

**CHARACTERISTICS OF ISOLATES OF MYCOBACTERIUM
TUBERCULOSIS IN EXTRAPULMONARY TUBERCULOSIS
IN KORLE-BU TEACHING HOSPITAL**

BY

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DECLARATION

I Michael Amo Omari hereby declare that this thesis is the results of research work undertaking by me at the Chest Clinic Laboratory of the Korle-Bu Teaching Hospital, under the supervision of Professor Kingsley Twum-Danso and Professor Mercy J. Newman all of Department of Microbiology, University of Ghana Medical School, College of Health Science.

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DEDICATION

This work is dedicated to all who have died of extrapulmonary tuberculosis in Ghana, especially my uncle Emmanuel Owusu Antwi, my supervisors, all those involved in management of tuberculosis worldwide, my wife Gloria, my daughter Maame Ofosua and my sons Kwasi Ofori and Kwame Owusu.



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LIST OF ABBREVIATIONS

AFB – Acid Fast Bacilli

AIDS – Acquired Immune Deficient Syndrome

BCG – Bacille Calmette-Guerin

BD – Becton Dickinson

CAS – Central and Middle-Eastern Asia

CD4 – Cluster of Differentiation 4

CDC – Centre for Disease Control

CSF – Cerebrospinal Fluid

DNA – Deoxyribonucleic Acid

DOTS – Directly Observed Therapy Short course

DST –Drug Sensitivity Testing

EAI – East-African Indian

EMB - Ethambutol

EPTB – Extrapulmonary Tuberculosis

FM – Fluorescence Microscopy

GAC-Ghana Aids Commission

HIV – Human Immunodeficiency Virus

INH – Isoniazid

IPT – Isoniazid Preventive Therapy

IUATLD – International Union Against Tuberculosis and Lung Diseases

LAM – Latino-American and Mediterranean

LJ – Lowenstein Jensen

LPA – Line Probe Assay

MAF 1-*Mycobacterium africanum* type 1 West Africa 1

MAF 2-*Mycobacterium africanum* type 1 West Africa 2

MDG – Millennium Development Goals

MDR-TB – Multi Drug Resistant Tuberculosis

MGIT – Mycobacteria Growth Indicator Tube

MRC – Medical Research Centre

MTBC – *Mycobacterium tuberculosis* complex

MUT - Mutation

NALC – N-acetyl L-cysteine

NaOH – Sodium Hydroxide

NTM – Non-tuberculous mycobacterium

NTP – National Tuberculosis Control Programme

OADC – Oleic acid, Albumin, Dextrose and Catalase

PCR – Polymerase Chain Reaction

P-Prevalence

PTB – Pulmonary Tuberculosis

PZA – Pyrazinamide

RIF – Rifampicin

RNA – Ribonucleic Acid

SA – South Africa

SIRE - Streptomycin, Isoniazid, Rifampicin and Ethambutol

SPSS – Statistics Package for Social Scientist

TB – Tuberculosis

TBL – Tubercular lymphadenopathy

WHO – World Health Organization

Xpert - GeneXpert

ZN – Zielh Neelsen

ABSTRACT

BACKGROUND

Tuberculosis (TB) is a worldwide disease. Annually, about nine million people contract tuberculosis and nearly two million are killed by the disease. The fatal synergy of human immuno deficiency virus (HIV) and tuberculosis, and the emergence of multi-drug resistant *Mycobacterium tuberculosis* have further contributed to the re-emergence of TB in many parts of the world. Extrapulmonary tuberculosis refers to the disease outside the lungs.

Extrapulmonary tuberculosis can occur alone or occur in combination with pulmonary TB in a person. About twenty-five percent (25 %) of TB cases are extrapulmonary.

In Ghana, studies on TB have focused more on pulmonary TB which is the most frequent form of the disease and no studies on extrapulmonary TB had been conducted. Therefore a study that aimed to understand the characteristics of extrapulmonary infections will enable the identification of risk factors contributing to the increase rate of extrapulmonary TB.

Identifying these factors is also crucial for policy makers to appropriately strategize to control the disease in order to reduce the global burden of TB. This study sought to describe the characteristics of isolates of *Mycobacterium tuberculosis* in patients with extrapulmonary tuberculosis

Methods: This study was a cross-sectional survey on extrapulmonary tuberculosis suspected patients and conducted between June 2013 and May 2014 at the Chest Clinic Laboratory of the Korle-Bu Teaching Hospital, Accra. All samples were analyzed bacteriologically using microscopy, culture and GeneXpert molecular technology. Drug susceptibility testing using MGIT 960 and Line Probe Assay and species identification were done on *Mycobacterium tuberculosis* complex isolates. Patient's possible risk factors were also assessed.

Results: One hundred and twenty-five (125) samples were analyzed in this study. Ninety one (72.8%) of the samples were pleural aspirates, 14 (11.2%) ascetic fluid, 8(6.4%)

cerebrospinal fluid, 5(4.0%) pericardial aspirate, 3(2.4%) urine, 2 (1.6%) lymph nodes, chestwall and sub-diaphragm aspirates 1(0.8%) each.

The mean age of the patients was 35.64 ± 21.12 years. There were 70 (56%) males with mean age of 36.74 ± 20.94 and 55 (44%) females with mean age of 34.23 ± 21.46 . The culture positive prevalence rate of extrapulmonary tuberculosis was 6.4% (8 out of 125). Out of the 8 culture positive extrapulmonary tuberculosis detected, 5 were males and 3 females.

HIV positive patients were 10 (8.0%) and only 1 out of the 10 had extrapulmonary tuberculosis. In this study there was no significant association between extrapulmonary tuberculosis and the socio-demographic characteristics such as HIV positive, alcohol use, age, smoking, previous treatment of TB and unemployment studied.

Also MDR-TB was not found in extrapulmonary tuberculosis; however 4 isolates out of the 8 were resistant to isoniazid, with 3 found on the katG wild -type gene located on codon 315.

Seven (87.5%) of the 8 *Mycobacterium tuberculosis* complex species were further confirmed as *Mycobacterium tuberculosis* and only 1(12.5%) confirmed as *Mycobacterium africanum*.

Conclusion: The prevalence rate of extrapulmonary tuberculosis is 6.4% and pleural tuberculosis is the predominant form of extrapulmonary tuberculosis at Korle-Bu Teaching Hospital. *Mycobacterium tuberculosis* strains predominate in extrapulmonary tuberculosis. The occurrence of extrapulmonary tuberculosis is not determined by one's socio-demographic risk factors and extrapulmonary tuberculosis is not associated with multi-drugs resistant tuberculosis at the Korle-Bu Teaching Hospital

CHAPTER ONE

1.0 BACKGROUND

1.1. Introduction

Tuberculosis (TB) is a worldwide disease. Annually, about nine million people contract tuberculosis and nearly two million are killed by the disease (World Health Organization, 2005; Shafi et al., 2008). The fatal synergy of human immunodeficiency virus (HIV) and tuberculosis, and the emergence of multi-drug resistant *Mycobacterium tuberculosis* have further contributed to the re-emergence of TB in many parts of the world and calls for concerted effort to fight the disease (Shafi et al., 2008; Chaisson et al., 2008).

The spread of tuberculosis is directly related to the socioeconomic and hygienic conditions of human populations (WHO, 2005). Except for the recent increase in the incidence of tuberculosis in the affluent West due to Acquired Immune Deficiency Syndrome (AIDS), tuberculosis is generally poverty related disease, having a high prevalence in the developing countries (WHO, 2005).

Tuberculosis is an airborne infectious disease caused by *Mycobacterium tuberculosis* (WHO, 2006). Tuberculosis occurs in both sexes, in all age groups and can affect virtually all organs of the body (Shafi et al., 2008; Chaisson et al., 2008). It commonly affects the lungs where it is called pulmonary TB. TB is one of the common fatal infectious diseases in the world: one-third of the world's population is currently infected and nearly two million people die each year due to tuberculosis (WHO, 2006).

Extrapulmonary tuberculosis refers to the disease outside the lungs (Kandola et al., 2014). Extrapulmonary tuberculosis (EPTB) can occur alone or occur in combination with pulmonary TB in a person (Fanning et al., 1999). Globally about twenty-five percent (25 %) of all TB cases are extrapulmonary (Fanning et al., 1999, Mostofa et al., 2010). In developed countries, ten to twenty-five percent (10-25%) of TB cases are extrapulmonary, but the rate is much higher in patients from high-incidence countries (Marjorie et al., 2005). People who are HIV positive and infected with TB also develop extrapulmonary disease much more frequently, up to fifty percent (50%) of cases (Ong et al., 2004; Marjorie et al., 2005). Depending upon the geographic locality and ethnicity of a population, variations have been reported in various studies regarding the occurrence and frequency of extrapulmonary tuberculosis in the two sexes, in different age groups and also in the organs involved (Kandola et al., 2014). EPTB is significantly more common in females, especially in their reproductive age (Shafi et al., 2008). EPTB is considered a diagnostic criterion in the case definition of the acquired immunodeficiency syndrome (Elder et al., 1997).

Extrapulmonary tuberculosis covers all forms of TB in which the disease process occurs outside the lungs. Many forms of extra-pulmonary tuberculosis originate from lymphatic or haematogenic spread of mycobacteria from a primary focus in the lung (Kandola et al., 2014). Diagnosis of extrapulmonary TB is often difficult, so the diagnosis may be presumptive after excluding other conditions and may require invasive procedures to obtain diagnostic specimens (Germa et al., 2002; Mostofa et al 2010). The most common types of extrapulmonary tuberculosis are: TB meningitis, TB lymphadenitis, disseminated tuberculosis, Pleural TB, empyema, pericardial effusion, Ascites and bones (Abdulrahman et al., 2002; Kandola et al., 2014).

The basic principles of treatment for pulmonary tuberculosis also apply to extrapulmonary forms of the disease (Wing-wai et al., 2006). Regimens of 6 months are supposed to be as effective in extrapulmonary as in pulmonary disease (Wing-wai et al., 2006). In some instances of severe disease longer therapy may be necessary.

Bacteriologic confirmation and evaluation of EPTB may be limited by the relative inaccessibility of the sites of disease (Abdulrahman et al., 2002). Thus response to treatment often must be judged on the basis of clinical and radiographic findings.

1.2. PROBLEM STATEMENT:

Tuberculosis is a major cause of morbidity and mortality in Ghana. An estimated ten thousand (10,000) deaths due to TB occur every year (Obiri-Danso et al., 2009). Sixty percent of cases occur in men and women of economically productive age, resulting in a four –seven percent (4-7%) cost of loss of productivity (Obiri-Danso et al., 2009).

Extrapulmonary TB accounts for about 10% to 25% of all cases of TB and its prevalence is believed to have significantly increased with the pandemic of HIV (Pefura-Yone et al., 2013). Extrapulmonary tuberculosis is common in children and people living with HIV (Fanning et al., 1999; Lalit et al., 2004). Extrapulmonary TB is often difficult to diagnose and most clinicians first think of other causes for patient's symptoms than EPTB, leading to delayed diagnosis (Lalit et al., 2004). Therefore the morbidity associated with EPTB is high especially among HIV infected patients (Lalit et al., 2004).

Again treatment of extrapulmonary TB, especially TB meningitis in children is between nine (9) to twelve (12) months and these patients are at risk of treatment failure, since the

probability of treatment default is high among these patients, leading to development of drug resistant tuberculosis which is also a global public health problem (Fanning et al., 1999 and WHO, 2008).

In Ghana, studies on TB have focused more on pulmonary TB which is the most frequent form of the disease (Agyeman et al., 2012) and no studies on extrapulmonary TB had been conducted. Again no study had assessed the possible interaction between demographic and socio-economic factors such as age, sex, alcohol abuse, smoking and extrapulmonary TB.

1.3. JUSTIFICATION / RATIONALE

In 2006, World Health Organization (WHO) launched the new Stop TB Strategy. The core of this strategy is the Directly Observed Therapy Short course (DOTS), the TB control approach launched by WHO in 1995. Since its launch, forty-one million patients have been treated under DOTS-based services (WHO, 2010). The Global Plan to Stop TB, 2010-2015, has new six-point strategy to build on this success. The six components of the Stop TB Strategy are:

- Pursue high-quality DOTS expansion and enhancement.
- Address TB/HIV, MDR-TB and the needs of poor and vulnerable populations.
- Contribute to health system strengthening based on primary health care.
- Engage all care providers.
- Empower people with TB, and communities through partnership.
- Enable and promote research.

The Global Plan is a comprehensive assessment of the action and resources needed to implement the Stop TB Strategy and to achieve the following targets (WHO, 2010):

- Millennium Development Goal (MDG) 6, Target 8: Halt and begin to reverse the incidence of TB by 2015;
- Targets linked to the MDGs and endorsed by the Stop TB Partnership:
 - By 2015: reduce TB prevalence and death rates by 50% relative to 1990;
 - By 2050: eliminate TB as a public health problem (1 case per million populations).

In Ghana the characteristics and risk factors of extrapulmonary TB are not known, and this pose challenges for implementation strategies for prevention of EPTB, since the risk factors for extrapulmonary TB may be different from those other countries. The high TB rates in Ghana have been attributed to HIV/AIDS, poverty, overcrowding, malnutrition, stress, drugs and alcohol abuse among others (Addo et al., 2007). Therefore a study of the characteristics of extrapulmonary infections will enable the identification of risk factors contributing to the increase rate of extrapulmonary TB. Identifying these factors is also critical and crucial for policy makers to appropriately strategize to eliminate the disease in order to meet the set goals and decrease the global burden of TB.

1.4. AIM

The main aim of the study is to characterize *Mycobacterium tuberculosis* isolates from patients with extrapulmonary tuberculosis.

1.5. SPECIFIC OBJECTIVES

- To determine the prevalence of *Mycobacterium tuberculosis* in patients with extrapulmonary tuberculosis.
- To identify the possible risk factors associated with *Mycobacterium tuberculosis* infection in patients with extrapulmonary tuberculosis.

- To determine the drug susceptibility pattern of isolates of *Mycobacterium tuberculosis* in patients with extrapulmonary tuberculosis.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Global burden of tuberculosis

The global burden of tuberculosis remains enormous and World Health Organization (WHO) estimates that approximately one-third of the global community is infected with *Mycobacterium tuberculosis* (Dye et al., 1999). More than two billion people are estimated to be infected with *Mycobacterium tuberculosis* (Lonnorth et al., 2008). The global incidence of tuberculosis peaked in 2003 and appears to be declining slowly (WHO, 2008). According to the World Health Organization (WHO), in 2012, 8.6 million individuals became ill with TB and 1.3 million died (WHO, 2013).

The global distribution of tuberculosis cases is skewed heavily toward low-income and developing countries. Asia has the highest prevalence of cases and countries like China, India, Bangladesh, Indonesia, and Pakistan together have more than 50% of the global burden (Dye et al., 1999, Alimuddin et al., 2013). In 2012 the South-East Asia and Pacific regions accounted for 58% of world TB cases (WHO, 2013). The global tuberculosis caseload is growing slowly. Meanwhile case numbers have declined more steadily in western and central Europe, the Middle East, North and South America. However, there have been striking increases in countries of the Russian Federation (former Soviet Union) and in sub-Saharan Africa (Frieden et al., 2003).

