Jimma University

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Department Of Biology

Prevalence and drug resistance patterns of pulmonary *Mycobacterium tuberculosis* isolated from outpatients visiting Robe Didea Hospital, Robe district, southeastern Ethiopia.

By

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A Thesis Presented to the School of Graduate Studies, Jimma University, in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biology

October, 2015

Jimma, Ethiopia

Prevalence and drug resistance patterns of pulmonary *Mycobacterium tuberculosis* isolated from outpatients visiting Robe Didea Hospital, Robe district; Southeastern Ethiopia.

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Declaration

I, the under signed, declare that this is my original work, has never been presented in this or other University, and that all the resources and materials used for the thesis have been dully acknowledged.

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LIST OF ACRONYMS AND ABBREVATIONS

AFB- Acid-fast bacilli

BCG- Bacillus Calmette-Guérin (vaccine)

BSC- Bio safety cabinet

BSL- Bio safety level

CTD- Central TB division of India

CXR- Chest x- ray

DM- Diabetes mellitus

DOT- Direct observed therapy

DST- Drug sensitivity test

EPHI- Ethiopian public health institute

FIND- Foundation for innovative new diagnostics

FMOH- Federal ministry of health

INH- Isoniazid

LED- Light Emitting Diode

LPA- Line Probe Assay

LTBI- Latent Tuberculosis infection

M- Mycobacterium

MDR-TB- Multidrug resistant tuberculosis

MTB- *Mycobacterium tuberculosis*

NALC- N-acetyl-L-Cysteine

OPHRCBQAL- Oromia Public Health Research Capacity building And Quality Assurance Laboratory

PTB- Pulmonary tuberculosis

RMP- Rifampicin

SPSS- Statistical Package for Social Science

XDR-TB- Extensively Drug-Resistant TB

OPERATIONAL DEFINITIONS

Biopsy- The removal of sample of tissue from a living person for laboratory examination

Fluorochrome- A molecule or part of a molecule that exhibits fluorescence.

Incidence- The number of new cases of a specific disease occurring during a given time in a Population.

MDR-TB - TB caused by MTB that is resistant to isoniazid and rifampicin.

Oligonucleotide – A polymeric chain containing ten nucleotide or fewer.

Pandemic- Existing in the form of a wide spread epidemic that affects people in many different Countries

Prevalence- The percentage of population that is affected by a particular disease in a given period.

Pathology- The scientific study of the nature, origin, progress, and cause of diseases

Radiology- Branch of medicine that deals with the use of radioactive substances in the diagnosis and treatment of diseases

XDR-TB - TB caused by MTB that is resistant to fluroquinlones and one of the three injectable second line drugs in addition to isoniazid and rifampicin

ABSTRACT

Tuberculosis is a major public health problem throughout the world. About a third of the world's population is estimated to be infected with MTB and hence at risk of developing active disease. Even though the prevalence and antibiotic susceptibility pattern of TB in Ethiopia is known, the trend of TB in most of the districts of the country is not known. To this effect, this study was designed to assess prevalence, drug resistance pattern, and risk factors of pulmonary MTB *among out patients visiting Robe Didea hospital; southeastern Ethiopia. The study design used to carry out survey on the prevalence of MTB was a cross- sectional study. Structured questionnaires were used to collect demographic data and risk factors for M. tuberculosis; and molecular line probe assay methodology for drug sensitivity test. Light emitting diode fluorescent microscopy was used to identify the presence of MDR-TB. A total of 147 suspected TB patients were included in the study. IBM SPSS software (version 20) was used to statistically analyze the data collected from the respondents. Chi square test was also used to identify the risk factor for prevalence of MTB. Categorical data was presented by means of percentages and frequencies. The result indicates that the prevalence of Mycobacterium tuberculosis was 6.12%. The risk factors for the prevalence of MTB are close contact with active TB patients 29.9% ($P<0.05$), crowded living condition 40.8% ($P<0.05$) and presence of family member with active tuberculosis 29.9% ($P<0.05$). Of all smear positive isolates, 11.1 % of the isolates were multi-drug resistant. Education and awareness development training should be given to the community on the risk factors for the disease transmission and cares to be taken.*

Key words: Drug sensitivity, *Mycobacterium tuberculosis*, prevalence, and risk factor

1. INTRODUCTION

1.1. Background

At least one out of three people in the world has latent TB infection, which increases the risk of becoming ill with TB (WHO, 2011a). TB remains a major global health problem and ranks second in being the cause of death from an infectious disease worldwide following human immunodeficiency virus (HIV) (WHO, 2014a).

TB is the leading cause of mortality among infectious diseases worldwide and 95% of TB cases and 98% of deaths due to TB occur in developing countries (FMoH, 2010a). The combination of high rates of TB infection with high Sero-positivity for HIV in sub-Saharan Africa adds new level of complexity to diagnosis and treatment and has raised the amount of money used for global TB control (Upshur *et al.,* 2009).

The national population based TB prevalence survey conducted in 2010/11 in Ethiopia revealed that the prevalence of smear positive TB among adults and all age group was found to be 108 and 63 per 100,000 populations, respectively. The prevalence of all forms of TB in Ethiopia is estimated to be 240/100,000 populations (FMoH, 2013).

1.2. Statement of the problem

According to estimates by WHO, the prevalence and mortality of tuberculosis is estimated to be 355 and 79 per 100,000 populations, respectively (FMoH, 2005). According to the WHO Annual Global TB Report, Ethiopia ranked seventh in the world for TB burden (WHO, 2009). Prevention and control of TB has created additional challenge and a major constraint to health care systems in many of developing countries including Ethiopia due its linkage with HIV/AIDS (FMoH, 2010b).

TB is affecting all sexes and age groups. Poverty is a risk factor for developing TB, which places Ethiopia as a high-risk environment. The country is one of the least developed in the world (FMoH and EPHI, 2011). According to the WHO global TB report 2012 which considered the findings from the national TB prevalence survey, there were an estimated 220,000 (258 per 100,000 populations) prevalent cases of TB in Ethiopia in 2011. According to the same report, the prevalence of TB was estimated to be 200,000 (237 per 100,000 populations). There were an estimated 15,000 deaths (18 per 100,000 populations) due to TB, excluding HIV related deaths, in Ethiopia during the same period. According to the 2011 health and health related indicators of the FMOH, tuberculosis is the third leading cause of death in Ethiopia. During the year 2010/11, a total of 159,017 TB cases were notified in Ethiopia. Among these 151,866 (95.5%) were new cases of TB (FMoH, 2013). Tuberculosis is one the first five diseases that are grouped as top 10 causes of death in Ethiopia. It is the fifth cause of death in Ethiopia next to lower respiratory infection, Cancer, Diarrheal diseases and Malaria. TB accounts for 5% of the deaths caused by diseases in Ethiopia (CDC, 2013a).

TB is a chronic infectious disease caused by MTB. Almost one-third of the world population (about 2 billion people) is infected with MTB and during the past decade there has been a resurgence of tuberculosis. Currently, TB is the leading cause of mortality among infectious diseases worldwide but 95 percent of TB cases and 98 percent of deaths due to TB occur in developing countries. TB remains one of the world's deadliest communicable diseases. In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease, 360 000 of whom were HIV-positive. About 60% of TB cases and deaths occur among men, but the burden of disease among women is also high. In 2013, an estimated 510 000 women died as a result of TB, more than one third of whom were HIV-positive. There were 80 000 deaths from TB among HIV-negative children in the same year (WHO, 2014a).

Drug resistant TB is one of the main factor affecting the country as well as different regions. The rate of MDR-TB was high 88/265 (33%) among suspected cases in the Oromia Region of Ethiopia (Mulisa *et al*., 2015)

As shown above, MTB is greatly affecting the world population including Ethiopians. Thus, periodic evaluation of the status of MTB is crucial to determine its current status and design control strategies before the impact get worse. Even though the prevalence of MTB was investigated and reported from some parts of Ethiopia, to our knowledge there is no report both on the prevalence and drug susceptibility pattern of the same in Robe woreda, Robe Didea hospital. So this study was significant to fill this gap. To this effect, this study was designed to assess the prevalence and drug resistance patterns of MTB in the study area with the assumption that the outcomes of the study will help concerned bodies for timely action.

This study focused on answering the following questions:

- What is the prevalence of MTB among outpatients visiting Robe hospital?
- What is the antibiotic susceptibility pattern of MTB isolated from the study population?
- What are the risk factors that make the study population exposed to tuberculosis?

1.3. Objective

1.3.1. General objective

The general objective of this study was to determine the prevalence, antibiotics susceptibility pattern and risk factors of MTB in the study area.

1.3.2. Specific objectives

- To determine the prevalence of MTB in the study area,
- To assess the antibiotic susceptibility pattern of MTB isolated from the Study Population,
- To determine the epidemiologic risk factors of MTB among the study population

1.4. Significance of the study

In this study the prevalence, drug susceptibility pattern and risk factors of MTB among outpatients visiting Robe Didea hospital was investigated. This study could help different levels of government offices to get current information on the status of prevalence of MTB in the area and take necessary measures to reduce the effect that it will impose on the community. The findings of this study will also help to know the risk factors of MTB and design strategy to control the pathogen. In addition, it could provide awareness to concerned bodies about antibiotic susceptibility pattern of MTB and to guide empirical and pathogen specific therapy. The finding of this study could also be used as a base-line data for further investigation on related issues in the future

2. LITERATURE REVIEW

2.1. General features of *M. tuberculosis*

MTB is a non- spore forming, non-motile bacilli (Barnard *et al*., 2012). MTB is not classified as either Gram-positive or Gram- negative (CTD, 2009). Tuberculosis is caused by H37Rv MTB which is a unique acid fast bacterium. It does not contain phospholipid outer membrane. (Rajani and Meena, 2011). TB is an infectious bacterial disease caused by MTB that most commonly affects the lungs and remains one of the world's deadliest communicable diseases (Girma, 2015). The dimensions of the bacilli have been reported to be 1-10 μm in length (usually 3-5 μm), and 0.2 -0.6 μm width (Velayati and Farnia, 2012). MTB is a prototrophic (i.e., it can build all its components from basic carbon and nitrogen sources) and heterotrophic (i.e., it uses already synthesized organic compounds as a source of carbon and energy). The most classical form of tubercle bacilli is a slender rod shape that seen in stained smears. (Velayati and Farina, 2011). MTB is an obligate aerobe microbe growing most successfully in tissues with high oxygen content, such as the lungs. They are intracellular pathogens usually infecting mononuclear phagocytes (e.g.macrophages), slow-growing with a generation time of 12 to 18 hours (20-30 minutes for *Escherichia coli*), hydrophobic with a high lipid content in the cell wall. Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains, e.g. Gram's stain. Mycobacterium tuberculosis is "acid-fast bacilli" because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resist decolorization with acidified organic solvents and are therefore called "acid-fast" (CTD, 2009)

2.2. Epidemiology

2.2.1. Transmission methods of MTB

MTB is carried in airborne particles called droplet nuclei that can be generated when persons who have pulmonary or laryngeal TB disease cough, sneeze, shout, or sing. The particles are approximately 1–5 μm. Normal air currents can keep them airborne for prolonged periods and

spread them throughout a room or building. Infection occurs when a susceptible person inhales droplet nuclei containing MTB, and the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli. Usually within 2–12 weeks after initial infection with MTB, the immune response limits additional multiplication of the MTB. However, certain bacilli remain in the body and are viable for multiple years. This condition is referred to as latent tuberculosis infection (LTBI). Persons with LTBI are asymptomatic (they have no symptoms of TB disease) and are not infectious (CDC, 2013c). A single cough can produce 3,000 droplets nuclei (FMoH, 2013). Two factors determine an individual's risk of exposure: the concentration of droplet nuclei in contaminated air and the length of time spent breathing that air (FMoH, 2012). Population migration due to wars and New World expedition accounts for the major transmission patterns of microbial pathogens, including MTB (Dou *et al*., 2011)

2.2.2. Risk factors of MTB

Risk factors include all factors that reduce immunity and make a person more susceptible to TB (Kleiva and Tingstveit, 2013). The selected groups know to have higher risk for tuberculosis infections are who have had contact with actively infected TB patients, who are HIV positive or have risk factor for HIV infection with past history of TB, drug users, low- income populations lacking access to adequate medical care and children who have contact with members of high-risk adult population (Cowan and Talaro, 2006). Several factors have been shown as risk factors of MTB as indicated in Table 1.

