routine conventional methods are time consuming (26) .

Regarding to drug susceptibility test, different studies were conducted on different technologies. As the study done to measure the reliability of the Becton Dickinson MGIT 960 system for rapid testing of *Mycobacterium tuberculosis* susceptibility to front-line drugs, MGIT 960 system was confirming good reproducibility between liquid-medium-based systems made by the same company(27).

In the other diagnostic method old MTBDR assay, the new GenoType MTBDR plus assay enhanced the rate of detection of INH resistance from 66 (88.0%) to 69 (92.0%) among the 75 INH-resistant strains and 36 (87.8%) to 37 (90.2%) among the 41specimens containing INH-resistant strains (28).

1.2 Statement of the Problem

In 2015, the percentage of TB patients with known HIV status was highest in the WHO African Region (81%) and the Americas (82%). The level of testing in the 30 high TB/HIV burden countries averaged 64%, but varied considerably from 11% in Indonesia to above 75% in 18 countries (29). Globally, the estimated prevalence of MDR-TB was 3.3% in newly diagnosed patients in the WHO 2015 report. This was higher (20%) in patients with a history of anti-TB treatment(30). MDR-TB is largely a consequence of poor supply management and quality of anti-TB drugs and inadequate or improper treatment which is further worsened by HIV. Recent studies have indicated that on average, new HIV-positive TB patients are at increased risk of MDR-TB compared with HIV-negative patients (31,32).

Poor infection-control practice has also been identified as a major contributing factor in the spread of drug-resistant TB (33,34).

The prevention, diagnosis, and treatment of TB have become more complicated because of HIV-associated TB and multidrug resistant (MDR) TB. Many people die of TB owing to delayed diagnosis, which makes people, mainly in the sub-Saharan region, unable to reduce transmission significantly, and thus the epidemic continues(34). A global TB report estimated that there were about 230,000 (247 per 100,000 population) incident cases of TB in Ethiopia. Ethiopia ranks seventh among the world's 22 high-TB-burden countries, 10th among high-TB-pandemic countries, and fourth in sub-Saharan Africa(30).This makes incidence of MDR-TB is increasing, Ethiopia is one of the 27 high MDR-TB countries, ranked 15th with more than 5,000 estimated MDR-TB patients each year(35,36).

Based on the 2005 nationwide survey in Ethiopia, the prevalence of MDR-TB was 1.6% among new cases and 11.8% in the retreatment cases and rifampicin resistant was lower than 2% in new cases(37).

In 2008, nearly 1 of 3 TB-related deaths (29%) worldwide was considered to be related to HIV infection, and TB contributed to 26% of the estimated deaths due to HIV infection. There were an estimated 1.4 million new cases of TB in HIV-infected persons and 520,000 deaths, which was double the previous estimates. This was not a true increase, but rather, the expansion of HIV testing and the availability of reliable and(38), representative data on prevalence of HIV infection among patients with TB necessitated revision of the estimates. According to the revisions, the number of HIV-infected patients with TB peaked in 2004, with 1.39 million cases and 550,000 deaths(39).

Countries in sub-Saharan Africa accounted for ∼80% of the estimated global burden of HIV infection–associated TB in 2007, followed by countries in Southeast Asia (10%). South Africa alone accounts for nearly one-third of the global burden.

The best estimate is that there were 1.4 million TB deaths in 2015, and an additional 0.4 million deaths resulting from TB disease among HIV-positive people. In terms of cases, the best estimates for 2015 are that there were 10.4 million new TB cases (including 1.2 million among HIV-positive people), of which 5.9 million were among men, 3.5 million among women and 1.0 million among children. Overall, 90% of cases were adults and 10% children, and the male to female ratio was 1.6:1.

Globally, children (aged <15 years) accounted for 6.3% of the new cases that were notified in 2015.

WHO has published a global TB report every year since 1997.The main aim of the report is to provide a comprehensive and up-to-date assessment of the TB epidemic, and of progress in prevention, diagnosis and treatment of the disease at global, regional and country levels (29).

Ethiopia is one of the high burden countries, reflected both in its TB incidence and the estimated rates of MDR TB.WHO estimates for Ethiopia indicates that the rate of MDR-TB is 1.6% for new cases and 12% for retreatment cases(40).

Therefore, the goal of this study was to determine magnitude of *Mycobacterium tuberculosis*, drug resistance pattern and its associated factors among TB-patients referred to St. Paul’s Hospital Millennium Medical College, Addis Ababa, Ethiopia.

1.3 Significance of the study

Ethiopia is one of the 22 high burden countries for TB and World Health Organization (WHO) estimates that 4.5 million people are co-infected with Human Immunodeficiency Virus (HIV) and TB globally. Patients co-infected with these diseases may be inappropriately treated; drug-resistant strains may continue to spread. So, this study provide information about early detection and appropriate treatment initiation for the patient, implementation MDR-TB control measures and magnitude of drug resistance to health care providers**,** ultimately reducing TB case incidence and decreases TB transmission and mortality in general.

2. Literature Review

Globally the latest 2014 WHO tuberculosis report has revised its estimates of new tuberculosis cases worldwide from previous years, and now shows that almost half a million more cases of tuberculosis occurred worldwide than in their 2013 estimate and that the problem of drug-resistant tuberculosis is worsening, with an estimated 480,000 new cases of multidrug-resistant (MDR) tuberculosis in 2013.Report since estimates for the true burden of drug-resistant tuberculosis across Asia, and eastern Europe are the fact that drug-resistance testing(41).

In contrast to western countries, where Pneumocystis jiroveci pneumonia was the commonest AIDS-defining illness, in developing countries TB is the most common life-threatening opportunistic infection (OI) in patients with HIV/AIDS with about 25 to 65 per cent patients with HIV/AIDS having tuberculosis of any organ(42).

The study of drug resistant test performed specifically for RIF to ascertain RIF resistant strain and the non-RIF resistant strains showed; only 6 (2.9%) were resistant to RIF while 197 (96.6%) were not resistant to RIF. Correlation analysis of MTB detected between sex, age and resistant to RIF showed non-significant association (p>0.05). The prevalence rates include: 2.9% and 7.4% for RIF Resistance and Non-RIF resistance respectively(43).

The study done in Tanzania showed from the total of 220, 130 were positive culture. Eight (7.2%) were mono resistant strains: 7 to isoniazid (INH) and one to streptomycin. Four strains (3.5%) were resistant to multiple drugs and the other three (2.7%) were MDR strains: one was resistant to INH, rifampicin and ethambutol and two were resistant to all four anti-TB drugs(14), whereas the other African country study in Zambia indicate Prevalence of rifampicin resistance was 5.9% and there was no statistical significant difference between being male or female (p = 0.721)(44).

The proportion of MDR-TB, RIF mono-resistant (RMR) TB and INH mono-resistant TB as estimated by LPA and DST, was as follows: MDR-TB: 1.38% / 1.26%; RMR-TB: 1.2% / 0.72%; INH mono-resistant TB: 2.1% / 2.4%, respectively(45).

Reports from different parts of Ethiopia suggest that the rate of drug resistant TB is highly variable across the country. For example study from Jimma, Southwest Ethiopia, 136 patients were enrolled in the study. Resistance to at least one drug was identified in 18.4%. The highest prevalence of resistance to any drug was identified against INH (13.2%) followed by STM (8.1%). There was no statistically significant difference in the proportion of any resistance by sex, age, HIV status and history of being imprisoned. The highest mono resistance was observed against INH (7.4%). Mono resistance to streptomycin was associated with HIV infection (crude OR 15.63, 95%CI: 1.31, 187). Multidrug-resistance TB (MDR-TB) was observed in two patients (1.5%)(40).

As the study conducted in Gondar, Ethiopia on the total of 1,820 *M. tuberculosis*-presumptive patients showed, the overall prevalence of *M. tuberculosis*-confirmed cases was 448 (24.6%, 95% CI 0.23–0.27). Of the 448 *M. tuberculosis*-confirmed cases, 71 (15.8%, 95% CI 1.12–1.19) were resistant to rifampicin. Rifampicin-resistant *M. tuberculosis* was observed among HIV seropositives (14 [18.7%]), males (45[17.3%]), and previously treated tuberculosis patients (61 [16.5%])(37).

Of 124 smear-positive pulmonary TB patients, 117 (94.4 %) were susceptible to Rifampicin, while 7 (5.7 %) were confirmed to be resistant to Rifampicin and Isoniazid. The overall prevalence of MDR-TB was 5.7 % (2.3 %among new cases and 13.9 % among previously treated cases). History of previous treatment (OR = 7, P = 0.025)was significantly associated risk factor for MDR-TB(36).

However the other study 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(46)

As the study done in debremarkos, of a total of 403 smear positive TB patients 248(61.2%), there was 48(11.9%) drug resistance TB cases. The prevalence of MDR-TB from both new and previously TB treated cases was found to be 1.5%. There was statically significant association between history of previous TB treatment and chance of developing MDR-TB (47).

In the study conducted Addis Ababa, Ethiopia, 17 of 121 isolates (14.0%) were resistant to one or more of the anti-tuberculosis drugs isoniazid (8.3%), streptomycin (7.4%), rifampin (2.5%), and ethambutol (1.7%). The high rate of drug-resistant isolates (29.6%) coincided with the peak prevalence of HIV infection (77.8%) in patients 35 to 44 years old (48).

Another study conducted on 60 TB lymphadenitis isolates, 8.3% were identified MDR-TB cases and one isolate was isoniazid mono-resistant. Eleven isolates in T3-ETH genetic sub lineage were sensitive to both RMP and INH, while only 2 isolates were MDR-TB. Most of the RMP- resistant isolates showed mutation in codon S531L and all isolates mutated in the katG gene conferring INH resistant strains had mutations in codon of S315T1(49).

In the study of Gondar hospital conducted a total of 344 extra pulmonary tuberculosis suspected clients were included in the study and specimens were taken from lymph node aspirates and body fluids. The overall prevalence of smear positive extra pulmonary tuberculosis was 34 (9.9%). Of these cases of extra pulmonary tuberculosis, lymph node tuberculosis constituted the largest proportion (82.4%). Among the 34 extra pulmonary tuberculosis patients, over half of them (52.9%) were positive for human immunodeficiency virus. The largest proportion of tuberculosis and human immunodeficiency virus cases occurred among persons with in the age group of 31–40 years(50).

Another study conducted in southern region a total of 450 tuberculosis patients aged 15 years and above were enrolled in the study. The overall HIV-seroprevalence rate was 44.4%. The highest rate was observed in the age group 20-39 years. A slightly higher HIV-infection rate was found in males (46%) than in females (41%). Those divorced and widowed patients had higher proportion of HIV sero-positivity. The HIV positivity rate was higher for extra-pulmonary than pulmonary form of tuberculosis (OR = 3.80; 95% CI: 1.49, 9.7)(51).

To date there is no report on the magnitude, anti-tuberculosis drug resistance in SPHMMC, Addis Ababa using gene X-pert and LPA. Therefore, the aim of the present study was to determine the magnitude of *Mycobacterium tuberculosis*, resistant pattern isolated from presumptive patients.

2.1 Conceptual framework

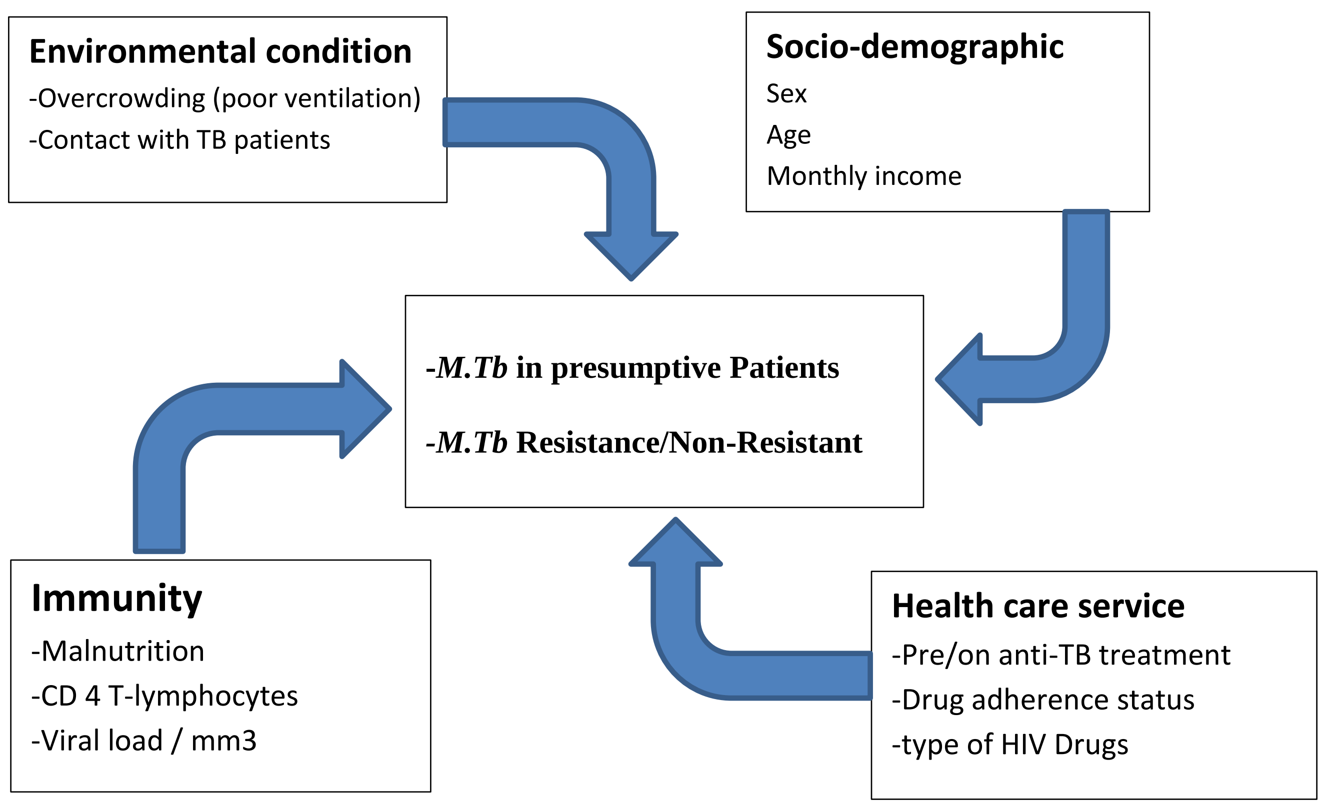

Figure 1: Conceptual framework for tuberculosis of risk factors.

