

ISOLATION OF RECURRENT *MYCOBACTERIUM* ISOLATES FROM SPUTUM  
SMEAR NEGATIVE RELAPSE AT THE CENTRAL REFERENCE TUBERCULOSIS  
LABORATORY IN NAIROBI, KENYA //

JOSPHINE N. WAHOGO

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Wahogo, Josphine N.  
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recurrent*



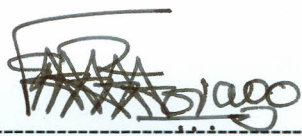
## DECLARATION

I declare that this thesis is my original work and has not been presented for a degree in any other university or for any other award.

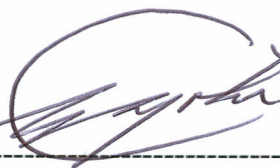
Signature -----  
 JOSPHINE N. WAHOGO  
 157/5760/2003

Date 5/5/09-----

SUPERVISORS: This thesis has been submitted after examination with our approval as University supervisors.

Signature -----  
 Prof. Alloys. S .S. ORAGO  
 National AIDS Control Council /Department of Pathology.

Date May 5, 2009.-----

Signature -----  
 Prof. Ephantus. W. KABIRU  
 Department of Pathology  
 Kenyatta University.

Date 05/05/09-----

## DEDICATION

I am grateful to my husband K .Mburu, son Eugene, and daughter Michelle for support and understanding when I was under taking this study .I am also very grateful to my Parents R.Wahogo and M.Wahogo, brothers, Karari, Jose and Rebo and sisters, Jane, Irene, Bena, Ndene and Wangari who were a great source of encouragement without their support and forbearance this work would not have been written. Your constant encouragement support, your prayers took me an extra mile. Lastly to my courageous and beautiful family whose light and love continue to shine through my life through this journey. Many blessings and I dedicate this work to all of you.

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**DEFINITION OF OPERATIONAL TERMS.**

- Definite case of TB:** A patient with a positive culture for *M. tuberculosis* complex  
Where culture is not routinely available, a patient with 2 sputum smear positive for AFB is also considered a “definite” case
- Case of TB:** A patient in whom TB has been bacteriologically confirmed
- Drug resistant:** The ability of strains of tubercle bacilli to survive and grow despite exposure to concentrations of drugs that inhibit or kill the parental cells, and transfer this characteristic to their progeny.
- Smear negative:** A patient with at least 3 sputum specimens being negative or there has been no response to course of antibiotics /anti cough agents or there is decision by the clinician to treat on anti –TB drugs.
- Default:** A patient who returns to treatment after having interrupted treatment for 2 months or more.
- Recurrence:** A patient having a positive culture less than 30 days after the last treatment.
- Relapse:** A patient who has been declared cured of any form of TB in the past by a clinician after one full course of chemotherapy ,and has become sputum smear Negative / positive and culture positive.
- Retreatment:** Previous TB treated patients for longer than a month, while on treatment remained smear positive; or became again smear positive at the 5<sup>th</sup> month or latter during the course of treatment.

## ACRONYMS AND ABBREVIATIONS

AID	Acquired Immune Deficiency Syndrome
AAFB	Acid Alcohol Fast Bacilli
AFB	Acid Fast Bacilli
DNA	Deoxyribonucleic Acid
DOTS	Direct Observed Treatment Strategy
HIV	Human Immunodeficiency Virus
HAART	Highly Active Antiretroviral Therapy
IULTD	International Union Against Tuberculosis and Lung Disease
LJ	Lowenstein Jensen
MTBC	<i>Mycobacterium tuberculosis</i> Complex
MTB	<i>Mycobacterium tuberculosis</i>
MDR-TB	Multi-Drug Resistant Tuberculosis
MGIT 960	<i>Mycobacterium</i> Growth Indicator Tube 960
MUT	Mutation
NLTP	National Leprosy Tuberculosis Programme
SPSS	Statistical Package for Social Sciences
TUB	Tuberculosis band
PTB	Pulmonary tuberculosis
EPTB	Extra Pulmonary tuberculosis
WT	Wild type
WHO	World Health Organization
QA	Quality Assurance
SRL	Supra reference laboratory
ZN	Ziehl Neelson