Table 1: Epidemiological risk factors for infection with MTB

Epidemiological risk group or factor	Reference
Immigration from high prevalent area	Boon *et al.,* 2006
Diabetes mellitus	Boon *et al*., 2006; CDC, 2013b; WHO, 2011b
Poverty	Wallace and Kohatsu, 2008; Millet *et al*., 2013.
Working in hospitals and correctional facilities	CDC, 2013b
Living in collective dormitories	Boon *et al*., 2006
Crowded condition	Wallace and Kohatsu, 2008
Alcohol	Narashiman *et al.,* 2013; CDC 2013b
Malnutrition	Lee *et al.,* 2013
Age group between 15 and 54	FMoH, 2012
Tobacco smoking	Narashiman *et al.,* 2013; CDC, 2013b;WHO,2011
Social deprivation	Boon *et al.,* 2006
Immune status	CDC, 2008
Working in residential facilities for patients with HIV	CDC, 2013b
Socio economics status	FMoH, 2012
Inadequately treated TB disease	CDC, 2013b
Transplant	Lee *et al.,* 2013
Imprisonment	Mohammed and Salami, 2009

The first report of the association between Diabetes mellitus and TB was documented by Avicenna over one thousand years ago. Since that time, the relationship between diabetes mellitus (DM) and TB, and the nature of their interaction with regards to co-morbidity are largely suggested by numerous epidemiological studies. Although the definite pathophysiological mechanism of the effect of DM as a predisposing risk factor for TB is unknown, some hypotheses are suggested: depressed cellular immunity, dysfunction of alveolar macrophages, and micronutrient deficiency (Baghaei *et al.,* 2013)

2.2.3. Prevalence of MTB

According to estimates of the World Health Organization (Feldman and Sarosi, 2005) which declared tuberculosis to be a global emergency in 1993, there were nearly 2 billion people in the world infected with MTB, with 8 million new cases of active disease and more than 2 million deaths in 1997. About 95% of cases of tuberculosis and 98% of tuberculosis deaths occur in developing countries. Tuberculosis has been estimated to cause 7% of all deaths and 26% of preventable deaths in the developing world. Most deaths occur in young adults between the ages of 15 and 40 years, during their most economically productive years. Tuberculosis is a disease of the poor and disadvantaged and is therefore concentrated predominantly in the developing world and in poor areas of major cities in the developed world. Although the greatest numbers of cases occur in certain parts of Southeast Asia, the highest prevalence of cases is found in sub-Saharan Africa .Nine of the 10 countries with the highest prevalence of tuberculosis are in Africa (Feldman and Sarosi, 2005). In 2007, there were an estimated 9.27 million new cases of TB (WHO, 2009). Despite excellent progress in expanding the Direct observed therapy strategy; the global TB prevalence rate continues to grow by 1% each year (WHO, 2006).

All countries are affected by TB, but 85% of cases occur in Africa (30%) and Asia (55%), while India and China alone represent 35% (WHO, 2011c). There were 12 million prevalent cases of TB in 2011 (FMoH, 2013). Most of the estimated number of cases in 2011 occurred in Asia (59%) and Africa (26%); 1 smaller proportions of cases occurred in the Eastern Mediterranean Region (7.7%), the European Region (4.3%) and the Region of the Americas (3%) (WHO, 2012a). In 2012, an estimated 8.6 million people developed TB and 1.3 million died from the disease (FMoH/EPHI, 2014). In 2013, an estimated 9.0 million people developed TB and 1.5 million died from the disease. Of the estimated 9 million people who developed TB in 2013, more than half (56%) were in the South-East Asia and Western Pacific Regions. A further one quarter was in the African Region, which also had the highest rates of cases and deaths relative to population. India and China alone accounted for 24% and 11% of total cases, respectively (WHO, 2014b) (Figure 1).

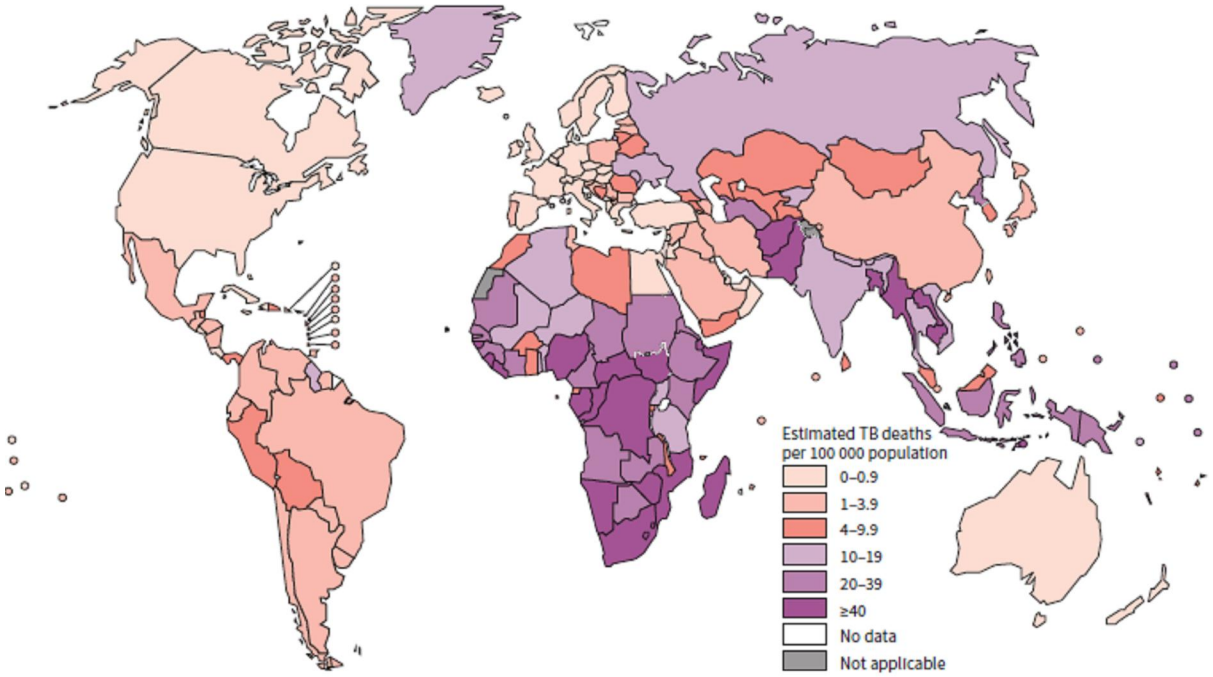

Figure 1: Estimated TB mortality rates in 2013 (WHO, 2014b).

According to the WHO Global TB Report 2009 (FMoH and EPHI, 2011), Ethiopia ranked seventh in the world for TB burden and third in Africa in 2008, with an estimated TB prevalence of 579 per 100,000 population (FMoH and EPHI, 2011). A report indicated that the prevalence of TB was estimated to be 310,000 (224 per 100,000 populations) (FMoH/EPHI, 2014). According to the 2014 WHO report, the prevalence of all forms of TB in Ethiopia is 211 /100,000 population (WHO, 2015).

2.3. Drug resistance in MTB

MDR-TB is a specific form of drug resistance TB due to a bacillus resistant to at least Isoniazid and rifampicin the two most powerful anti-TB drugs (Getachew and Tegegn, 2006; WHO, 2014a; Palomino and Martin, 2010; CDC, 2013b). Shortly after the first anti-tuberculosis drugs were introduced into clinical practice in the late 1940‟s, resistance to these drugs was observed (Barnard *et al*., 2012; Zang and Yew, 2009). Multidrug resistant tuberculosis (MDR-TB) is caused by the transmission of multidrug resistant MTB strains in new cases, or by the selection of single and drug resistant strains induced by previous treatment (Faustini *et al.,* 2006; Esmael,

et al.,2014). The primary reason why treatment of tuberculosis fails is that patients do not comply with the drug therapy (Mullins, 2006).

MTB is particularly prone to becoming antibiotic resistant. Prescribing a single antibiotic to treat it often leads to becoming mutant bacteria that are not affected by the drug eventually dominate the population in the lungs. TB infections are always treated with multiple drugs simultaneously for this reason (Cowan and Talaro, 2006). The re-emergence of TB has been accompanied by a marked increase in drug- resistant strains, particularly in the poorest countries, is closely linked to inadequate treatment. Cure is possible but prolonged treatment with less effective, more toxic and more expensive therapies is often necessary. Infection with MDR-TB substantially increases the risk of treatment failure, further acquired resistance and death (Boon *et al.,* 2006).

Primary drug-resistant TB is drug-resistant TB caused by person-to-person transmission of drug-resistant organisms while secondary drug-resistant is referred as acquired drug-resistant TB because it develops during TB treatment, either because the patient was not treated with the appropriate treatment regimen or because the patient did not follow the treatment regimen as prescribed (CDC National Center for HIV/AIDS, Viral Hepatitis, STD, and TB Prevention Division of TB Elimination**,** 2008). Development of drug resistance has been associated with different risk factors such as being male/female (Esmael *et al*., 2014).

The annual global MDR-TB burden is estimated at around 490 000 cases, or 5% of the global TB burden; however, less than 5% of existing MDR-TB patients are currently being diagnosed as a result of serious laboratory capacity constraints (FMoH, 2010). In 2008, The World Health Organization (WHO) estimates that each year 440,000 new cases of MDR-TB are emerging globally, and 150,000 patients with MDR-TB die (Lem *et al*., 2013). Globally, 3.5% of new TB cases were estimated to have had MDR-TB in 2013. This translates into an estimated 480 000 people developed MDR-TB in 2013. If all notified TB patients (6.1 million, new and previously treated) had been tested for drug resistance in 2013, an estimated 300 000 cases of MDR-TB would have been detected, more than half of these in three countries alone: India, China and the Russian Federation. (WHO, 2014a) (Figure 2). 150 000 patients with multidrug-resistant TB initiated on appropriate treatment every year by 2015 (Dowdy and Chaisson, 2013).