3. Objectives:

3.1. General objective

To assess the magnitude of *Mycobacterium tuberculosis,* drug resistance pattern and its associated factors among patients referred to SPHMMC, Addis Ababa, Ethiopia.

3.2. Specific objectives

- ✓ To determine magnitude of ***Mycobacterium tuberculosis infection*** among patients referred to SPHMMC, Addis Ababa, Ethiopia.
- ✓ To determine drug resistance patterns of ***Mycobacterium tuberculosis*** isolates from presumptive TB patients referred to SPHMMC, Addis Ababa, Ethiopia.
- ✓ To identify associated factors of ***Mycobacterium tuberculosis*** among patients referred to SPHMMC, Addis Ababa, Ethiopia

4. Hypothesis

Ho= There is no magnitude of ***Mycobacterium tuberculosis*** among presumptive patients refered to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia

Ho= There is no drug resistant ***Mycobacterium tuberculosis*** among presumptive patients refered to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia

5. Materials and methods

5.1. Study area

The study was conducted in St Paul’s Hospital ,St. Paul's Hospital was built in 1961 (was named St Paul General Specialized Hospital until 2008) by Emperor Haile Selassie in collaboration with the German Evangelical Church, as a source of medical care for underserved populations. It currently has 392 beds, with an annual average of 200,000 patients and a catchment population of more than 5 million. Approximately 75% of the patients receive medical services free of charge. It has over 1300 clinical and non-clinical staff in over 13 departments, most recently launching its new hemodialysis unit. Starting 2007, the name is changed to St. Paul’s Hospital Millennium Medical College, Addis Ababa, Ethiopia(52).

St. Paul’s receives referrals from around the country and is under the guidance of the Ethiopian Federal Ministry of Health (FMOH).Concerning the laboratory professionals staff members totally who were actively engaged for laboratory work;1M.Sc./Laboratory Manager/, 2 M.Sc/microbiologist/, 30 B.Sc medical laboratory technologists, 7 diploma holders/ laboratory technicians/, 4 phlebotomist, 5 data clerk, 3 runner and 5 cleaners, which gives totally, 56 staff members.

5.2. Study design and period

A cross-sectional study was conducted at SPHMMC, Addis Ababa, Ethiopia from Jan to July 2019.

5.3. Population

5.3.1. Source population

The source population was all patients who visited the SPHMMC, Addis Ababa, Ethiopia during the study period.

5.3.2. Study Population

The study population was all Mycobacterium tuberculosis presumptive patients visited microbiology laboratory and fulfil the inclusion criteria, SPHMMC, Addis Ababa, Ethiopia.

5.4. Inclusion and exclusion criteria

5.4.1. Inclusion criteria

All presumptive *Mycobacterium tuberculosis* patients visiting Microbiology laboratory of St. Paul's Hospital Millennium Medical College.

5.4.2. Exclusion criteria

Patients who have inadequate specimen, previous history of known multidrug resistance for *Mycobacterium tuberculosis*.

5.5. Study variables

5.5.1. Dependent variables

Magnitude of *Mycobacterium tuberculosis* and its drug resistance pattern among presumptive patients.

5.5.2. Independent variables

Independent variables were socio-demographic characteristics (age, sex, etc), symptoms of Mycobacterium tuberculosis infection (radiological sign, fever, weight loss, cough, chest pain diarrhea, lymphadenopathy), and possible clinical risk factors (TB contact history, previous treatment for TB, presumptive DRTB, BCG vaccination status, CD4, Viral load)

5.6. Sample size calculation and Sampling method

5.6.1. Sample size calculation

The sample size was estimated based on the assumption of single population proportion formula, considering the previous studies for both pulmonary and extra pulmonary tuberculosis. For pulmonary tuberculosis study we considered Rifampicin Resistance pattern of *Mycobacterium tuberculosis* done in Debremarkose, Northwest Ethiopia taken as 23%, 5% marginal error, and 95% confidence level, for extra pulmonary tuberculosis we considered a study done in University of Gondar, Ethiopia, which was 9.9%, then by taking the proportion of pulmonary tuberculosis that was done in Debremarkose, Northwest Ethiopia to get the highest sample size, the calculation result determined as:

$$n = \frac{(z^{\alpha}/_{2})^2\ p\ (1-p)}{d^2} \Longrightarrow \frac{(1.96)^2\ 0.23(1-0.23)}{(0.05)^2} = 272 \text{ samples were taken.}$$

Where: n = minimum sample size,

P = estimated rifampicin resistance pattern of *Mycobacterium tuberculosis* for the study population, taken from the previous study (53) for *Mycobacterium tuberculosis*.

d= the margin of sample error, $z^{\alpha}/_{2}$= the standard normal variable at $1-^{\alpha}/_{2}$ confidence level and for EPTB,

$$n = \frac{(z^{\alpha}/_{2})^2\ p\ (1-p)}{d^2} \Longrightarrow \frac{(1.96)^2\ 0.09(1-0.09)}{(0.05)^2} = 126 \text{ EPTB samples were expected (54).}$$

5.6.2. Sampling Method

Non probability consecutive sampling technique was used to select the study population.

5.7. Measurement and Data collection

5.7.1. Data collection procedure

Data collectors were trained and informed how to collect the data for pre-tested and the researcher was explain the aim of the study using information sheet and to obtain consents from the study participants.

Structured questionnaire was used to the socio-demographic status and associated risk factors of the study participants were collected. From each presumptive *Mycobacterium tuberculosis* patients, 2-4 ml of clinical sputum sample was collected. In the case of presumptive extra-pulmonary tuberculosis, 2-4 ml of pus, CSF, lymph node aspirate or peritoneal and pleural fluid samples were collected to detect magnitude of *Mycobacterium tuberculosis* and drug resistance pattern on gene X-pert and LJ culture media.

These data were collected based on the WHO symptom screen (one or more of the following symptoms: current cough, fever, and night, sweats or weight loss). CD4 cell counts or plasma viral load were taken from their log book in ART clinic, SPHMMC. Some of associated risk factors were collected from patient registration card and log book from this hospital, SPHMMC. Half of the collected clinical specimens were analyzed by X-pert in SPHMMC hospital laboratory whereas the remaining half of the clinical specimens were transported to EPHI for culture using LJ culture media.

Transportation specimens: transportation of specimens were transported to the laboratory as quickly as possible. specimens like sputum sample and extra-pulmonary sample other than blood to collect more than 0.5ml(at least) then by using triple packaging, cold chain system to transport and storage in which temperature was maintained as low as possible**.**

5.7.2. **Laboratory procedures and analysis**

Purpose of this standard operating procedure (SOP) describe the procedure for detecting *Mycobacterium tuberculosis* complex bacteria and their rifampicin susceptibility using the Gene X-pert MTB/RIF system; methods of Extra-pulmonary specimen processing for testing in the X-pert MTB/RIF assay and culturing on solid media.

5.7.2.1 Principle and procedure of Gene x-pert

The Gene X-pert® MTB/RIF purifies and concentrates *M. tuberculosis* bacilli from clinical samples. Genomic material isolated from the captured bacteria by sonication and subsequently amplifies the genomic DNA by polymerase chain reaction (PCR). Furthermore, the process identifies all the clinically relevant rifampicin resistance inducing mutations in the RNA polymerase beta (rpoB) gene in the M. tuberculosis genome in a real time format using fluorescent probes Gene X-pert is capable of detecting rifampicin resistance in pulmonary and extra-pulmonary specimens from clinical cases of TB. The Gene X-pert can detect mutations in the rpoB gene and show the results in <2 hours. Finally the results will be recorded (55).

Procedures: Sputum pellets was tested by trained laboratory personnel using the Gene X-pert MTB/RIF. Sample reagent (1.5 ml) was added to 0.5 ml of the re suspended sputum pellet and manually agitated twice at room temperature during a 15-min period and Clinical body fluid samples were treated with a sodium hydroxide and isopropanol-containing (SR). The sample reagent (SR) is added to the sample 2:1 ratio and incubated at room temperature for 15 min. This step is designed to reduce the viability of M. *tuberculosis* in sputum at least 10^6-fold to reduce biohazard risk .The inactivated material then transferred to 2ml to the test cartridge and inserted into the automated machine and subsequent processing is fully automated.

Sample Preparation of CSF, Effusions (synovial, pleural, pericardial, peritoneal, or hydrocele fluid), Lymph nodes.

- ✓ **CSF and Effusions (synovial, pleural, pericardial, peritoneal, or hydrocele fluid)**
- ✓ **If there is more than 5 ml of CSF and Body fluids (sterile)**

For Gene X-pert test, transfer the entire specimen to a conical centrifuge tube, and concentrate the specimen at 3000 g for 15 minutes , Carefully pour off the supernatant through a funnel into a discard can containing 5% sodium hypochlorite ,re-suspend the deposit to a final volume of 2 ml by PBS , Using transfer pipette, add a double volume of the X-pert MTB/RIF Sample Reagent (1.4 ml) to 0.7 ml (2:1) of suspension then transfer 2 ml of the concentrated CSF specimen to the X-pert MTB/RIF cartridge finally load the cartridge into the GeneX-pert instrument following the manufacturer's instructions. For procedure details refer to annex-V.

- ✓ **If there is 2- 5 ml of CSF and Body fluids**

For Gene X-pert testing, add an equal volume of sample reagent to CSF or Body fluids (1:1 ratio), Add 2 ml of the sample mixture directly to the X-pert MTB/RIF cartridge and load the cartridge into the Gene X-pert instrument following the manufacturer's instructions. This procedure is the same as that of LJ or solid culture.

5.7.2.2 Procedure of LJ culture media

The purpose of this procedure is to isolate and semi-quantify growth of M. tuberculosis on LJ medium. It incorporates congo red and malachite green to inhibit unwanted bacteria Slants will be inoculated with decontaminated and concentrated sputum specimens. Once good growth is obtained, these positive slants will be stored in a cool, dark place to archive the positive M. tuberculosis isolates (for more information about the principle, see the annexed section) (41).

Sputum Specimen Processing

Sputum was processed using methods recommended by the United States Centers for Disease Control and Prevention (USCDC), including specimen volume adjusted to 10 ml; equal volume of N-acetyl-l-cysteine w/NaOH-citrate (final concentration of NaOH 1%) added; specimen mixed well and incubated for 15 to 20 minutes at room temperature; PBS (pH 6.8) added up to the 45-ml solution mixed well and centrifuged (3,000 × *g* at 4°C for 15–20 minutes); specimens decanted and re-suspended in 1 to 2 ml PBS (pH 6.8); suspension mixed thoroughly and directly inoculated to solid media (Lowenstein-Jensen [LJ]).

Non sputum Specimen Processing (extra-pulmonary)

Body fluids, such as CSF, synovial fluid and pleural fluid, Lymph node aspirates are collected aseptically and thus can be inoculated into an LJ tube, and a smear was prepared without decontamination.

Mycobacterial Culture and identification

From the sediment 2 or 200µl drop of sediment, one loopful each was inoculated onto two slopes of LJ medium the tubes were incubated at 37°C , LJ cultures were evaluated twice within the first week of inoculation and then once per week for 42 days. Cultures with growth were confirmed as AFB positive by visual inspection or smear. Cultures showing no growth after 8 weeks of incubation were reported as negative. Liquefied or discolored (dark green) LJ media or LJ slants with colonies of non-acid-fast bacteria were considered contaminated .The isolates obtained were confirmed as M. tuberculosis complex by performing an immunochromatography test for the detection of MPT64 antigen(56).For procedure details refer to annex-VI.

5.7.2.3 Acid-Fast Bacilli Microscopy (AFB) Preparation and Staining

The purpose of AFB microscopy is to detect acid-fast bacilli (AFB) by microscopic examination of clinical specimens and cultures. Both living and dead (viable and non-viable) bacilli were stained and be counted. A semi-quantitative grading system is used to report the number of AFB observed in stained smears(57).

The ZN stain confirms the acid-fast property of mycobacteria with ZN and were examined using microscopy. Bacillary density were graded as negative (no AFB seen per 100 fields), and scanty (1-9 per 100 fields, 1+ (10–99 AFB per 100 fields), 2+ (1–10 AFB per 100 fields), and 3+ (>10 AFB in at least 20 fields). 1+, 2+, and 3+, all such smears were defined as ‘‘smear-positive’’.

5.7.2.4 Principle of capilla TB antigen test

MPT64 is one of the major culture filtrate protein, encoded by the RD2 region genes and has been shown to be a specific antigen that differentiates the *M. tuberculosis* complex from the mycobacteria other than tuberculosis (MOTT) species. An MPT64-based, simple and rapid immune-chromatographic assay was developed by the Standard Diagnostics, known as the SD Bioline TB Ag MPT64 RAPID test (SD bioline kit).

This lateral flow test has been reported to identify the *M. tuberculosis* complex from the MOTT using the mouse monoclonal anti-MPT64 antibody were immobilized on a nitrocellulose membrane as the captured material.