Africa, specifically sub-Saharan Africa, has the highest incidence rate of TB (Dye et al., 1999). In the WHO's 2013 report, the African region had about one quarter of the world cases and the highest rates of cases and deaths in relation to population (255 incidence cases

per 100,000, which is more than double the global rate of 122). South Africa and Swaziland had the highest incidence rate per capita (WHO, 2013). The increase in tuberculosis incidence in Africa is strongly associated with the prevalence of HIV infection. Rates of HIV infection among tuberculosis patients are correspondingly high, exceeding 60% in Botswana, South Africa, Zambia, and Zimbabwe. (Frieden et al., 2003; Alimuddin et al., 2013). Worldwide, 80% of the 8 million TB cases occurring annually are in 22 high burden countries. Out of the 10 countries with the highest incidence, nine are in Africa (Mwinga et al., 2004).

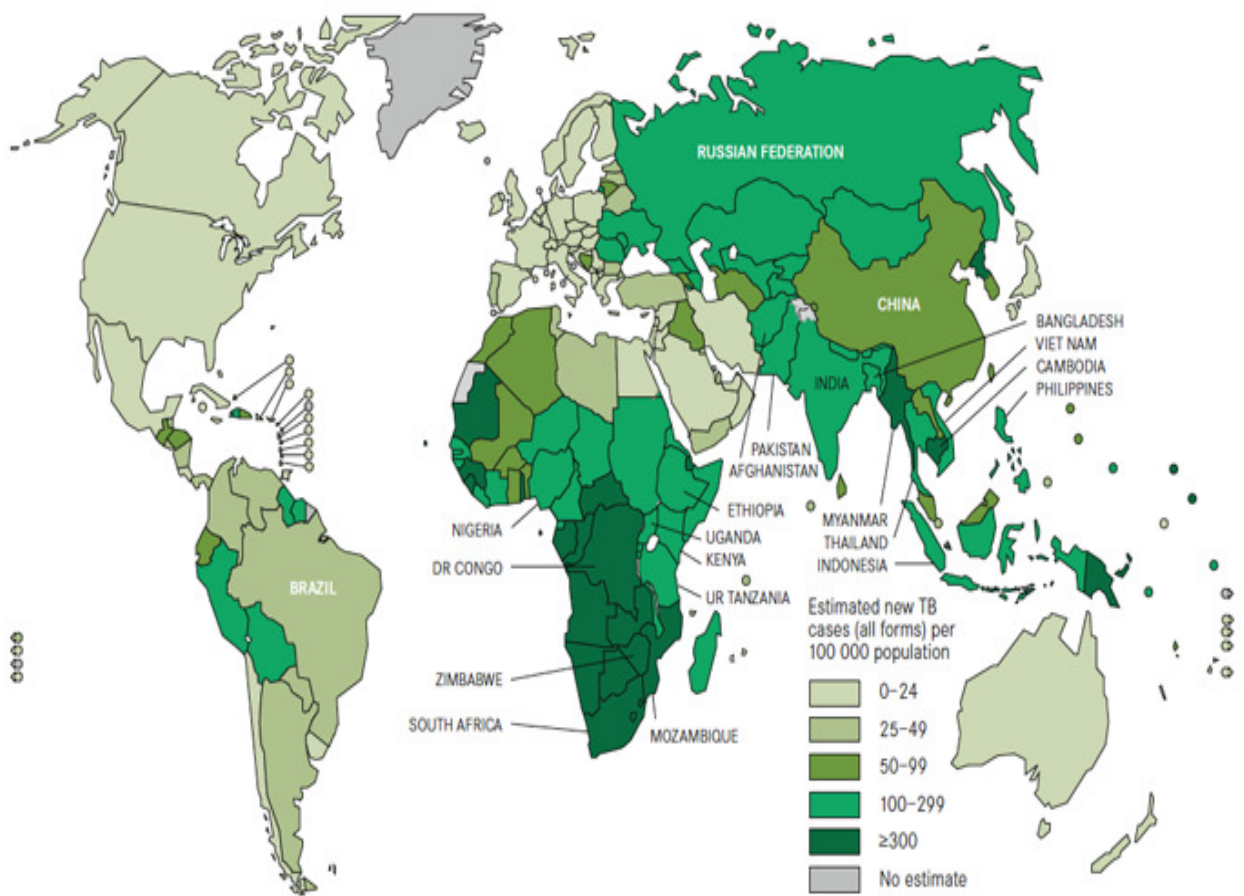


Figure 2. 1. Estimated Tuberculosis incidence rate 2010 (WHO Report, 2011)

2.2. The epidemiology of Tuberculosis worldwide

Prevalence of the infection with *M. tuberculosis* varies according to demographic factors such as age, sex and country as well as socio-economic factors such as crowding, poverty, confinement, etc. In 1993, WHO declared TB a global emergency in recognition of the growing importance of the disease as a worldwide public health problem (Espinal et al., 2003; WHO, 2013). Although the TB mortality rate, (deaths per 100,000 populations per year) has fallen globally by 45% since 1990 and TB incidence rates have also fallen in most of the world, the numbers of TB deaths are still unacceptably large (WHO, 2013).

Most of TB cases and deaths occur among men but the disease burden is high among women. The estimates of cases by WHO in 2012 were 2.9 million cases and 410,000 TB deaths among women and 530,000 cases among children with 74,000 deaths (WHO, 2013). WHO again reports that about 12.1% of all TB deaths involve men (WHO, 2013). Most of TB cases (56 million) occur predominantly in the economically most productive age group of 15-50 years in developing countries (Dye et al., 1999 and Yahaya et al., 2014) and Sub-Saharan Africa has the highest incidence rate (290 per 100,000 populations) (Frieden et al., 2003).

2.3. Mycobacterium tuberculosis

Mycobacterium tuberculosis is a bacterium species of the family Mycobacteriaceae, suborder Corynebacterineae of the order Actinomycetales and phylum Actinobacteria. *Mycobacterium tuberculosis* is a non-motile, obligate aerobe and rod-shaped bacteria with two distinguishing characteristics: acid-fastness and slow growth (Grange et al., 1998).

Mycobacterium tuberculosis has circular chromosomes of about 4,200,000 nucleotides long. The G+C content is about 65% (Ouellet et al., 2010). The bacterial genome contains almost 4000 genes, those that are responsible for lipid metabolism are a very important part of the bacterial genome, and 8% of the genome is involved in this activity (Mohn et al., 2008).

M. tuberculosis has very tough cell wall that prevents passage of nutrients into and excreted from the cell, this tough cell wall gives it the characteristic slow growth rate. The cell wall of *M. tubercuclosis* looks like a Gram-positive cell wall. Its cell envelope also contains polypeptide layer, free lipids and a peptidoglycan layer. There is also a complex structure of fatty acids such as mycolic acids that appear glossy (Van der Geize et al., 2007). The mycolic acids of *M. tuberculosis* cell wall are in three classes of namely: alpha-, keto- and methoxymycolates. The cell wall also contains lipid complexes including acyl glycolipids and others such as free lipids and sulfolipids. There are porins also in the membrane to facilitate transport. Beneath this cell wall, are layers of arabinogalactan and peptidoglycan that lie just above the plasma membrane (Thomas et al., 2011).

Mycobacterium tuberculosis belongs to *Mycobacterium tuberculosis* complex (MTBC) which causes tuberculosis. *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium microti* and *Mycobacterium canetti* are the other members of this MTBC. The *Mycobacterium* genus includes more than 50 environmental species, which can also cause disease which resemble tuberculosis. These *Mycobacterial* species are collectively referred to as *non-tuberculous mycobacterium* (NTM) (Van Soolingen et al., 1997; Addo, 2001).

M. africanum is commonly found in West African countries; it causes up to a quarter of cases of tuberculosis (TB) in the Gambia (De Jong et al., 2010). The symptoms of *M. africanum* infection resemble those of *M. tuberculosis*. Its infectivity is also similar to that of *M. tuberculosis*, and it is an important opportunistic pathogen in the immunosuppression due to HIV or other causes patients (De Jong et al., 2010).

In 2007, Addo et al. reported that three (3) species of *Mycobacterium tuberculosis complex* caused pulmonary tuberculosis in patients visiting the Chest Clinic of Korle-Bu Teaching Hospital. It was reported that *M. tuberculosis* was responsible for 73% of all the cases, *M. africanum I* 20%, *M. africanum II* 3% and *M. bovis* 4% of all cases. (Addo et al., 2007).

2.4. Tuberculosis and its transmission

Tuberculosis (TB) is a chronic infectious disease caused by the obligate human pathogen, *Mycobacterium tuberculosis*. It primarily affects the lungs causing pulmonary tuberculosis but can cause extra pulmonary disease of virtually all organs of the body (Glickman et al., 2001). Tuberculosis is one of the oldest infectious diseases known to man. It was the cause of the “White Plague” of the 17th and 18th centuries in Europe. During this period nearly 100 percent of the European population was infected and 25 percent of all adult deaths were caused by tuberculosis (Todar et al., 2009; Schiffman et al., 2009).

Tuberculosis was responsible for almost one-seventh of all the deaths in Europe at the time its etiology was identified in 1882 (Brock et al., 1988). Today it remains the most common infectious cause of death in adults worldwide (W.H.O. Report, 2008).

Patients with active pulmonary tuberculosis are the source of *Mycobacterium tuberculosis*. In more than 90% of persons infected with *M. tuberculosis*, the pathogen is contained as asymptomatic latent infection (Alimuddin et al., 2013).

Tuberculosis is spread from person to person through the air by droplet nuclei, 1 to 5 μm in diameter containing *M. tuberculosis* bacilli. These tiny droplet nuclei float in air, the fluid evaporates and the living tubercle bacillus may remain airborne for long periods until inhaled (American Thoracic Society, 2000; Sonal et al., 2008). Droplet nuclei are produced when persons with pulmonary or laryngeal tuberculosis cough, sneeze, speak, or sing (American Thoracic Society, 2000). They may also be produced by aerosol treatments, sputum induction, and creation of aerosols during bronchoscopy, and through procedures done on lesions or processing of tissue or secretions in the hospital or laboratory. Droplet nuclei, containing few *M. tuberculosis* organisms, are so small that air currents normally present in any indoor space can keep them airborne for long periods of time. The small size of droplet nuclei enables it to reach the alveoli within the lungs, where the organisms replicate (American Thoracic Society, 2000).

Infants can acquire the tubercle bacilli through aspiration or ingestion of their mother's infected amniotic fluid either before or during pregnancy (eCureMe, 2003). Tuberculosis is not transmittable through shaking hands with or touching the clothes of the infected person (Houghton et al., 2002).

2.5. Pathogenesis of *Mycobacterium tuberculosis*

The pathogenesis begins when a droplet nucleus, generated by an index case, is inhaled by a respiratory contact and it passes through the airstream to a site in the lung. Initially, there is rapid inflammation at the alveolar site where the tubercle bacillus is deposited, usually in the subpleural and in the mid-lung zones where greater air flow favours bacilli deposition. The initial inflammation does not usually inhibit the growth of the organism (Sonal et al., 2008).

This droplet nucleus is ingested by an alveolar macrophage at the site of implantation, and after few days, the bacillus multiplies in the alveolar macrophage cell (Balasubramanian et al., 1994; American Thoracic Society, 2000). The macrophage eventually dies and the bacilli are released and ingested by other macrophages. This process continues, and that leads to the formation of primary lesion which can be identified after calcification (Balasubramanian et al., 1994). Because a single droplet nucleus is sufficient to initiate the development of the primary lesion, the infectious dose in TB is exceedingly low (Balasubramanian et al., 1994).

In most individuals only a single primary lesion is observed. As the primary lesion enlarges, bacilli then spread through the pulmonary lymphatics and reach lymph nodes, which may become enlarged. The lymph nodes enlarge as the bacilli multiply intracellularly, creating a situation in which bacilli escape from the leaky, swollen lymph node. The term progressive primary tuberculosis is often used to describe disease arising directly from either the parenchymal or the lymph node component of the primary infection (Balasubramanian et al., 1994). Although primary lesions can occur anywhere in the lung, developments of post-primary disease most commonly occur in the apical regions (Stewart et al., 2003).

2.6. Clinical manifestation of tuberculosis

Clinical manifestations of TB are able to vary and depend on a number of factors that are related to the microbe characteristics, the host, the environment as well as the host and microbe interactions (American Thoracic Society, 2000, Zhenhua et al., 2004).

Early symptoms of active tuberculosis (pulmonary and extrapulmonary) normally include loss of weight, fever, night sweats and loss of appetite (CDC, 2009). The most common clinical manifestation of TB is pulmonary disease which is observed in about 80% of patients (Frieden et al., 2003). Symptoms of advanced stage TB include cough, hemoptysis, pleuritic pain, dyspnea and respiratory failure (American Thoracic Society, 2000).

2.7. Extrapulmonary Tuberculosis and its distribution

The epidemiology of extrapulmonary tuberculosis is not well understood (Ong et al., 2004). Extrapulmonary infections with *Mycobacterium tuberculosis* complex (MTBC) remain a diagnosis that is often difficult to establish, since the number of bacilli found in specimen is often lower than is in pulmonary specimens (Hillemann et al., 2011). The epidemiology of extrapulmonary tuberculosis (EPTB) differs from that of pulmonary tuberculosis (PTB) and that of pleural TB is also distinct from other forms of extrapulmonary TB (Ong et al., 2004).

In 2006, Bryan et al., reported that extrapulmonary tuberculosis is more common than pulmonary tuberculosis in Somalis in Minnesota. Fifty percent (50%) of Somali patients who have extrapulmonary tuberculosis have lymphatic disease (Bryan et al., 2006).

In 2010, Mostofa et al., reported that the most common form of EPTB in Bangladesh is Tubercular lymphadenopathy (TBL) while the most common samples sent for analysis are pleural fluids (Mostofa et al., 2010). Similarly Huang et al., ranked lymph node TB as the leading extrapulmonary TB in Shanghai in 2000 (Huang et al., 2000) which also have a similar trend to a study reported by Lowieke et al., in 2006 at Netherland (Lowieke et al., 2006).

In 2006, Torgerson et al., in a review of extrapulmonary tuberculosis at Maryland State, reported that cervical lymphatic tuberculosis represents the largest proportion of EPTB cases, followed by pleural tuberculosis and then other lymphatic sites (Torgerson et al., 2006).

Heather et al, also in their study reported that meningeal tuberculosis is more frequent in US-born patients (Heather et al., 2009).

2.8. Risk Factors of Extrapulmonary Tuberculosis

In 2004, a study of extrapulmonary TB in San Francisco reported that extrapulmonary TB as compared to pulmonary TB is more common in female patients, younger-aged persons, and HIV-infected individuals (Ong et al., 2004). Again impaired host immunity has been suggested as a risk factor for extrapulmonary TB in HIV-uninfected persons (Ong et al., 2004).

In 2010, Fiske et al., also reported extrapulmonary tuberculosis as a marker of underlying immune compromise. They also reported that the highest incidence of extrapulmonary tuberculosis is among black people.

Their study reported about 5-fold increase in rates of extrapulmonary TB in blacks compared to whites. They further emphasized that among all persons with tuberculosis, the highest proportion of extrapulmonary disease is among black women as compared to black men, non-black women, and non-black men (Fiske et al., 2010). Similarly in 2008, Shafi et al., in a study conducted in Pakistan reported that EPTB is significantly more associated with females, especially those in their reproductive age (Shafi et al., 2008).

Zhenhua et al., also in 2004 identified three independent risk factors for EPTB as being female, being a non Hispanic black and being HIV positive (Zhenhua et al., 2004). They did not report any association between excessive alcohol use and EPTB (Zhenhua et al., 2004) which is similar to the report by Heather et al., that there is negative association between selected risk factors such as alcoholism, incarceration, drug use and homelessness (Heather et al., 2009).