TB remains a major public health problem claiming the lives of thousands of Ethiopians every year. Ethiopia is among the 22 high TB burden and 27 high MDR TB burden countries in the world. The prevalence of MDR TB is increasing at an alarming rate from a baseline rate of 1.6% among new TB cases in 2005 to current level of 2.3% in 2014 ((FMoH, 2014).

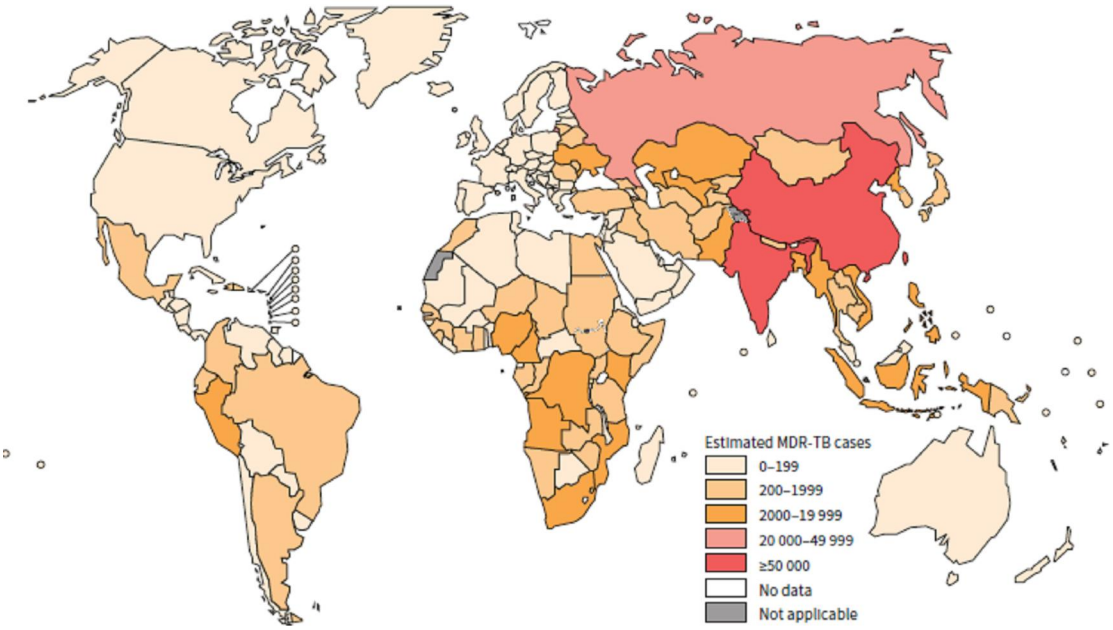

Figure 2: Number of MDR-TB cases estimated to occur among notified pulmonary TB cases, 2013. (WHO, 2014a).

Drug-resistant TB is a man-made problem, largely being the consequence of human error as a result of individual or combination of factors related to management of drug supply, patient management, prescription of chemotherapy, and patient adherence. Poor infection control practice has also been identified as a major contributing factor for the spread of DRTB. More recently the emergence of Extensively Drug-Resistant TB (XDR-TB) has added to the complexity of TB care and treatment. An increased risk for drug resistance are previous exposure to anti-TB treatment, exposure to a known MDR-TB case**,** history of using poor or unknown quality TB drugs**,** treatment in poorly performing control program**,** co-morbid conditions associated with mal-absorption and HIV/AIDS (FMoH, 2013). The contributing

factors to drug resistance include poor compliance in taking therapy, irregular supplies of drugs, addition of single drugs to failing regimens in the absence of bacteriological control, unacceptably high cost to the patient, difficult journeys to the clinic and time off work leading to irregular intake, use of time-expired, impure, mishandled or even fake drugs Use of poorly formulated combination preparations, and prescription of inappropriate drug regimens (Johnson, *et al*., 2009)

Mechanism of drug resistance

The genetic mutations responsible for resistance to TB therapy are so many. The majority of rifampicin-resistant clinical isolates of MTB harbor mutations in the *rpoB* gene that codes for the β-subunit of the RNA polymerase. The two main molecular mechanisms of Isoniazid resistance are associated with gene mutations in *katG* and *inhA* or its promoter region. The recognized mechanism of resistance to ethambutol has been linked to mutations in the gene *embB*. Mutations in the gene *pncA* remain as the most common finding in pyrazinamide resistant strains (Palomino and Martin, 2010). Genetic resistance to an anti-tuberculosis drug is due to spontaneous chromosomal mutations at a frequency of 10^{-6} to 10^{-8} mycobacterial replications (Zang and Yew, 2009).

2.4. Method of detection of MTB

The most important tool in the diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for acid-fast bacilli (CTD, 2009). There are two procedures commonly used for acid-fast staining, Carbol fuchsin methods which include the Ziehl-Neelsen methods (direct microscopy) and Fluorochrome procedure using Auramine-O dyes (fluorescent microscopy) (CDC, 2013b). According to WHO guidelines, the recommended diagnostic tool for confirming PTB in Ethiopia is sputum microscopy, which detects acid fast bacteria (Kleiva and Tingstveit, 2013).

Culture-based tests are difficult to implement in the field. They require dedicated facilities and staff, with specific requirements for training, quality assurance, bio-safety, and equipment, which

can take time and significant local resources to set up (Guillermo *et al.*, 2006; Barnard *et al*., 2012). In order to culture MTB from clinical specimen there are various kinds of solid and liquid media such as Lowenstein –Jensen, Kirchner, and the various middle brook formulations. The growth of solid culture media is 6 weeks or longer, where as that of liquid culture media is usually 7-21 days (Barnard *et al*., 2012).

Chest X-rays will be helpful in the diagnosis of smear negative pulmonary TB when interpreted in conjunction with presenting signs and symptoms (FMoH, 2005). The chest X-ray in active pulmonary TB typically demonstrates infiltrates within the apical and/or posterior segments and often the infiltrate contain variably sized cavities (Wallace and Kohatsu, 2008). X-ray is a useful method to support the diagnosis of TB. It is sensitive but less specific. CXR remains an important tool for diagnosis of PTB in children who are sputum smear-negative or who cannot produce sputum (FMoH, 2013).

The diagnostic technology recommended in current control strategies is sputum microscopy, which was developed in the 1880s and has remained essentially unchanged since then. In many countries it is based only on the examination results of Ziehl-Neelsen (ZN) stained smears. Since the first description of the auramine O fluorescence microscopy technique by Hagemann in 1937, numerous reports have confirmed the superior diagnostic performance of fluorescence microscopy, compared with Ziehl-Neelsen (ZN) staining and light microscopy. Fluorescence microscopy of auramine-stained smears provides similar specificity and increased sensitivity (mean improvement of 10%), compared with light microscopy of ZN-stained smears. In addition to increased sensitivity, fluorescence microscopy also allows more-rapid screening of sputum smear specimens. From an operational perspective, this is highly advantageous, particularly when high numbers of samples are screened per day, because the majority of laboratory time is spent confirming negative smear results (FIND, 2008). Compared to ZN, timing data showed that LED has similar gains in efficiency as conventional fluorescence microscopy, while requiring around half the time than ZN for smear examination (WHO, 2010). In fluorescence microscopy, the same area that needs examination for 10 minutes with a light microscope can be examined in 2 minutes (Bhavan, 2011).

Light emitting diode (LED) technology has been developed over recent years to allow the benefits of fluorescent microscopy without the associated costs. In 2009, the evidence base for LED microscopy was assessed by the world health organization (WHO) following standards appropriate for evaluating both the accuracy and patient/public health impact of new TB diagnostics. Results showed equivalent accuracy of LED microscopy to international reference standards, improved sensitivity over conventional ZN microscopy, and qualitative, operational and cost advantages of led relative to both conventional fluorescent and ZN microscopy. Based on these findings, WHO recommend that conventional fluorescent microscopy be replaced by LED microscopy, and that LED microscopy be phased in as an alternative for conventional ZN light microscopy. Compared to conventional mercury vapor fluorescent microscopes, LED microscopes are less expensive, require less power and are able to run on batteries, the bulbs have a very long half-life and do not pose the risk of releasing potentially toxic products if broken. LED microscopes are reported to perform equally well without a dark room. These qualities make LED microscopy feasible for use in resource-limited settings, having the potential to bring the benefits of fluorescent microscopy (improved sensitivity and efficiency) where needed most (WHO, 2010). Microscopic examination of sputum is the only widely available diagnostic tool for identifying TB in most high-burden countries (WHO, 2011a).

2.5. Anti-tuberculosis therapy

MTB is grows extremely slowly and a long period of antimicrobial therapy is therefore required to treat the infection. In addition the MTB may be protected by closed cavities in the lung or macrophages. Treatment with a combination of drugs is therefore used to prevent resistant organisms emerging and to kill intracellular bacteria. (Wilson, 2000). The requirements for adequate chemotherapy include an appropriate combination of drugs, prescribed in the correct dosage, taken regularly by the patient and for a sufficient period of time (FMoH, 2013). For cases of MD-RTB diseases, the treatment regime should include four anti-tuberculosis drugs to which the organism is susceptible (Committee of infectious diseases of American academy of pediatrics, 2009). Drug-resistant TB disease should always be treated with a daily regimen and under direct observation. There are no intermittent regimens for treatment of multidrug-resistant (MDR) TB (CDC, 2013b).

The aims of TB treatment is to cure the TB patient and restore quality of life and productivity, to prevent death from active TB or its late effects, to prevent relapse of TB, to prevent the development and transmission of drug resistance and to decrease TB transmission to others. First line drugs for the treatment of TB in Ethiopia include Rifampicin(R), Ethambutol (E) Isoniazid (H), Pyrazinamide (Z) and Streptomycin (S) (FMoH, 2013). According to the Ministry of Health of Ethiopia, the treatment regimens for the new TB cases consist of Isoniazid (INH) - rifampicin (RMP) - pyrazinamide (PZA) - ethambutol (EMB) for the first two months followed by INH-RMP for four months whereas for previously treated TB cases an eight month regimen containing streptomycin (SM)-RMP-INH-PZA and EMB for two months followed by INH-RMP-PZA-EMB for one month during the intensive phase, followed by five months INH-RMP-EMB, is recommended (Current anti TB therapy consists of a combination of drugs taken over a period of at least 6 months for new patients and 8 months for retreatment patients (Sintayehu *et al*., 2014). There has been good progress in DOTS (Direct observed therapy) expansion in the African Region in recent years. Nine of the world's 22 TB high-burden countries are in Africa, and all nine (Democratic Republic of Congo, Ethiopia, Kenya, Mozambique, Nigeria, South Africa, Uganda, United Republic of Tanzania and Zimbabwe) have a DOTS program (Stop TB partnership and WHO, 2006). DOT is a component of case management that helps ensure patients adhere to therapy. It is the method whereby a trained health-care worker or another trained designated person watches a patient swallow each dose of anti-TB drugs and documents it (CDC, 2013c).