The test cassettes strips were incubated for 15-30 minutes at room temperature (RT). The pink band in the 'C' region confirmed the test validity. An additional pink band in the 'T' region was interpreted as positive for the MPT64 Ag. Only the pink band in the 'C' region and no band in the 'T' region were considered negative for the MPT 64 antigen. No band in 'C' region was interpreted as an invalid test. H37Rv was taken as a positive control for each new kit.(26)

5.7.2.5. DST principle for LPA

The GenoType MTBDRplus test is based on DNA strip technology and allows for the molecular identification of the Mycobacterium tuberculosis complex (which includes M. tuberculosis, M. Canetti, M. africanum, M. microti, M. bovis and M. bovis BCG) and its associated genotypic susceptibilities to Rifampicin (RMP) and Isoniazid (INH) (25).

The molecular LPA can be divided into three procedures

1. DNA extraction from NaOH-NALC decontaminated smear positive specimens, or from cultured isolates (solid or liquid media);

2. A multiplex PCR with conditions that are specific for the type of specimen that was extracted and;

3. Reverse hybridization, where probes (reaction zones or bands) on the strips are used to interrogate the M. tuberculosis target DNA associated with RMP and INH resistance by detecting sequences complementary to the probes on the strip (detailed procedure refer to annex)(28).

Line Probe Assay interpretation and reporting

The LPA has two internal controls on the strip: the **Conjugate Control** (line 1), and the **Amplification Control** (line 2). The Conjugate Control line should always be visible to document the efficiency of conjugate binding and substrate reaction. The Amplification Control serves as reference for the interpretation of WT and MUT probes.

5.8. Data Quality Assurance

The questionnaire was pre-tested and proper training prior to the actual data collection was given for data collectors. The necessary adjustments were made after the pre-test. The quality of data was maintained through strictly following the pre-analytical, analytical and post-analytical steps.

Pre-analytical quality assurance

During the laboratory data collection period, the study participants were identified by unique code number, then specimens were collected, labeled with legible hand writing and follow standard operating procedures (SOPs).

Analytical quality assurance

Daily internal quality controls (IQC) run, checked for each test, SOPs was strictly followed and laboratory tests were performed at the right time.

Post-analytical quality assurance

At the end of each day, the collected data were checked by the principal investigator for completeness. Data were documented, entered to statistical software and analyzed.

5.9. Data analysis and interpretation

The collected data were entered to EPI info 2002 version 3.32 after data editing and cleaning it was exported to SPSS version 23 windows software computer program for analysis. The logistic regression was employed to assess the association between of different factors. A p-value of less than 0.05 was considered as statistical significance.

5.10. Ethical considerations

This study was approved by Department of Medical Laboratory Sciences**,** College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia. Then official permission was obtained from Institutional Review Board (IRB) of St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia, and then submitted to laboratory department. Written informed consent was secured from each participant. Patients' names were not recorded on the questionnaire to guarantee confidentiality of the information and privacy of the patients. Infected patients and/or those who had resistance M. *tuberculosis* were informed to their health care provider for better care and management.

5.11. Dissemination of the results

The findings of the study was presented to thesis defense day, to all concerned bodies such as St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia annual research conference. Effort will be made to publish the findings in a peer reviewed journal.

5.12. Operational definitions

MDR-TB is non-susceptibility of *Mycobacterium Tuberculosis* at least two first line drugs or as most rifampin-resistant isolates are also resistant to isoniazid, rifampin resistance can be used as a Julyker for MDR *M. tuberculosis.*

5.13. Work flow

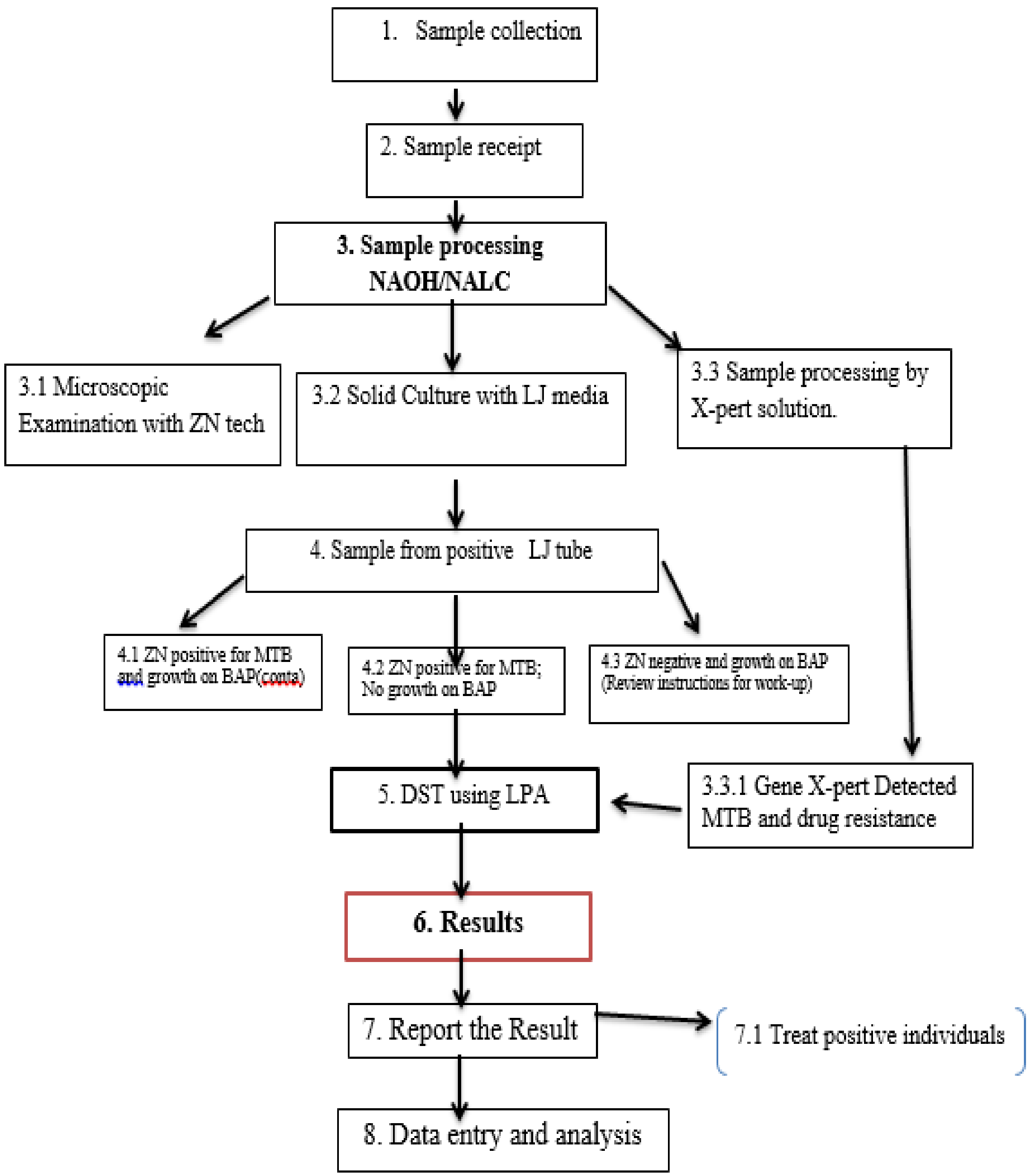

Figure 2: flow chart for workflow.

6. Results

6.1.Socio-demographic characteristics

The total of 436 respondents were included in the study, of this 223(51%) were male. The mean ± SD age the participants was 38±17years. The highest age category was 35-49 years and the least was less than 15 years old. Majority of the respondents were 240(55%) were urban resident, 214 (49%) had monthly income 100-1000 Ethiopian Birr, 278 (63.8%) used carbohydrate as a usually monthly food item, Table 1.

Table 1: Socio-demographic characteristics and magnitude of M. *tuberculosis* drug resistance pattern and its associated factors among patients referred to SPHMMC, Addis Ababa, Ethiopia, 2019.

Variables/ characteristics		M. Tb Present	M. Tb Absent	No. of Participants	Percentages (%)
Sex	Male	16	208	224	51.3
	Female	11	201	212	48.6
Age groups	<15 yrs	3	36	39	8.9
	15-24	4	52	56	12.8
	25-34	6	92	98	22.5
	35-49	9	118	127	29.1
	>50 yrs	5	111	116	26.6
Residence	Urban	13	227	240	55.0
	Rural	14	182	196	45.0
Family size/house	1-3	8	144	152	34.9
	4-6	16	204	220	50.5
	>6	3	61	64	14.6
Julyital status	Single	10	136	146	33.5
	married	12	226	238	54.6
	Divorced	3	17	20	4.6
	Widowed	2	30	32	7.3
Occupational status	Laborer	8	89	97	22.2
	Gov’t Workers	6	91	97	22.2
	Private workers	5	58	109	25.0
	House wife	5	104	70	16
	Student	3	67	63	14.4
Educational status	Illiterate	7	112	119	27.3
	1-8th grades	7	140	147	33.7
	9-12th grades	8	98	106	24.3
	>12th grade	5	59	64	14.7
Monthly Income	<100 Birr	4	56	60	13.8
	100-1000 Birr	4	79	83	19.0
	1001-2000 Birr	8	147	155	35.6
	2001-3000 Birr	5	54	59	13.5
	3001-4000 Birr	0	32	32	7.3

4001-5000 Birr	5	20	25	5.7
>5001 Birr	1	20	22	5.0

6.2 Clinical data

From the total 374 (85.8%) were suspected for Pulmonary Tuberculosis and 62 (14.2%) were suspected for extra-pulmonary tuberculosis, 130 (30 %) were HIV positive individuals. About 422 (96.8 %) of the participants were presumptive TB whereas 14(3.2 %) were presumptive DRTB. In this study 33(7.5%) had history of TB patient/family contact, 68(15.6%) history of alcohol drinking, 22 (5%) history of cigarettes smoking, 319 (73.1%) fever, 311 (71.3%) night sweating and 365(83.7%) had cough. Out of 130 HIV positive participants, 104 (81%) were on anti-HIV treatment and monitored their CD4+ T-cells count, in addition, 119 (91.5%) participants tested for HIV viral load. Higher magnitude seems to appear for those who have $CD4^{+}$count 200-350/mm^3 (5/34) and their viral load was ≥1000/mm^3 (6/90), Table 2.

Table 2:- Clinical characteristics for magnitude of M. *tuberculosis, drug* resistance pattern and its associated factors among patients referred to SPHMMC, Addis Ababa, Ethiopia, 2019.

Variables/ Characteristics		M. Tb present	M. Tb Absent	Number of participants	Percentages (%)
Reason for Diagnosis	Presumptive TB	25	397	422	96.8
	Presumptive DRTB	2	12	14	3.2
BCG Vaccination	Vaccinated	9	147	156	35.8
	Non-Vaccinated	18	262	280	64.2
TB contact History	Yes	5	28	33	7.5
	No	22	381	403	92.5
Alcohol Drinking	Yes	6	62	68	15.6
	No	21	347	368	84.4
Cigarette Smoking	Smokers	2	20	22	5.1
	Non-smokers	25	389	414	95.0
Night Sweating	Yes	23	287	310	71.1
	No	4	122	126	28.9
Presence of Fever	Yes	22	296	318	73.0
	No	5	113	118	27.0
Weight loss	Yes	20	180	200	46.0
	No	7	229	236	54.0
Presence of Cough	Yes	24	340	364	83.5
	No	3	69	72	16.5
Loss of Appetite	Yes	20	265	285	65.4
	No	7	144	151	34.6
Presence of Chest Pain	Yes	16	190	206	47.2
	No	11	219	230	52.8
Presence of Diarrhea	Yes	3	54	57	13.0
	No	24	355	379	87.0
Presence of Dyspnea	Yes	9	131	140	32.1
	No	18	278	296	67.9
External-Adenopathy	Yes	3	60	63	14.4
	No	24	349	373	85.6
Anti-TB Treatment	Previously treated	7	103	110	25.2
	Previously untreated	20	306	326	74.8
Presumptive DRTB	New	24	362	384	88.1
	Relapse	2	44	46	10.6
	Failure	1	3	6	1.4
HIV Status	Positive	10	120	130	29.8
	Negative	17	289	306	70.2
Tuberculosis Type	PTB	24	349	373	85.6
	EPTB	3	60	63	14.4
$CD4^+$ Count	<200 cells/mm^3	0	16	16	15.5
	200-350/mm^3	5	29	34	33.0
	>350/mm^3	1	52	53	51.5
HIV Viral load	<1000/ mm^3	2	27	29	24.4
	≥1000/ mm^3	6	84	90	75.6

6.3 Magnitude of M. *tuberculosis* and resistance pattern.

Out of the total participants, 36(8.3%) were detected with X-pert, and of this figure only 2 (0.5 %) of them were RIF resistant. Regarding culture result, 27(6.2%) were positive and one M. *tuberculosis* strain was resistant for Isozianide drug (mono-resistant) and 2 were resistant for Isozianide and RIF (Multidrug resistant TB). The bivariate logistic regression analysis of socio-demographic characteristics showed, age of less than 15 years old has 1.8 times (95%CI: 0.4, 8.1)) more likely to develop M. *tuberculosis* as compared to age greater than 50 years old, and regarding Julyital status, widowed participants were 2.6 times (95%CI: 0.4, 17) more likely to have M. *tuberculosis* than single, and Gov't Workers were 1.8 times (95%CI: 0.6, 5.9) more likely to have M. tuberculosis than house wife, however, none of the socio-demographic characteristics significantly associated with M. tuberculosis, table 3.

Table 3:- Bivariate analysis of socio-demographic characteristics among patients referred to SPHMMC, Addis Ababa, Ethiopia, 2019.