## ABSTRACT

Rates of smear-negative pulmonary tuberculosis have been rising in countries with HIV epidemics. The mortality rate among HIV-infected tuberculosis patients is higher than that of non infected tuberculosis patients, particularly for those with smear-negative pulmonary and extra pulmonary tuberculosis. Tuberculosis (TB) is an ancient infectious disease with high mortality rates. The World Health Organization (WHO) estimates that 2 billion people have latent TB, while another 3 million people worldwide die each year due to TB. The diagnosis of pulmonary tuberculosis (TB) relies on the bacteriological examination of sputum. However, microscopy of smears made directly from sputum has a low sensitivity and there is an urgent need for improved methods. This study intends to investigate the proportion of smear positive TB cases which were misdiagnosed as smear negative TB cases and also determine positivity rate amongst the smear negative relapses and the proportion of tubercle. The main objective of the study was to isolate *Mycobacterium tuberculosis* from patients with recurring infections due to smear negative relapse amongst smear negatives, which are actually due to *Mycobacterium tuberculosis* referred at the Central Reference Laboratory Nairobi, Kenya. The study was conducted at the TB Central reference laboratory a referral public facility Located in Nairobi, Kenya, next to Kenyatta National hospital which cultures all retreatment cases in the whole country. The study was a prospective, cross-sectional descriptive study, which was under taken at TB reference laboratory as routine laboratory based activity. TB register was used to identify samples which were sputum smear negative tuberculosis relapse. 4% Sodium hydroxide (NaOH) was used as a decontaminant agent and Lowenstein Jensen culture medium used as "gold standard" few as ten viable tubercle bacilli were detected, highly sensitive and specific test. P-nitro benzoic (PNB) acid was used as a bio-chemical test with Molecular method (DNA-HAIN), and all culture positive samples were genotyped to identify the *Mycobacterium* species. Data was collected using appropriate structured laboratory culture request forms taking into consideration parameters of interest e.g. Age, Sex. Of the 273 initially confirmed direct Ziehl Neelsen (ZN) smear negative sputum specimens 47 (17%) were culture positive. The ZN sensitivity, specificity, Positive and Negative predictive values were 60%, 92%, 60% and 92% respectively after centrifugation with 4% Sodium hydroxide ( $p=0.001$ ). There was a significant increase in sensitivity ( $\chi^2=67.93$ ), this means that a fifth of the samples screened using direct ZN method. However, using 4% NaOH (Modified Petroff's method) centrifugation method 47/273 (17%) specimens were smear positive for acid fast bacilli. DNA method identified the entire isolates, which are due to *Mycobacterium Tuberculosis* except one, which was due to environment *Mycobacterium* with 1 Multi drug resistant sputum specimen. This study intends to inform policy makers on diagnosis of sputum smear negative relapse cases and suggest the way forward on better diagnosis for active case finding to the Ministry of Health through the TB Program.

## CHAPTER ONE: INTRODUCTION

### 1.1 Background information

Tuberculosis (TB) is an infectious disease with high mortality rates; The World Health Organization (WHO) estimates that 8 Million people become ill, while 2 million people worldwide die each year from the disease. Tuberculosis was present in Egypt from early Pharaonic times, perhaps as early as 3700 BC (Morse *et al.*, 1964). It is postulated that tuberculosis was well recognized by the time of Hippocrates (377-460 BC) who gave an excellent clinical description of the disease (*ibid*). The history of sputum examination dates back to 1882 when Robert Koch discovered the tubercle bacillus and confirmed the bacterial etiology of tuberculosis. The causative agent of TB was first isolated in 1882 among the *Mycobacterium tuberculosis complex* (Cook, 2003).

The only definitive way to diagnose TB is by direct sputum microscopy for acid fast bacilli (AFB), or culture for *Mycobacterium tuberculosis*. (Perkins 2000). The sensitivity of microscopy diagnosis is about 50-70% of patients with active pulmonary TB cases when 2-3 smears are examined over 2 day and patient with negative sputum smear for AFB is still not captured and represent diagnostic dilemma. The gold standard for TB diagnosis is culture which is highly sensitive and specific. (WHO, 2006) Culture for MTB should be done to confirm the diagnosis of TB which is more sensitive and the only way to demonstrate viable TB bacilli in Lowenstein Jensen Medium. (Wolinsky 1994). Therefore smear microscopy remains the cornerstone of TB diagnosis in high burden countries like Kenya (IUATLD 2004). The bacilli in the sputum can be detected by Ziehl Neelson (ZN) which is commonly used throughout the world and still remains the standard method against which new tests must be measured (Jain *et al.*, 1996)

In Kenya despite the efforts to control TB over the past 14 years, still the prevalence of TB remains very high. Cases notified in Kenya has increased ten fold since 1990 with 11,5234 TB cases being notified to the Kenya's TB control programme in the year 2006 (NLTP Annual report 2006) with a projected annual increase of 16%. In Kenya, Tuberculosis (TB) remains a major cause of morbidity and mortality. It affects all age groups but has its greatest toll in the most productive age group of 15 to 45 years. The major factor responsible for the large TB disease burden in Kenya is the current HIV epidemic. Other factors that have contributed to this large TB disease burden include poverty and social deprivation that has led to mushrooming of peri-urban slums, congestion in prisons and limited access to general health care services.

## **1.2 Problem statement**

Direct smear examination with Ziehl- Neelsen (ZN) staining for diagnosis of TB used in low in-come countries is cheap and easy to use, but its low sensitivity is major drawback. The low specificity of chest X-rays, used for the diagnosis of sputum smears negative TB, risks high levels of mis-diagnosis at this era of HIV and AIDS. The control of the re-emerging smear negative TB relapse is further complicated by emergence of HIV and AIDS, and continued decline socio-economic conditions. Currently Tuberculosis is a priority on a list of the WHO communicable disease of the poor, Because of the lifestyle and poor health infrastructure, the malady is still high especially with the escalating rate of poverty in Kenya, moreover the health system is also poorly developed and the facilities are in adequate especially the health centers. Therefore the health centers care system is incapable of carrying out certain conventionally TB screening procedures.

These scenarios have led to sharp upsurge and emergence of multi drug resistant TB. The current sharp increase in the infectious is also due to misdiagnosis, treatment defaults and non compliance. Sputum smear microscopy remains the cornerstone of TB diagnosis in developing countries. The method depends upon the quality and bacterial load of the sputum specimen and the training and motivation of laboratory technicians. Although culture is highly specific and more sensitive than smear microscopy but its major limitation includes high cost, lack of availability in peripheral facilities and the duration is quite long thus limits access to health care services.