2.6. Drug Susceptibility test for MTB

MTB is a slow- growing bacterium, resistant to most conventional antimicrobial agents partly due to its impermeable cell wall (Worku and Befikadu, 2015). Mycobacterial susceptibility testing is important in determining the appropriateness of treatment given against mycobacterial infection. Among the methods used for susceptibility testing of MTB, the agar proportion method (MOP) is universally accepted as the gold standard. The result of this method takes 3-4 weeks. The preferred medium for this test is Middle brook 7H10 agar plate because it has simple composition, is easy to prepare and allows the easy detection of colonies. A smear positive specimen is used as the source of inoculums. The number of colony forming units (CFU) on the

drug- containing plates is compared with the number of colony forming units on a drug- free plate. Strains of tubercle bacilli that exceed greater than 1% growth on drug-containing media, compared with growth on a drug-free media, are considered resistant to that agent (Markova *et al*., 2012).

MDR-TB is a particularly threatening infection that is difficult to diagnose without a proper laboratory facility. The most critical factor in addressing MDR-TB in Ethiopia is the lack of laboratory infrastructure and transport networks that can provide rapid diagnosis. In addition, transmission dynamics of MDR-TB in Ethiopia are not well understood. In fact, laboratory services in Ethiopia remain in a stage of Ziehl Nielsen (ZN) smear in clinical specimen. ZN smear lack sensitivity and specificity. Moreover, these methods are limited due to lack of species identification and drug susceptibility testing. Culture-based drug susceptibility testing methods can provide definitive results, but are labor intensive, time consuming and generally unavailable in resource-limited settings where TB is endemic (Biadglegn, *et al.,* 2014b) The envelope of tuberculosis bacilli is a major determinant of exceptional impermeability that limits nutrient uptake and is responsible for the long doubling time and slow growth of mycobacteria (Markova *et al*., 2012).

Conventional methods for mycobacteriological culture and drug susceptibility testing (DST) are slow and cumbersome, requiring sequential procedures for isolation of mycobacteria from clinical specimens, identification of MTB complex, and *in vitro* testing of strain susceptibility to anti-TB drugs. During this time patients may be inappropriately treated, drug resistant strains may continue to spread, and amplification of resistance may occur. Due to the slow growth of mycobacterial, novel technologies for rapid detection of anti-TB drug resistance become a priority in TB research and development and molecular line probe assays focused on rapid detection of Rifampicin resistance (alone or in combination with Isoniazid) are most advanced (WHO, 2008).

Line probe assay is a rapid and accurate test to identify cases with MDR-TB (FMoH, 2013). Rapid diagnosis of drug-resistant TB will have several benefits. These include earlier initiation of treatment of patients, which will in turn save more lives, reducing the time spent on

inadequate treatment when infected by drug-resistant strains (therefore promoting development of additional drug resistance), and reducing the spread of MDR-TB in the community. Moreover, early identification of MDR-TB will lead to faster initiation of laboratory testing for XDR-TB. Systematic reviews and meta-analyses of the performance of LPAs compared to conventional DST methods showed that LPAs are highly sensitive (≥97%) and specific (≥99%) for the detection of RMP resistance, alone or in combination with INH (sensitivity ≥90%; specificity ≥99%), on isolates of MTB and on smear-positive sputum specimens. When RMP resistance alone was used as a marker for MDR, the overall accuracy for detection of MDR was equally high, at 99%. These results were confirmed by laboratory validation and field demonstration data in several countries, most notably in the large-scale demonstration project in South Africa, executed by FIND (Foundation for Innovative New Diagnostics), the South African Medical Research Council (SAMRC) and the South African National Health Laboratory Service (NHLS) (Barnard *et al.,* 2012).

In wild-type MTB strains (susceptible strains), RMP (Rifampicin) and INH (Isoniazid) inhibit the activity of enzymes involved in RNA and cell wall synthesis respectively, thereby killing the bacilli. However, resistant strains have mutations in genes encoding these critical enzymes, thereby altering the structure of the proteins so that a complex cannot be formed between the altered enzymes and the drugs. The bacilli with these specific mutations (resistant bacilli) can therefore still replicate and multiply in the presence of the drug (Barnard *et al.,* 2012). Genotype MTBDR*plus* assay identifies these mutations in the *rpoB* gene (coding for the *β*-subunit of the RNA polymerase) for detection of RIF resistance, mutations in the *katG* gene (coding for the catalase peroxidase) for high-level INH resistance, and mutations in the promoter region of *inhA* gene (coding for the NADH enoyl ACP reductase) for low levels INH resistance (Sharma *et al*., 2014).

The Genotype MTBDR (Hain Lifescience, Nehren, Germany) is a commercial LPA developed for the detection of RMP and/or INH resistance in MTB (Bernard *et al.,* 2012). Line probe assay Genotype MTBDR *plus* assay is validated for both direct use on smear-positive pulmonary specimens and on isolates of MTB grown on liquid medium or in solid medium. (Sharma *et al*., 2014; FMoH, 2012). The molecular LPA is divided into three procedures. These are DNA

extraction from NaOH-NALC decontaminated smear positive specimens, or from cultured isolates (solid or liquid media), A multiplex PCR with conditions that are specific for the type of specimen that was extracted and Reverse hybridization, where probes (reaction zones or bands) on the strips are used to interrogate the MTB target DNA associated with RMP and INH resistance by detecting sequences complementary to the probes on the strip (Barnard *et al.,* 2012).

MTB is classified by WHO as a risk group 3 laboratory pathogen, causing serious human disease but with limited possibilities of (laboratory) spread and with effective treatment and preventive measures available. Mycobacteriological culture and DST procedures generate high concentrations of organisms that pose an increased risk of aerosol spread. Bio-safety level 3 containment requires the strengthening of laboratory operations and safety programs, specifically those related to laboratory design, use of specialized equipment to prevent or contain aerosols, and health surveillance of laboratory staff (WHO the stop TB department, 2008).

3. MATERIALS AND METHODS

3.1. Description of the study area

The study was conducted in Robe Didea hospital found in Robe woreda (Figure 3), Arsi zone, Oromia National Regional State. Around 201, 445 people live in Arsi Robe woreda. Robe town is located about 223 km southeast of Addis Ababa. The area comprises mid land area with an altitude ranging from 1150 to 2659 meter above sea level. The annual mean rainfall ranges from 700 to 1300 mm and annual ambient temperature varies from15- 30 ^{o}C (Hamusse, *et al.* 2014).

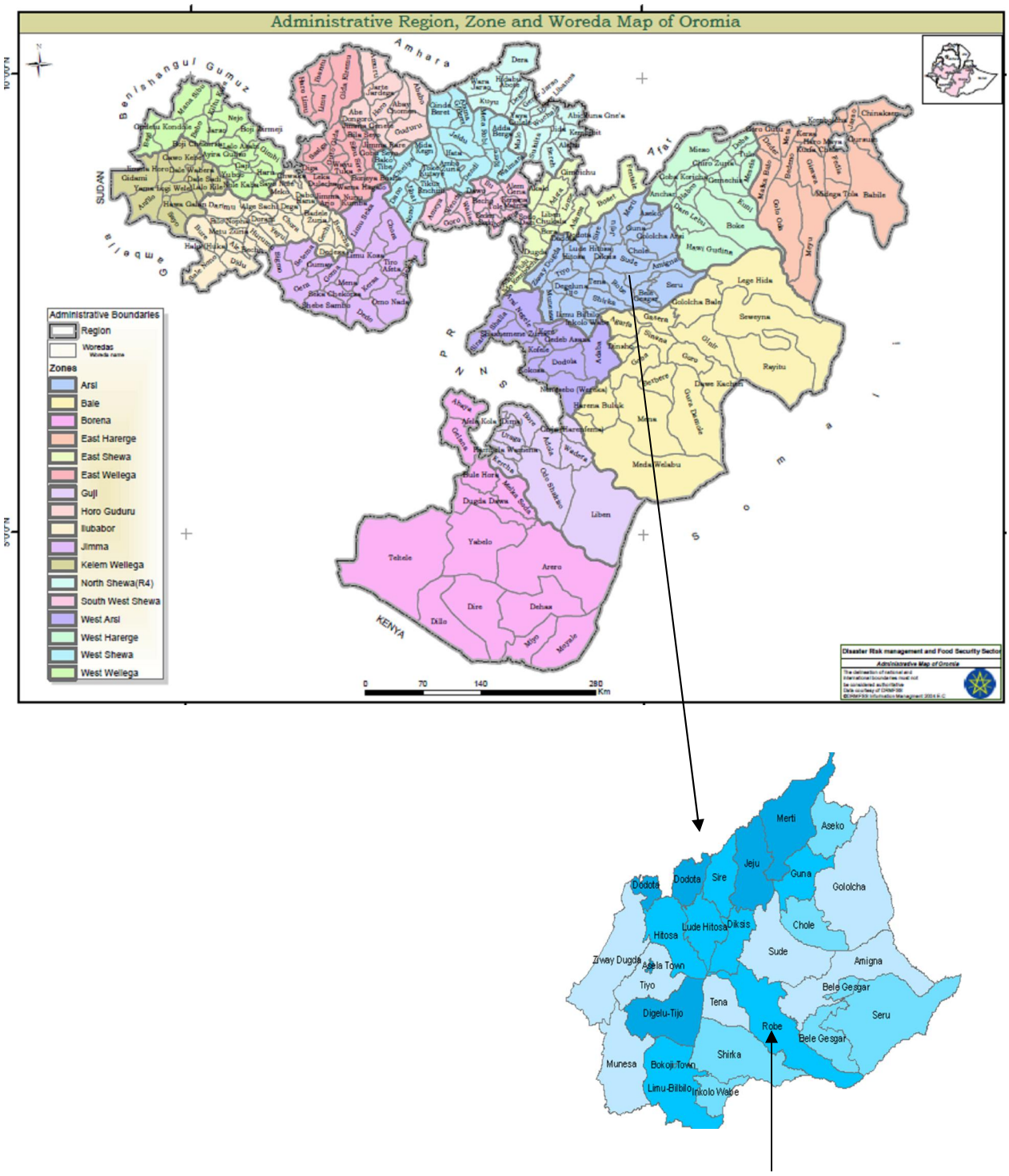

Fig. 3 Map of the study site (Hamusse, *et al.* 2014).

3.2. Study design

The study design for carrying out a survey on the prevalence of MTB was a cross- sectional study. It was a health facility based study. The study included laboratory activities and study of demographic and medical histories by means of questionnaires.

The laboratory experiments for identifying TB patients were carried out at the medical laboratory of Robe hospital. Drug susceptibility test was carried out in Oromia Public Health Research, Capacity building and Quality assurance Laboratory Center.