Variables/ characteristics		MTB Negative	MTB Positive	Total	COR (95% CI)	P-value
Sex	Male	208	16	224	1.4(0.6-3.1)	0.4
	Female	201	11	212	1	
Age groups	<15 yrs	36	3	39	1.8(0.4, 8.1)	0.41
	15-24	52	4	56	1.7(0.4, 6.6)	0.44
	25-34	92	6	98	1.5(0.4, 4.9)	0.55
	35-49	118	9	127	1.7(0.6, 5.2)	0.36
	>50 yrs	111	5	116	1	
Residence	Urban	227	13	240	1	
	Rural	182	14	196	1.4(0.6, 2.9)	0.4
Family size/house	1-3	144	8	152	1	
	4-6	204	16	220	1.4(0.6, 3.4)	0.4
	>6	61	3	64	0.9(0.3, 3.5)	0.8
Julyital status	Single	136	10	146	1	
	Julyried	226	12	238	1(0.2, 5.3)	0.9
	Divorced	17	3	20	0.8(0.2, 3.7)	0.7
	Widowed	30	2	32	2.6(0.4, 17)	0.3
Occupational status	Laborer	89	8	97	1.4(0.4, 4.6)	0.6
	Gov't Workers	91	6	97	1.8(0.6, 5.9)	0.3
	Private workers	58	5	63	1.7(0.4, 6.4)	0.4
	Student	67	3	70	0.9(0.2, 4.0)	0.9
	House wife	104	5	109	1	
Educational status	Illiterate	112	7	119	0.7(0.3, 2.4)	0.6
	1-8th grades	140	7	147	0.6(0.2, 1.9)	0.4
	9-12th grades	98	8	106	0.9(0.3, 3.0)	0.9
	>12th grade	59	5	64	1	
Monthly Income	<100 Birr	56	4	60	1.4(0.2, 13)	0.8
	100-1000 Birr	79	4	83	1(0.1, 9)	0.9
	1001-2000 Birr	147	8	155	1(0.3, 9)	0.9
	2001-3000 Birr	54	5	59	1.8(0.2, 16)	0.6
	3001-4000 Birr	32	0	32	-	0.9
	4001-5000 Birr	20	5	25	5(0.5, 46)	0.2
	>5001 Birr	20	2	22	1	

6.4 Logistic regression analysis

Presence of contact history with tuberculosis infected patients, patient weight loss, pneumonia result with chest-X-ray examination, and $CD4^{+}$results were symptoms and associated factors for *M. Tuberculosis* in the bivariate logistic analysis (Table 4).

Table 4:-Bivariate analysis symptoms and factors for *M. tuberculosis* among patients referred to SPHMMC, Addis Ababa, Ethiopia, 2019.

Variables/ characteristics		**Result of M.TB**			**COR**	**P-**
		Negative	**Positive**	**Total**	**(95%CI)**	**value**
Reason for Diagnosis	Presumptive TB	397	25	422	1	
	Presumptive DRTB	12	2	14	2.6(0.6, 12)	0.2
BCG Vaccination	Vaccinated	147	9	156	1	
	Non-Vaccinated	262	18	280	1.1(0.5, 2.6)	0.7
TB contact History	Yes	28	5	33	3.1(1.1, 8.7)	0.03
	No	381	22	403	1	
Alcohol Drinking	Yes	62	6	68	1.6(0.6, 4.1)	0.3
	No	347	21	368	1	
Cigarette Smoking	Smokers	20	2	22	1.6(0.3,7.0)	0.5
	Non-smokers	389	25	414	1	
Presence of Night Sweating	Yes	287	23	310	2.4(0.8, 7.2)	0.1
	No	122	4	126	1	
Presence of Fever	Yes	296	22	318	1.7(0.6, 4.5)	0.3
	No	113	5	118	1	
Weight loss	Yes	180	20	200	3.6(1.5, 8.8)	0.004
	No	229	7	236	1	
Presence of Cough	Yes	340	24	364	1.6(0.5, 5.5)	0.43
	No	69	3	72	1	
Loss of Appetite	Yes	265	20	285	1.6(0.6, 3.7)	0.33
	No	144	7	151	1	
Chest Pain	Yes	190	16	206	1.6(0.8, 3.7)	0.2
	No	219	11	230	1	

	Yes	54	3	57	0.8(0.2, 2.8)	0.75
Diarrhea	No	355	24	379	1	
	Yes	131	9	140	1.1(0.5, 2.4)	0.88
Dyspnea	No	278	18	296	1	
	Yes	60	3	63	0.7(0.2, 2.4)	0.61
External-Adenopathy	No	349	24	373	1	
	Pneumonia	25	3	28	3(33, 319)	0.02
	Interstitial	28	3	31	3(0.3, 30)	1.0
Chest X-ray	Bronchiectasis	11	1	12	2.6(0.3, 27)	0.34
	Bilateral	6	8	14	9(0.9, 8)	0.4
	Unilateral	14	5	19	0.5(0.6, 4.3)	0.5
	Normal	334	7	331	1	
	Untreated	103	7	110	1	
Anti-TB Treatment	Previously treated	306	20	326	1.1(0.4, 2.5)	0.9
	New	362	24	386	1	
Presumptive DRTB	Relapse	44	2	46	0.7(0.2, 3)	0.6
	Failure	3	1	4	5.0(0.5, 5.0)	0.2
	Positive	120	10	130	1.4(0.6, 3.1)	0.4
HIV Status	Negative	289	17	306	1	
CD 4 Count/ mm^3	<200	16	0	16	1.2(0.9, 2.4)	0.9
blood	200-350	29	5	34	8.9(0.5, 0.9)	0.049
	≥350	52	2	54	1	
Viral Load /mm^3	<1000	27	2	29	1	
blood	≥1000	84	6	90	0.9(0.2, 5.0)	0.9

7. Discussion

The highest TB frequency was observed in age groups of 35-49 years old, living in 4-6 family size / house, regarding to occupation; laborer workers, having monthly income 1001-2000 Ethiopian Birr. This TB magnitude among the productive age group (35-49) years of study participants (9 [33.3%]). This might be due to more exposure to the high workload, and wide range of mobility in these age-groups.

In this study it seems that as the number of family size per house hold increase the prevalence of smear positivity also increases. Family size 5-6 was highly affected by Mycobacterium tuberculosis. Different studies indicated individuals living in larger family size members and malnutrition are at higher risk of developing pulmonary tuberculosis (54), however our study revealed that no association family size/house hold and *Mycobacterium tuberculosis*.

Higher *Mycobacterium tuberculosis* was detected from participants diagnosed the reason for presumptive tuberculosis 25/436 (5.7%), from non-vaccinated for BCG 18/436(4.1%), in non-alcoholic drinkers 21/436 (4.8%), and non-cigarette smokers 25/436 (5.7%).

Again higher *Mycobacterium tuberculosis* result observed in tuberculosis symptoms like in those who have night sweating 23/436(5.2%), fever 22/436(5.0%), weight loss 20/436(4.5%), cough 24/436(5.5%), loss of appetite 20/436(4.5%), and chest pain 16/436(3.7%). The least results were observed those who have diarrhea 3/436(0.7%), dyspnea 9/436(2.0%), and external-adenopathy 3/436 (0.7%) sign and symptoms of *Mycobacterium tuberculosis.*

The distribution of pulmonary tuberculosis was also measured in terms of contact history with chronic coughers, smoking habit and alcoholism to trace the epidemiological features of the disease. In this study, the magnitude of pulmonary tuberculosis was not significantly high in those who had contact with TB infected patients, previous history of anti-TB treatment, drinking of alcohol and in those who were smokers. This findings were different from the studies done in Addis Ababa, Ethiopia in 2011 and north Gondar in 2015 (58,59).

The possible reason might be due to lower number of participant diagnosis the reason for presumptive DRTB in our cases and using more number of participants from urban resident.

Higher result again observed in previously treated patients 20/436(4.5%) with anti-TB drugs and in new patients for presumptive drug resistance tuberculosis 24/436(5.5%).

Statistically significant association was observed between culture positive pulmonary tuberculosis and TB contact History and some of tuberculosis patient symptoms weight loss, having pneumonia and CD 4$^+$ counts. The previous study also indicated that pulmonary tuberculosis associated with the level of CD4$^+$ in HIV patients and the amount of virus present in the participants blood (58,60).

In the current study we found that the overall culture confirmed 27/436(6.2%) *Mycobacterium Tuberculosis*, 3/436(0.7%) non-*Mycobacterium tuberculosis* and 15/436(3.4%) observed as contaminated in Lowenstein Jensen culture media.

The current result seems similar with reports of 10% (20 individuals smear positive) study conducted in Addis Ababa, Ethiopia in 2017(61), prisons settings of East Gojjam Zone, Northwest Ethiopia using using Gene Xpert MTB/RIF, 9(3.4%) (62) and 9.9 % of the study conducted in extra pulmonary tuberculosis at University of Gondar, Northwest Ethiopia (63).

This overall culture confirmed *Mycobacterium Tuberculosis,* 27/436(6.2%) magnitude is lower than the study conducted in the Health Centers of Addis Ababa, Ethiopia reported as 46.0% (233/506) (58), from Metehara sugar factory hospital, eastern Ethiopia (14.2%) (63) and 124 (32.2 %) of studied in two public hospitals in East Gojjam zone, northwest Ethiopia(64).

As compared to retrospective study report, from the University of Gondar Hospital from January 2013 to August 2015, prevalence of (24.6%), we found low result (65).

Our finding also lower than 23.2% of the study conducted in Debre markos Referral Hospital, Ethiopia using Gene Xpert MTB/RIF assay.

The possible reason for the difference might be associated with the variation of the diagnostic methods we used, for example in our cases we used sputum sedimentation concentration technique for microscopic smear examination, Gene Xpert assay and finally LJ culture for confirmation whereas, a single diagnostic tool used in the previous study like; stained by Ziehl-Neelsen staining and examined by Microscopy in the case of Metehara (63), using GeneXpert MTB/RIF in the case of prisons settings of East Gojjam Zone (62). This low prevalence may also mean that there might be a comparatively good TB infection control around our study area, Addis Ababa, Ethiopia.

From the overall confirmed *Mycobacterium Tuberculosis* 6.2% (27/436), a total of three *Mycobacterium Tuberculosis* strain showed resistance pattern to anti-tuberculosis drug, of which two of them were multi drug (INH and RIF) resistance strains. This result lower than the study conducted in the University of Gondar Hospital, northwest Ethiopia which is resulted as 71(15%) resistant to rifampicin (65)among tuberculosis-presumptive cases at University of Gondar Hospital, northwest Ethiopia, 15.58 % of two public hospitals in East Gojjam zone, northwest Ethiopia(64), and 12 (10.3%) patients referred to Debre markos Referral Hospital, Ethiopia (66).

From a total of 130 HIV positive status, only 10 (7.7%) of the participants MTB detected. Out of this sero-positive figure, one mono (INH) resistant and one MDR- TB (INH+RIF) resistant strains were detected. Regarding participants' viral load and TB relation, only one mono resistant strain was found in the participant serum which contain high copies of viral load count ($\geq$1000/mmm^3). This might be due to HIV infection; HIV infection may cause malabsorption of anti-TB drugs and immune suppression which lead to resistance and our result is supported by other findings (67,68).

Similarly, the proportion of our MDR-TB result was lower than from 89 (39.4%) of among multi drug-resistant tuberculosis suspected patients from Ethiopia (69). This may implies the existence of ongoing transmission of drug-resistant strains in our study area due to more participants coming from urban settings and many number of them were educated greater than 8^{th} grade in the educational status.

We found only two (2) Mycobacterium tuberculosis strain resistance to RIF and INH. This result in line with other study done in southwest Ethiopia, 2012 which showed two (1.5%) MDRTB strains (70), but lower than, 23% in the Eastern Ethiopia(71), and reports of northwest Ethiopia 15.58 %, 12 (10.3 %) (65,66) resistant MTB, also lower than 89 (39.4%) to rifampicin Addis Ababa's study in 2019 (69) of the country Ethiopia. This variation might be due to high number of new study participants 384/436 (88 %) for presumptive DRTB, high number of previously untreated study population 326/436(74.8%) in our cases, whereas the already tuberculosis patient used in the previous studies.

The bivariate logistic analysis showed that presumptive drug resistance tuberculosis two times more likely (2.6 (95% CI 0.6, 12, p=0.2)) to develop tuberculosis than presumptive tuberculosis; also having the symptoms of night sweating two times more likely (2.4(95% CI 0.8, 7.2, p=0.1)) to develop tuberculosis than those who did not the symptoms of night sweating. Having the presence of chest pain also (1.6(95%CI 0.8, 3.7, p=0.2)) times more likely to develop *Mycobacterium tuberculosis* than from those who did not have chest pain.

8. Conclusion and Recommendations

8.1. Conclusion:-Presence of contact history with previous tuberculosis infected patients, patient weight loss, presence of pneumonia with radiological examination, and $CD4^+$ results were the identified symptoms and factors associated from M. *Tuberculosis* in the bivariate logistic analysis.

In general, this study highlights low magnitude Mycobacterium tuberculosis and MDR-TB among patients visited to SPHMMC, Addis Ababa, Ethiopia, however from the total of three strains, two of MDR strains were observed on those who have history of failure, relapse and previously treated with anti-TB treatment.

8.2 Recommendation:-Health education about tuberculosis, TB control programs should be continued and large community based study also recommended to sustain these low result of the disease. Strengthening TB infection control activities and proper implementation of DOTS are also recommended to reduce the burden of MDR-TB.

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10. Annexes

Annex- I: Participant Information Sheet

Name of Investigator: Melkayehu Kassa

Institution Name: Addis Ababa University

Title of Project: **Magnitude of *Mycobacterium tuberculosis* (TB), drug resistance pattern ,and associated factors among patients referred to St. Paul's Hospital Millennium Medical College, Addis Ababa, Ethiopia.**

Introduction:

Tuberculosis (TB) is an airborne transmission of MTB is responsible for Mycobacterium tuberculosis (TB) infection which can evolve in immune-competent, but more frequently in immune-compromised hosts into TB. Tuberculosis (TB) is one of the world's most important infection causes of morbidity and mortality in the population When TB is detected and effectively treated, the disease is largely curable.