### **1.3 Justification of the study**

The high prevalence of TB has created an urgent mandate for rapid isolation, diagnosis and effective therapeutic measures to patient with smear negative sputum. The diagnosis can only be made reliable on demonstrating the presence of tubercle bacilli in the sputum by bacteriological culture which provides the definitive diagnosis. Until recently, smear negative TB cases were thought not to contribute significantly to transmission. However, there is evidence that infective threshold is dependent on other factors in addition to number of organism. Over reliance on smear negativity to determine infectiousness may contribute to ongoing transmission. This is the reason why it is necessary to use a more sensitive diagnostic test like culture which increases the number of TB cases detection by 20-25% over microscopy, thereby reducing sources of transmission. It also allows early appropriate interventions to be undertaken reducing sources of transmission. Furthermore, outside referral centers, little is available in most disease endemic countries beyond microscopy. It's therefore important to promote more sensitive microscopy tests e.g. use of concentration method in such setting. Depending on the decontamination

method and the type of culture medium used, as few as ten viable tubercle bacilli can be detected. 5000 -10000 tubercle bacilli per millimeter of sputum are required for direct microscopy to be positive. The gain by Culture over microscopy increases the number of tuberculosis cases, often by 20--25% (WHO 2004).

#### **1.4 Null Hypothesis**

There is no difference in sputum smear negative status of the specimens when subjected to Lowenstein Jensen (LJ) culture medium in diagnosis of *Mycobacterium tuberculosis*.

#### **1.5 Research Questions**

- a). What is the proportion of smear positive cases amongst the reported smear negative relapse referred at the Central Reference Laboratory diagnosed with TB?
- b). What is the positivity rate of *Mycobacterium tuberculosis* amongst all smear negative relapse referred at the Central Reference Laboratory diagnosed with TB?
- c). What is the proportion of *Mycobacterium tuberculosis* among the smear negative samples is actually due to *Mycobacterium tuberculosis*?

#### **1.6.0 Objectives of this study**

##### **1.6.1 General Objective**

To isolate recurrent *Mycobacterium* from sputum smear negative relapse at the Central Reference Tuberculosis Laboratory Nairobi, Kenya.

##### **1.6.2 Specific objectives**

- a) To determine smear negative relapse cases by Age and Sex of the study subject.

- b) To determine the proportion of smear negative sputum samples that are bacteriological culture positive amongst the referred cases.
- c) To compare the sensitivity and specificity of ZN with Culture method using concentrated sputum treated with 4%NaOH.
- d) To determine if recurrent or re-infection due to *Mycobacterium tuberculosis* or other *Mycobacterium* species.

## **1.7 Significance and Anticipated output of the study.**

### **1.7.1 Significance of the study**

Improved diagnosis provided to patients, and to improve the screening services on patient using more specific /sensitive methods to detect TB from sputum smear negative during this era of HIV and AIDS.

### **1.7.2 Anticipated output**

The study will assist in enhancing better quality diagnosis services for the purpose of improving treatment and control measures, surveillance on the recurrent TB by differentiating actual TB cases and opportunistic infections.

## CHAPTER TWO: LITERATURE REVIEW

### 2.1 A historical perspective of Tuberculosis:

Tuberculosis, like all infectious diseases, involves exposure to pathogen resulting in an asymptomatic period of incubation or latency that may progress to active disease. Unlike most other infectious diseases, tuberculosis involves a delay between infection and disease that is extremely variable, ranging from few weeks to lifetime. (Verver, 2005). Tubercle bacillus, the causative agent of TB and a member of *Mycobacterium* complex was first isolated by Robert Koch in 1882 (Cook 2003). Other members of the *Mycobacteria tuberculosis* complex (MTBC) include *Mycobacterium africanum*, *Mycobacterium canetti* and *Mycobacterium microti*. *Mycobacterium tuberculosis* is the most important cause of morbidity and mortality in humans in the complex group. Early diagnosis of smear negative pulmonary TB (PTB) and extra-pulmonary TB (EPTB) followed by prompt initiation of treatment are likely to have a favorable impact on over all TB mortality. In resource-limited settings, however, methods of TB diagnosis based on sputum microscopy or chest radiography are unreliable, (Harris *et al.*, 1998).

Tuberculosis is a contagious respiratory infection that follows poverty and urban crowding, infecting 30 million each year and killing 5,000 people every day. Worldwide, TB is the leading killer of people with HIV, and the course of both HIV and TB is much more rapid and deadly in persons with both infections. In Africa, half of all TB cases are associated with HIV, due to robust prevention and treatment efforts, 10 to 20 percent of TB cases are associated with HIV (CDC, 2004)

## 2.2 The global tuberculosis disease burden

According to the World Health Organization (WHO), nearly 2 billion people one third of the world's population have been exposed to the tuberculosis pathogen (WHO, 2006). Annually, 8 million people become ill with tuberculosis, and 2 million people die from the disease worldwide (CDC, 2006). In 2004, around 14.6 million people had active TB disease with 9 million new cases. The annual incidence rate varies from 356 per 100,000 in Africa to 41 per 100,000 in the America (WHO, 2006). The World Health Organization (WHO) has identified 22 high-burden TB countries that combined and contributes 80 percent of the global burden of TB. These include (in order from highest burden of TB): India, China, Indonesia, Nigeria, South Africa, Bangladesh, Pakistan, Ethiopia, Philippines, Kenya, DR Congo, Russian Federation, Vietnam, Tanzania, Uganda, Brazil, Afghanistan, Thailand, Mozambique, Zimbabwe, Myanmar, and Cambodia. India has the largest number of TB cases in the world, accounting for nearly one-third of the global burden (WHO, 2006).

In real life situation the malady has three distinct but overlapping dimensions: Economic, humanitarian, and public health. The belief in public health is that with proper diagnosis and treatment of tuberculosis will lead to a decrease in infection and transmission in the communities. From a public health perspective, poorly supervised or incomplete treatment of TB is worse than no treatment at all. When victim of the disease fail to complete standard treatment regimens they may remain infectious (WHO, 2004).