3.3. Study population

The study population was all patients with sign and symptoms of TB seeking medication at Robe Didea hospital during the study period (from February to July 2015).

3.4. Study participants

The study participants were those patients suspected for TB and willing to participate in the study. The participants of the study were also those that were agreed to give informed consent. FMOH (2008) defined Tuberculosis suspect as a person who presents with symptoms and/or signs suggestive of TB, in particular, cough for two weeks or more.

3.5. Sample size determination

Based on the data obtained from the hospital, 321 patients suspected for Tb visited the hospital in the previous year. Based on this data, a total of 147 suspected patients were estimated by single population proportion formula (Cochran, 1977).

$$n = \frac{n_0}{1 + \frac{n_0}{N}} \qquad \text{Where } n_o = \frac{Z^2_{\alpha/2} P(1-P)^2}{d^2}$$

Z= Standard normal deviation (1.96 for 95% confidence interval)

n=Total sample size

d=margin of error

N=total number of the population

P=proportion of the population

n_o =required sample size

α=level of significance

d=0.04, P= 0.13 and α=0.05

$$n_o = \frac{(1.96)^2 \times 0.13 \times (0.87)}{(0.04)^2} = 271$$

$$\text{Therefore } n = \frac{271}{1 + \frac{271}{321}} = 147$$

A total of 147 patients suspected for TB were involved in the study.

3.6. Sampling technique

Convenient sampling technique was implemented to include all suspects of TB who were sent to Robe-Didea hospital laboratory.

3.7. Data collection

The diagnosis of TB was based on the national TB program designed by the Federal ministry of health (FMOH) of Ethiopia. TB was considered in the presence of suggestive sign and symptom, and pathological finding compatible with pulmonary tuberculosis from acid- fast bacilli isolated from the sputum.

Identification of TB suspects was done according to guidelines for clinical and programmatic management of TB, leprosy and TB/HIV in Ethiopia (FMoH, 2012). It involved screening of

patients for signs & symptoms of TB, in particular cough of two weeks or more duration. Other symptoms that were used to identify TB suspects include fever, night sweating, and weight loss, chest pain, shortness of breath, Hemoptysis (coughing up of blood), loss of appetite, malaise fever, weight loss, chills, and fatigue. All clinically suspected cases of PTB who were sent to the laboratory with a request form for AFB examination during the data collection period were involved in the study. Clinical manifestations of the study participants were registered using clinical data record form by health professionals of the hospital. The data was collected for the period extending from February to July, 2015.

The epidemiologic risk factors for exposure to MTB were investigated using questionnaires. The questionnaires on demographic and medical history of the study population including age, sex, contact with actively infected patient, the completion of taking provided drug, previous drug usage, housing condition, previous time exposure to the disease, presence of diabetes mellitus, being a health worker, presence of family member with active TB, presence of healed TB and being in prison were filled by suspected TB patients. It had mainly closed type of questions. The questionnaires were prepared in English and Amharic languages, and were translated to local language Afan Oromo so that respondents easily understand the questions. The questionnaire was filled by the respondents assisted by health professionals.

3.8. Microbiological analysis of specimen

3.8.1. Sample sputum collection and preparation

Sputum samples were collected from 147 participants by trained laboratory technologist following the national tuberculosis and leprosy control program (NTLCP) manual standard procedures on the collection of sputum sample for PTB suspect cases (i.e. spot-early morning - spot) by using sputum cap. For this particular study data were collected from sputum sample because it consists of the highest number of mycobacterium important for diagnosis and there are no facilities to collect other types of specimens in the study site.

Fluorescence microscope of acid fast stain was used to detect the presence of MTB in the specimen collected from sample population following the Training Manual for Fluorescent based AFB microscopy by Foundation for innovative new diagnostics (2008). New, clean and unscratched frosted-end slides were used for smear preparation. The laboratory serial number and order number of the sputum specimen was recorded on the frosted end of the slide. The sputum smears were prepared by using an applicator stick in the center of the slide. Slides were arranged in serial order, smear side up and a finger thickness was kept between the smears. They were fixed with flame by passing three times under flame. Then they were flooded with filtered 0.1 % Auramine solution. After flooding, the slides were left for 20 minutes, rinsed with water and drained. The next procedure was applying decolorizing solution of 0.5 % acid alcohol made from 0.5 ml of hydrochloric acid and 100 ml of ethanol, for 3 minutes. Then, they were rinsed, drained and air dried. After that the entire surface of the slides were covered with potassium permanganate solution for 1 minute, rinsed, drained and air dried.

Finally, the slides were placed onto stage of the florescent microscope smear facing upward and examined first using 20x objectives and if confirmation is necessary a 40x objective were used. MTB appeared bright yellow against dark background material.

3.9. Antibiotic susceptibility testing

Those sputum samples positive for AFB were collected in a sterile falcon tube and stored in a refrigerator at a temperature of 2-4^{o}C before transported to where drug susceptibility test were conducted.

After temporary storage sputum samples were transported to the Oromia Public Health Research, Capacity building and Quality Assurance Laboratory (OPHRCBQAL) found at Adama located 103 Km away from the study site in sterile falcon tube using a cold (Ice) box (at 4°C) on every four days of collection after the specimen ID, collection date of the specimen and time of collection is registered on plaster and fixed into each of the falcon tubes.

The method used for drug susceptibility test was a Molecular method known as Genotype MTBDR*plus* line probe assay (LPA) (HLEA, 2012). The Genotype MTBDR (Hain Lifescience, Nehren, Germany) is a commercial LPA developed for the detection of RMP and/or INH resistance in MTB (Barnard *et, al.,* 2012). This particular study focused on the resistance of the above two drugs. Genotype MTBDR*plus* line probe assay was performed on smear positive sputum according to manufacturer's instructions (HLEA, 2012). The Line probe assay technology involved the following major steps.

DNA extraction

DNA extraction was performed in biosafety level 3 laboratories according to the manufacturers, HLEA, protocol. At the time of DNA extraction equal amounts of specimen and activated NALC (N-acetyl-L- Cystein) -NaOH solution (approximately 10 ml of each) were added a sterile, aerosol-free 50 ml centrifuge tube with screw cap to decontaminate the sputum sample. Next, the mixtures were centrifuged using a Vortex-type mixer until the specimen was liquefied. Finally, the mixtures were allowed to stand at room temperature for 15 min with occasional gentle shaking. After decontamination, 500 ml of decontaminated sputum sample was transferred into 1.5 ml screw capped tubes and the tubes were centrifuged at 10,000 x g for 15 minutes. Then the supernatant in each tube was discarded and the pellets were re-suspended in 100 ml of lyses buffer. The next process was incubating the pellets for 5 minutes at 95^{O}C and letting them to stand for 5 minutes to cool. Then after, 100ml of neutralization buffer was added to the sample. Finally, they were made to spin at full speed and 10 ml of the supernatant was used for PCR.

Polymerase chain reaction

Polymerase chain reaction (PCR), amplification of the resistance-determining region of the gene under question was performed using thermo cycler machine. At the beginning of this process, master mix used in the process of amplification was prepared. The master mix was made up of 5 components with a total volume of 45 ml for each PCR reaction (35 µl of the Primer Nucleotide mix, 5 µl of buffer, 2 µl of MgCl2, 3 µl of distilled water and 0.2 µl of *Taq* polymerase). Before

the PCR tubes are placed into the thermal cycler, they were mixed slightly and spun down for 5 - 10 seconds in a mini-centrifuge.

A total 5 ml of respective DNA was transferred into 45 ml of master mix and the caps were closed tightly and placed in thermo-cycler. The Polymerase Chain Reaction (PCR) included 30 cycles of repeating three steps comprising denaturation of the double-stranded DNA into single-stranded DNA at 95ºC for 25 seconds, annealing of the forward and reverse primers at 53ºC for 40 seconds, and DNA polymerase mediated Elongation/Extension of the DNA by incorporation of the dNTPs (nucleotides) at 70ºC for 40 seconds.

Figure 4: Amplification of DNA by PCR using Thermo cycler machine.

Hybridization

The LPA used Reverse Hybridization. The biotin-labeled amplicons (amplified DNA of the genes of interest generated during amplification of the target DNA) were in the fluidic state and the wild-type and/or mutated probes (reaction zones) are unlabeled and immobilized as bands onto the positively charged nitrocellulose membrane strips. The hybridization step involved two different steps: denaturation and hybridization. During denaturation, 20 ml of denaturation solution (DEN) was added in each well of the TwinCubator tray containing 20 ml of DNA PCR products and the mixture was mixed. Chemical denaturation with sodium hydroxide (DEN buffer) of the biotinylated double-stranded DNA (dsDNA) into biotinylated single stranded DNA

(ssDNA) allowed the ssDNA to bind to a specific single-stranded probe (reaction zone) on the strip. During hybridization, 1 ml of hybridization solution (Buffer) (HYB) was added to each well containing DEN and amplified biotinylated ssDNA (Amplicons). Labeled strips of DNA (membrane-bound probes) was placed to each tray containing DEN, amplicons and HYB and incubated for 20 minutes at 45 ºC in the TwinCubator. Due to addition of labeled strips of DNA, hybridization of the single-stranded-biotin-labelled amplicons to membrane-bound probes took place. The hybridization buffer was used to stabilize the exact nucleotide match between the amplicons and the membrane-bound probes. After this, the HYB was removed completely by aspiration. Then, 1 ml of STR (Stringent wash buffer) was added to each tray and incubated for 10 minutes. Stringent wash buffer removed the non-specifically bound amplicons. Following this, the whole quantity of STR was removed and 1 ml of RIN (rinsing solution) was added and incubated. The rinsing solution was used to remove the soapiness and saltiness caused by the addition of hybridization and stringent wash buffer. Finally the whole Rinsing solution (RIN) was removed.

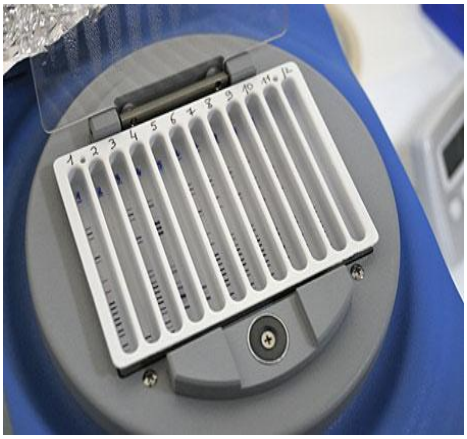

Figure 5: A TwinCubator tray where hybridization takes place

Figure 6. When buffers and DNA were added into the TwinCubator tray

Detection

Following hybridization of the biotin-labeled amplicons to the reaction zones, the strips were exposed to a conjugation buffer. About 1ml of conjugation buffer was added to the mixture and it was incubated for 20 minutes at 37 ºC in the TwinCubator. The conjugation buffer consisted of streptavidin-alkaline phosphatase. The biotin of the primers of the amplicons and streptavidin

had a high affinity for one another and binding took place at band sites on the strip where hybridization had occurred. Then, the conjugation buffer was aspirated using filter tip. Next, 1 ml of RIN (rinsing solution) was added to tray and those trays were incubated for 1 minute at room temperature. Followed by removal of RIN, 1 ml of substrate was added to each tray and incubated for 2-10 minutes in the twicubator. The substrate contained hydrogen peroxide that reacted with the alkaline phosphatase and undergo a calorimetric change that turns the bound amplicons purplish-brown. Finally, the substrate was aspirated completely and the strip was rinsed.