We are conducting a research on it and we need your voluntary participation. Your participation has useful component for yourself and in the community. Before you decide whether to take part, it is important for you to understand why we are collecting this information and what it will involve. Please take time to hear this paper carefully and discuss it with friends and relatives if you wish to. Ask us if there is anything that is not clear or if you would like more information.

Purpose of the study:

The study aimed at prevalence of TB and drug resistance in among patients referred to SPHMMC. Ethiopia is one of the 22 high burden countries for TB. Patients co-infected with these diseases may be inappropriately treated; drug-resistant strains may continue to spread. In contrast, the X-pert MTB/Rif assay is a rapid test which identifies both the presence of *M. tuberculosis* and resistance to rifampicin in a single test.

So, accurate detection of MTB and diagnosis is useful for timely initiation of appropriate treatment monitoring, decreases TB transmission and mortality in general and knowing of its resistance pattern is useful in particular.

Procedure:

If you decide to participate in the study, you will be asked a series of questions about you and your family. If you have TB, we will also ask questions related to the disease and finally we would like to take **Sputum, Plural fluid, Peritoneal, CSF and Ascetic fluid sample**. The clinical samples will be analyzed in SPHMMC Microbiology Laboratory by X-pert and half of the samples will be going to EPHI for culture.

Benefits: The study will identify accurate detection of MTB and is useful for timely initiation of appropriate treatment monitoring, decreases TB transmission and mortality and is an input to design control strategies of the disease, especially drug resistant pattern. However, you will not get any incentive for your involvement in this study.

Discomfort :this study has no any harm or discomfort on you except little pain during **Plural fluid, Peritoneal, CSF and Ascetic fluid samples collection done by physician and** it may take 10-15 minutes to collect the clinical samples and to fill some of socio-demographic questions.

Confidentiality: The information that we collect from this research will be kept confidential. Information about you that will be collected from the study will be stored in a file, which will not have your name on it, but a code number assigned to it. It will be kept under lock and key, and it will not be revealed to anyone except the principal investigator and the health professional following you.

Right to refuse or withdraw

You have full right to refuse from participating in this research if you do not wish to participate; and this will not compromise the health services you get at the health institutions in any way at any time.

Whom to contact

If you have any questions contact with the following address:

Contact Address:

Melkayehu Kassa= Cell Phone: +251 921613354, email=kassamelkayehu@gmail.com, or

Kassu Desta= Cell phone:+251 911 10 70 99, email= kassudesta2020@gmail.com

Addisu Gize= Cell phone: +251 911809173, email- konjoaddisu@gmail.com

Code No.__________

Annex- II: Consent Form

I have been informed about a study. For this study I have been requested to give any clinical sample for the diagnosis of Tuberculosis. It has been read to me all the information stated in the introductory part and I have had an opportunity to ask any ambiguous question. I got satisfactory answer for all of my concerns. I have fully understood and gave my consent to give the requested clinical specimen.

- Sputum sample ☐
- Plural fluid ☐
- Peritoneal ☐
- CSF ☐
- Ascetic fluid ☐
- Others ☐

It is therefore, with full understanding of the situation that I gave my informed consent and cooperate in the course of the study.

Participant's name______________________ Sign. ________________Date____________

If finger print (illiterate): name of the independent witness, and

Participant's name___________________ Finger print. ________________Date____________

Name of researcher, date and signature of researcher

Melkayehu Kassa_________________ ______/____/_____ (dd/mm/yy) _________

Assent form for children aged 12-17 years

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. **I voluntarily assent that I would participate in this study provided my parents/guardians give their consent on:**

- ❖ Sputum sample ☐
- ❖ Plural fluid ☐
- ❖ Peritoneal ☐
- ❖ CSF ☐
- ❖ Ascetic fluid ☐

and be a participant in this study and understand that I have the right to withdraw from the study at any time.

N*ame of participant, date and signature or thumb impression of participant*

____ ____ /____ /____ (dd/mm/yy) ___________

If illiterate;

Print name of independent literate witness, date and signature of witness (if possible, this person should be selected by the participant and should have no connection to the research team)

____ ____/____/_____ (dd/mm/yy) ___________

Phone number (parents/guardians)____

Name of researcher, date and signature of researcher

Melkayehu Kassa________________ ______/____/_____ (dd/mm/yy) _________

Consent form for parents/guardians

I have read the information above, or it has been read to me. I have been given the opportunity to ask questions and my questions have been answered to my satisfaction. **I voluntarily consent that my child participates** in this study provided he/she gives assent.

- ❖ Sputum sample ☐
- ❖ Plural fluid ☐
- ❖ Peritoneal ☐
- ❖ CSF ☐
- ❖ Ascetic fluid ☐

and be a participant in this study and understand that I have the right to withdraw my child from the study at any time.

Name of participant, date and signature or thumb impression of participant

____ ____ /____ /____ (dd/mm/yy) ________

If illiterate;

Print name of independent literate witness, date and signature of witness (if possible, this person should be selected by the participant and should have no connection to the research team)

____ ____/____/_____ (dd/mm/yy) ___________

Name of researcher, date and signature of researcher

Melkayehu Kassa_________________ ______/____/_____ (dd/mm/yy) _________

Code number__________

Consent form to transport clinical samples to EPHI

Name of main researcher: Melkayehu Kassa, MLS; AAU, Ethiopia.

Advisors/Co-investigators: Kassu Desta and Addisu Gize

Name of institute: AAU and SPHMMC

Funded by: AAU

Reviewed by: DREC (AAU) and SPHMMC, Addis Ababa, Ethiopia

RESEARCH TITLE: **Magnitude of *Mycobacterium tuberculosis,* drug resistance pattern and its associated factors among patients referred to SPHMMC, Addis Ababa, Ethiopia.**

If you agree to take part, please read this form and sign the consent sheets at the end.

1. My questions concerning this study have been answered
2. I know that there is no special payment for being participating in the study.
3. I agree to take part in this study and I have no any objection if half of my clinical sample is transported to EPHI.

Name:________________________ Signature:_________________ Date: ___________________

The participant is unable to sign. As a witness, I confirm that all the information about the study was given and the participant consented to taking part.

Signature____________________________Date_______________________________

We thank you for consenting to take part in the study

Annex-III: Questionnaire

Name of data collector ________________ Code of the client________ Date __________

S. No	I. Demographic Data	
1.2	Age of Respondent	______________________
1.3	Sex	a. Male b. Female
1.4	Marital status	a. Single b. Married c. Divorce d. Widowed
1.5	Resident	a. Urban b. Rural
1.6	Education status	________________________
1.7	Occupational status	A. Laborer B. Gov't worker C. House wife D. Self employer
1.8	Family number (overcrowding)/house	____________________
1.9	Monthly income	______________________
1.10	The usual type of food intake at home	A. Protein (meat, egg, peans & beans

		B. Carbohydrates (injera, bread, cake C. Fats and Lipids (butter, D. Vegetables & fruits (banana, orange..	
II. Clinical Data			
2.1	Reason for diagnosis	A.Presumptive TB	B.Presumptive DR TB
2.2	TB contact history	A.Yes	B. No C. Unknown
2.3	History of alcohol drinking	A. Yes	B.No
	If Qs No 2.3 is Yes, Number of days per week____________________?		
2.4	History of Cigarettes smoking	A. Yes	B. No
	If Qs2.4 is Yes, Number of Cigarettes per day__________?		
2.5	Past BCG vaccination	A.Yes	B. No
2.6	Presence of Fever	A. Yes	B. No
2.7	Loss of Appetite	A. Yes	B. No
2.8	Presence Weight loss	A Yes	B. No
2.9	Presence of Cough	A. Yes	B. No
	If yes Qs 2.9; the duration of cough is ________________________ ?		
2.10	Presence Chest pain	A. Yes	B. No
2.11	External adenopathy	A. Yes	B. No
2.12	Presence Diarrhea	A. Yes	B. No
2.13	Presence Dyspnea	A. Yes	B. No

2.14	Radiological signs	A. Interstitial B. Thoracic lymphadenopathy C. Bronchiectasis, D. Cavity E. Bilateral, F. Unilateral G. Normal	
2.15	Treatment history with anti-TB drugs	A. Previously treated	B. Previously untreated
If previously treated in 2.15; duration of treatment ______________________________?			
2.16	Presumptive DRTB	A. New B. Relapse C. Failure D. Lost to follow-up D. MDR-contact	
2.17	Site of TB	A. Pulmonary. B. Extra-pulmonary	
2.18	Type of specimen	A. Respiratory (sputum). B. Non-respiratory. C. Lymph node aspirate. D. Pus. E. Pleural fluids. F. Others	
2.19	HIV status of the patient	A. Positive	B. Negative
2.20	Treatment of anti HIV drug	C. Pre-treatment	D. On treatment E. Defaulter
2.21	Duration of treatment time for HIV	_______________________	
2.22	Type of the current HIV Drug	_______________________	
2.23	CD4 count	_______________/mm3	
2.24	Viral Load	_______________/mm3	
	III. Laboratory Result		

3.1	Gen x-pert TB	A. Detected	A. Not detected
3.2	If Gen x-pert result positive in Q3.1:	A. Rif Resistance	B. Rif non Resistance
3.3	LJ /Solid media results	A. +ve B. -ve	C. If +ve: D. Resist, Non- Res
3.4	Microscopic results	A. AFB seen	B. Not AFB see

Annex IV: Amharic Version of Annexes

እዝል

እዝል- 1: ***የተሳታፊዎች መረጃ መስጫ ቅጽ***

የተመራማሪው ስም፡ *መልካዬሁ ካሳ*

የመ/ቤቱ ስም፡ አዲስ አበባ ዩኒቨርስቲ

የጥናቱ ርዕስ፤ የሳንባ ና ከሳንባ ውጭ ለሚያጠቃ የነቀርሳ በሽታ ብዛቱን ወይም የሚያመጣውን ጫና፤ መድሃኒት የተላመደ ባህሪውን እንዲሁም ተያያዥ ችግሮችን ከቅዱስ ጳውሎስ ሆስፒታል ህክምና ኮሌጅ ከሚታከሙ ኤች አይ ቪ በሽተኞች ላይ ማጥናት፣ አዲስ አበባ፣ ኢትዮጵያ፡፡

መግቢያ፡-

የሳንባ ነቀርሳ ከሰው ወደ ሰው በዋናነት በእስትንፋስ የሚተላለፍ ማንኛውንም የሰውነት መቋቋም ችሎታ ያለውን የሚያጠቃ ሲሆን በተደጋጋሚ ግን የሰውነት አቅም መቋቋም የማይችሉት ላይ ጎልቶ ይገኛል፡፡ የሣንባ ነቀርሳ በዓለም ላይ ካሉት በሽታዎች በጣም ዋነኛ ህመም አምጭ ና ገዳይ በሽታ ሲሆን በአግባቡ ከታወቀ ና በደንብ ከታከሙት ግን በቀላሉ ሊድን የሚችል ነው፡፡

እኛ በዚህ በሽታ ላይ ምርምር ጀምረናልና የእናንተን በጎ ፈቃድ ተሳትፎ ልናገኝ ይገባናል፡፡ ሥለዚህ በጥናቱ ክመሳተፍዎ አስቀድመው ለምን መረጃው እንዳስፈለገን ከዚህ በታች በተዘረዘሩት ነጥቦች ማዳመጥና መረዳት ይችላሉ፣ ግልጽ ካልሆነለዎት ከሚቀርቡትን ሰው ወይ ጓደኛ ጋር መመካከር ይችላሉ፡፡

የጥናቱ ዓላማ፡-

የዚህ ጥናት ዓላማ **የሳንባ ና ከሳንባ ውጭ ለሚያጠቃ የነቀርሳ በሽታ ብዛቱን ወይም የሚያመጣውን ጫና፤ መድሃኒት የተላመደ ባህሪውን እንዲሁም ተያያዥ ችግሮችን ከቅዱስ ጳውሎስ ሆስፒታል ከሚታከሙ ኤች አይ ቪ በሽተኞች ላይ** ዳሰሳ ማድረግ ነው፡፡ ጥናቱ የሚያካትተው ሁሉንም የእድሜ ክልል የሆኑትን ሰዎች ነው፡፡ ይህ ጥናት የሳንባ ና ከሳንባ ውጭ ለሚያጠቃ የነቀርሳ በሽታ ብዛቱን ወይም የሚያመጣውን ጫና፤ መድሃኒት የተላመደ ባህሪውን እንዲሁም ተያያዥ ችግሮችን ከቅዱስ ጳውሎስ ሆስፒታል ከሚታከሙ ኤች አይ ቪ በሽተኞች ላይ የሚያጠና ሲሆን፣ ውጤቱም ታማሚዎችን ትክክለኛውን መድሃኒት እንዲጀምሩ ያደርጋል፣

መድሃኒት የተላመደን የነቀርሳ በሽታ ለመቀነስም ሆነ በሙያው ላሉት ባለድርሻ አካላት ለማሳወቅ ከፍተኛ ሚና ና ጉልህ ድርሻ ይኖረዋል።.