Another study by Xinyu et al., involving 5,684, of bacteriologically confirmed TB patients (including 1,925 EPTB cases) diagnosed in Denmark and Greenland during 1992–2007 reported that among patients from Somalia and Asia, persons 25–44 and 45–64 years of age were more likely to have EPTB than persons 15–24 years of age (Xinyu et al., 2011).

In contrast, among persons from Greenland, the two oldest age groups (25–44 and 45–64 years) were significantly less likely to have EPTB than the youngest age group (15 – 24 years). Also for all the age groups, the odds for having EPTB were significantly higher among patients from Somalia and Asia. The odds were also significantly lower among the patients from Greenland than among patients from Denmark. Again, the occurrence of specific types of EPTB significantly varied among different age groups or origins (Xinyu et al., 2011).

2.9. Prevalence of extrapulmonary tuberculosis

Many studies have reported that between 10-25% of tuberculosis infections worldwide occur in extrapulmonary sites (Haley et al., 2013). In 2004, Zhenhua et al., reported that the proportion of extrapulmonary tuberculosis cases in the United States increased from 16% of TB cases in 1991 to 20% in 2001 (Zhenhua et al., 2004). This collaborates with a similar report from Heather et al. in 2009 which reported that EPTB in United States increased from 15.7% in 1993 to 21% in 2006 (Heather, et al., 2009). From the European Union, Sandgren et al., in 2013 reported that the proportion of EPTB increased from 16.4% of all TB cases in 2002 to 22.4% in 2011 (Sandgren et al., 2013).

2.10. Clinical manifestation of extrapulmonary tuberculosis

Symptoms of extrapulmonary tuberculosis vary according to site of infection in the body (Germa et al., 2002; CDC, 2009). Extrapulmonary tuberculosis can affect any organ in the body and has a wide variety of clinical manifestations (Alimuddin et al., 2013; Kandola et al., 2014). Tuberculosis involving any site may produce symptoms that are not specifically related to the organ or tissue involved but, rather, is systemic in nature. Of the systemic effects, fever is the most easily quantified (American Thoracic Society, 2000)

2.10.1. Tuberculous meningitis

Tuberculous meningitis is a particularly devastating disease. Meningitis can result from direct meningeal seeding and proliferation during a tuberculous bacillema either at the time of initial infection or at the time of breakdown of an old pulmonary focus, or can result from breakdown of an old parameningeal focus with rupture into the subarachnoid space

(American Thoracic Society, 2000; Kandola et al., 2014). Tuberculous meningitis has three clinical phases. The prodromal phase involves several weeks of nonspecific malaise, fever, and headache. The meningitic phase involves worsening head-ache, nausea, vomiting, and early signs of meningismus. The paralytic phase; at this stage the process of disease is located primarily at the base of the brain and is associated with high fever, confusion, stupor and coma (American Thoracic Society, 2000; Kuo et al., 2010).

2.10.2. Tuberculous lymphadenitis

Lymph node TB is seldom complicated by systemic symptoms, except in people with HIV infection, in whom the bacterial load is large. The nodes are usually discrete, firm and non tender, but with continuing disease they may become matted and the overlying skin inflamed. Rupture of the node can result in formation of a sinus tract, which may be slow to heal (Fanning et al., 1999; American Thoracic Society, 2000). Tuberculous lymphadenitis usually presents as painless swelling of one or more lymph nodes. The nodes involved most commonly are those of the posterior or anterior cervical chain or those in the supraclavicular fossa (American Thoracic Society, 2000).

2.10.3. Pleural tuberculosis

There are two mechanisms by which the pleural space becomes involved in tuberculosis (American Thoracic Society, 2000). In early infection of tuberculosis a few organisms may gain access to the pleural space and, in the presence of cell-mediated immunity, cause a hypersensitivity response. Often this form of tuberculous pleuritis goes unnoticed, and the process resolves spontaneously.

However, in some patients, tuberculous involvement of the pleura is manifested as an acute illness with fever and pleuritic pain. If the effusion is large enough, dyspnea may occur (American Thoracic Society, 2000).

The second mechanism of tuberculous involvement of the pleura is empyema. This is much less common than tuberculous pleurisy with effusion and results from a large number of organisms spilling into the pleural space, usually from rupture of a cavity or an adjacent parenchymal focus. A tuberculous empyema is usually associated with evident pulmonary parenchymal disease on chest films and air may be seen in the pleural space (American Thoracic Society, 2000).

2.10.4. Genitourinary tuberculosis

Genitourinary TB occurs with the hematogenous spread of tubercle bacilli to the glomeruli (Fanning et al., 1999). The symptoms of genitourinary TB are those of bacterial pyelonephritis, recurring in spite of treatment; sterile pyuria is frequent, dysuria, hematuria, and frequent urination are also common, and flank pain may also be experienced (Fanning et al., 1999, American Thoracic Society, 2000). In women genital involvement is more common without renal tuberculosis than in men and may cause pelvic pain, menstrual irregularities, and infertility. In men painless or slightly painful scrotal mass is probably the most common presenting symptom of genital involvement, but symptoms of prostatitis, orchitis, or epididymitis may also occur (American Thoracic Society, 2000).

2.10.5. Skeletal tuberculosis:

The most common symptoms of skeletal TB are pain, tenderness and limitation of motion. Swelling of the involved joint may be present. Systemic symptoms of infection are not common (Fanning et al., 1999; American Thoracic Society, 2000).

2.10.6. Pericardial tuberculosis

The symptoms associated with tuberculous pericarditis may be the result of either the infectious process itself or the pericardial inflammation causing pain, effusion, and eventually hemodynamic effects. The systemic symptoms produced by the infection are quite nonspecific. Fever, weight loss, and night sweats are the common ones. Symptoms such as dyspnea, orthopnea, ankle swelling, cough, and chest pain tend to appear later (American Thoracic Society, 2000).

2.11. Diagnosis of extrapulmonary tuberculosis

Diagnosis of extrapulmonary continues to be a challenge to both clinicians and microbiologist mainly due to the pauci-bacillary nature of tissues and fluids at the sites of infection (Hesselink et al., 2003). Signs and symptoms of extrapulmonary tuberculosis are non specific; however there are some clinical clues that may prompt the suspicion of the disease. The most common clinical clues that may prompt the suspicion of extrapulmonary tuberculosis include, lymphocytes predominance ascites with negative bacterial cultures, chronic lymphadenopathy, lymphocytes predominance pleural effusion with negative bacterial cultures, HIV infection, joint inflammation with negative bacterial cultures, persistent sterile pyuria, vertebral osteomyelitis involving the thoracic spine, unexplained

pericardial effusion, constructive pericarditis or pericardial calcification and lymphocytic pleocytosis of cerebrospinal fluid (CSF) increased protein and decreased glucose (American Academy of Family Physicians, 2005).

Extrapulmonary tuberculosis diagnosis depends much on the physician considering the possibility of tuberculosis in patient at risk, based on history, clinical symptoms and signs and submitting appropriate clinical specimen for bacteriological examination (Fanning et al., 1999). Tissue and/or fluids from the suspected site of infection are collected and used in the bacteriological diagnosis. In extrapulmonary TB diagnosis some of the more common types of specimens used are: biopsy tissue, pleural fluids, peritoneal fluid, and needle aspirates/ biopsy of lymph nodes, cerebrospinal fluid, and urine (Fanning et al., 1999; American Thoracic Society, 2000).

Extrapulmonary TB diagnosis like pulmonary TB also relies on smear microscopy and culture. Culture of *Mycobacterium tuberculosis* is the standard diagnostic tool for extrapulmonary tuberculosis. Molecular methods, such as Line probe assays (LPA), genexpert (Xpert MTB/RIF), Amplicor *Mycobacterium tuberculosis* test (Amplicor) and Amplified *Mycobacterium tuberculosis* test are also often used in diagnosing extrapulmonary TB. However the sensitivity (66.7% for Genexpert and 79.4% for Amplicor) of these methods is often crucial since few bacteria may be present in extrapulmonary TB (WHO, 2007).

2.11.1. Microscopy – Direct Smear Examination

The direct smear examination is an inexpensive test that can be carried out rapidly. The smear is vitally important clinically. The detection of acid fast bacilli (AFB) in stained smears examined microscopically provides the physician with a preliminary confirmation of the diagnosis (Frieden et al., 2003; Sonal et al., 2008).

There are two standard methods of staining smears for acid fast bacilli microscopy. The Ziehl-Neelsen (ZN) technique which requires an ordinary light microscope and the fluorescence microscopy (FM) technique which requires fluorescence microscope (an instrument with ultraviolet illumination) (Collins et al., 1997). Where both microscopes are available, the choice of method is influenced by number of slides to be examined at anytime (Collins et al., 1997) The International Union Against Tuberculosis and Lung Disease (IUATLD) and WHO recommend the Ziehl-Neelsen method under most circumstances (Frieden et al., 2003)

2.11.1.1. Ziehl-Neelsen method

Paul Ehrlich discovered the acid fastness property of tubercle bacillus a year after its discovery by Robert Koch. Subsequently, the Ehrlich's method was modified by Ziehl and Neelsen whose names the method bears (Collins et al., 1997). The acid-fast staining procedure depends on the ability of Mycobacteria to retain dye when treated with mineral acid or an acid-alcohol solution (Sonal et al., 2008).

Smears prepared directly from clinical specimen or concentrated preparation when stained using ZN technique for detection of the acid-fast bacilli shows strong red acid fast bacilli against blue background as shown in plate 2.1 below (Collins et al., 1997). In the ZN method,

heat fixed smears are flooded with 0.3% carbol-fuchsin stain and heated until steam rises. After five minutes the smear is washed with clean water and then decolourised with 20% sulphuric acid or 0.3% acid-alcohol (hydrochloric acid and ethanol) can also be used. The smear is again washed after five minutes and flooded again with 0.3% methylene blue which is the counter stain after one minute (Collins et al., 1997)

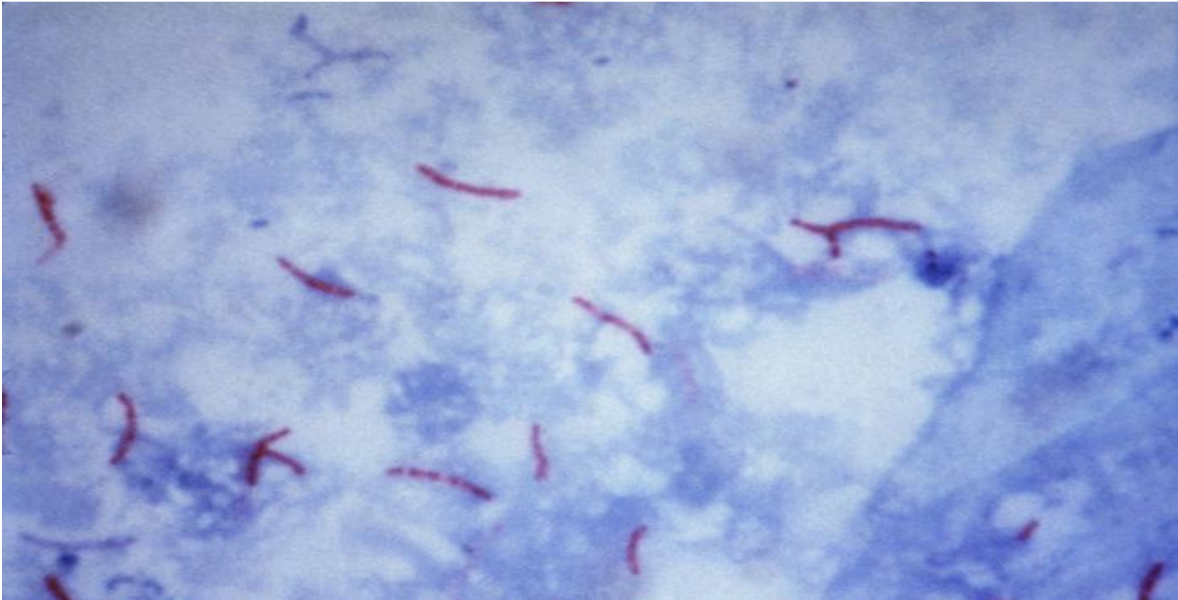


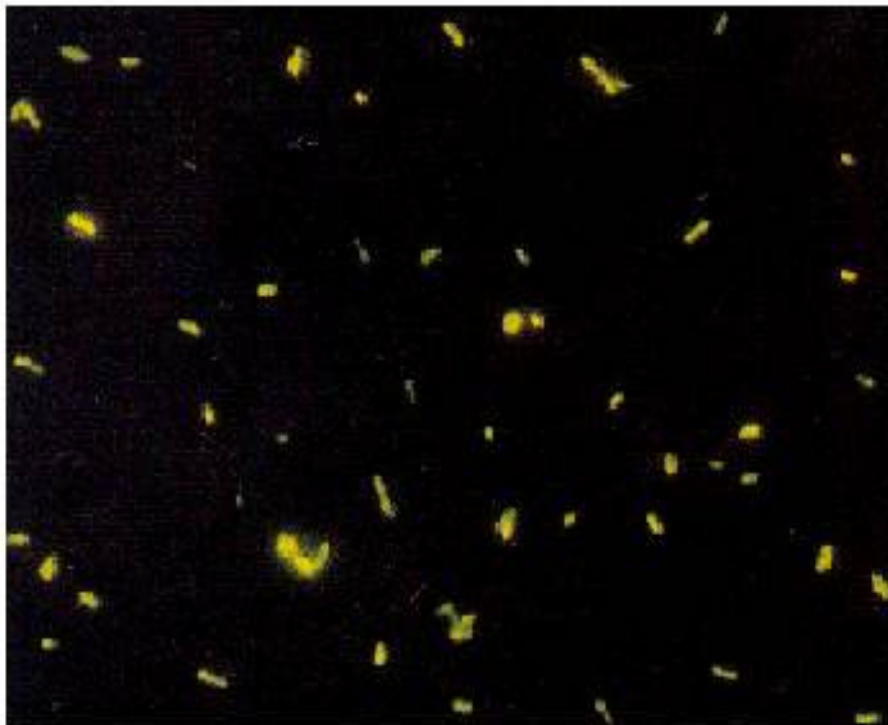
Plate 2.1. Ziehl-Neelsen stained smear showing Acid-Fast Bacilli as red rods (adapted from www.slideshare.net)

2.11.1.2. Fluorescence Microscopy method

There are several variations of the basic fluorescence staining method. Some methods use auramine and others use a mixture of auramine and rhodamine. In a well stained smear using this method, AFB appears as yellow to orange fluorescent bacilli against a dark background as shown in PLATE 2.2. The background should not show much remaining fluorescence, but some fluorescing artifacts such as hair, crystals and cells will often be present (Collins et al.,

1997). In this technique heat fixed smears are flooded with auramine for at least fifteen minutes and not more than thirty minutes.

It is then washed with clean water and completely decolourized with 0.5% acid-alcohol for about four minutes. The smear is again washed and counter stained with 0.5% potassium permanganate for one minute (Collins et al., 1997). The auramine stain technique is noted to give more “false positives” than the Ziehl-Neelsen technique; therefore positive FM smears are confirmed by overstaining the smear by the ZN method (Collins et al., 1997).