Mounting and interpretation

After band or color formation, the DNA strips were removed from the tray and air dried on absorbent paper. A pair of clean or disposable tweezers was used to remove the strips from the TwinCubator tray and place them onto absorbent paper. At the end, dried strips were made to stick to the result shit GenoType MTBDR*plus* score sheet and interpreted. During interpretation, an isolate was considered sensitive when all wild type probes produce band but no such bands in mutation probes. Missing of band development in any of the wild types probes or band development in any of the mutation probes suggested resistant type of isolates.

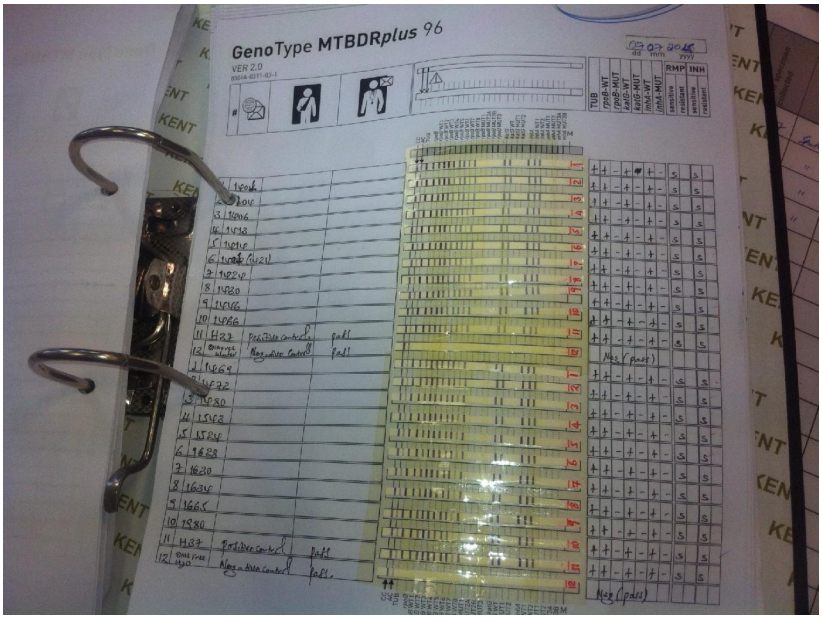

Figure 7. When dried strips were made to stick to the result shit for interpretation

3.10. Quality Assurance

Quality assurance of LED microscopic examination

Prepared positive and negative controls which are supplied to the laboratory, where the sputum examination of participants took place, by national regional laboratory were used during the sputum examination. Positive and negative controls were also prepared as stated by training manual for fluorescence based acid fast bacilli microscopy (FIND, 2008). Positive control was made using smears with low positive (1+) sputum. Negative controls was made using sputum that had been has been extensively examined to ensure that there is no AFB is present. Quality control was performed for each batch of freshly prepared staining reagents so that they do not contain artifacts or contaminating AFB. Quality control was performed by using each of freshly prepared reagents and the normal staining procedure as descried for positive controls. All the controls were examined for number, completeness and intensity of color of AFB, as well as color and complete distaining of background. All reagents including Auramine O were tested in this way before used for examination.

Quality assurance of Line probe assay

The quality assurance of line probe assay was performed as stated in HAIN life science east Africa Limited trainee manual (HLEA, 2012). The specimen preparation area (DNA extraction lab), reagent preparation area (pre-amplification lab), template addition, amplification, and hybridization/detection area (post-amplification lab) were individually enclosed and separated from one another to reduced risk of contamination. Positive pressure system was used for the reagent preparation area to prevent introduction of contaminants, while negative pressure system was used for the specimen preparation and hybridization/detection areas to adhere to the BSL-3 guidelines on Biosafety, and to keep the exogenous DNA and amplicons in the area itself. Contaminating solutions was avoided by pipette tips by changing pipettes when working with DNA amplicons to avoid contamination. All the materials in the DNA contaminated areas (PCR/Hybridization) areas were not taken back to the pre PCR area.

3.11. Inclusion criteria

Patients that had ≥ 2 weeks duration of cough and those who agreed to give informed consent were included in the study.

3.12. Exclusion criteria

Patients who provided incomplete information were excluded from the study. Patients that refused to give informed consent were also excluded from the study.

3.13. Data analysis

IBM SPSS software version 20 was used to statistically analyze the data collected from the respondents. Categorical data was presented by means of percentages and frequencies. The presence of risk factor association with MTB was tested using chi-square test. In this study p-value <0.05 was considered as statistically significant. Relative risk was used to describe the strength of association between risk factors and prevalence of MTB.

3.14. Ethical considerations

The study was ethically approved by Research Review and Ethical committee of Jimma University, College of Natural sciences. Letter of permission was given to concerned officials in Robe hospital. Oral and written permission was also obtained from participants of the study before data collection.

4. RESULT

4.1. Sociodemographic characteristics of the study participants

A total of 147 sputum samples were collected from the patients and examined during the study period. About 3.4% of the study population was aged between 1 and15 years while 42.2% were between 31 and 45.9 years. About 57.8 % were males and the remaining 42.2 % were females (Figure 4).

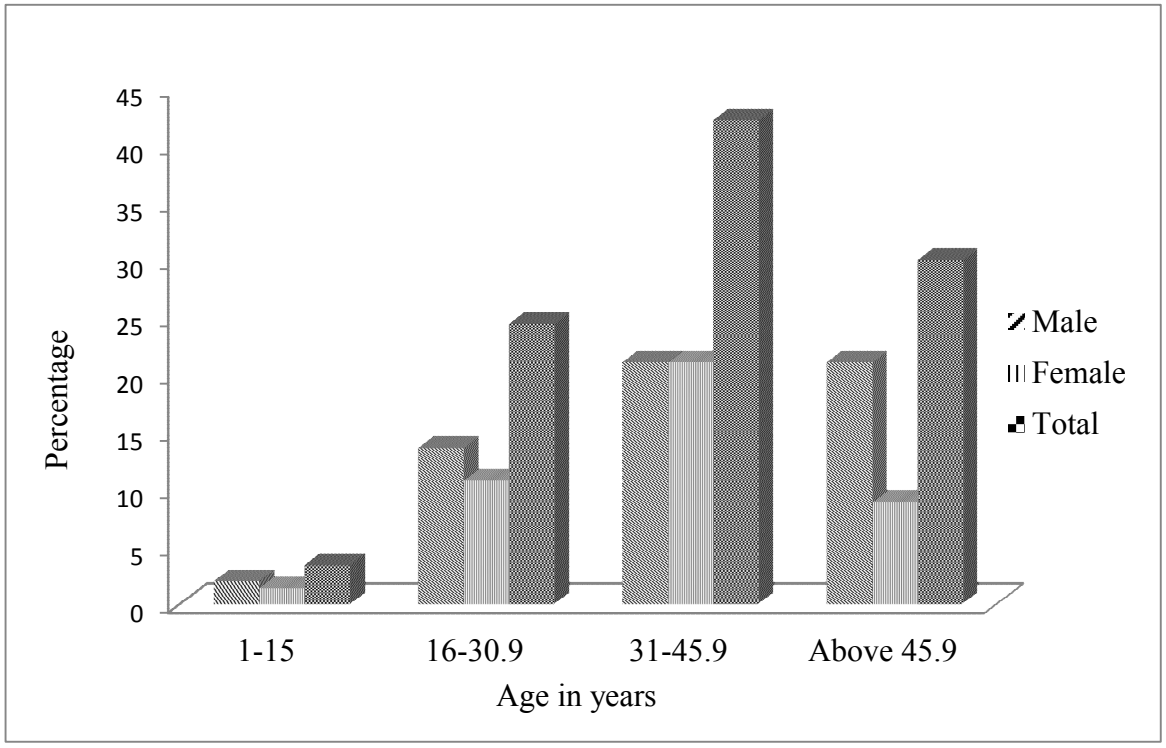

Figure 8: Socio-demographic characteristics of the study population, Robe Didea Hospital, Arsi zone, 2015

4.2. Prevalence of *Mycobacterium tuberculosis*

MTB were detected among 9 of the 147 suspected TB patients. Accordingly, the overall prevalence of MTB was 6.12%. About 22.2% of the MTB positive were aged between 16-30.9

while 55.6% were between 31 and 45.9 years. 66.7% of the smear positive patients were males and 33.3% of them were females (Table 2).

Table 2: Prevalence of MTB among patients seeking medication at Robe Didea, Arsi zone, Robe Didea Hospital, 2015 (n=147)

Socio-demographic Character	Alternatives	AFB- Positive	
		Number	Percentage (%)
Sex	Male	6	4.08
	Female	3	2.04
	Total	9	6.12
Age (years)	≤ 15.9	-	-
	16-30.9	2	1.36
	31-45.9	5	3.4
	Above 45.9	2	1.36

4.3. Analysis of antibiotic susceptibility test

A total of 9 isolates were subjected to antibiotic susceptibility test against the two first line drugs used to treat TB: Isoniazid and rifampicin. A total of 88.9 % were sensitive to Isoniazid and rifampicin. Accordingly, equal mono-resistance was observed for isoniazid and rifampicin (Table 3). MDR-TB is caused by strains of MTB that are resistant to at least Rifampicin and Isoniazid, two key drugs in the treatment of the disease. Of the total 9 isolates, only 1 (11.1 %) of the isolates was resistant to both Rifampcin and Isoniazid (Table 3).