የጥናቱ ሂደት፡-በጥናቱ ለመሳተፍ ከወሰኑ፣ የተወሰኑ ተከታታይ ጥያቄዎችን ስለ እርስዎ እና ስለቤተሰብዎ፣ስለኑሮዎ ሁኔታ ቃለመጠይቅ እንጠይቅዎታለን። ሣንባ ና ከሳንባ ውጭ በሽታ ጥርጣሬ ካለብዎት ደግሞ ከበሽታው ጋር የተያያዙ ጥቂት ጥያቄዎችን ከተጠየቁ በኋላ በመጨረሻም የአክታ ወይንም ሌላ የሰውነት ፈሳሾችን ናሙና ይሰጣሉ። የናሙናው ምርመራም የሚካሄደው ጳውሎስ ሆስፒታልና የተወሰነው ደግሞ በኢትዮጵያ ህብረተስብ ጤና ምርምረ ማዕከል አዲስ አበባ፣ ኢትዮጵያ ነው።

የጥናቱ ትቅም፡-በጥናቱ ወቅት ትክክለኛው ነቀርሳ ይታወቀል፣ ከታወቀም በኋላ ትክክለኛውን መድሃኒት በአግባቡ እንዲወስዱ ይመከራል፣ የነቀርሳ መተላለፊያ መንገዱን፣ ህመሙን ና ሞትን ይቀንሳል ብሎም የቁጥጥር ስራዎችንም ያጠናክራል፣ በተለይም ደግሞ መድሃኒት ለተላመደ ነቀርሳ በሽታ ጉልህ ሚና ይኖረዋል። ነገር ግን በጥናቱ ስለተሳተፉ ምንም አይነት የገንዘብ ጥቅማጥቅም አይኖረውም ።

የጥናቱ ጉዳት፡- ከ10-15 ደቂቃ ማህበራዊ ነክ የሆኑ ቃለመጠይቆችን ሲጠየቁ ሊወስድብዎት ይችላል። የሰውነት ፈሳሽ በሚሰጡበት ጊዜ በመርፌ ስለሚቀዱ ትንሽ የህመም ስሜት ሊኖር ይችላል፤ ነገር ግን ልምድ ያላቸው ባለሙያዎች ስላሉን አይጨነቁ።

የሚስጥር አጠባበቅ፡- ማንኛውም ስለእርስዎ በጥናቱ የሚሠበሰብ መረጃ ሚስጥርነቱ በተጠበቀ መልኩ ይቀመጣል።ማንኛውም እርስዎን የሚመለከት መረጃ ከጥናት ክፍል ሲወጣ ስምና አድራሻዎ እንዳይኖረው ይደረጋል።

የማቋረጥና የመቃወም መብት፡-በማንኛውም ጊዜ ቃለመጠይቅ ላለማድረግና ናሙና ላለመስጠት ከፈለጉ ማቋረጥ ይችላሉ ነገርግን የሚሠጡን መረጃ ለጥናቱ በጣም ጠቃሚ መሆኑን አይዘንጉ። በዚህም ምክንያት በሚያገኙት የጤና አገልግሎት ላይ ምንም አይነት ተጽእኖ አይኖረውም።

ተጨማሪ መረጃ ከፈለጉ፤

ከዚህ በታች በተገለፀው አድራሻ መጠየቅ ይችላሉ፡፡

መልካዬሁ ካሳ = የሞባይል ስ.ቁ. +251 921613354, **ኢሜል**፡=kassamelkayehu@gmail.com,

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በዚህ ጥናት ላይ ለመሳተፍ ፈቃደኛ በመሆንዎ አስቀድሜ ላመሰግንዎት እፈልጋለሁ፡፡

የተሳታፊዎች የስምምነት ቅፅ **መለያ ቁጥር--------------**

ከላይ የተጠቀሰውን መረጃ በአጥኚው ወይም ተወካዮች አስፈላጊውን ገለፃና ማብራሪያ ተደርጐልኛል፡፡ ማንኛውንም ግልፅ ያልሆነ ጥያቄ የመጠየቅና መልስ የማግኘት ዕድል ተሰጥቶኛል፡፡ ላልገባኝና ለጠየቁት ጥያቄም በቂ መልስ ስላገኘሁ፤ በጥናቱ ለመሳተፍ ሙሉ ፈቃደኛ መሆኔን በፊርማዬ እያረጋገጥሁ የተጠየቅሁትን የአክታና የሰውነት ፈሳሾችንም ናሙና እንደአስፈላጊነቱ ለመስጠት ወስኛለሁ፡፡

የጥናቱ ተሳታፊ ስም__________________________ ፊርማ__________________________

ቀን________

መፃፍና ማንበብ ለማይችሉ፤

የጥናቱ ተሳታፊ ስም________________________ የጣት አሻራ ፊርማ_____ ቀን ________

የተመራማሪው ስም፣ ቀን ና ፊርማ፡

መልካዬሁ ካሳ ________________/ ___________

እድሜያቸው ከ12-17 አመት ለሆኑት ህፃናት ተሳታፊዎች የስምምነት ቅፅ

ከላይ የተጠቀሰውን መረጃ በአጥኚው ወይም ተወካዮች አስፈላጊውን ገለፃና ማብራሪያ በቤተሰቦቼ ወይንም በአሳዳጊዎቼ ተደርጎልኛል፡፡ ማንኛውንም ግልፅ ያልሆነ ጥያቄ የመጠየቅና መልስ የማግኘት ዕድል ተሰጥቶኛል፡፡ ላልገባኝና ለጠየቁት ጥያቄም በቂ መልስ ስላገኘሁ፤ በጥናቱ ለመሳተፍ ሙሉ ፈቃደኛ መሆኔን በፊርማዬ እያረጋገጥሁ የተጠየቅሁትን የአክታና የሰውነት ፈሳሾችንም ናሙና እንደአስፈላጊነቱ ለመስጠት ወስኛለሁ፡፡

የጥናቱ ተሳታፊ ስም________________________ ፊርማ________________________ ቀን________

መፃፍና ማንበብ ለማይችሉ፤

የጥናቱ ተሳታፊ ስም______________________ የጣት አሻራ ፊርማ_____ ቀን ________

የተመራማሪው ስም፣ ቀን ና ፊርማ፡

መልካዬሁ ካሳ ______________/ __________

እድሜያቸው ከ12 አመት በታች ለሆኑት ህፃናት ተሳታፊዎች የወላጆቻቸው የስምምነት ቅፅ

ከላይ የተጠቀሰውን መረጃ በአጥኚው ወይም ተወካዮች አስፈላጊውን ገለፃና ማብራሪያ በቤተሰቦቼ ወይንም በአሳዳጊዎቼ ተደርጎልኛል፡፡ ማንኛውንም ግልፅ ያልሆነ ጥያቄ የመጠየቅና መልስ የማግኘት ዕድል ተሰጥቶኛል፡፡ ላልገባኝና ለጠየቁት ጥያቄም በቂ መልስ ስላገኘሁ፤ ልጄ በጥናቱ እንዲሳተፍ/እንድትሳተፍሙሉ ፈቃደኛ መሆኔን በፊርማዬ እያረጋገጥሁ የተጠየቅሁትን የአክታና የሰውነት ፈሳሾችንም ናሙና እንደአስፈላጊነቱ ለመስጠት ወስኛለሁ፡፡

የጥናቱ ተሳታፊ ስም_________________________ ፊርማ_________________________ ቀን________

መፃፍና ማንበብ ለማይችሉ ከሆነ፤

የጥናቱ ተሳታፊ ስም______________________ የጣት አሻራ ፊርማ_____ ቀን ________

የተመራማሪው ስም፣ ቀን ና ፊርማ፡

መልካዬሁ ካሳ _______________/ ___________

መለያ ቁጥር _______________

ወደ ኢትዮጵያ ህብረተሰብ ጤና ማዕከልናሙና ለማጓጓዝ የስምምነት ቅጽ

የተመራማሪው ስም፡ መልካዬሁ ካሳ፤

የት/ቤቱ ስም፡ አዲስ አበባ ዩኒቨርስቲ

እርዳታ አድራጊው መስሪያ ቤት ስም፤ አዲስ አበባ ዩኒቨርስቲ

የአረጋገጠው መስሪያ ቤት ስም፤ አዲስ አበባ ዩኒቨርስቲ እና ቅዱስ ጳውሎስ ሆስፒታል ኮሌጅ

አማካሪዎች ስም ዝርዝር፤ ካሱ ደስታ እና አዲሱ ጊዜ

የሚሰሩበት መስሪያ ቤት፤ አዲስ አበባ ዩኒቨርስቲ እና ቅዱስ ጳውሎስ ሆስፒታል ህክምና ኮሌጅ

የጥናቱ ርዕስ፤ የሳንባ ና ከሳንባ ውጭ ለሚያጠቃ የነቀርሳ በሽታ ብዛቱን ወይም የሚያመጣውን ጫና፤ መድሃኒት የተላመደ ባህሪውን እንዲሁም ተያያዥ ችግሮችን ከቅዱስ ጳውሎስ ሆስፒታል ከሚታከሙ ኤች አይ ቪ በሽተኞች ላይ ማጥናት፣ አዲስ አበባ፣ ኢትዮጵያ።

በጥናቱ መስማማት ከቻሉ፤ ከዚህ በታች ያሉትን አንብበው ወይም ተነቦለዎት መጨረሻ ላይ መፈረም ይችላሉ ።

1. ስለ ናሙናዬ መጓጓዝበተመለከተ ጥያዬ ተመልሶልኛል ።
2. በጥናቱ ተሳታፊ በመሆኔ ና ናሙናየ ወደ ኢትዮጵያ ህብተሰብ ጤና ማዕከል ተልኮ

   ስለተሰራ ምንም አይነት ክፍያ እንደሌለው አውቃለሁ ።

3. በጥናቱ ለመሳተፍ እና ግማሹ የሰጠሁት ናሙና ወደ ኢትዮጵያ ህብተሰብ ጤና ማዕከል እንደሚሄድ አውቄ ተስማምቻለሁ።

የጥናቱ ተሳታፊ ስም_________________________ ፊርማ_________________________

ቀን________

መፃፍና ማንበብ ለማይችሉ ፤

የጥናቱ ተሳታፊ ስም______________________ የጣት አሻራ ፊርማ_____ ቀን ________

የተመራማሪው ስም፣ ቀን ና ፊርማ፡ መልካዬሁ ካሳ _______________/ __________

በጥናቱ ፈቀደኛ ሆነው ስለተሳተፉ እናመሰግናለን።

እዝል 3- ቃለ መጠይቅ

የጠያቂ ስም _____________________ቀን:___________ ፊርማ_______________የተሳታፊ መለያ ቁጥር______

I	**ክፍል 1: የተሳታፊው ማህበራዊ ነክመረጃዎች**		
	ጥያቄዎች	**መልሶቻቸው**	
1.2	እድሜ	____________________________	
1.3	ጾታ	c. ወንድ d. ሴት ____________________________ ____________________________	
1.4	የጋብቻ ሁኔታ?	1. ያላገባ 2. ያገባ 3. የተፋታ 4. የሞተበት	
1.5	አድራሻ ?	1. ከተማ 2. ገጠር	
1.6	ከጥናቱ በፊት የነቀርሳ መድሃኒት ወስደዋል?	1. አዎ፣ ወስጃለሁ 2. የለም፣ አልወሰድኩም	
1.7	ከነቀርሳ ታማሚ ጋር ንክኪ አሉዎት ?	1. አዎ 2. የለም 3. አይታወቅም	
1.8	ሥለ ነቀርሳ ተከትበዋል?	1. አዎ 2. የለም	
1.9	የቤተሰብ ቁጥር ብዛት ?	________________________	
1.10	የወር ገቢዎ?	________________________	
II.	**የጤና ምርመራ መረጃ**	1. ትኩሳት አለዎት?	1. አዎ 2. የለም
		2. ክብደት መቀነስ	1. አዎ 2. የለም

		3. ሳል አለዎት?	1. አዎ 2. የለም
		4. የደረት ህመምስ	1. አዎ 2. የለም
		5. ከሳንባ ውጭ ያለ አካል ዕብጠት ና ማቃጠል	1. አዎ 2. የለም
		6. ተቅማጥ አለዎት?	1.አዎ 2. የለም
		7.ቶሎቶሎ መተንፈስ	1. አዎ 2. የለም
		8.የራጅ ምርመራ ምልክቶች	1.አንጀት 2. ደረት ላይ እብጠት ና ማቃጠል 3. ሳንባን ማቃጠል 4. ባይላተራል የራጅ ምልክት 5. ዩኒላተራል የራጅ ምልክት
		9. የፀረ-ቲቢ መድኃኒት አጠቃቀም ታሪክ	1.ካሁን በፊት ወስጃለሁ 2.እስካሁን አልወሰድኩም
		10. የፀረ-ኤች አይ ቪ መድኃኒት አጠቃቀም ታሪክ	1. ወስጀ አላውቅም 2. እየወሰድኩ ነው
		11. የፀረ-ኤች አይ ቪ መድሃኒቱን እየወሰዱ ከሆነ ለምን ያህል ጊዜ ቆዩ	____________________
		12. የሚወስዱት ፀረ-ኤች አይ ቪ መድሃኒት አይነት	____________________

		13. መድሃኒቱን የተላመደ ነቀርሳ በሽታ የመሆን ዕድል	1. አዲስ ታካሚ 2. በድጋሜ ያገረሸ 3. ስኬት አልባ 4. ክትትል ማቋረጥ 5. መድሃኒቱነ የተላመደ ነቀርሳ ጋር ንክኪ
		14. በሽታው የሚገኝበት አካል	1. ሣንባ ውስጥ 2. ከሣንባ ውጭ
		15. የናሙናው አይነት	1. አክታ 2. ከመተንፈሻ አካል ውጭ 3. ከእባጭ አካባቢ የተወሰደ 4. ፈሳሺ 5. መግል 6. የሳንባ ፍሳሺ 7. ሌላ
		16. የሲዲ4 ቁጥር	____________/ሚሜ^3
		17. ቫይራል ሎድ	______________/ሚሜ^3
III	**የላቦራቶሪ ውጤት**	1. ጅን ኤክስፐርት	2. ተቢ የተገኘ/የታወቀ 3. ቲቢ ያልተገኘ/ያልታወቀ
3.1	በጅን ኤክስፐርት ውጤት ከሌለው	1.ካልቸር ላይ የታወቀ 2.በካለቸር ያልታወቀ	
	ጅን ኤክስፐርት ውጤት ካለው፣	1. መድሃኒቱን የተላመደ ነወ 2. መድሃኒቱን ያልተላመደ ነው	
	ካልቸሩ ውጤት ያለው ከሆነ፤ መድሃኒቱ ፍቱን መሆኑን መስራት	1. መድሃኒቱን የተላመደ ቲቢ በ LJ 2. መድሃኒቱን ያልተላመደ ቲቢ በ LJ	

አመሰግናለሁ

Annex- V: SOPs for detection of TB using Gene x-pert MTB/RIF test.