A uramine-O staining of AFB under Fluorescence Microscopy

Plate 2.2. A fluorescent stained smear of *Mycobacterium tuberculosis* (adapted from medicinembbs.blogspot.com)

2.11.2. Culture of *Mycobacterium tuberculosis*

Bacteriological culture provides a definitive diagnosis of tuberculosis. Culture is much more sensitive than microscopy and is able to detect as few as 10 bacteria/ml of material (Sonal et al., 2008). Culture thus increases the potential for diagnosis of TB at early stages of disease, treatment failures, extrapulmonary TB, and TB in children and in HIV-positive patients who usually have low bacilli loads and are usually smear-negative. Culture enables both species identification and drug susceptibility testing (DST) (Sonal et al., 2008; Bonsu et al., 2012).

Culture is carried out in containment laboratory (Biosafety level 2 or 3 laboratory) usually on egg-based media e.g. Lowenstein-Jensen (LJ) and Ogawa media. Liquefied medium such as Middlebrook 7H9 is also used for culturing mycobacteria (Bonsu et al., 2012). Clinical specimen is digested and decontaminated, if required, using the mucolytic agent N-acetyl-L-Cystein (NALC) and sodium hydroxide and neutralized using phosphate buffer solution of pH 6.8. The decontaminated specimen is inoculated onto slopes of egg based Lowenstein-Jensen media and Middlebrook 7H9 liquid media and incubated at 35 – 37 °C. Growth of a positive tuberculosis culture is seen within eight weeks, apparently two to four weeks (Collins et al., 1997).

Typical *Mycobacterium tuberculosis* growth on Lowenstein Jensen (LJ) media show colonies that are dry, rough, appearance of bread crumbs or cauliflower and buff-coloured (never pigmented) (Bonsu et al., 2012). In liquid culture medium signs of *M. tuberculosis* growth are observed at least one week after inoculation and the growth is in the form of visible floccules

in the medium which tend to remain isolated after shaking the medium (Bonsu et al., 2012).

The limitations of culture as the diagnostic tool of tuberculosis are; slow growth of *M.*

tuberculosis complex and technically demanding nature of the technique (Bonsu et al., 2012).



Plate 2. 3. *Mycobacterium tuberculosis* colonies growing on Lowenstein Jensen media (adapted from Biology Image Library)

2.11.3. Nucleic acid amplification techniques (molecular techniques)

Nucleic acid amplification techniques have been used for diagnosis of *Mycobacterium tuberculosis* and its identification mainly because diagnosis can be achieved rapidly with high degree of sensitivity and specificity (Collins et al., 1997).

The majority of molecular tests have focused on (i) detection of nucleic acids, both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), that are specific to *Mycobacterium tuberculosis*, by amplification techniques such as polymerase chain reaction

(PCR); and (ii) detection of mutations in the genes that are associated with resistance to anti-tuberculosis drugs by sequencing or nucleic acid hybridization (Seetha et al., 2009).

Molecular technologies allow for the amplification of specific target sequences of nucleic acids that can then be detected through the use of a nucleic acid probe.

For *M. tuberculosis*, a number of nucleic acid amplification techniques are available and most of them target 16SrRNA gene sequencing for identification (American Thoracic Society, 2000; Seetha et al., 2009). The current molecular based tests, such as GenoType MTBDR*plus* assay (LPA) (Hain-Lifescience, Germany), which tests for both rifampicin (RIF) and isoniazid (INH) susceptibility, and the more recently introduced, GeneXpert MTB/RIF assay (Cepheid, USA), which tests for rifampicin susceptibility only, are being increasingly used in developed countries, for the rapid detection of resistance (Yacoob et al., 2013).

The Hain (Life Science) test is a Line Probe Assay (LPA) which is based on solid-phase hybridization technique. Hain have different Mycobacteria diagnosis products for different purposes namely: Genotype Mycobacteria Direct for detection of *Mycobacterium tuberculosis* complex and non-tuberculous mycobacteria (NTM) differentiation, Genotype Mycobacterium CM/AS for NTM species identification, Genotype MTBC for MTB complex species differentiation, Genotype MTBDR*plus* for detection of MTB complex resistance to rifampicin and isoniazid and many more products (Yacoob et al., 2013).

The GeneXpert is a molecular based PCR (real time PCR) assay that detects *Mycobacterium tuberculosis* and rifampicin resistance in the bacteria. Rifampicin resistance is detected by mutations in the 81-base pair rifampicin resistance-determining region of the *rpoB* gene.

Some studies had indicated that rifampicin resistance most often is proxy for isoniazid resistance and therefore MDR-TB (WHO, 2014).

2.12. Management of extrapulmonary tuberculosis

The appropriate treatment of tuberculosis is chemotherapy consisting of a combination of several anti-tuberculosis drugs (Ait-Khalsed et al., 2003). The duration of treatment has decreased considerably since 1960: initially given for 24 months, it now lasts for 6–8 months and is known as “short-course chemotherapy” (Ait-Khalsed et al., 2003; Wing-wai et al., 2006). However major stakeholders in TB control agree that some forms of disease, such as meningitis, may benefit from a longer treatment course (Frieden et al., 2003), since treatment of all forms of tuberculosis has proved to be very difficult.

There are five key anti-tuberculosis drugs isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol. Inappropriate use of any of these drugs leads to the development of resistant strains that normally make up the bacterial populations. This is why several anti-tuberculosis drugs must be given together in order to achieve cure in a patient with tuberculosis (Ait-Khalsed et al., 2003; Wing-wai et al., 2006).

In most cases of extrapulmonary tuberculosis, there are fewer organisms present. In general, regimens used for pulmonary tuberculosis are effective in the treatment of extrapulmonary tuberculosis (Frieden et al., 2003). Steroids should be used for patients with large pleural effusions, pericardial disease, and meningitis, particularly with neurological impairment, because these drugs are likely to decrease morbidity and mortality in such patients (Frieden et al., 2003).

2.13. Drug sensitivity pattern in extrapulmonary tuberculosis

Multidrug-resistant tuberculosis (MDR-TB) is defined as resistance to both isoniazid (INH) and rifampicin (RIF), with or without resistance to other anti-tuberculosis drugs (Hillemann et al, 2011). Unlike most other bacteria, resistance in *Mycobacterium tuberculosis* develops primarily through mutations in chromosomal genes. These mutations develop spontaneously and are sustained in the bacterial population mainly through selective pressure with inappropriate treatment (Yacoob et al., 2013).

Some studies have confirmed that multidrug-resistant tuberculosis is less frequent among EPTB patients. In the European Union surveillance study, MDR-TB was identified in 1.3% of the EPTB cases compared with pulmonary tuberculosis (Sandgren et al., 2013).

Heather et al, in their study in 2009 also found out that MDR-TB occurs less often among extrapulmonary tuberculosis than pulmonary tuberculosis (Heather et al., 2009).

2.14. Molecular Distribution of *Mycobacterium tuberculosis* strains

Mycobacterium tuberculosis complex genotyping is used to identify and distinguish them into distinct lineages and sub-lineages that are used for tracking and control of tuberculosis epidemic (Ozcaglar et al., 2010). The major lineages of MTBC are; *M. africanum*, *M. canetti*, *M. bovis*, *M. microti* and *M. tuberculosis*. The *M. tuberculosis* has sub-groups which include; sub-group Indo-Oceanic, subgroup East-Asian (Beijing), subgroup Euro-America and subgroup East-African Indian (EAI) (Ozcaglar et al., 2010).

The *Mycobacterium africanum* has two sub-types, *M. africanum* type I and *M. africanum* type II. The type I is further sub-divided into *M. africanum* type I West Africa 1 (MAF1) and *M. africanum* type I West Africa 2 (MAF2) (De Jong et al., 2010; Bonsu et al., 2012). The *Mycobacterium africanum* is said to have originated from West Africa (De Jong et al., 2010) and is a major causative agent of human tuberculosis in Africa. Studies had confirm that the *M. africanum* is predominant in West Africa, with Benin having the highest prevalence rate of 39% and Ghana 21% (De Jong et al., 2010), The *Mycobacterium bovis* also has sub-species, which include *M. bovis bovis*, *M. bovis caprae*, and *M. bovis* Bacillus Calmett-Guerin (BCG) (Bonsu et al., 2012).

A total of 36 potential subfamilies or sub-clades of *M. tuberculosis* complex have been tentatively identified, leading to the definition of major and minor recognition rules. The ancestral East-African Indian (EAI) family is made up of at least five main sub-clades, whereas at least three major spoligotyping patterns are found within the Haarlem family (Filliol et al., 2002 and Kremer et al., 1999). Two families found in Central and Middle Eastern Asia (CAS1 and CAS2) are newly defined.

The X family (Filliol et al., 2002) is also currently split into at least three well-defined sub-clades. Similarly, the Latino-American and Mediterranean family (LAM) is tentatively split into sub-clades LAM1–LAM10 (Filliol et al., 2002). In a study conducted by Manisankar et al in 2012 in New Delhi, it was reported that CAS family isolates were the most prevalent genotype in extrapulmonary tuberculosis (Manisankar et al., 2012). Meanwhile in Turkey, Salami et al reported that majority of Beijing strains identified in their study were related to extrapulmonary TB (Salami et al., 2011).

CHAPTER THREE

3.0. MATERIALS AND METHOD

3.1. Study design

This study was a cross-sectional survey on extrapulmonary tuberculosis suspected patients and conducted from June 2013 and May 2014. A standardised protocol (appendix I) was used to collect data on patients socio-demographic and clinical information, including sex, age, previous diagnosis of TB, sites of disease, human immunodeficiency virus (HIV) status and residential address. Again information on patients risk behaviour, such as alcohol use and smoking habit were collected.

3.2. Study site

This study was conducted at the Chest Clinic Laboratory of the Korle-Bu Teaching Hospital, Accra. The Korle-Bu Teaching Hospital is a referral hospital which sees patients from all over the country. The Chest Clinic is a specialist clinic of the hospital that attends to both in-patients and out-patients with tuberculosis and other chest diseases.

3.3. Subjects

Samples were taken from suspected EPTB patients when they sought for treatment at the Korle-Bu Teaching Hospital.

3.3.1 Inclusion Criteria

Patients of all ages and sexes who were suspected of having extrapulmonary TB were enrolled in this study after they gave their formal consent.

3.3.2. Exclusion Criteria

Patients suspected of having only pulmonary TB were excluded from this study.

3.4. Sample size

With incidence rate of tuberculosis in Ghana as 351 per 100,000 (WHO, 2011) and case detection of extrapulmonary tuberculosis as 10%, a prevalence of 10% , together with a standard (z) score of 1.96 at 96% confidence level and 5% allowable error margin was used to determine the sample size.

Using the equation; $n = z^2 p (1-p) / e^2$

where n- the minimum sample size, z-the standard score, p- the prevalence of extrapulmonary TB, and e- the allowable error margin, the minimum number of study participants that was required for the study was 137.

However 125 participants consented to be enrolled in this study during the period. This number of participants did not result in any significant change in the 5% allowable error.

3.5. Biosafety consideration

Smear preparation, decontamination, inoculation of culture media, identification and sub culturing of specimen and isolates were all conducted in a class II biosafety cabinet.

Protective clothing was worn and all other biosafety protocols for individual and staff protection were observed.

3.6. Reagents and chemicals

The following commercially prepared media and reagents were obtained from BD diagnostics through National TB Programme (NTP): Lowenstein Jensen (Glycerol) media, MGIT culture tubes, MGIT growth supplement, MGIT PANTA, TBcID cards, MGIT SIRE

kits, Pyrazinamide tubes, Mycoprep and supplement and Auramine fluorescence stains.

Again the GeneXpert cartridges were purchase from Cepheid also through NTP as well as the HAIN's reagents from HAIN Life Science (Nehren, Germany). All other reagents used were prepared from the laboratory.

3.7. *Mycobacterium tuberculosis* control strains

Mycobacterium tuberculosis H₃₇Rv ATCC 27294 and *M. bovis* BCG control strains were obtained from Medical Research Council (MRC), The Gambia for internal quality control.

3.8. Sample Collection

Depending on the presentations, appropriate sample was collected from the participants. The sample types obtained from consented participants for this study include pericardial fluid, ascitic fluid, pleural fluid, cerebrospinal fluid (CSF), urine, lymph node aspirate, chestwall aspirate and sub-diaphragmatic fluid.

These samples were collected by the attending clinicians. The samples were then sent to the Chest Clinic laboratory for bacteriological confirmation of clinical diagnosis and characterization of the obtained isolates.

3.9. Conventional methods

3.9.1. Digestion and decontamination of specimen

All specimens collected except CSF were digested and decontaminated to remove unwanted fast growing bacteria using 4% sodium hydroxide (NaOH) and N-acetyl L-cysteine (NALC)

method. Cerebrospinal fluids (CSF) were not subjected to this procedure due to the paucibacillary nature of it coupled with inadequate sample volume.

Mycoprep, a commercially formulated NaOH-NALC-sodium citrate solution was used for the digestion and decontamination. The digestion and decontamination of the specimen was done by adding equal volumes of sample and NaOH-NALC solution and allowing the preparation to stand for fifteen minutes at room temperature (25°C), and then the reaction is neutralized by adding about 40ml of phosphate buffer solution of pH 6.8 for twenty minutes. The whole preparation was then centrifuged at 3000g for fifteen minutes to concentrate the specimen and also wash the NaOH solution. The supernatant was then discarded to obtain the sample inoculums.

3.9.2. Smear preparation and staining

Two smears were carefully prepared from each concentrated inoculums after culture media had been inoculated. One smear was stained for microscopic examination using Ziehl-Neelsen (ZN) staining technique and the other smear also stained using fluorochrome (auramine) staining method. These smears were air dried after staining and examined microscopically. The ZN stained smear was examined using objective lens of 1000 magnifications and the auramine stained smear also examined using objective lens of 400 magnifications. Smears were graded using the IUALTD/WHO standards.

3.9.3. Culture - Solid Media

Mycobacterium tuberculosis grows slowly and it does not grow on any simple media. It grows well on some media containing albumin, oleic acid and egg with glycerol or pyruvate .

Two Lowenstein-Jensen media, one containing pyruvate to support the growth of *Mycobacterium bovis* and the other one containing glycerol which also enhance the growth of *Mycobacterium tuberculosis* were inoculated with 0.5ml each of the decontaminated and concentrated inoculums. These were incubated at 37°C aerobically for maximum of eight weeks. All solid cultures were examined after forty-eight hours of incubation to check for absorption after inoculation and detect early contamination. Cultures were thereafter examined every week for growth. Positive cultures were identified using presumptive colonial characteristics like the dry, rough and crumbly nature of isolates. The demonstration of acid-fast bacilli (AFB) in a smear provides a preliminary diagnosis of mycobacterial disease.

3.9.4. Liquid culture – (MGIT 960)

The Mycobacteria Growth Indicator Tube (MGIT) containing 7.0 ml of modified Middlebrook 7H9 broth base was inoculated with 0.5ml of the inoculums. An enrichment of the MGIT tube, known as MGIT 960 Growth Supplement (Oleic acid, Albumin, Dextrose and Catalase), was added to make the medium complete. Again panel of antibiotics, Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim and Azlocillin (PANTA) was added to the medium to suppress growth of contaminating bacteria.

These cultures were placed in an automated BACTEC MGIT 960 instrument where they were incubated and monitored for increasing fluorescence every 60 minutes. Growth of bacteria as well as mycobacteria increases the fluorescence.