Table 3: Drug resistance pattern in MTB isolated from outpatients visiting Robe Hospital, Arsi Zone, 2015

Antimicrobial drug	Condition	Isolates of MTB	
		No	%
Isoniazid	Susceptible	8	88.9
	Resistant	1	11.1
Rifampcin	Susceptible	8	88.9
	Resistant	1	11.1
Isoniazid and Rifampcin	Susceptible	8	88.9
	Resistant	1	11.1

4.4. Analysis of epidemiologic risk factors of *M. tuberculosis*

Table 4: A summary of Analysis of epidemiological risk factors for MTB among out Patients visiting Robe Didea hospital, 2015 (n=147)

Risk factor	Characteristic	Sputum examination		P value
		Positive	Negative	
		%	%	
Age	1- 15	-	100	>0.05
	15-30.9	5.6	94.4	
	31-45.9	8.1	91.9	
	Above 45	4.5	95.5	
Sex	Male	7.1	92.9	>0.05
	Female	4.9	95.1	

……..Cont'd

Living condition	Over crowded	11.6	88.3	<0.05
	Sparse	2.3	97.7	
Presence of family member with active TB	Present	13.6	35.84	<0.05
	Absent	2.91	97.08	
Completing taking prescribed drug	Yes	12.5	87.5	-
	No	100	-	
Previous treatment	Present	9.2	90.8	>0.05
	Absent	3.7	96.3	
Close contact with active TB patients	Present	13.6	35.84	<0.05
	Absent	2.91	97.08	
Presence of healed TB case	Present	8.3	91.7	>0.05
	Absent	4.6	95.4	
Being in prison	Present	16.7	83.3	>0.05
	Absent	5.2	94.8	
Number of prisoners in a cell	1-25	-	-	-
	26-50	-	100	
	51-70	22.2	77.8	
	Above 70	-	100	
Diabetic	Yes	9.1	90.9	>0.05
	No	5.9	94.1	
Health professional	Yes	-	-	-
	No	6.12	93.88	

The prevalence of MTB is the highest in age groups between 31 and 45 (62/147). It is also higher in males than females (85/147). But, significant association was not demonstrated between age group and prevalence of MTB ($p>0.05$). Likewise, there was also no significant association between sex of individual and prevalence ($p>0.05$) (Table 4).

Regarding close contact with active TB patients, minority of the respondents (44/147) responded that they have close contact and the rest (103/147) responded oppositely (Table 4). There was significant association between the respondents practice of having close contact with active TB patient and prevalence of MTB ($p<0.05$).

In relation to housing condition, 60/147 (40.8%) of the participants made a statement that they live in crowded condition and the rest of them 87/147 (59.2%) said they live in a sparse condition. From the respondents that live in crowded condition, 7/60 (11.6%) were diagnosed as smear positive patients (Table 4). There is significant relationship between housing condition and prevalence of MTB ($p<0.05$).

With regard to presence of family member with active TB, 44/147 (29.9 %) of the respondents gave positive answer as they have a family member with active TB. From this respondents with positive answer, 6/44 (13.6%) were diagnosed as smear positive for *M. tuberculosis* (Table 4). There is significant relationship between Presence of family member with active TB and prevalence of MTB as indicated by the p-value of 0.013.

With regards to previous treatment, the resulted indicated that 6/65 (9.2%) of participants who used treatment of TB previously were diagnosed as smear positive for MTB while only 3/82 (3.7%) of participants who did not used treatment before were diagnosed as smear positive for MTB. Statistically significant association was not observed between previous usage of treatment and prevalence of MTB with p value equals >0.05

Regarding completing taking prescribed drug the result reveals that 8/64 (12.5%) of respondents that took their treatment correctly were diagnosed as smear positive for *M. tuberculosis* (Table 4). There is no association between prevalence of MTB and completing taking prescribed drug.

Considering presence of healed pulmonary TB, 5/60 (8.3%) of study participants' who were recovered from pulmonary TB were diagnosed as smear positive for MTB while 4/87 (4.6%) of participants who were not recovered from pulmonary TB are diagnosed as smear positive for the bacilli (Table 4). Statistically significant association was not observed between presence of healed TB and prevalence of MTB with $p>0.05$

In this study, 12/147 (8.2%) of the participants of the study were prisoners. From this participants 2/12 (16.7%) were diagnosed as smear positive patients. The P value of 0.112 indicates that there is no statistical significant association between being prisoner and prevalence of MTB.

Concerning number of prisoners in a cell, information was collected from participants that are prisoners. The majority of prisoners 9/12 (75%) stated that the number of prisoners were 51-70 per cell. From this prisoners, 2/9 (22.2%) were diagnosed as smear positive for MTB. Furthermore, 2/12 (16.7%) of the prisoners responded that there are 26-50 prisoners per cell. From this group, no one was diagnosed as smear positive case.

With regard to being diabetic, the majority of patients 136/147 (92.5%) were not diabetic. Out of patients who were not diabetic, 8/136(5.9%) were diagnosed as positive for MTB. From those who were diabetic, 1/11 (9.1%) was diagnosed as smear positive for MTB (Table 4). The P value of $p>0.05$ indicates that there was no significant statistical association was observed between being a diabetic and prevalence of MTB

In this particular study none of the participants were health professional. About 9/147 (6.12%) of the participants who were not health professionals was tested smear-positive for MTB (Table 4). No association was found between being health professional and prevalence of MTB.

Housing condition (including over crowdedness), presence of active TB case in the family, and close contact with active TB patients are the high risk factors ($RR > 1$, $P< 0.05$) contributing to the high prevalence of MTB in the study area (Table 5). [$RR>1$ indicates important risk factor; $RR<1$ indicates that the factor is either less important or protective]. From among the suspected

risk factors, the significance of some of the factors such as presence of healed TB case, diabetic, and being maleness or femaleness appeared less important.

Table 5: Strength of association between risk factors and prevalence of MTB

Risk factors	Alternatives	Relative Risk (95% CI)
Sex	Male	1.45 (0.38-5.6)
	Female	
Housing condition	Over crowded	5.07(1.09-23.59)
	Sparse	
Presence of family member with active TB	Present	4.68 (1.22-17.88)
	Absent	
Completing taking prescribed drug	Yes	0.125(0.065-0.239)
	No	
Previous treatment	Present	2.52 (0.6559- 9.705)
	Absent	
Close contact with active TB patients	Present	4.68 (1.22- 17.88)
	Absent	
Presence of healed TB case	Present	1.81 (0.5-6.47)
	Absent	
Being in prison	Present	3.21 (0.749- 13.79)
	Absent	
Diabetic	Yes	1.54 (0.212- 11.26)
	No	
Health professional	Yes	7.78 (0.998- 60.70)
	No	

5. DISCUSSION

In this particular study, 147 sputum samples were collected from patients suspected for TB. The result reveals that 9 of these 147 samples were smear- positive for MTB. Accordingly, the prevalence of MTB in the study site is 6.12%.

The prevalence of TB among patients attending Seka Health center, Jimma, was 10.625% (Gebre and Mimano, 2010), in Nekemte referral hospital was 9.41% (Ejeta *et al.,* 2013), in south east Ethiopia hospitals was`9.21% (Tulu *et al.,* 2014), in three governmental hospitals in East Ethiopia was 21.6% (Mekonen, 2014) and in outpatients in Shashigo woreda was 4.6% (Eliso *et al.,* 2015). The prevalence of MTB in the study area was lower to the one reported from Seka heath center, Nekemte referral hospital, south east Ethiopian hospitals and three governmental hospitals in east Ethiopia but more or less similar to the one found on out patients of Shashigo woreda. To the contrary, related study conducted in Gojam (Adane *et al.,* 2013) and Metehara Sugar Factory Hospital (Yohannes *et al.,* 2012) reported much higher prevalence of MTB (32.2% and 14.2%, respectively) than the current finding. Thus, the prevalence of MTB in the study was relatively less severe than the prevalence in Gojam and Metehara Sugar Factory although it still needs attention.

Several risk factors could contribute to the current prevalence of MTB. Among the main risk factors were close contact with active TB patient, presence of family member with active TB and crowded living condition. In a similar study done by Yohannes *et al*. (2012), the isolation of MTB was significantly high in those who had close contact with chronic coughers (TB infected patients). As reported by FMoH (2008), and CDC (2013) persons living in the same household, or who otherwise are in frequent and close contact with an infectious patient have the greatest risk of being exposed to MTB. According to Ejeta *et al.* (2013), previous history of contact of TB patients was one of the high risk factors in outpatients visiting Nekemete referral hospital.

This study further reveals that crowded condition was one of the main risk factor for prevalence of MTB. In agreement with our findings, Yohannes *et al*. (2012) also showed that family size is one of the main factors that risked patients of Metehara sugar factory hospital to infection with

MTB. In a related study reported by WHO (2011), Soborog, *et.al* (2011), and Millet *et al*. (2013), crowded condition and number of household residents are among factors associated with the risk of TB infection among children.

The other epidemiological risk factor which the finding of the study reveals it as one of the main factors that exposes the participants of this particular study was the presence of family member with active TB. Under conditions of poor hygienic practices, the active TB cases could easily expose healthy individuals to the disease. Close contacts of infectious TB cases including household contacts and care givers/health care workers are at a higher risk of becoming infected with MTB and development of primary active tuberculosis (Narashiman *et al.,* 2013). One of High Risk for Progression from LTBI to TB Disease is failure to cover the mouth and nose when coughing (CDC, 2005). In a related study by Tulu *et al.* (2014) family size and history of contact with active TB patients were among the risk factors significantly associated with acquiring tuberculosis in TB suspects.

From the assessment made on strength of association between risk factors and prevalence of MTB, it can be observed that patients that live in crowded living condition were five times more likely to be affected by TB than those that live in sparse living condition, suspected patients who have family member with active TB have a five times more chance to be infected with MTB than those that do not have and study participants that had close contact with active TB patient were affected by TB five times more likely than that do not have contact with active TB patient.

Based on the present study, the overall resistance rate to isoniazid and rifampicin is 11.1 %. According to the 2005 nationwide survey done by FMoH/EPHI (2014), Rifampicin resistance in Ethiopia was lower than 2% in new cases. This indicates the resistance rate of rifampicin is higher in the study area than the overall pattern in the nation. In a related study conducted in Nairobi, thirty-seven isolates (30.2%) were resistant to isoniazid and four (1.4%) to rifampicin (Ndung'u *et al.,* 2012). According to the recent report made by Seyoum *et al*. (2014), frequencies of resistances to isoniazid and Rifampicin recorded from Dire Dawa, Harar and Jijiga were 50 (14%), 10 (2.8%), and 41 (11.5%) respectively. The finding of this study revealed that resistance to isoniazid in the study area was lower than all cases reported from Kenya, Dire Dawa and

Jigjiga but higher than Harar. Concerning resistance to rifampicin, the resistance rates observed in Dire Dawa and Jigjiga were higher and in Kenya and Harar were lower than the case at the study area. According to Lem *et al*. (2013) resistance to isoniazid in Lao republic was 6.8 %. The most recent study conducted in Gojam (Adane *et al*., 2015) revealed that rifampicin resistance was 3.89% among newly diagnosed patients. Accordingly, the isolates resistance to isoniazid in the study area was higher as compared to report from Lao republic. The rifampicin resistance in the study area is higher than the one obtained from Gojam. This might be due to the presence of failing to completely take the prescribed drug by the patients.

In other studies the rifampicin resistance was reported 3.4% in Yirgalem hospital (Worku and Befikadu, 2015) and 12% in Bangladesh (Mottalib, 2011). Mottalib *et al.* (2011) also indicated that resistance to isoniazid was 26% in Bangladesh. The 11.1 % resistance to both rifampicin and isoniazid in our current study indicated that the resistance to isoniazid was lower from that of Bangladesh but resistance to rifampicin was higher than Yirgalem and more or less similar to that of Bangladesh. In related study, resistance to rifampicin was reported 17.7% in South Africa (Green et al., 2010), 12.1% in Tanzania (Ranger *et al.,* 2012). The overall resistance rate (11.1%) towards rifampicin observed in this study indicated similar pattern to the one encountered in Tanzania but lower than the South African case.