Infectious diseases remain the largest cause of death in the world, and among infectious diseases tuberculosis is responsible for the greatest number of deaths. Each year, 54

million people are infected with the tubercle bacillus (*Mycobacterium tuberculosis*), 6.8 million develop clinical disease and 2.4 million people die of tuberculosis. Tuberculosis is responsible for 5% of all deaths worldwide, and 9.6% of adult deaths in the 15-59 age groups. Tuberculosis kills more women worldwide than all causes of maternal mortality. The case fatality rate of tuberculosis is high; approximately 50% of untreated cases die of the disease. (MMWR 1998; Murray 1996; Blower 1996)

Tuberculosis is transmitted by the respiratory route, and the principal risk factor for acquiring infection is breathing. Most infected individuals develop a latent or persistent infection that can reactivate at any time during the individual's lifetime; on average, 10% of infected individuals will develop active disease over their lifetime. It is estimated that there are currently 2.1 billion people worldwide who are infected with the tubercle bacillus and whose infection could reactivate. Thus, the major current strategy of treating only patients with active disease limits our ability to control tuberculosis and will require decades to reduce the incidence of disease significantly (MMWR, 1998; Murray, 1996; Blower, 1996)

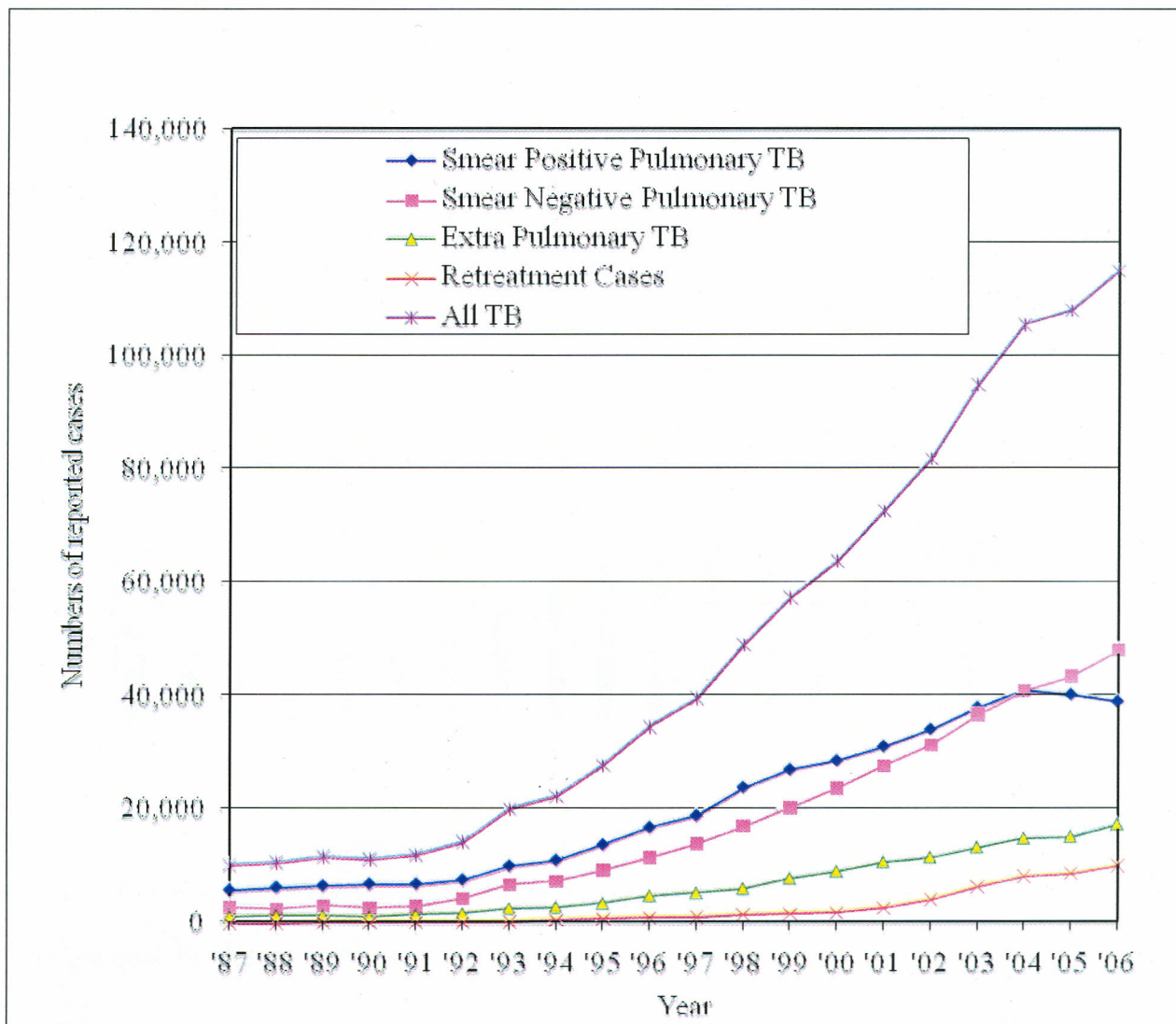
### **2.3 African perspective on TB**

The incidence of TB varies with age. In Africa, TB primarily affects adolescents and young adults (WHO, 2006). There are a number of known factors that make people more susceptible to TB infection: worldwide the most important of these is HIV. Co-infection with HIV is a particular problem in Sub-Saharan Africa, due to the high incidence of HIV in these countries (WHO, 2006). Africa has the highest rates of TB and the worst treatment outcomes globally, driven by weak health systems and fueled by the TB-HIV

co-epidemic. At the same time, Africa faces the largest funding gap of any region in the world. TB has been declared an emergency in Africa after 46 Ministers of Health unanimously adopted a resolution at the WHO Africa Regional Committee in Maputo, Mozambique, on 25 August 2005. Recognizing the deep concern about the gravity of the epidemic, the resolution warned that unless "urgent extraordinary actions" are in place, the situation will worsen and the 2015 Millennium Development Goal TB targets will not be met. There are 1500 TB deaths every day in Africa (WHO, 2006).