Specimens containing no mycobacterial growth (indicated by little or no fluorescence) were reported negative after six weeks of incubation. Specimens containing mycobacterial growth (indicated by increased fluorescence) became apparent after two to four weeks and were reported positive culture (machine positive). The detection of growth was also visually observed by the presence of a non-homogeneous light turbidity or small granular appearance in the medium.

3.9.5. Confirmation of Mycobacterial Growth

Phenotypic methods were used for the tentative differentiation of culture positive cases. Smears were prepared from the culture positive specimen and stained with ZN technique for acid fast bacilli to confirm growth of mycobacteria.

Smears made from a positive MGIT broth also helped in the tentative differentiation of *M. tuberculosis* complex from other mycobacteria. Some cultures belonging to the *M. tuberculosis* complex showed serpentine cords while other mycobacteria appeared loose in the stained smear. See plate 3.1



Plate 3. 1. Ziehl-Neelsen stained smear of culture positive showing the serpentine cord of *M. tuberculosis* (adapted from labmedx.ucsfmedicalcentre.org)

Again the BD MGIT TBc identification test (TBcID test) was also used to differentiate *M. tuberculosis* complex from non tuberculous mycobacteria. See plate 3.2. The TBcID test is a rapid chromatographic immunoassay for the qualitative detection of *Mycobacterium tuberculosis* complex antigen from AFB smear positive BD MGIT tubes. The test detects the following species of MTBC; *M. tuberculosis*, *M. bovis*, *M. africanum* and *M. microti*.

This product detects MPT64, a mycobacterial protein fraction that is secreted from MTBC cells during culture. When samples are added to the test device, MPT64 antigen binds to anti-MPT64 antibodies conjugated to visualize particles on the strip. The antigen-conjugate complex migrates across the test to the reaction area and is captured by a second specific MPT64 antibody applied to the membrane. If the MPT64 antigen is present in the sample, a colour reaction is produced by the labeled colloidal gold particles and is visualized as a pink to red line.

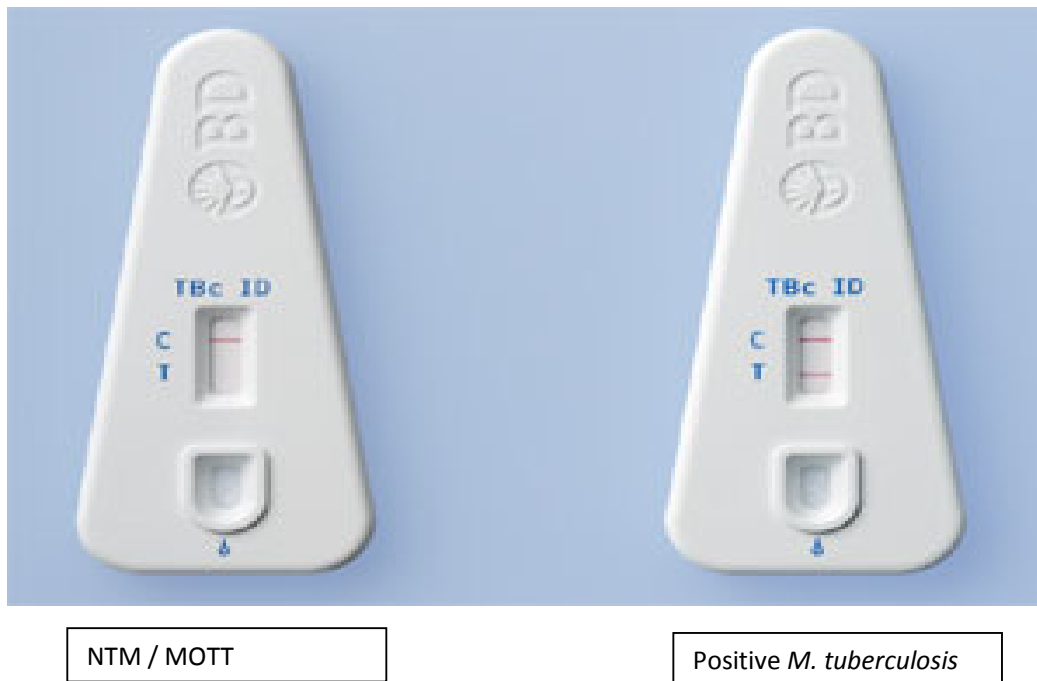


Plate 3. 2 TBcID cassettes for immunochromatographic identification of *M. tuberculosis* (adapted from www.bd.com)

3.9.6. Drug Sensitivity Testing

The drug susceptibility patterns of the isolate were determined using the conventional (phenotypic) sensitivity testing method and the MDRTBplus (Genotypic) method.

The BACTEC MGIT 960 susceptibility testing for Streptomycin (S), Isoniazid (I), Rifampin (R) and Ethambutol (E), called SIRE, was used to determine the *Mycobacterium tuberculosis* strain's phenotypical sensitivity to drugs.

Isolates from the cultures were subjected to growth in the presence of known concentrations (Streptomycin = 1.0 μ g/ml, Isoniazid = 0.1 μ g/ml, Rifampicin = 1.0 μ g/ml, and Ethambutol = 5.0 μ g/ml) of the drugs. The standard critical concentration of all the drugs was 1 μ g/ml.

0.8ml of the MGIT SIRE supplement is added to each vial of antibiotics to re-constitute the lyophilized antibiotics. 100 μ L of each re-constituted antibiotic is added to the MGIT culture tube. Controls were also included with no drug added. The isolates that grew in the controls

but did not grow in presence of the drugs were considered susceptible. On the other hand, those that grew in both the control and the drug containing medium were considered to be resistant to that drug.

Susceptibility testing against pyrazinamide (PZA) was also carried out using same technique and principle as the SIRE but at a lower pH (5.5) of the medium, since pyrazinamide (PZA) is active only at the low pH *in vitro*.

3.9.7. Quality control

All procedures performed and culture media used were quality controlled using reference culture strains of *Mycobacterium tuberculosis* H37Rv ATCC27294 and *Mycobacterium bovis* BCG.

3.10. Molecular methods

3.10.1. GeneXpert test

The GeneXpert (Xpert MTB/RIF) detects *Mycobacterium tuberculosis* and rifampicin resistance by using hemi nested PCR to amplify the rifampicin region of the rpoB nucleic acid. It detects the presence of *M. tuberculosis* DNA in specimen and mutations in the rpoB gene that leads to rifampicin resistance.

The GeneXpert system consists of modules, cartridges and computer system and uses multiplex, rapid real-time PCR technique. Real-time PCR techniques are based on hybridization of amplified DNA or RNA with fluorescent-labeled probes in the DNA region of interest and monitored inside thermal cycler. The amount of amplicons that is made in the reaction tube is directly proportional to increase in the fluorescent signal.

Each GeneXpert cartridge is a self-contained unit test device and contains liquid sample-processing, PCR buffers and freeze-dried (lyophilized) PCR reagents.

Cartridges were prepared as follows;

- PCR buffer which is sample reagent was added to the clinical samples in a ratio of 2 to 1, to digest specimen.
- The mixtures were vortexed for five second each and then allowed to stand for fifteen minutes.
- 2ml of the mixtures were transferred into the cartridges.

The cartridges were then loaded into the machine; at that point the tests began. It took approximately two hours for a result to come out.

3.10.2. Hain's test (LPA)

This study used Genotype MTBC for species identification and Genotype MTBDR_{plus} for drugs susceptibility testing. Genotype MTBC is based on the DNA-STRIP technology:

Mycobacterial DNA was extracted from the specimen, amplified using PCR and detected on a membrane strip using reverse hybridization and an enzymatic color reaction. The test was used for the differentiation of the *Mycobacterium tuberculosis* complex:- *M. africanum*, *M.*

bovis BCG, *M. bovis ssp. bovis*, *M. bovis ssp. caprae*, *M. microti* and *M. tuberculosis* from cultivated samples.

The obtained hybridized bands were compared with standard template provided in plate 3.3 below for results interpretation of results.

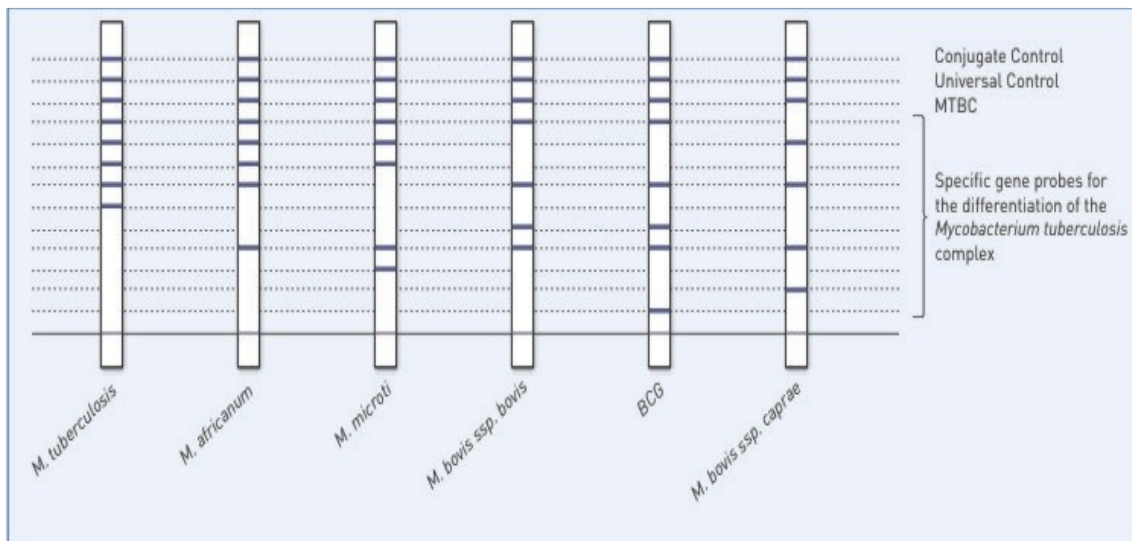


Plate 3.3. Standard template for MTBC identification and differentiation

The Genotype MTBDR $plus$ test is also based on the DNA-STRIP technology and allows for identification of *M. tuberculosis* complex and it test for susceptibility to rifampicin and isoniazid. The identification of rifampicin resistance is based on the detection of mutations of the *rpoB* gene which codes for the beta-subunit of the RNA polymerase. Mutations in the *katG* gene which codes for catalase peroxidase and the promoter region of the *inhA* gene which code for the NADH enoyl ACP reductase were tested for isoniazid resistance.

In performing the procedure the following major three steps were followed;

- DNA was extracted from isolates using chemical (Genolyse) method.
- A multiplex amplification of the DNA with biotinylated primers was done.
- The amplicons were hybridized onto the DNA strip by reverse hybridization.

The hybridization process involved; chemically denaturing of the amplification products, hybridization of single-stranded biotin-labeled amplicons to membrane bound probes, stringent washing, addition of streptavidin/alkaline phosphatase conjugate and an alkaline phosphatase mediated staining. Afterward, the obtained banding patterns of the tests were compared with provided template as in plate 3.4 below and results were interpreted.

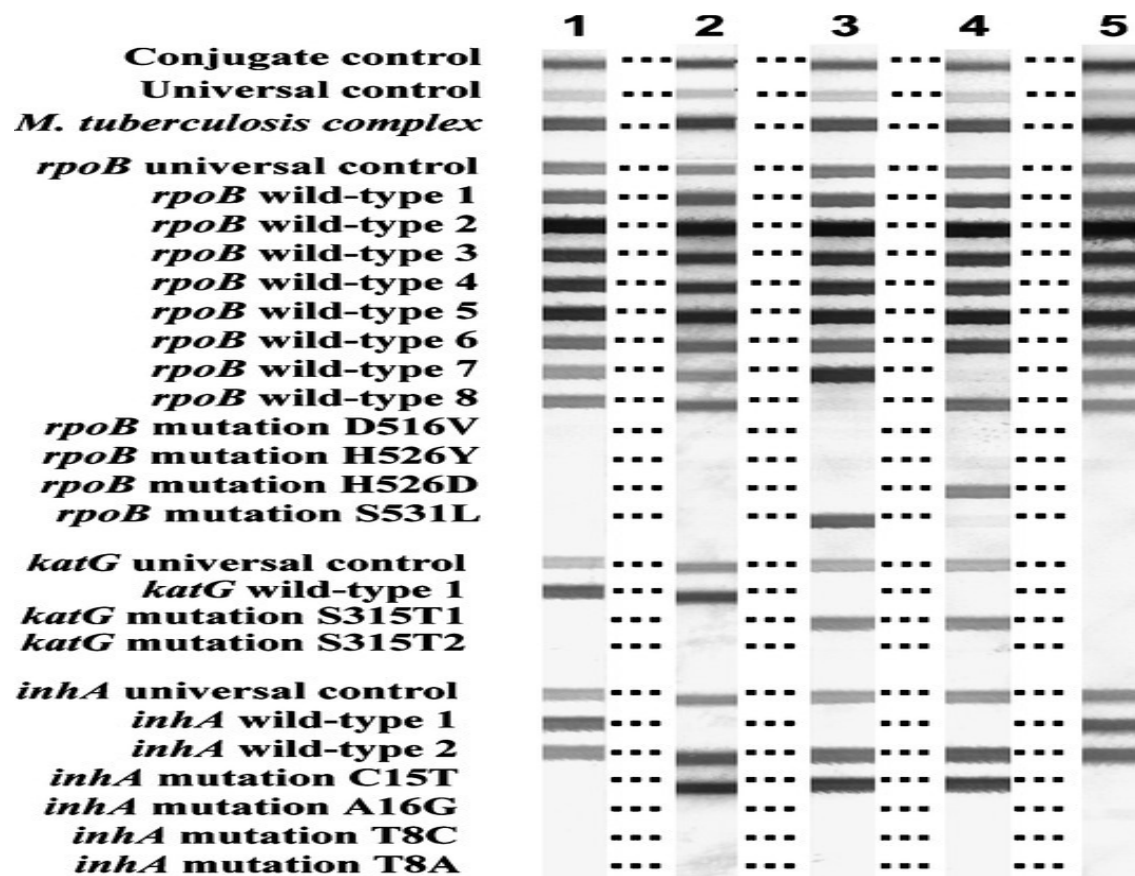


Plate 3. 4. Standard template for the interpretation of MDRTBplus assay results

3.11. Statistical analysis

The data was analyzed with Statistical Package for Social Scientist (SPSS) statistical software version 16.0 and MedCalc statistical software version 13.3. To determine the association between extrapulmonary tuberculosis and risk factors, odds ratio, p-value and 95% confidence intervals were calculated. To test the difference between male's age and female's age Means and Standard deviations were calculated. The sensitivity and specificity of the Microscopy and Genexpert were calculated using the following formulae with culture as the reference:

$$\text{Sensitivity} = \frac{\text{total number positive cases}}{\text{Total number of suspected cases}} \times 100$$

$$\text{Specificity} = \frac{\text{total number negative cases}}{\text{Total number uninfected suspects}} \times 100$$

CHAPTER FOUR

4.0. RESULTS

4.1. Samples Distribution

Between June 2013 and May 2014, one hundred and twenty-five (125) clinical samples were collected and analyzed. Figure 4.1 below is the distribution of clinical specimen collected by anatomical sites.