Purpose

These standard operating procedures (SOPs) describe the procedure for detecting Mycobacterium tuberculosiscomplexbacteriaandtheirrifampicinsusceptibilityusingthe Gene Xpert MTB/RIF system.

1. Principle

The Gene Xpert MTB/RIF system is a fully automated nested real-time PCR system, which detects MTB complex DNA in smear positive and negative sputum samples and some types of non-sputum samples. It simultaneously identifies mutations in the rpoB gene, which are associated with rifampicin resistance.

The Gene Xpert MTB/RIF system consists of the instrument, a computer, a barcode scanner and requires single-use disposable Xpert MTB/RIF cartridges that contain assay reagents. Following a3-stepsample preparation in the laboratory, the specimen is transferred in to the MTB/RIF cartridge and entered into the Gene Xpert instrument.

By starting the test on the systems of tware, the Gene Xpert automates all following steps, including sample work-up, nucleic acid amplification, detection of the target sequence and result interpretation.

The primers in the Xpert MTB/RIF assay amplify a portion of the rpoB gene containing the 81 base pair "core" region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with RIF resistance.

Mutations in the rpoB gene encoding for β-subunit of RNA polymerase:

- Rifampicin prevents binding to RNA polymerase to DNA, which stops RNA synthesis and thus protein synthesis, leading to a loss of essential functions for the cell and killing the bacilli. Mutations in rpoB, the target of Rifampicin, allow the enzyme and the cell to function normally.
- 95% of all resistance to rifampicin are due to mutations in the rpoB gene and 5% due to mutations outside the gene
- > 90% of mutations in the rpoB gene are located in the 81 base pairs region (codons 507 – 533).

Furthermore, the assay includes a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor the presence of inhibitor (s) in the PCR reaction. A Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability.

2. Specimens

Note: Any in coming specimen must be properly labeled, as a minimum with a unique identification number. This identification is also written on the request form and must correspond with the identification in the laboratory register.

2.1 Type of Specimen

- A single sputum specimen is recommended for Xpert MTB/RIF:
- An additional sputum specimen may be needed in case of an error or invalid Xpert MTB/RIF result.
- 2-4ml of sputum should be collected**.**
- X-pert MTB/RIF is NOT recommended for monitoring patient's treatment.
- The Gene X-pert MTB/RIF is only validated for sputum and concentrated sputum but other samples may be used (pleural, lymph node aspirate or tissue, CSF, gastric fluid and tissue other than lymph node).

2.2 Storage of Specimens

If immediate processing is not possible, the specimens can be stored at room temperature for 3 days (without addition of sample reagent) or at 2-8°c for 10 days, at -70 for longer time.

It can be incubate sputum in sample reagent (SR) for up to 5 hours before loading the cartridge at room temperature, or up to 12 hours if refrigerated at 2-8 ^{O}C, (Note: Once sample is added to cartridge, testing must start within 4 hours).

2.3 To Receive specimen in the Laboratory

Check the quantity and quality of specimens:

- ✓ Check the volume (Ideally 1-4 ml for X-pert MTB/RIF testing)
- ✓ Record sputum consistency (Mucoid, purulent, Bloody or Watery)
- ✓ Ensure that specimens do not contain food particulates.
- Check that the patient's information is complete, and that Xpert MTB/RIF testing has been requested in accordance with the NTP's guidelines.

- Follow guidelines for rejecting samples.
- Ensure that the patient's information is complete on both the request form and the specimen container, and ensure that the information matches.
- Record the specimen in the laboratory register and allocate a laboratory serial number to the specimen.

3. Equipment and Materials

3.1. Equipment

- Place the Gene X-pert on a hard, level surface away from any vibrations (e.g. centrifuge).
- Provide at least 10X15 cm of clearance on each side of the Gene X-pert.
- Do not block the exhaust fan or the air intake on back side of the unit.
- Do not place Gene X-pert in direct sun.
- Do not place next to a window.
- Do not place Gene X-pert directly under A/C unit
- Ambient temperature in the room must be between 15-30 ^{O}C.
- Make sure the power cord connection and the power switch are accessible.

3.2 Gene X-pert MTB/RIF assay Reagent

- MTB/RIF cartridges(50x) and Sample Reagent(50x 8mlbottles)
  - ✓ Keep in a clean, dry, well-ventilated, organized and secured storage area
  - ✓ X-pert MTB/RIF kits must be kept at 2-8°Cto maintain shelf-life.
  - ✓ Store away from direct sunlight.
  - ✓ Keep the bin cards (inventory) updated.
  - ✓ Organize existing and new shipments by expiry date (supplies with the soonest expiry date must be used first).
- **MTB/RIF cartridges contain**
  - ✓ Processing Chambers which contain reagents, primers and probes, buffers and extracted samples.
  - ✓ Amplification tubes to perform rapid thermal cycling and optical excitation/detection.
  - ✓ Valve body: - it directs the fluids into the different chambers and PCR tubes.

3.3 Material required

- ✓ Sterile screw-capped specimen collection containers
- ✓ 30-50 ml capacity

- ✓ Translucent or clear material
- ✓ Leak-proof with a screw-cap
- ✓ Wide mouth
- ✓ Label with the patient's name, identification number and the date of collection on the side of the container.

- Disposable gloves, Plastic bag for waste disposal, Timer, Disinfectant solution, Labelsand/orindeliblelabelingJulyker,*Optional:*Sterilepipettesforsampleprocessing

3.3 Storage and handling of equipment and materials

- Store the X-pert MTB/RIF cartridges and reagents at room temperature.
- Do not use reagents or cartridges that have passed the expiration date.
- The cartridge is stable upto7days after opening the package.

4. Preparation of Sample

1. Disinfect the working area.
2. Label each X-pert MTB/RIF cartridge with the sample ID.
3. Leave specimen in leak-proof sputum collection container.
4. Unscrew lid of sputum collection container, add Sample Reagent 2:1(v/v)to sample and close the lid again.
5. Shake vigorously10 – 20 times.
6. Incubate for 10minutes at room temperature.
7. Shake the specimen again vigorously10–20 times.
8. Continue incubation for another 5minutes.

Note: Samples should be liquefied with no visible clumps of sputum. If there are still clumps of sputum, shake again vigorously and incubate for another 3-5 minutes.

4.1 Preparing the Cartridge

Note: Start the test <u>within 30 minutes</u> of adding the sample to the cartridge.

1. Using the sterile transfer pipette provided, aspirate the liquefie sample into the transfer pipette until the meniscusis above the minimum Julyk(= 2ml).
2. Open the cartridge lid.
3. Transfer sample into the open port of the X-pert MTB/RIF cartridge.
4. Dispense slowly to minimize the risk of aerosol formation.
5. Close the cartridge lid. Make sure the lid snaps firmly into place.

Note: Remaining liquefied sample may be kept for up to 12 hours at 2-8°C should repeat testing be required.

5. **Start the test on the Gene x-pert Instrument**

1. In the Gene X-pert System window, click "CREATE TEST". The Scan Cartridge Barcode dialog box appears.
2. Scan the barcode on the X-pert MTB/RIF cartridge.
3. The Create Test window appears.
4. Using the barcode information, the soft ware automatically fills the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date.
5. In the Sample ID box, scan or type the sample ID. Make sure you type the correct sample ID. The sample ID is associated with the test results "View Results" window and all there ports.
6. Click "Start Test".
7. In the dialog box that appears, type your password.
8. Open the instrument module door with the blinking green light and load the cartridge.
9. Close the door.
10. The test starts and the green light stops blinking.
11. When the test is finished wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.
12. Dispose of used cartridges in the appropriate specimen waste containers according to your institutions standard practices.

6. **Reporting**

- Report"MTB not detected" for Negative result.
- Report "MTB Detected, Rif resistance Detected".
- Report "MTB Detected, Rif resistance not detected".
- If the report is "MTB Detected, Rif resistance Indeterminate" because of the concentration of MTB in the sample was very low and resistance could not be determined due to insufficient data collected to interpret resistance-related signals.

- Accurate recording and reporting of results is absolutely critical because:-

✓ False-negatives mean that the results that were reported as negative were truly positive and can result in patients with TB may not be treated resulting in on-going disease, disease transmission, or death.

- ✓ False-positives mean that the results that were reported as positive were truly negative and can result in patients are treated unnecessarily or treatment may continue longer than necessary
- ✓ Medications will be wasted
- ✓ Patient has a different underlying condition that requires treatment**.**

7.0 Troubleshooting

7.1. Invalid result due to:-

- Sample Processing Control (SPC) did not meet acceptance criteria.
    - ✓ Sample was not properly processed.
    - PCR was inhibited due food particles or blood in the sample.

Prevention

- Before mixing with sample reagent (SR) for decontamination, check whether the sample contains food particles or blood.
- Allow food particles to settle to bottom of specimen before adding sample to cartridge.

Solution(s)

Collect another specimen if necessary.

NB :- Two times invalid on analytical phase repeat the sample .

Annex-VI: SOPs for Lowenstein Jensen (LJ solid media)

The purpose of this procedure is to isolate and semi-quantify growth of M. tuberculosis on LJ medium. Slants will be inoculated with decontaminated and concentrated sputum specimens (Processing Sputum for Smear Microscopy and Qualitative Culture). Slants will also be inoculated from each positive LJ tube. Once good growth is obtained, these positive slants will be stored in a cool, dark place to archive the positive M. tuberculosis isolates.

Principle

Many different solid media are available for cultivating mycobacteria. Most are variations of egg-potato base or albumin-agar base media. There is no general consensus on which medium is best for routine isolation. The advantages of egg-based media such as LJ are: 1) it is easy and economical to prepare, 2) it is associated with lower contamination rates, and 3) isolated colonies with characteristic colony morphology for MTB can be observed. Disadvantages are: 1) when contamination occurs, it often involves total surface of medium, 2) if contamination is slight, it is not evident when mycobacterial growth is confluent, and 3) drug susceptibility tests are more difficult to perform using egg-based media because some drugs must be adjusted to account for their loss by heating or by interaction with certain components of the egg.

As with all media preparation, attention must be given to purity of chemical components, including quality of eggs; preparing and sterilizing medium and glassware; exposure of final product to excessive heat or sunlight; and method and length of storage. All lab-prepared media must be tested for sterility and performance characteristics before being used.

Sputum Specimen Processing

Sputum was processed using methods recommended by the United States Centers for Disease Control and Prevention (USCDC), including specimen volume adjusted to 10 ml; equal volume of N-acetyl-l-cysteine w/NaOH-citrate (final concentration of NaOH 1%) added; specimen mixed well and incubated for 15 to 20 minutes at room temperature; PBS (pH 6.8) added up to the 45-ml Julyk; solution mixed well and centrifuged (3,000 × *g* at 4°C for 15–20 minutes); specimens decanted and re-suspended in 1 to 2 ml PBS (pH 6.8); suspension mixed thoroughly and directly inoculated to solid media (Lowenstein-Jensen [LJ]).

Non sputum Specimen Processing (extra-pulmonary)

Other body fluids

Body fluids, such as CSF, synovial fluid and pleural fluid, Lymph node aspirates are collected aseptically and thus can be inoculated into an LJ tube, and a smear was prepared without decontamination. However, since sterility is not guaranteed, it is recommended these specimens should be lightly decontaminated. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000-3500x g for 15-20 minutes. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100 mg). After centrifugation, resuspend the sediment in about 5 ml of saline and then decontaminate following the procedure similar to that for sputum.

Mycobacterial Culture and identification

From the sediment 2 or 200µl drop of sediment, one loopful each was inoculated onto two slopes of LJ medium the tubes were incubated at 37°C , LJ cultures were evaluated twice within the first week of inoculation and then once per week for 42 days. Cultures with growth were confirmed as AFB positive by visual inspection or smear. Cultures showing no growth after 8 weeks of incubation were reported as negative. Liquefied or discolored (dark green) LJ media or LJ slants with colonies of non-acid-fast bacteria were considered contaminated .The isolates obtained were confirmed as M. tuberculosis complex by performing an immunochromatography test for the detection of MPT64 antigen

Materials required

- LJ medium
- Sterile, transfer pipettes with graduations Julyking volume (individually packaged)
- Tuberculocidal disinfectant
- Discard bucket with biohazard bag insert, containing appropriate disinfectant
- Sterile loop or disposable applicator stick
- Ziehl-Neelsen stain (carbol fuchsin, 3% acid alcohol, methylene blue)
- Parafilm
- Microscope slides, frosted at one end, new and clean • Paper towel soaked in disinfectant
- Sterile distilled water
- Incubator etc.

Inoculation and Incubation of Solid Cultures.

1. Remove any excess water in the slant using a sterile transfer pipette.

2. Inoculate the tube with 200 µl of the sample (either well-mixed, processed sputum: Processing Sputum for Smear Microscopy and Qualitative Culture; using a sterile graduated disposable pipette. Spread inoculum evenly over entire surface of medium.

3. Replace cap and ensure there are no droplets around the rim of the tube. Wipe off the outside of the tube with a paper towel soaked in tuberculocidal disinfectant.

4. Leave tube in slanted position with cap loosened until inoculum is absorbed (about a week), then tighten cap securely and incubate in upright position at 37°C (±1°C). Alternatively, the tube can be incubated immediately in an upright position with cap loose for the first week of incubation.

5. Examine and record results for the cultures weekly, for 8 intervals. Cultures can be read on the bench, as long as the caps are NOT loosened.

6. To observe fine growth, a strong direct light from the angle poise lamp must be shone onto the slant surface. M. tuberculosis usually grows as a buff-colored, dry colony, which is very distinctive.

Recording Results of primary Solid Culture

1. Record weekly growth results on the laboratory worksheet.

2. At weeks 1 through 7 if there is no growth, record "neg". If at the 8th read date the culture is negative, record "no growth" on the laboratory worksheet.