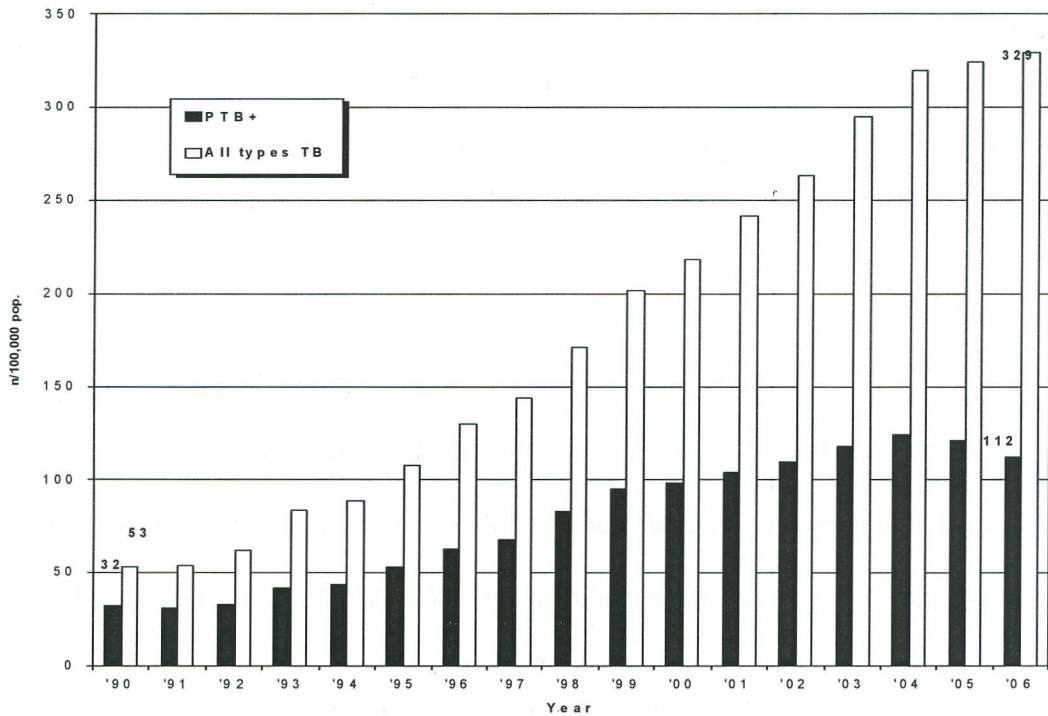
## **2.4 The Kenyan Perspective on TB**

Kenya was ranked 10<sup>th</sup> among 22 high burdened countries in the Tuberculosis world (WHO, 2005). There were 115,234 new cases of TB diagnosed during that year, among whom 42686 were sputum smear negative, 39154 smear positive TB having extra pulmonary TB (EPTB). Detection rate of smear negative TB was only 37% and 34% for smear positive. The overall case detection is below 50% falling short of the expected 85%. In the year 2006, the National Leprosy/ Tuberculosis Program reported 115,234 cases of Tuberculosis and the retreatment cases reported were 10299 and only 23% of the retreatment cases were sent for smear microscopy and culture at the central reference laboratory (NTLP, 2006). To date Tuberculosis remains a major Health problem in the world; resurgence of *Mycobacterium tuberculosis* infections has been seen in the last two decades. Tuberculosis case findings has increased more than tenfold from a figure of 10,000 cases in 1990 to 115,234 TB cases in 2006 as illustrated in figure 2.1 while the incidence of TB through case notification has increased from 50/100,000 population per year in 1990 to 329/100,000 population per year in 2006 as shown in figure 2.2.



**Figure 2.1: All forms of TB cases Notified in Kenya**

(Source: Republic of Kenya, Ministry of Health 2006 NLTP Annual Report.)



**Figure 2.2: Notification Rates of TB in Kenya**

(Source: Republic of Kenya, Ministry of Health 2006 NLTP Annual Report.)

This increase has been attributed to the sputum smear negative. The spread is enhanced by poverty and deprivation (JAMA; 1995). Recently interaction of TB with HIV and AIDS have contributed to re-infection, relapse or reactivation. In developing countries the disease is a major cause of morbidity and mortality even in Kenya, too affects all age groups, although the majority of cases are reported in the cohorts of 15-49 years with male preponderance in the last decade (WHO, 1999). Kenya has observed a five-fold increase in reported cases 14% annual increase (Republic of Kenya, 2005).

## 2.5 Aetiology, Morphology and Clinical Manifestation of Human Tuberculosis

### 2.5.1 Aetiology

Tuberculosis is caused by a group of five closely related species, which form the *Mycobacterium tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*. *M. tuberculosis* (Koch's bacillus) is responsible for the vast majority of TB. The main defining characteristic of the genus *Mycobacterium* is the property called 'acid-fastness', which is the ability to withstand decolorization with an acid-alcohol mixture after staining with carbol fuchsin or auramine-rhodamine. Once inside the human host cell, *Mycobacterium tuberculosis* inflicts a contagious-infectious disease called tuberculosis (TB), although the disease could either be latent or active depending on the ability of the person's immune system to defend against the pathogen. (Ducati *et al.*, 2006).