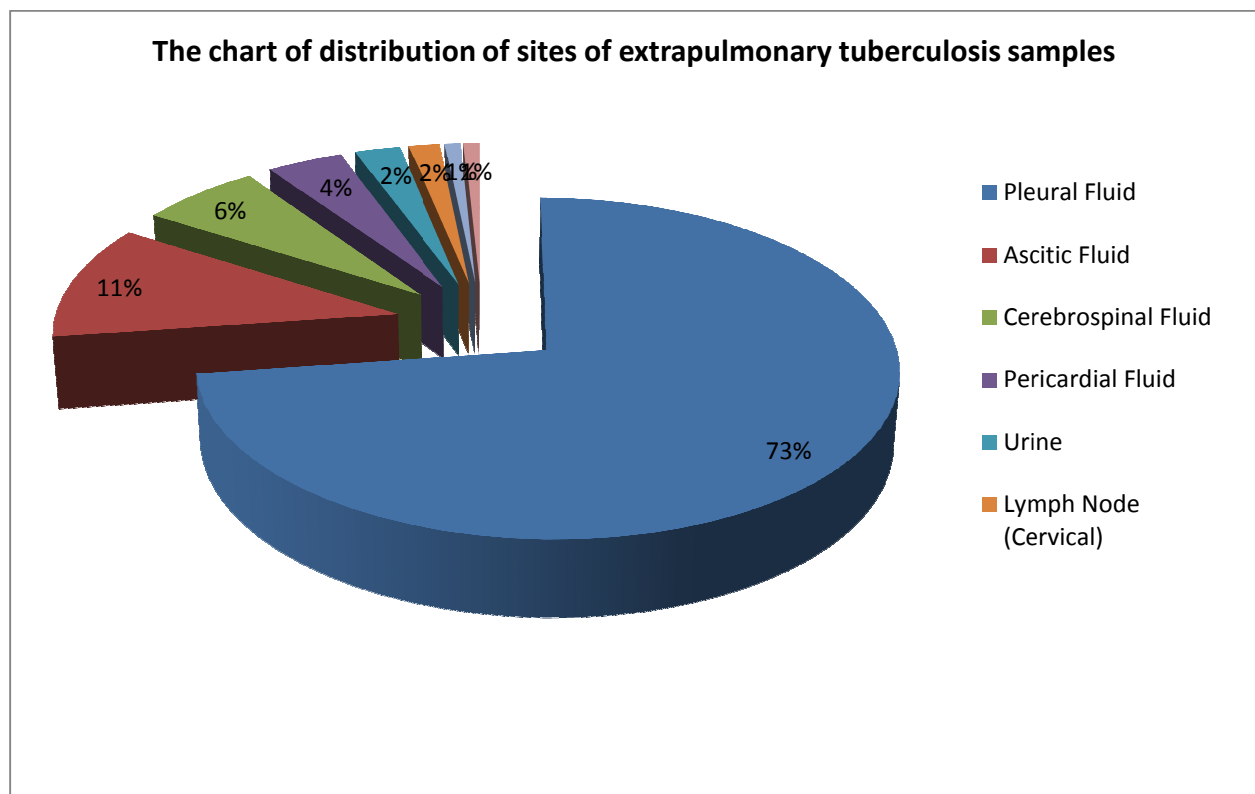


Figure 4. 1. The distribution of samples of extrapulmonary tuberculosis by anatomical sites

Pleural aspirate was the most common sample (91), followed by ascitic fluid (14). The least were sub-diaphragmatic aspirate and chestwall aspirate (1 each).

Due to the difficulty in obtaining extrapulmonary samples, the most common and probably less risky to obtain was Pleural aspirate.

4.2. Study Participants

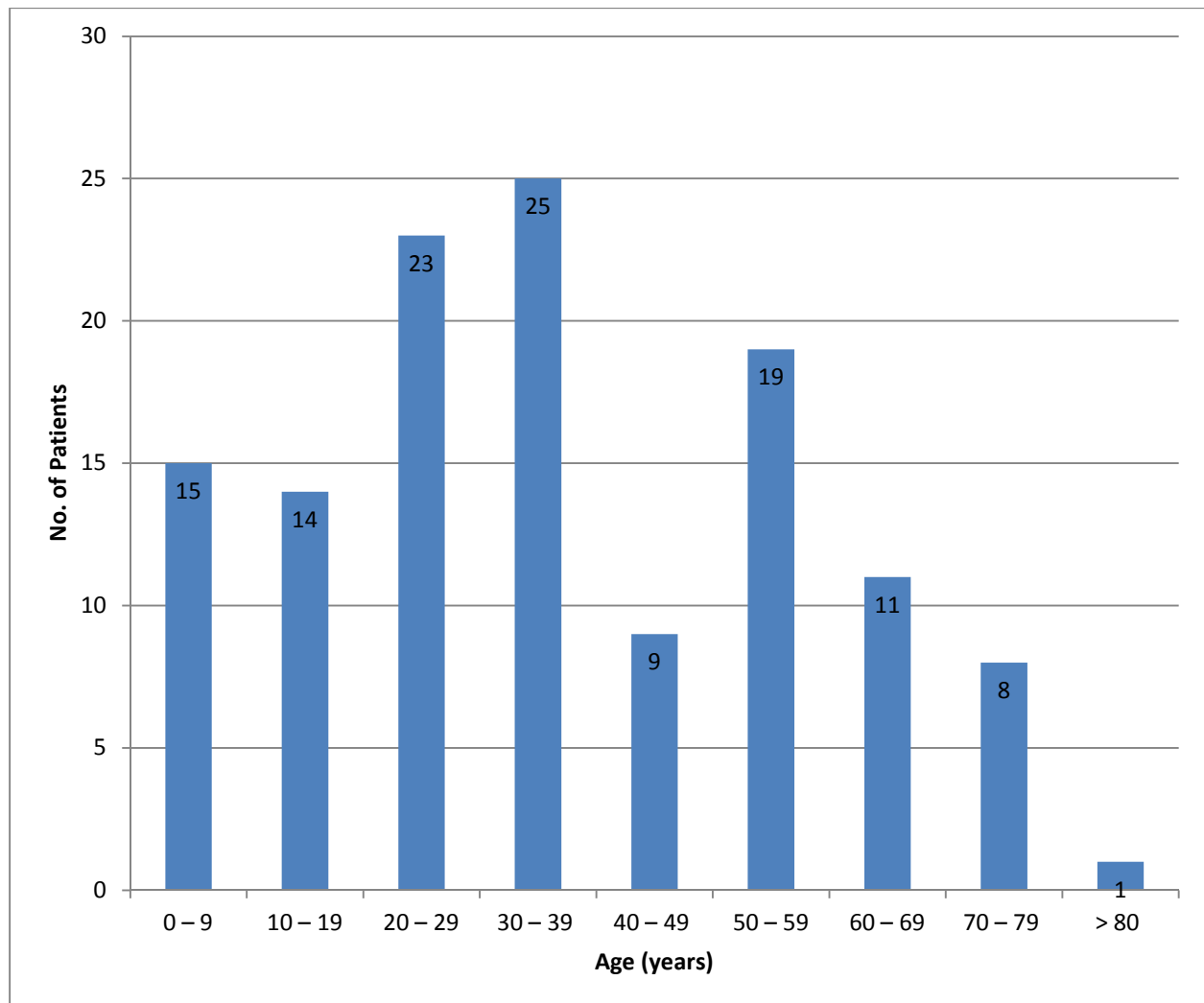


Figure 4. 2. Age distribution of persons whose samples were tested for extrapulmonary tuberculosis.

Figure 4.2 also represent the age distribution of suspected patients involved in the study. The range was 0.5 to 82 years with mean age of 35.64 ± 21.12 years.

There were 70 (56%) males with mean age of 36.74 ± 20.94 and 55 (44%) females with mean age of 34.23 ± 21.46 . The highest numbers of participants in the study were males and the largest age groups for the study were aged between 30 – 39 years. The least group is the most aged (80 years and above)

4.3. Bacteriological Examination

Table 4.1. Sites of cases of bacteriologically proven extrapulmonary tuberculosis

Site of sample	No. of Samples	Culture (MTB)
Pleural Fluid	91	6
Ascitic Fluid	14	1
Cerebrospinal Fluid	8	0
Pericardial Fluid	5	1
Urine	3	0
Lymph node aspirate	2	0
Chestwall aspirate	1	0
Sub-diaphragmatic F.	1	0
Total	125	8

Using culture as the gold standard for the diagnosis of extrapulmonary tuberculosis, the prevalence rate of extrapulmonary tuberculosis among the study participants is 6.4% (8 out of 125) as shown in Table 4.1 above. The most common type of extrapulmonary tuberculosis in this study was pleural tuberculosis (6 cases) with ascitic and pericardial tuberculosis being one (1) case each. There were no meningeal, lymphatic and genitourinary tuberculosis in this study as shown in Table 4.1 above.

Two (1.6%) of the isolates were Non-tuberculous mycobacteria (NTM) one each from pleural fluid and ascetic fluid.

The molecular Genexpert technique detected 12 *Mycobacterium tuberculosis* complexes out of 100 samples (due to the small volume of some of the samples). The microscopy also recorded 17 acid fast bacilli out of the 125 samples.

Table 4.2. The positivity of the methods used with the number of sample.

	Microscopy(%)	Culture(%)	GeneXpert(%)
Positive cases	17(15.2)	8(6.4)	12(12.0)
Negative cases	108(84.8)	117(93.6)	88(88.0)
Total cases	125(100)	125(100)	100(100)

The sensitivity and specificity of the other diagnostic tools were evaluated with reference to the culture as the gold standard. The GeneXpert had sensitivity of 66.67% (95% CI: 23.04% - 72.14%) and specificity of 96.46% (95% CI: 91.18% - 99.01%). The microscopy also had sensitivity of 47.06% (95% CI: 23.04 – 72.14%) and specificity of 91.67% (95% CI: 84.77% - 96.11%). This confirms that the GeneXpert molecular technique has higher sensitivity and specificity compared to that of smear microscopy.

4.4. Demographic Characteristics

Table 4.3 Risk characteristics of patients with culture-confirmed extrapulmonary tuberculosis.

Variables	Total samples (%)	Culture EPTB (%)	P-value	OR	95% CI
SEX			0.72	1.31	0.30 - 5.72
Male	70 (56.0)	5 (7.1)			
Female	55 (44.0)	3 (5.5)			
HIV STATUS			0.66	1.64	0.18 -14.72
Positive	10 (8.0)	1 (10.0)			
Negative	104 (83.2)	7 (6.7)			
Unknown	11 (8.8)	0 (0.0)			
Alcohol use			0.91	1.10	0.21 - 5.76
Yes	29 (23.2)	2 (6.9)			
No	96 (76.8)	6 (6.25)			
Smoking Habit			0.83	0.72	0.04 - 13.48
Yes	9 (7.2)	0 (0.0)			
No	116 (92.8)	8 (6.9)			
Employment			0.78	1.23	0.28 - 5.40
Yes	84 (67.2)	5 (6.0)			
No	41 (32.8)	3 (7.3)			
History of TB Treatment/Contact			0.36	2.83	0.30 - 26.80
Treatment / contact	6 (4.8)	1 (16.7)			
None	119 (95.2)	7 (5.9)			
AGE			0.82	0.84	0.19 - 3.68
0-9	15 (12.0)	0 (0.0)			
10-19	14 (11.2)	1 (7.1)			
20-29	23 (18.4)	1 (4.3)			
30-39	25 (20.0)	1 (4.0)			
40-49	9 (7.2)	2 (22.2)			
50-59	19 (15.2)	1 (5.3)			
60-69	11 (8.8)	1 (8.3)			
70-79	8 (6.4)	1(12.5)			
>80	1 (0.8)	0 (0.0)			

Table 4.3 above also shows the various risk characteristics of patients with extrapulmonary tuberculosis.

In the study only 1 (10%) of the HIV positive patients was diagnosed to have extrapulmonary tuberculosis. None of the smokers had extrapulmonary tuberculosis.

Again there was no association between extrapulmonary tuberculosis and demographic factors such as alcohol use, unemployment and history of either TB treatment or contact with TB patient. This study recorded no extrapulmonary tuberculosis among children of age 9 years and below, whereas the 40 – 49 years group recorded the highest (22.2%) percentage of extrapulmonary tuberculosis.

Therefore it is confirmed in this study that there is no significant association between extrapulmonary tuberculosis and the risk characteristics studied such as sex, age, HIV status, smoking, alcohol use, unemployment and previous exposure to TB.

4.5. Drugs Susceptibility Pattern of *Mycobacterium tuberculosis* isolates from EPTB

Patients

Using the rapid molecular identification and susceptibility technique (GeneXpert MTB/RIF), 12 *Mycobacterium tuberculosis* were detected among 100 participants. Out of the 12 detected, 11 were susceptible to rifampicin and only one resistant. The GeneXpert, which detects *Mycobacterium tuberculosis* specific sequence of the rpoB gene, was able to detect all the culture positive cases and additional positive cases that were missed by culture.

This shows that GeneXpert assay is reliable for the detection of *Mycobacterium tuberculosis* in extrapulmonary specimen.

Using the phenotypic method, 3 isolates were found to be resistant to streptomycin and 4 isolates resistant to isoniazid as shown in Table 4.4. Two isolates were resistant to both streptomycin and isoniazid

Table 4.4. Drug susceptibility pattern of MTB in extrapulmonary tuberculosis

ISOLATES	PHENOTYPIC (CULTURE) DST					MTBDR- PLUS		GENEXPERT	
	STR	INH	RIF	EMB	PZA	RIF	INH	RIF	COMMENTS
S/0505	S	S	S	S	S	S	S	S	All susceptible
S/0470	S	R	S	S	S	S	S	S	Resistant to Isoniazid
S/4061	R	S	S	S	S	S	S	S	Streptomycin resistance
S/0016	R	R	S	S	S	S	R	S	katG WT absent MUT1 present
C/707	R	R	S	S	S	S	R	S	katG WT absent MUT2 present
PA/477	S	S	S	S	S	S	S	S	All susceptible
S/0888	S	S	S	S	S	S	S	S	All susceptible
C/582	S	R	S	S	S	S	R	S	katG WT absent MUT1 present

Key: S- Susceptible, R- Resistant, STR- streptomycin, INH- Isoniazid, RIF- Rifampicin, EMB- Ethambutol and PZA- Pyrazinamide

Using the genotypic method, 3 isolates were resistant to isoniazid. Although one isolate was determined to be sensitive to isoniazid by the genotypic method, it was however resistant by the phenotypic method.

Again using the phenotypic method, 4 (50%) isolates were resistant to isoniazid: isoniazid mono-resistance isolates were 2 (25%) and poly-resistance to isoniazid and streptomycin were also 2 (25%).

The MDRTB_{plus} genotype method detected isoniazid resistance in three (3) *M. tuberculosis* isolates and the pattern is shown in the Figure 4.3 below.