3. If there is growth at any reading interval, re-incubate the tube and read again the following week.

- If the same approximate count is seen, reading can be stopped and this count can be considered final.

- However, if the count increases substantially (e.g., 1+ at week 2, 2+ at week 3) the following week, continue to read the culture weekly until growth stabilizes.

4. Use the following standardized reporting scheme to report growth from the solid culture on the laboratory worksheet.

Growth	Laboratory Report	ZN Result	ID Result	Study Report – Solid Culture
None	No growth	N/A	N/A	Negative for MTB complex
1-9 colonies	Record actual number	POS	MTB	TB growth (1-9 colonies); record ID result and test method

10-100 colonies	1+	POS	MTB	TB growth (10-100 colonies); record ID result and test method
> 100-200 colonies	2+	POS	MTB	TB growth (more than 100 colonies); record ID result and test method
> 200 colonies (too numerous to count or confluent)	3+	POS	MTB	TB growth (innumerable or confluent) record ID result and test method
Other mycobacterial growth	Positive for other mycobacteria	POS	NEG	No MTB complex growth positive for other mycobacteria; record ID result
Contaminated	Contaminated	N/A	N/A	Contaminated
ZN+ growth in presence of contamination	Positive for MTB and contamination	POS	POS	Positive for MTB complex and contaminated; record ID result

Annex-VII: SOP Ziehl-Neelsen (Z-N) Sputum Smear Microscopy

The purpose of AFB microscopy is to detect acid-fast bacilli (AFB) by microscopic examination of clinical specimens and cultures. Both living and dead (viable and non-viable) bacilli will stain and be counted. A semi-quantitative grading system is used to report the number of AFB observed in stained smears. All sputum smears are prepared from decontaminated and concentrated specimens. Processing Sputum for Smear Microscopy and Qualitative Culture. These smears are stained with Ziehl-Neelsen stain can be used to confirm fluorescent smear results, but these results will not be reported. The Ziehl-Neelsen stain is used to confirm the presence of AFB in positive cultures (LJ).Microscopic examination of the sputum smear must conform to the reading and reporting procedures described in Acid-fast Bacilli Microscopy (AFB) Examination (57).

Principle

The Ziehl-Neelsen method uses a carbolfuchsin stain, acid alcohol decolorizer, and methylene blue counter stain (stains background). Acid-fast organisms stain red, while the background of debris stains blue. The ZN stain confirms the acid-fast property of mycobacteria with ZN and will be examined using microscopy. Bacillary density were graded as negative (no AFB seen per 100 fields), and scanty (1-9 per 100 fields, 1+ (10–99 AFB per 100 fields), 2+ (1–10 AFB per 100 fields), and 3+ (>10 AFB in at least 20 fields). 1+, 2+, and 3+, all such smears were defined as ‘‘smear-positive’’.

Annex-VIII: SOPs for Line Prope Assay

Principle of Line Probe Assay

Line probe assays are a family of DNA strip-based tests that determine the drug resistance profile of a MTBC strain through the pattern of binding of amplicons (DNA amplification products) to probes targeting the most common resistance associated mutations to first- and second-line agents and to probes targeting the corresponding wild-type (WT) DNA sequence.

LPAs are WHO-approved tests for rapid detection of drug resistance to first- and second-line agents. They can be used for testing of culture isolates (indirect testing), as well as direct testing of acid fast bacilli (AFB) smear microscopy positive specimens, and both smear positive and smear negative sputum specimens.

Mutations are detected by: (i) the binding of amplicons to probes targeting the most commonly occurring mutations (MUT probes) or (ii) inferred by the lack of hybridization (i.e. lack of binding) of the amplicons to the corresponding WT probes. The post-hybridization reaction leads to the development of colored bands on the test strip detecting probe binding.

LPA has three procedures in their process

1. DNA extraction: using GenoLyse chemical method. This procedure provides instructions for chemical extraction of mycobacterial DNA from NALC-NaOH decontaminated smear positive specimens or cultured isolates (solid or liquid media). The extracted DNA from the specimen under investigation is used to make diagnosis of genotypic drug susceptibility to Rifampicin and Isoniazid.

Principle: DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular biology analysis. With the Genolyse chemical method test, this implies that: the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a lyses buffer.

2. Master Mix preparation: it is a premixed, ready to use solution containing Taq DNA polymerase, dNTPs, and $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

Principles: All reagents needed for amplification are included in the Amplification Mixes A and B 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 deoxynucleoside 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 Mg^{2+} in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognize them as substrates during the amplification procedure.

3. DNA addition, amplification and detection: This procedure provides instructions for determining genotypic drug susceptibility of *M. tuberculosis* complex to isoniazid and rifampicin through PCR amplification and binding of amplicons to specific oligonucleotide probes immobilized on a membrane strip.

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 deoxynucleoside 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 Mg^{2+} in the salts forms

soluble complexes with the free nucleotides allowing for the DNA polymerase to recognize 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 phosphatise transforms an added substrate into a dye which becomes visible on the membrane strips as a colored precipitate.

Line Probe Assay interpretation and reporting

The LPA has two internal controls on the strip: the **Conjugate Control** (line 1), and the **Amplification Control** (line 2). The Conjugate Control line should always be visible to document the efficiency of conjugate binding and substrate reaction. The Amplification Control serves as reference for the interpretation of WT and MUT probes: only those bands whose intensities are about as strong as or stronger than that of the Amplification Control band are to be considered. In case of a positive test result, the signal of the Amplification Control zone can be weak or even vanish totally. This can occur more frequently upon indirect testing, whereas it is rare upon direct testing. The absence of Amplification Control might be due to competition of the single reactions during amplification. In this case the test has been carried out correctly and can be interpreted. In case of a negative test result, both Conjugate Control and Amplification Control bands should always be visible (i.e. valid negative result). The absence of Amplification Control in a negative test indicates mistakes during setup and/or performance of the amplification reaction, or presence of amplification inhibitors. In this case, the test result is **invalid** and must be repeated.

The **TUB reaction band** (line 3) is only present if the DNA amplified is from members of the MTBC. The presence of non-tuberculous mycobacteria (NTM) in the specimen can result in random banding patterns, with several species testing positive at some *rpoB* WT bands due to the gene similarities among the species. Therefore, in the presence of NTM rather that MTBC bacteria, the TUB band will always be absent, and the result reported as MTBC not detected.

The gene **locus control** bands for the different target regions analyzed on the DNA strip are located just before their respective WT and MUT bands. These locus control bands must always be present for the assay to be considered valid for the corresponding target. However, when only one gene locus control band is missing, the results for the other genes for which the gene locus control band is present can be interpreted.

The LPA is defined as **indeterminate** for a specific drug or group of drugs if the corresponding locus control for that specific drug or group of drugs is missing while the test is valid (i.e. Conjugate Control and TUB bands are visible with or without the Amplification Control). In this case, the assay should be repeated before reporting the results. If the same result is obtained upon re-testing, report the results for the interpretable loci following the guideline, while report as indeterminate the result for the drugs or group of drugs for which the locus control is missing. Systematic reasons for these indeterminate results could be mutations or deletions in the locus control region, as well as the complete or partial deletion of a target gene. In this cases, sequencing should be requested to identify the specific mutation.

The **WT reactions zones** comprise regions of the genome with known resistance mutations. The **MUT reaction zones** correspond to probes that identify the most common resistance mutations of the gene interrogated. Resistance is detected when MUT probes are developed, whereas in the absence of WT probes, resistance can only be inferred.

- Presence of all WT and absence of MUT = Sensitive
- Presence of all WT and presence of MUT = Resistant
- Absence of any WT and presence of MUT = Resistant
- Absence of any WT and absence of MUT = Resistant

Standard operating procedure for DNA addition, amplification and detection.

This procedure provides instructions for determining genotypic drug susceptibility of *M. tuberculosis* complex to isoniazid and rifampicin through PCR amplification and binding of amplicons to specific oligonucleotide probes immobilized on a membrane strip. Genotypic DST is used for rapid confirmation of drug resistance tuberculosis in suspected patients groups. MDR-TB diagnosis can be made in as short as 48 hours as compared to conventional DST which can take as much as 1-2 months.

Principles 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 deoxynucleoside 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 Mg^{2+} 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 phosphatise transforms an added substrate into a dye which becomes visible on the membrane strips as a coloured precipitate.

Materials used

Reagents preparation:

Prepare master mix reagent

Master mix reagent should be prepared fresh for each batch of DNA to be amplified.

Detection reagents are ready to use except for substrate and conjugate which have to be diluted. Prepare working Conjugate and Substrate in 15 ml conical vials by diluting 1:100 with corresponding Con-D and Sub-D. Wrap Substrate dilution in aluminium foil and keep at room temperature. Prepare fresh Conjugate and Substrate dilutions every day of detection and for every batch. Reagents stability and storage:

- Prepared master mix reagents should be prepared fresh on each day of amplification and should never be stored for future use
- The detection reagents should be stored at 2-8°C and should be used before expiry date

  Equipment's required

- Timer
- PCR workstation or hood
- Adjustable pipette, 200µl
- Adjustable pipette, 1000µl
- Twincubator
- Thermal cycler with heated lid

Sample preparation

Sample type	Amount required	Transport and Storage	Stability
DNA samples	5µl	Avoid direct exposure to sunlight; cold chain	2-8°C/7 days -20°C/1 year

DNA addition procedures

.Apply 70% ethanol to all the surfaces PCR hood

. Obtain DNA samples from the refrigerator or freezer and allow to reach room temperature

. Assemble PCR tubes containing 45µl master-mix reagent

. Add 5µl of DNA to corresponding master mix PCR tubes in the PCR hood.

Result interpretation

- Presence of all WT and absence of MUT = Sensitive
- Presence of all WT and presence of MUT = Resistant
- Absence of any WT and presence of MUT = Resistant
- Absence of any WT and absence of MUT = Resistant

Standard operating procedure for preparation of master mix reagent for GenoType MTDR*plus* Technique.

Purpose of this procedure provides instructions for the preparation of reagents required for the PCR steps of the GenoType MTBDR*plus* technique

Abrebations

DNA= Deoxyribonucleic acid

SOP= Standard Operating Procedure

dATP= Deoxyadenosine triphosphate

dGTP= Deoxyguanosine triphosphate

dTTP= Deoxythymidine triphosphate

PCR= Polymerase Chain Reaction

MSDS= Material Safety Data Sheet

N/A= Not applicable

dCTP= Deoxycytidine triphosphate

Mg^{2+}= Magnesium ion

Definition: Master Mix: it is a premixed, ready to use solution containing Taq DNA polymerase, dNTPs, and MgCl2 and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

Principles of the test: All reagents needed for amplification are included in the Amplification Mixes A and B 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 deoxynucleoside 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 Mg^{2+} in the salts forms soluble complexes with the free nucleotides allowing for the DNA polymerase to recognise them as substrates during the amplification procedure.

Standard operating procedure for DNA extraction using Genolysis chemical method.

This procedure provides instructions for chemical extraction of mycobacterial DNA from NALC-NaOH decontaminated smear positive specimens or cultured isolates (solid or liquid media). The extracted DNA from the specimen under investigation is used to make diagnosis of genotypic drug susceptibility to Rifampicin and Isoniazid.

Principles : DNA extraction is a procedure whereby DNA is fetched from bacterial cells or fragments of bacterial cells to be used for molecular biology analysis. With the Genolyse chemical method test, this implies that: the bacterial cells in the decontaminated patient sample or culture samples are chemically broken to expose the DNA by using a lyses buffer. Materials: reagents : GenoLyse kit reagents

- A-LYS (lysis buffer) and A-NB (neutralization buffer
- Reagents stability and storage: The reagents should be stored at 2-8 °C and be used before expiry date

Sample	Culture isolate, solid culture	1µl loopful	Triple packaging, cold chain	2-8°C/1 month

				-20°C/1 year -80°C/10 years	

Procedure of the test : DNA extraction from decontaminated smear positive sediments

Work on no more than 10 sediments and 2 controls at a time

Vortex thoroughly for 15-20 seconds each of the 50ml conical tubes containing the sediments

Using a sterile disposable Pasteur pipette, transfer 500µl of each decontaminated sample to labelled 1.5ml screw cap tube

Centrifuge for 15 minutes at 10,000 RCF or 10263RPM

Discard supernatant from each tube by use of a 1000µl adjustable pipette

Resuspend each pellet in 100µl Lysis Buffer (A-LYS)

Incubate the tubes for 5 minutes at 95 °C in a thermo block

Add 100 µl Neutralization Buffer (A-NB) and vortex the sample for 5 seconds

Load the tubes into a micro centrifuge and spin for 5 minutes at 13,000 x g

Carefully carry the tubes to the BSC. Uncap tubes one at a time, and transfer 100µl of DNA-containing

supernatant to a sterile 1.5ml screw cap tube

Store DNA at 2-8 °C for not more than 7 days. For longer storage, keep at -20 °c

DNC extraction from solid culture isolate

Steps

Pipette 100µl lysis buffer (A-LYS) into sufficient number of 1.5ml screw cap tubes (1 tube per culture)

Use 1µl sterile disposable inoculation loop to collect bacteria from solid media with sufficient growth

Inoculate the bacteria into the lysis buffer. Break the clumps by aid of the inoculation loop

For 15-20 seconds thoroughly vortex to adequately mix

Proceed similarly to the procedure for **decontaminated smear positive sediments**

Declaration

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

M.Sc. candidate: Melkayehu kassa (B.Sc.)

Signature: ___________________

Date of submission: ___________________

This thesis has been submitted with our approval as advisors.

Advisor: kassu desta (MSc, PhD Candidate)

Signature: _____________________

Date: _____________________

Place: Addis Ababa, Ethiopia.

Advisor: Addisu Gize (BSc, MSc)

Signature: ____________________

Date: ____________________

Place: Addis Ababa, Ethiopia.