### 2.5.2 Morphology

*Mycobacterium* is primarily intracellular pathogens, has slow growth rates, is obligate aerobes, and produce in normal hosts a granulomatous reaction. The bacillus is a non motile, non- sporing, non-capsulated and measures 2-5um by 0.3-0.5 um in diameter. The cell wall has thick lipids; hydrophobic wall renders resistance to dryness and structural integrity of lipid rich cell wall containing mycolic renders acid fast staining. In cultures, *M. tuberculosis* does not produce significant amounts of pigment, has a buff-colored, smooth surface appearance, and biochemically produces niacin. These characteristics are useful in differentiating *M. tuberculosis* from non-tuberculous *Mycobacterium*. One characteristic but not distinctive morphologic property of *M. tuberculosis* is the tendency

to form *cords* or dense clusters of bacilli aligned in parallel. The biochemical background of *cording* is called '*cord factor*' (a trehalose dimycolate), and its contribution to bacterial virulence is still unclear (Blumberg *et al.*, 2000).

### **2.5.3 Clinical Manifestations and Pathology of TB**

TB is a disease with protean /diverse manifestation which can mimic other disease .The clinical presentation can be typical with constitutional symptoms of fatigue, weight loss, anorexia, low grade fever and night sweats, or atypical depending on the immune system of the patient. Pulmonary symptoms include cough, which initially dry but latter productive of purulent cough. There are various manifestations of the disease in humans infected with TB bacilli which incur a 10% risk of developing active TB. Although most people may carry TB bacilli passively without becoming sick, any weakening of the immune system or malnutrition enhances the chance that the TB bacilli will become active.

#### **2.5.3.1 Clinical symptoms of TB**

Majority of patients experience pulmonary TB characterized by fever, night sweats, weight loss, difficulty breathing, and cough., chest pains ,absence of expectoration of sputum. Recurrence of active TB usually occurs after treatment due to relapse of infection with the same strain or re-infection with a new strain of *Mycobacterium tuberculosis*.

Several studies conducted in sub-Saharan Africa have pointed to an increased risk of recurrent TB in patients (Harries *et al.*, 2003). Routine case notification data from the

Malawi Tuberculosis Programme, which has improved its registration practices in the last two years, shows that recurrent TB (smear- negative TB) constitutes 9% of total notifications. Smear microscopy of sputum remains the cornerstone of diagnosis of TB in most high burden countries. The sensitivity of smear microscopy of sputum to identify all cases of PTB is only 70% is dependent on the type and quality of the specimen. The patients who are smear negative sputum are not detected. Clinical decision have been hindered by the TB-HIV and AIDS co-infection broadening the diagnosis of smear negative or infected with *Mycobacterium tuberculosis* (WHO, 2002). In countries with high TB incidence, recurrent TB accounts for a significant proportion of all cases, and HIV infection is a strong risk factor for recurrent TB as are post-TB scarring, cavities, drug regimen used to treat the initial episode of TB. Africa bears the additional burden of high levels of poverty and some of the lowest health indicators in the world (Mwinga *et al*, 2004).

### **2.5.3.2 Pathogenesis of TB**

Miliary TB occurs when tubercle bacilli enter the bloodstream and are carried to all parts of the body, where they grow and cause disease in multiple sites. Inflammations will be present at the primary site of infection (Ghon focus) and in the hilar lymph nodes; referred to as primary complex. Transmission of *Mycobacterium tuberculosis* to some groups of people is at a higher risk for TB because they are more likely to be exposed. Other groups are at higher risk of developing tuberculosis once infected; for example, people with certain medical conditions, especially HIV infection. For people infected with HIV, the risk of developing TB is about 7% - 10% each year (in contrast, people

with latent TB, the risk of developing TB is 10% over a lifetime. These bacteria primarily infect the lungs and are transmitted from person-to-person by inhalation of droplet nuclei containing the bacteria. Patients with pulmonary or laryngeal TB generate nuclei when they talk, cough, or sneeze.

## **2.6. Types of TB**

### **2.6.1. Pulmonary tuberculosis (PTB)**

Pulmonary tuberculosis is a contagious bacterial infection caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). The lungs are primarily clinically manifested, but the infection can spread to other organs. Pulmonary tuberculosis is caused by *Mycobacterium tuberculosis*. Transmission by breathing in air droplets, droplet infection from a cough or sneeze of an infected person. However, in some cases, the disease may become active within weeks after the primary infection, or it may lie dormant for years and later reappear. In addition, smear-negative TB is infectious and early diagnosis and treatment reduces the risk of transmission (Behr *et al.*, 1999).

### **2.6.2. Extra pulmonary tuberculosis**

Extra pulmonary tuberculosis (EPTB) is an infection caused by tuberculosis bacteria that have spread beyond the lungs. Extra pulmonary sites of infection commonly include lymph nodes, pleura, and osteoarticular areas; although any organ can be involved extra pulmonary involvement can occur in isolation or along with a pulmonary focus as in the case of patients with disseminated tuberculosis (TB). The recent human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) pandemic has resulted in changing epidemiology and has once again brought extra

pulmonary tuberculosis (EPTB) into focus. The risk of EPTB *tuberculosis* is Mycobacteremia increases with advancing immunosuppressant (Behr *et al.*, 1999).