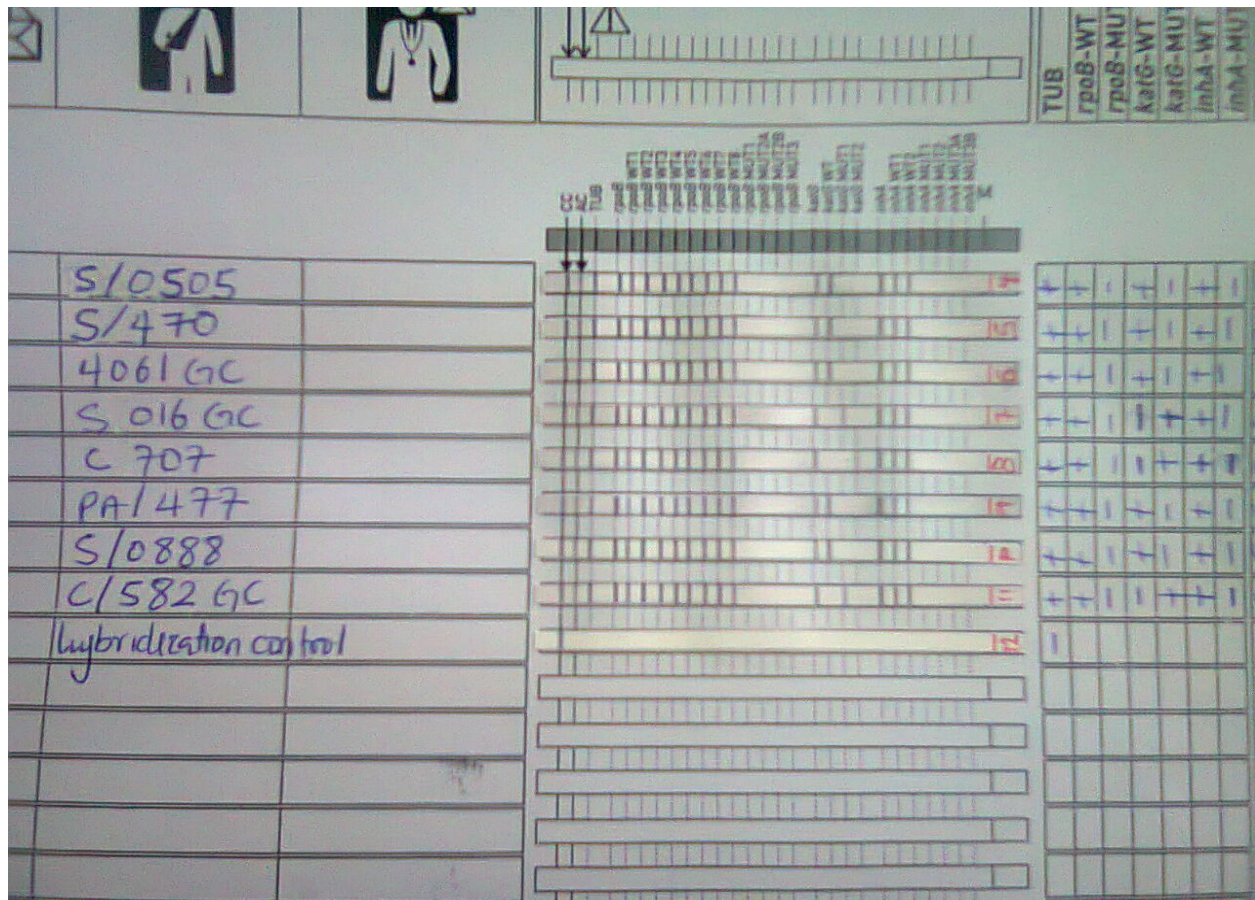


Figure 4. 3. Valid results obtained by MDRTB_{plus} assay

Comparing the results with the standardized band, it showed that in all the three isoniazid resistance strains, the *katG* wild type gene is absent, but in two isolates mutation 1 is present and the other one mutation 2 is present.

In this study MDR-TB was not detected among the extrapulmonary tuberculosis patients.

4.6. Identification of *Mycobacterium tuberculosis* complex (MTBC) species

As shown in figure 4.4 below, 7 of the 8 *Mycobacterium tuberculosis* complex species were confirmed as *Mycobacterium tuberculosis* and only 1 as *Mycobacterium africanum*.



Figure 4. 4. Results of characterization of *Mycobacterium tuberculosis* complex using Genotype MTBC

CHAPTER FIVE

5.0. DISCUSSION

The primary aim of this hospital based study was to describe the characteristics of *Mycobacterium tuberculosis* isolates obtained from extrapulmonary tuberculosis patients.

From this study, culture positive prevalence rate of extrapulmonary tuberculosis (EPTB) in Korle-Bu Teaching Hospital was 6.4%. Comparatively this rate is lower than the worldwide rate of between 10 – 25% that many studies had reported (Haley et al., 2013; Heather et al., 2009; Sandgren et al., 2013). However, the result of this current study is similar to a study in Ibadan, Nigeria by Kehinde which reported a prevalence rate of 4.1% among extrapulmonary patients, though Nigeria is among the 22 high TB burden countries (Kehinde, et al., 2010).

Another study in Ethiopia, also a high TB burden African country, using smear microscopy reported 9.9% prevalence rate of extrapulmonary tuberculosis (Zenebe et al., 2013). However, by smear microscopy, this current study recorded 17(15.2%) smear positive cases out of the 125 participants.

Observations have shown that often in the hospital, patients suspected of extrapulmonary tuberculosis are registered and put on anti-tuberculosis drugs before clinical samples are sent to the laboratory for bacteriological confirmation of clinical diagnosis. Therefore the low culture positive prevalence rate of 6.4% may be due to the administered drugs inhibiting the growth of *Mycobacterium tuberculosis*.

There is the need therefore for clinicians to wait for bacteriological confirmation before putting suspected EPTB patients on antibiotics. Again for the culture detection rate (6.4%) to be lesser than that of smear microscopy (15.2%), then it may be as a result of harsh decontamination process for the extrapulmonary samples.

In this study the sensitivity and specificity of the GeneXpert were calculated to be 66.67% and 96.46% respectively. A similar study in the German National Reference Laboratory for Mycobacteria (NRL), in 2011 recorded sensitivity and specificity of the GeneXpert assay as 77.3% and 98.2% respectively (Hillemann et al., 2011). With these results it is evident that Genexpert can be used in the diagnosis of EPTB, notwithstanding the small sample size.

Again in this study, MTBC isolated from pleural aspirate had been noted to be the highest form of extrapulmonary tuberculosis in Korle-Bu Teaching Hospital. This finding is contrary to the study by Huang et al, in 2000, in Shanghai, which reported tuberculous lymphadenitis as leading extrapulmonary tuberculosis (Huang et al., 2000). Similarly Lowieke et al., and Mostofa et al. reported tuberculous lymphadenitis as the leading extrapulmonary tuberculosis in Netherlands and Bangladesh respectively (Lowieke et al., 2006; Mostofa et al., 2010). However, in both studies the proportions of lymph node samples were high compared to other samples. The findings of this study may be as a result of the proportions of samples in this study since the most common sample sent to the laboratory for the study was pleural fluid.

Previous studies have demonstrated that female patients are more likely to have extrapulmonary tuberculosis than male patients (Zhenhua et al., 2004; Ong et al., 2004 and Heather et al., 2009). It was suspected that this difference is due to immunological factors in females (Heather, et al., 2009). However, in the current study there was no significant difference between the sexes.

Many studies have identified HIV infection or impaired host immunity as major risk factors of EPTB (Ong et al., 2004; Fiske et al., 2010), indeed extrapulmonary tuberculosis was once a defining condition for AIDS (Ong et al., 2004). It has been established that reduced immune system predispose to tuberculosis especially EPTB. This is because the HIV targets of CD4 cells are unable to completely fight TB infection and carries greater risk of causing endogenous reactivation of TB (De Backer et al., 2006). It is reported that younger age groups, being sexually active, are at risk of having HIV compared to the older and countries where tuberculosis is low HIV does not cause increased tuberculosis (De Backer et al., 2006).

In this study there was no significant association between HIV and EPTB, just as Heather et al report in 2009, which reported no strong association between EPTB and HIV status or age (Heather, et al., 2009). This result is probably related to the fact that Ghana is not considered to have high burden of tuberculosis (Bonsu et al., 2012) as well as having a low prevalence rate of HIV (1.3%) (El-Adas, 2013).

In this study, there was no significant association between extrapulmonary tuberculosis and risk characteristics such as smoking, alcohol use, unemployment, history of previous exposure and contact with tuberculosis patients. This result is consistent with the studies of Nissapatorn et al., Garcia-Rodriquez et al. and Zhenhua et al., which found no association between EPTB and alcohol use, smoking of tobacco, contact with TB patient or low social status (Nissapatorn et al., 2004; Zhenhua et al., 2004; Garcia-Rodriquez et al., 2011).

Alcohol use and tobacco smoking are known to reduce the functions of the immune system and result in increased risk of microbial infections including *Mycobacterium tuberculosis* (Zhenhua et al., 2004; Jianming et al., 2009). Though studies have established the strong association between alcohol abuse and pulmonary tuberculosis, such is yet to be established between alcohol use and extrapulmonary tuberculosis (Zhenhua et al., 2004). Again no consensus had been reached at, as to whether tobacco smoking increase risk of tuberculosis development (Bates et al., 2007), though few studies have provided evidence that tobacco smoking is a risk factor for tuberculosis (Bates et al., 2007; Arcavi et al., 2004).

Contrary to the general expectation of high extrapulmonary tuberculosis among children (Addo et al., 2007; Fanning 1999), this study found no extrapulmonary tuberculosis among children of age 9 years and below. Chandrashakhar et al. reported from Nepal that EPTB was more common at 25 years and less (younger age) than in older adults. Another study also reported age as a risk factor for EPTB for people from sub-saharan Africa (Chandrashakhar et al., 2008). Meanwhile this current study shows that EPTB can occur in any age group.

The members of *Mycobacterium tuberculosis* complex include *M. tuberculosis*, *M. bovis* (subspecies *bovis* and *caprae*), *M. bovis* Bacillus Calmette-Guerin (BCG), *M. africanum* (subspecies I and II), *M. microti*, *M. canetti* and *M. pinnipedii* (Cousins et al., 2003; Brooks et al., 2007). These can be differentiated using few phenotypic and genotypic characteristics. The 8 MTBC isolated were classified into species. It was shown from this study that *Mycobacterium tuberculosis* is the predominant specie of the complex causing extrapulmonary tuberculosis in Korle-bu Teaching Hospital followed by *M. africanum*.

The relatively low rate of *M. africanum* in this study was a surprise since it is of West African origin and a higher prevalence rate was expected. However this result is consistent with a study by Lawn et al., which used 26 isolates of MTBC from pulmonary tuberculosis in Kumasi, Ghana and reported 92.0% as *M. tuberculosis* and only 8.0% as *M. africanum* but not with that of Van der Werf et al., which characterized MTBC causing pulmonary tuberculosis in the middle belt of Ghana and reported 57.6% *M. tuberculosis* and 42.4% *M. africanum* (Van der Werf et al., 1989; Lawn et al., 2001).

Unlike the suggestion by Addo et al., that *Mycobacterium bovis* is presumed to be the highest strain causing disseminated and extrapulmonary tuberculosis, especially in children (Addo et al., 2007), this study found no *M. bovis* causing extrapulmonary tuberculosis. This is probably due to the age group in which culture positive extrapulmonary tuberculosis were found. No child was found to have extrapulmonary tuberculosis.

It is noteworthy that no multi-drugs resistance tuberculosis (MDR-TB) was detected in this study. However in a study on drug susceptibility pattern of mycobacterium isolates from Ghana by Yeboah-Manu in 2012, MDR-TB rate of 2.5% was recorded (Yeboah-Manu et al., 2012), an indication of low rate of MDR-TB in Ghana. This may be the reason why MDR-TB is not yet associated with EPTB in the Korle-Bu Teaching hospital, though available data at the hospital indicates that MDR-TB is high among month two and treatment failure cases (Poster Presentation, 2012). Again the reason why MDR-TB was not detected in EPTB could be because of low proportion of patients previously exposed or treated for tuberculosis included in the study, since previous exposure increases the chance of developing MDR-TB.

This study also found out that half of the isolates were resistant to isoniazid and 3 were resistant to streptomycin. Again 3 of the isolates were mono-resistance; 2 and 1 to isoniazid and streptomycin respectively. Two of the isolates were poly-resistance to streptomycin and isoniazid (STR/INH). These resistance patterns observed in this study is also consistent with that of Yeboah-Manu et al., which also reported high level of drug resistance particularly in streptomycin and isoniazid. This high rate of isoniazid resistance noted can be worrying, due to the fact that isoniazid is one of the most effective and major drugs in the standard four drugs regimen for tuberculosis treatment and development of isoniazid resistance is a common first step in the evolution of MDR-TB (Manzour et al., 2006), however the Yeboah-Manu et al., study in 2012 established that treatment outcome of *Mycobacterium tuberculosis* primarily depends on the isolates' susceptibility to rifampicin (Yeboah-Manu et al., 2012).

Three of the 4 isoniazid resistances observed in this current study have mutations in the *katG* wild-type gene and all are located on the codon 315 (Ser315Thr). This is consistent with earlier studies elsewhere which reported that 40% - 95% of isoniazid resistant *Mycobacterium tuberculosis* isolates have mutations in *katG* gene and most are located on the codon 315. (Escalante et al., 1998; Somoskovi et al., 2001; Mokrousov et al., 2004). The high rate of isoniazid resistance could be attributed to the institution of isoniazid preventive therapy (IPT) in HIV patients. Persistence of isoniazid resistance to *Mycobacterium tuberculosis* will jeopardize the continued use of this drug in tuberculosis treatment.

The one isolate that showed discordance of results between phenotypic DST and MDRTBplus assay, is considered to be true resistance to isoniazid. There is evidence that isoniazid blocks the synthesis of mycolic acids in *M. tuberculosis* (Somoskovi et al., 2001). The intracellular targets for this drug are the fatty-acid enoyl-acyl carrier protein reductase (*inhA*), and a complex of an acyl carrier protein (*acpM*) and a β -ketoacyl-ACP synthase (*kasA*). These enzymes are involved in the synthesis of mycolic acids and mutations have been found in the genes that encode for these proteins (*inhA*, *acpM* and *kasA*) (Slayden et al., 2000; Zhang et al., 2000; Vilcheze et al., 2000). The MDRTBplus assay detects mutations only in the *katG* gene and the *inhA* gene; therefore mutation that may occur in different regions of the genome such as the *acpM* and *kasA* could not be detected with this genotypic assay but could be detected by the phenotypic method. This result therefore is likely to be seen in isolates which have different gene mutations other than mutations in the *katG* and the *inhA* genes.

Limitations of the study

- The difficulty in getting the adequate number of samples coupled with some patients starting antibiotics therapy before samples were taken for analysis. Considering the fact that the study period was limited and could not be prolonged for greater number of isolates to be obtained, though more isolates would have made the study more interesting.
- Again most sample volumes obtained for the study were inadequate and that could not allow for repeat procedures and further investigations

CHAPTER SIX

6.0. CONCLUSIONS AND RECOMMENDATIONS

6.1. CONCLUSIONS

In this study, about 90% of cases suspected to be extrapulmonary tuberculosis at the hospital could not be confirmed using bacteriological methods. It is therefore concluded that the culture positive prevalence rate of extrapulmonary tuberculosis is 6.4% and pleural tuberculosis is likely to be the predominant form of extrapulmonary tuberculosis at Korle-Bu Teaching Hospital.

At the Korle-Bu Teaching hospital, *Mycobacterium tuberculosis* species predominate in extrapulmonary tuberculosis. Development of extrapulmonary tuberculosis is independent of socio-demographic risk factors.

Extrapulmonary tuberculosis is not associated with multi-drugs resistant tuberculosis at the Korle-Bu Teaching Hospital, even though isoniazid resistance is emerging. The presence of isoniazid resistance in extrapulmonary cases is a threat to the treatment of extrapulmonary tuberculosis.

Notwithstanding the small sample size, the high sensitivity and specificity of Genexpert obtained has confirmed that GeneXpert technique can also be a useful tool for the rapid detection of extrapulmonary tuberculosis.