In Ethiopia, single drug resistance to Isoniazid and rifampicin has been reported to be 13.8 and 5.8 per cent, respectively (Biadgilign *et al.,* 2014a). Various reports demonstrated that, in Africa, resistance to one or more anti-TB and MDR-TB (resistance to both isoniazid and rifampicin) ranges between 3% to 37.3% and 1.4% to 11.6% , respectively (Seyoum *et al.,* 2014). The problem of drug resistant TB exists in different parts of Ethiopia, and data on patterns of resistance among Ethiopian isolates ranges between 2%-21% (isoniazid), 2%-20% (streptomycin) and 14%-15% for any of the drugs tested (Desta *et al*., 2008). The resistant rate obtained from the study site is lower in the case of Isoniazid but higher in the case of rifampicin when compared with the result obtained from the nation.

The rate of MDR_TB (resistance to both isoniazid and rifampicin) in the study area was 11.1%. The rate of MDR_TB in Ethiopia is 0.341% (FMOH *et. al.,* 2008). In similar study the rate of

multi-drug resistant tuberculosis (MDR-TB) in Iran was 7.8% (Farazi, *et al.,* 2013), (58.4%) cases in Mpumalanga province of South Africa (Green *et al*., 2010), 17.9% in Tanzania (Range *et al*., 2012) and 5% globally (WHO, 2014). The finding of this study showed that the rate of MDR-TB is higher than that of Iran and much lower than that of South Africa. However, the rate is related to the report from Tanzania.

The prevalence of TB in Ethiopia is decreasing from time to time. One of the reasons is increasing implementation of BCG vaccination in the country. The other reason for the decrease of prevalence of TB is implementation of DOT strategy in the prevention of the effect of MTB. As a case in point, the introduction and expansion of DOTS in Arsi zone has improved the overall TB case notification (Hamusse *et al.,* 2014). The estimated global prevalence rate fell to 128 cases per 100,000 populations in 2010, after peaking at 141 cases per 100,000 in 2002. The rate is falling but very slowly (WHO, 2012). Globally, the TB mortality rate fell by an estimated 45% between 1990 and 2013 and the TB prevalence rate fell by 41% during the same period (WHO, 2014). The prevalence of MTB is higher in the study area. This might be the reasons that individuals with TB do not visit health facilities and submit their sputum for examination for long time and there is a higher chance of transmitting disease to more number of healthy individuals. The other reason might be the presence of poverty that increases the transmission of TB in the community as poverty causes crowdedness,

6. CONCLUSION

- According to the current study, the prevalence of MTB among out patients visiting Robe Didea hospital is 6.12%.

- With respect to the risk factors that expose participants to MTB close contact with active TB patient, presence of family member with active TB, and crowded living condition, are the major contributing risk factors among out patients of Robe Didea hospital, Arsi zone.

- Concerning antibiotic susceptibility pattern of MTB, 11.1 % of the isolates from the participants were resistant to Isoniazid, one of the first line drug used to treat TB patients. Similarly, 11.1 % of the isolates were resistant to the other first- line drug, Rifampcin. About 11.1. % of the isolates was resistant to both Rifampcin and Isoniazid. As MTB is referred as multi-drug- resistant if the bacillus is resistant to at least Isoniazid and Rifampcin, a total of 11.1. % of the isolates was multi-drug resistant.

7. RECOMMENDATION

It is vital to take actions in preventing the community from problems associated with MTB. Understanding the incidence prevalence, drug resistance pattern and risk factors of MTB permit the responsible bodies to take necessary measures as the need arise in order to make the burden decrease in the community. Accordingly, the following recommendations are forwarded based on the current study:

1. Awareness development on the mode of transmission of MTB including the significance of contact with active TB patients should be given to the community to reduce the risk of infection with the bacillus.
2. Periodic surveillance of the status of drug resistance in MTB is recommended.
3. Health care workers should advice and teach families having member with Tb so that the patient use protective measures, such as face masks and tissue to cover their mouth anytime they laugh, sneeze or cough.
4. TB patients should be advised to finish their entire course of medication as this is the most important step that can protect them deleterious effect of TB. Stopping treatment early or skipping the doses could end-up in the development of mutations that allow TB bacterium survives the most potent TB drugs. The resulting drug-resistant strains are much more deadly and difficult to treat.
5. People should not spend long periods of time in stuffy, enclosed rooms with anyone who has active TB until that person has been treated for at least 2 weeks. This information should be disseminated in the community by health workers and all other responsible stake holders.
6. Crowding has been identified as the main a risk factor for TB transmission. Living with persons with TB disease and crowded housing condition lead to an increased risk in terms of exposure to MTB. The risk of exposure is also increased if there is limited air movement in an enclosed space. The risk of contracting TB should be reduced by living and working in well-ventilated rooms where there is an adequate supply of fresh air and sunlight.

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APPENDENCES

Appendix I. Questionnaire (to be filled by the patients)

Jimma University

College of Natural science

Department of Biology (General Biology)

This questionnaire is prepared for the study entitled "**Prevalence and drug resistance pattern of *Mycobacterium tuberculosis* among out patients in Robe woreda visiting Robe Didea hospital"**. You are sincerely asked to respond to the following questions accordingly.

Direction – Mark the symbol "√**"** on the given space that best elucidate about you.

1. Age A. < 15.9 _____ B. 16- 30.9 _____ C. 31- 45.9 D. > 45.9 _____
2. Sex A. Male _____ B. Female _____
3. Close contact with active TB patient A. Yes _____ B. No_____
4. Previous treatment for TB A. Present _____ B. Absent _____
5. If your answer for question number 6 is A, did you complete your treatment?
   A. Yes_____ B. No_____
6. Presence of healed TB. A. Present _____ B. Absent _____
7. Being in prison A. Present _____ B. Absent _____
8. If your answer for question number 9 is A, how many individuals are present in your room?
   A. 1-25_____ B. 26-50 _____ C. 51-70_____ D. >70_____
9. Presence of Diabetes mellitus A. Present _____ B. Absent _____
10. Are you a health care worker? A. Yes _____ B. No____
11. Presence of family member with active TB A. Present _____ B. Absent _____
12. Housing condition A. Over Crowded _____ B. Sparse _____

Thank you

Appendix II- Consent form for adult study participants

PIN _____

Name of study participant _____

I have been informed about a study that plans to investigate the prevalence and drug resistance pattern of *Mycobacterium tuberculosis* in Robe Didea hospital will help in investigating the extent to which tuberculosis is a public health problem in the area.

For the study I have been requested

1. To give sputum and
2. Respond to questionnaires.

They told me that experienced health professionals according to the established aseptic procedure would do the sputum collection. Based on this, I have agreed to continue the examination. The investigator also informed me that all the laboratory results would be kept confidential.

I have been given enough time to think over before I signed this informed consent. It is therefore, with full understanding of the situation that I gave my informed consent and cooperates in the course of the study.

Participants' signature _____ Date _____

Health professional's name _____ Signature _____Date _____

Appendix III- Consent form for parents of study participant

PIN _____

Name of study participant child _____

Name of the parent _____

I have been informed about a study that plans to investigate the prevalence and drug resistance pattern of *Mycobacterium tuberculosis* in Robe Didea hospital which will help in investigating the extent to which tuberculosis is a public health problem in the area.

For the study I have been requested to make my child

1. Give sputum and
2. Respond to questionnaires.

They told me that experienced health professionals according to the established aseptic procedure would do the sputum collection. Based on this, I have agreed to continue the examination. The investigator also informed me that all the laboratory results would be kept confidential.

I have been given enough time to think over before I signed this informed consent. It is therefore; with full understanding of the situation that I gave my informed consent to make my child cooperates in the course of the study.

Parent's signature _____ Date _____

Health professional's name _____ Signature _____Date _____

Appendix IV-Case Record for registering Clinical manifestation of tuberculosis

Patients code	Persistent cough	Coughing up blood (Hemoptysis)	Chest pain	Loss of appetite	Unexplained weight loss	Night sweats	Fever	Fatigue

Data collector

Name _____sign_____ Date_____

Appendix V-TB laboratory request format of OPHRCBQAL

OROMIA PUBLIC HEALTH RESEARCH, CAPACITY BUILDING AND QUALITY ASSURANCE LABORATORY

ADAMA, ETHIOPIA

Address:P.O. Box 866 Adama Tele: +251-22-112-7962 Fax: +251-22-111-3522 oregionallab@gmail.com

TB LABORATORY TEST REQUEST FORM

Referring health facility: ……..
Patient full name: ……
Age: …… Sex: ……. Card number: ……..
Specimen ID……
Address: Region ……Zone……..
Woreda…… kebele……..
House No……..Tel. pat ……..

Ordering clinician: ……
Phone: clinician…… HF ……..
Patient TB registration No……
Co-infection ☐ No ☐ Yes
Clinical relevant information: ……..
……
……

TB disease type and treatment history; Site ☐ Pulmonary ☐ Exra pulmonary (specify) ……
Registration group: First line ☐ New ☐ Relapse ☐ Lost to follow up ☐ After failure of 1st treatment
☐ After failure of re- treatment ☐ MDR TB contact ☐ Other(previously treated with unknown)
Privious TB drug use: ☐ First Line ☐ Second Line
Resean for request : ☐ Diagnosis ☐ Follow up at ……months **during** treatment
☐ Follow up at ……months **after** treatment

Type of specimen: ……..
Specimen collection date: …… Time: ……..
Collected by: ……..
Date of specimen received ……..Time……

Test Requested: ☐ Routine ☐ Urgent
☐ Microscopy ☐ TB culture only
☐ Genexpert
☐ TB culture and DST (LPA)

Cost: ☐ Free ☐ Paid: …….. Et.Birr. Receipt number ……
(For lab use only) **LABORATORY REPORT** Reception Specimen ID……

Microscopy result								
Method	**Microscopy Result**					Smear type		Remark
	Negative	Actual no	1+	2+	3+	Direct	Concentrated	
☐ Ziehl-Neelsen								
☐ Fluorescence								

GeneXpert result							
☐ MTB not detected ☐ Invalid ☐ No result ☐ Error							
☐ MTB detected, Rifampicin: ☐ Sensitive ☐ Resistant ☐ Indeterminate							
TB culture and Drug susceptibility testing(DST)							
Culture result		Positive ,Mycobacterium tuberculosis complex(MTBC)					
Contaminated	Negative	Non-TB bacteria	<50colonies Actual count	50-100 colonies- 1+	100-200 colonies -2+	200-500 colonies -3+	>500 colonies- 4+
Drug susceptibility test result (LPA)			Rifampicin				
			Isonizid				
Comment ……							
Test done by :			Signature		Date ……/……/…….E.C		
Final report verified by :			Signature		Date ……/……/…….E.C		

SF 38 Version 2 Effective date: 01 Nov 2007 E.C
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