## 2.7 Transmission of TB

Until recently, smear-negative TB cases were thought not to contribute significantly to secondary transmission. Intuitively this dogma is suspect, as it remains plausible that sputum isolates with 5000–10 000 bacilli/ ml, the minimum number required for smear positivity are capable of transmission as the infective threshold is likely to depend on other factors in addition to the number of organisms. The frequency of coughing, viscosity of the sputum, organism virulence and host factors are likely to play a role. This issue is important as it has pragmatic public health implications. Over-reliance on smear negativity to determine infectiousness may contribute to ongoing transmission (Hobby *et al.*, 1973)

Transmission of tuberculosis is virtually entirely by droplet nuclei created through coughing by untreated persons suffering from tuberculosis, in a confined environment. Infected droplets remain airborne for a considerable time, may be inhaled by a susceptible person. Pulmonary tuberculosis usually occurs in the apex of the lungs. This develops cavities which contain large populations of tubercle bacilli that can be detected in sputum specimen by direct sputum smear microscopy for acid fast bacilli (AFB), or early morning sputum culture for viable *Mycobacterium tuberculosis*.

In natural circumstance MTB, the causative agent of TB is transmitted by expulsion of nasal droplets which contain tubercle bacilli from infected human individual to uninfected one (Clack *et al.*, 2003). When infectious TB patients, coughs or sneezes, droplet

nuclei containing tubercle bacilli are expelled in to the air. Other people may inhale the air containing these droplet nuclei and become infected (American thoracic society, 2000). The disease usually occurs in the lungs (pulmonary TB), but it can also occur in other place in the body (extra pulmonary TB).

## **2.8 Control of Tuberculosis.**

The greatest concern of Tuberculosis control is the appearance of Multi-drug resistant (MDR) which encodes resistance not only to Isoniazid (INH) and Rifampicin (RIF) but also to at least the other three of the six main classes of both first /second line drugs (CDC , 2004). The cornerstones for effective control of drug resistance tuberculosis are immediate isolation of patients and rapid detection of drug resistant strains followed by prompt implementation of an adequate anti-tuberculosis therapy that is based on laboratory findings. Laboratory surveillance of drug resistance and early identification of resistant strains are critical steps for the beginning of appropriate treatment. Any delay in resistant strain identification jeopardizes the efforts to control the transmission of the disease.

The goal for tuberculosis treatment/control is to ensure relapse free cure, prevent relapse due to reactivation of metabolically inert organisms, while preventing the emergence of drug resistance. The effect of treatment should therefore be judged not by the anatomical healing of lesions but by their sterilization, or at least by the elimination of bacilli from sputum. *Mycobacterium tuberculosis* is a slow growing aerobic organism that can remain dormant for a prolonged period .Consequently prolonged treatment with multiple drugs is required to ensure relapse –free cure and to prevent the emergence of resistance.

The effect of treatment is determined mainly by bacteriological ; the number of tubercle bacilli varies widely with the type of lesion and the larger the bacterial population the higher the probability that resistant mutants strain are present before treatment is started, environmental ; the type o tissue harboring tubercle bacilli may attack drug action or the PH and partial oxygen pressure are important biochemical factors that influence the antimicrobial effect of the drug and Pharmacological factors ; drugs must be given in doses large enough to produce inhibitory concentration at the sites where bacilli are found ,regimens should contain a combination of three or more drugs particularly at the initial phase (Canetti, 1955). Recurrence of active tuberculosis after treatment can be due to relapse of infection with the same strain or re-infection with a new strain of *Mycobacterium tuberculosis*. The proportion of recurrent tuberculosis cases caused by re-infection has varied widely in previous studies (Jasmer *et al.*, 2004). The disease can remain dormant for long periods (Frieden *et al.*, 2003). Four possible options for reducing recurrent TB are, using Rifampicin and Isoniazid (RH) in the continuation phase eliminating most residual bacilli and reduce numbers of failures and relapses, extending the duration of the continuation phase, providing post-treatment Isoniazid prophylaxis. The World Health Organization introduced the direct observation and treatment strategy (DOTS), (Blomberg *et al*, 2001) to help in the management of tuberculosis.

The global resurgence of TB has re-emergence, rekindled interest in improving the efficiency with which laboratories detect *Mycobacterium tuberculosis* in clinical specimens, especially in resource poor settings (MOH.2000; Tenover *et al.*, 1993). Much of the interest is directed to optimizing the entire diagnostic process, which includes an

evaluation of the clinical picture of suspects patient, sputum smear examination and if necessary, examination of CXR if sputum results are negative.

## **2.10 Health care seeking behaviour**

Health care seeking behavior for TB includes the recognition of TB related symptoms, presentation to health facilities and/or alternative medical resources (e.g. family, community healers) and adherence in effective treatment regimens, treatment monitoring. Individual factors, such as knowledge, attitudes, gender, sex, ethnicity income and education, in addition to health service barriers including accessibility and often delay prevent a person from seeking TB care and treatment. Stigma associated with TB appears to be universal. The consequence of stigma can be seen affecting care seeking behaviors, as persons have been known to hesitate or choose not to disclose their TB status to family, friends and co-workers out of fear of being socially ostracized. Personal rejection occurs as a result of the stigma surrounding TB. This belief that TB is extremely contagious leads to isolation of the sufferers of TB. The fear and stigma associated with TB have a greater impact on women than men, often leaving social and economic position. (Connolly *et al.*, 1996)

## **2.11 Treatment and Drug resistance**

Treatment for TB uses antibiotics to kill the bacteria. The two antibiotics most commonly used are Rifampicin and Isoniazid. However, instead of the short course chemotherapy typically used to cure other bacterial infections, TB requires much longer periods of treatment (6-12 months) to entirely eliminate *Mycobacterium* from the body. (CDC, 2003) Latent TB treatment usually uses a single antibiotics, while active TB disease is

best treated with combination of several antibiotics, to reduce the risks of the bacteria to develop resistance (O'Brien, 1994)