6.2. RECOMMENDATIONS

The number of isolates obtained for this study was small and sample collection was limited to Korle-Bu Teaching Hospital only, it is therefore recommended that further studies based on larger number of isolates that have the ability to examine much details of extrapulmonary tuberculosis be conducted.

The high rate of isoniazid resistance in extrapulmonary tuberculosis is unacceptable, therefore recommended that much more investigations into the cause and also if possible, review the isoniazid preventive therapy system.

Again it is recommended that the gold standard of Mycobacteria culture is time consuming and not very sensitive for extrapulmonary tuberculosis diagnosis, therefore there is the need to compliment it with GeneXpert technique (molecular method) which has shown improved diagnosis for *Mycobacterium tuberculosis* in extrapulmonary specimen.

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APPENDICES**APPENDIX A****PATIENT AND SAMPLE INFORMATION SHEET**

Name.....

Sample ID.....

Residential Address.....

.....

AgeSex.....

Date of specimen collection.....

Occupation: Farmer, Trader, Skilled Labourer..... Civil Servant.....

Businessman / womanProfessional.....

Education: Illiterate....., Elementary, Secondary....., Tertiary.....

SOCIAL LIFE HISTORY

1. Do you drink alcohol? Yes..... No....., if yes for how long have you been drinking?

.....

2. Do you smoke? Yes..... No....., if yes for how long have you been smoking?

MEDICAL HISTORY

Have you been treated for TB before? Yes..... NoIf yes, the year.....

Have you come in contact with TB patient before? Yes..... No.....

HIV status; Positive, Negative.....

APPENDIX B**INFORMATION SHEET****CHARACTERISTICS OF ISOLATES OF MYCOBACTERIA TUBERCULOSIS
IN EXTRAPULMONARY TUBERCULOSIS IN KORLE-BU TEACHING
HOSPITAL**

You are being invited to participate in a study on the Characteristics of isolates on Mycobacteria tuberculosis in Extrapulmonary Tuberculosis in Korle-Bu Teaching Hospital, Accra Ghana. We will use the extrapulmonary sample collected from you by your doctor for diagnosis of tuberculosis. The staff at the reception of the laboratory will ask you few questions on your social life including your contact number that will last about five (5) minutes. The possible risk and discomfort of the sample collection will be explained by the attending doctor. The researcher is a student of University of Ghana Medical School, College of Health Sciences, University of Ghana. The study forms part of the requirements for the award of Master of Philosophy (M. Phil) degree in the institution.

The study is to describe the characteristics of isolates of Mycobacteria tuberculosis in extrapulmonary tuberculosis in Korle-Bu Teaching Hospital.

When completed, it will provide information on the distribution and risk factors to extrapulmonary TB and the immediate benefit will be the opportunity to appropriate treatment when you are confirmed bacteriologically as having extrapulmonary TB. The results of this study also will help in future management of extrapulmonary TB in the country.

Your participation in this study is completely voluntary. You are free to refuse permission to participate and this will never affect the services that will be rendered to you at this facility. If at any point in time during the study you take the decision not to participate any further, you are at liberty to do so immediately without any further discussion and it will have no consequences for you.

All information related to your participation would be kept strictly confidential. If you have any problems or questions about the study you can contact;

Michael Amo Omari – Chest Clinic Laboratory, Korle-bu Teaching Hospital.

Tel: 0277527187; 0248164778;

APPENDIX C**CERTIFICATE OF VERBAL INFORMED CONSENT****CHARACTERISTICS OF ISOLATES OF MYCOBACTERIA TUBERCULOSIS****IN EXTRAPULMONARY TUBERCULOSIS IN KORLE-BU TEACHING HOSPITAL**

I of

having understood the content of the information sheet of this study, after I have thoroughly read it and / or it had been thoroughly explained together with this consent form to me in..... (Specify language) have agreed to participate in the Characteristics of isolates of MTB in Extrapulmonary Tuberculosis in Korle-Bu Teaching Hospital study been carried out at the chest clinic laboratory of the Korle-Bu Teaching Hospital.

Name of Participant.....

Sex Age

Signature / Thumbprint of Participant.....

Witness

Date

For an illiterate participant, an impartial literate will act as a witness

I have witnessed the accurate reading and translation of the consent form to the participant. The participant has had the opportunity to ask questions which have been honestly answered. I confirm that the individual has given consent freely to participate in the study.

Name of witnessSignature of witness.....

Thumbprint of ParticipantDate

APPENDIX D: Media and Reagents Preparation

1. NaOH-NALC Digestion and Decontamination solution (Mycoprep)

- NaOH ----- 20.0g
- Trisodium citrate ----- 14.5g
- NALC ----- 0.370g
- Distilled water ----- to 1 litre

2. Phosphate buffer (pH 6.8)

- Disodium Phosphate ----- 2.37g
- Monopotassium Phosphate --- 2.27g
- Distilled Water ----- to 500mL

3. Ziehl-Neelsen staining solutions

- 0.3% Carbol Fuchsin
- 20% Sulphuric Acid
- 0.3% Methylene Blue

4. Fluorescent stain kits

- 0.2% Auramine O
- 0.5% Acid-Alcohol (Hydrochloric acid-isopropanol)
- 0.5% Potassium Permanganate

5. Blood Agar Plate

6. MGIT Medium

- Modified Middlebrook 7H9 broth base -----5.9g
- Casein peptone ----- 1.25g
- Distilled water ----- to 1L

7. MGIT Growth supplement (Enrichment)

- Bovine Albumin ----- 50.0g
- Dextrose ----- 20.0g
- Catalase ----- 0.03g
- Oleic Acid ----- 0.1g
- Polyoxyethylene state (POES) ---- 1.1g
- Distilled water ----- to 15ml

8. MGIT PANTA (Lyophilized drugs per vial)

- Polymyxin B ----- 6,000 units
- Amphotericin B ----- 600 μ g
- Nalidixic Acid ----- 2,400 μ g
- Trimethoprim ----- 600 μ g
- Azlocillin ----- 600 μ g

9. Drugs**SIRE**

- Streptomycin – lyophilized drug per vial ----- 332 μ g
- Isoniazid – lyophilized drug per vial ----- 33.2 μ g
- Rifampicin – lyophilized drug per vial ----- 332 μ g
- Ethambutol – lyophilized drug per vial ----- 1,660 μ g

Pyrazinamide (PZA) – lyophilized drug per vial---- 20,000 μ g

10. SIRE Supplement

- Bovine Albumin ----- 50.0g
- Dextrose ----- 20.0g
- Catalase ----- 0.03g
- Oleic Acid ----- 0.6g

11. PZA Supplement

- Bovine Albumin ----- 50.0g
- Dextrose ----- 20.0g
- Catalase ----- 0.03g
- Oleic Acid ----- 0.1g
- Polyoxyethylene state (POES) ---- 1.1g

12. L-J Medium

Preparation of L-J medium

- Homogenized whole eggs ----- 1L
- L-J base ----- 600mls

Homogenized whole eggs

Fresh eggs less than one week old were used. The shells are scrubbed with soap and kept in the soap solution for about 30 minutes. They were rinsed under running tap water and soaked in 70% alcohol for 15 minutes.

The eggs were broken into a sterile container and homogenized by shaking in a laboratory blender. The solution obtained was filtered through a four layers of sterilized gauze into a sterile graduated measuring cylinder.

L-J Base

- Dehydrated L-J powder ----- 37.2g
- Glycerol ----- 12mls
- Distilled water up to -----600mls.

The resultant suspension is autoclaved at 121°C at 15lbs pressure for 15 minutes.

1 Litre of the homogenized egg is then added after it had cooled to 45-55° C and mixed thoroughly without air bubbles.

The media was then dispense into sterile screw capped tubes, slant tubes and coagulate medium at 85°C (inspissations) for 45 minutes.

APPENDIX E: PROCEDURES

1. NaOH-NALC Digestion and Decontamination

- Specimens were transferred into 50ml Falcon tubes with screw caps.
- Equal volumes of NaOH-NALC solution was added to the specimen and timed for 15minutes.
- The caps were tightened and the specimen-NaOH-NALC mixtures were lightly mixed by vortex for 30 seconds.
- After 15minutes, Phosphate buffer of pH 6.8 was added to the 50ml mark and centrifuged at 3000g for 15 minutes.
- The supernatants were discarded into 5% phenol disinfectant and the pellets (sediments) were re-suspended with 1-2ml of phosphate buffer depending on the quantity of sediment.
- 0.5ml of the sediments was used to inoculate culture medium.

2. Smear Preparation

- The frosted ends of clean grease free microscope slides were labeled with the specimen numbers.
- A drop of the re-suspended sediments was carefully transferred onto the slides and gently smeared to give smears of even thickness and size.
- The smears were arranged in a slide rack and allowed to air-dry in the Biosafety cabinet.

3. Zielh-Neelsen staining procedure

- The smears were heat-fixed by passing over flame three times.
- The slides were arranged on a staining rack and flooded with carbol fuchsin stain.

- The under-side of the slides was gently heated until steam arises.
- The slides were left for 5 minutes to stain and gently washed with running tap water thereafter.
- The slides were decolourized with 20% sulphuric acid for 5 minutes and copiously rinsed with running water.
- The smears were counter stained with 0.3% methylene blue stain for 1 minute.
- The slides were washed gently under running tap water, drained of excess water and air-dried in a slide rack.

4. Fluorescence staining procedure

- The smears were heat-fixed by passing over flame three times.
- The slides were arranged on a staining rack and flooded with TB auramine M stain for 15 minutes and gently washed with tap water thereafter.
- Smears were decolorized with TB decolourized TM for 2-3minutes and thereafter washed gently with running water.
- Smears were counter stained with TB Potassium Permanganate for 2 minutes
- Smears were gently washed with running tap water and air-dry.

APPENDIX F: Statistical data**Crosstabs****sex * F.liquid Crosstabulation**

Count				
		F.liquid		Total
		NG	MTB	
sex	M	64	6	70
	F	53	2	55
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.252 ^a	1	.263		
Continuity Correction ^b	.564	1	.453		
Likelihood Ratio	1.324	1	.250		
Fisher's Exact Test				.464	.230
Linear-by-Linear Association	1.242	1	.265		
N of Valid Cases ^b	125				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.52.

b. Computed only for a 2x2 table

Crosstabs**hiscon * F.liquid Crosstabulation**

Count				
		F.liquid		Total
		NG	MTB	
hiscon	No	112	7	119
	Yes	5	1	6
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.109 ^a	1	.292		
Continuity Correction ^b	.039	1	.843		
Likelihood Ratio	.807	1	.369		
Fisher's Exact Test				.333	.333
Linear-by-Linear Association	1.100	1	.294		
N of Valid Cases ^b	125				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is .38.

b. Computed only for a 2x2 table

Occupation * F.liquid Crosstabulation

Count		F.liquid		
		NG	MTB	Total
Occupation	unemployed	39	2	41
	employed	78	6	84
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.236 ^a	1	.627		
Continuity Correction ^b	.009	1	.923		
Likelihood Ratio	.247	1	.619		
Fisher's Exact Test				1.000	.478
Linear-by-Linear Association	.234	1	.629		
N of Valid Cases ^b	125				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.62.

b. Computed only for a 2x2 table

Crosstabs

alc * F.liquid Crosstabulation

Count				
		F.liquid		
		NG	MTB	Total
alc	No	90	6	96
	Yes	27	2	29
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.016 ^a	1	.901		
Continuity Correction ^b	.000	1	1.000		
Likelihood Ratio	.015	1	.902		
Fisher's Exact Test				1.000	.593
Linear-by-Linear Association	.015	1	.901		
N of Valid Cases ^b	125				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 1.86.

b. Computed only for a 2x2 table

Crosstabs

smk * F.liquid Crosstabulation

Count				
		F.liquid		
		NG	MTB	Total
smk	NO	108	8	116
	YES	9	0	9
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.663 ^a	1	.415		
Continuity Correction ^b	.012	1	.914		
Likelihood Ratio	1.237	1	.266		
Fisher's Exact Test				1.000	.540
Linear-by-Linear Association	.658	1	.417		
N of Valid Cases ^b	125				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is .58.

b. Computed only for a 2x2 table

Crosstabs

HIVstat * F.liquid Crosstabulation

Count		F.liquid		Total
		NG	MTB	
HIVstat	NEG	97	7	104
	POS	9	1	10
	Unknown	11	0	11
Total		117	8	125

[DataSet1] C:\Users\OMARI\Documents\EPTB- MPHIL Project\Final Data.sav

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.987 ^a	2	.610
Likelihood Ratio	1.660	2	.436
Linear-by-Linear Association	.397	1	.529
N of Valid Cases	125		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is .64.

Crosstabs

sex * F.liquid Crosstabulation

Count		F.liquid		Total
		NG	MTB	
sex	M	64	6	70
	F	53	2	55
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.252 ^a	1	.263		
Continuity Correction ^b	.564	1	.453		
Likelihood Ratio	1.324	1	.250		
Fisher's Exact Test				.464	.230
Linear-by-Linear Association	1.242	1	.265		
N of Valid Cases ^b	125				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.52.

b. Computed only for a 2x2 table

Crosstabs

Age1 * F.liquid Crosstabulation

Count		F.liquid		Total
		NG	MTB	
Age1	0-9	15	0	15
	10-19	13	1	14
	20-29	22	1	23
	30-39	24	1	25
	40-49	7	2	9
	50-59	18	1	19
	60-69	10	1	11
	70-79	8	1	9
Total		117	8	125

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.709 ^a	7	.574
Likelihood Ratio	5.279	7	.626
Linear-by-Linear Association	1.388	1	.239
N of Valid Cases	125		

a. 8 cells (50.0%) have expected count less than 5. The minimum expected count is .58.

sex**Cases with positive (bad) outcome**Number in exposed group: a= Number in control group: c= **Cases with negative (good) outcome**Number in exposed group: b= Number in control group: d= **Results**

Odds ratio	1.3095
95 % CI	0.2998 to 5.7200
z statistic	0.359
	P = 0.7200

HIV**Cases with positive (bad) outcome**Number in exposed group: a= Number in control group: c= **Cases with negative (good) outcome**Number in exposed group: b= Number in control group: d= **Results**

Odds ratio	1.6429
95 % CI	0.1834 to 14.7191
z statistic	0.444
	P = 0.6572

ALCOHOL USE

Cases with positive (bad) outcome

Number in exposed group: a=

Number in control group: c=

Cases with negative (good) outcome

Number in exposed group: b=

Number in control group: d=

Results

Odds ratio	1.1034
95 % CI	0.2112 to 5.7649
z statistic	0.117
	P = 0.9071

SMOKING HABIT

Cases with positive (bad) outcome

Number in exposed group: a=

Number in control group: c=

Cases with negative (good) outcome

Number in exposed group: b=

Number in control group: d=

Results

Odds ratio	0.7214
95 % CI	0.0386 to 13.4809
z statistic	0.219
	P = 0.8269

TB CONTACT**Cases with positive (bad) outcome**Number in exposed group: a= Number in control group: c= **Cases with negative (good) outcome**Number in exposed group: b= Number in control group: d= **Results**

Odds ratio	2.8333
95 % CI	0.2986 to 26.8846
z statistic	0.907
	P = 0.3643

Employment**Cases with positive (bad) outcome**Number in exposed group: a= Number in control group: c= **Cases with negative (good) outcome**Number in exposed group: b= Number in control group: d= **Results**

Odds ratio	0.8135
95 % CI	0.1853 to 3.5711
z statistic	0.273
	P = 0.7845