Drug resistant tuberculosis is transmitted in the same way as regular TB. Primary resistance occurs in persons who are infected with a resistant strain of TB. A patient with fully susceptible TB develops secondary resistance (acquired resistance) during TB therapy because of inadequate treatment, not taking the prescribed regimen appropriately or due to using low quality medication (O'Brien, 1994). Drug resistant is a public health issue in many developing countries, as treatment is longer and requires more expensive drugs. (*MMWR*, 2004)

## **2.12 Diagnosis of Tuberculosis**

People with these latent infections are treated to prevent them from progressing to active TB disease later in life. However, treatment using Rifampicin and Pyrazinamide is not risk-free. The CDC, 2003 notified the health care professionals of revised recommendations against the use of Rifampicin plus Pyrazinamide for treatment of latent TB infection, due to high rates of hospitalization and death from liver injury associated with the combined use of these drugs. (*MMWR*, 2003)

## **2.13 Conventional Diagnostic Techniques**

### **2.13.1 Sputum Microscopy**

Direct Microscopic examination of sputum for AFB remains the cornerstone for the diagnosis of Pulmonary TB in both industrialized and low income countries (Rieder *et al.*, 1997). The technique of staining AFB now attributed to Ziehl Neelson (ZN) has evolved with combinations from many workers (Bishop *et al.*, 1970) and has remained a

mainstay of TB diagnosis for nearly 100 years. Although less sensitive than culture, the ZN is considered to be most reliable, rapid, cheap and highly specific method for the diagnosis of Pulmonary TB as a microbiological tool in resource poor setting (Aber 1980; Braun 1992).

### **2.13.2 Microscopy**

In order to detect *Mycobacterium tuberculosis* in a sputum sample, an excess of 10,000 organisms per ml of sputum are needed to visualize the bacilli with a 100X microscope objective (1000X). One acid-fast bacillus/slide is regarded as "suspicious" of an MTB infection. More than 90% of TB patients live in low and middle –income countries, where the diagnosis of TB relies on primarily on identification of acid –fast bacilli on sputum smears using conventional light microscope. In these countries, most laboratories use smears of unconcentrated sputum (direct smears) with Ziehl-Neelsen (ZN) staining. (Dye *et.al* 2005). Microscopy is relatively simple, inexpensive, widely applicable and highly specific for *Mycobacterium Tuberculosis* in TB endemic countries. In addition it identifies the most infectious patients (Lulemo 2004; Van 2004)

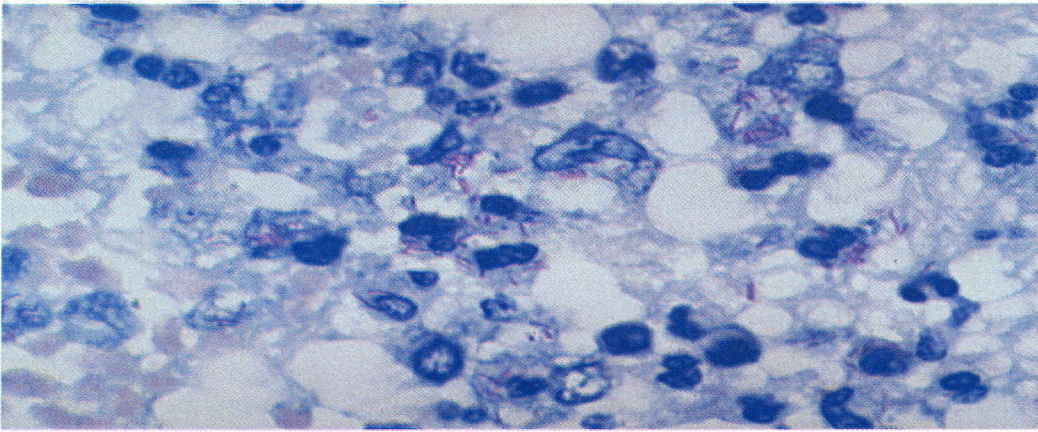
The diagnosis of pulmonary Tuberculosis, the most common type of TB, has over the years relied on sputum smear microscopy especially high burdened countries like Kenya. Sputum smear microscopy by use of Ziehl Neelsen stain has a variable sensitivity and may miss TB in up to 40-80% of patients suspected to have Tuberculosis (Tomon, 2004). The only definitive way of diagnosis of pulmonary TB is by direct sputum smear microscopy for AFB (acid fast bacilli) or culture for *Mycobacterium tuberculosis*. Early morning sputa should be collected prior to initiating treatment. The yield is optimal in the morning, as bacilli accumulate overnight in the lungs. Direct microscopy of the sputum,

using special stains for acid fast bacilli is the cornerstone of diagnosis. Microscopy carried out using ZN detects approximately  $10^4$  bacilli per ml sputum to achieve positive results, with a sensitivity of 60-70% with PTB. The culture allows confirmation of cases and also facilitates species identification.

It is acid alcohol fast bacilli, resistant to harsh physiological chemical environment, slow growth: generation time, 18-24 hours, Ivory colored dry surfaced colonies appear after 4 weeks incubation bacilli aggregates/forms "serpentine cord". The gold standard method for TB diagnosis is sputum culture on Lowenstein Jensen medium. Culture is both highly sensitive and highly specific but requires special equipments and takes 4-8 weeks of incubation before scored negative culture.

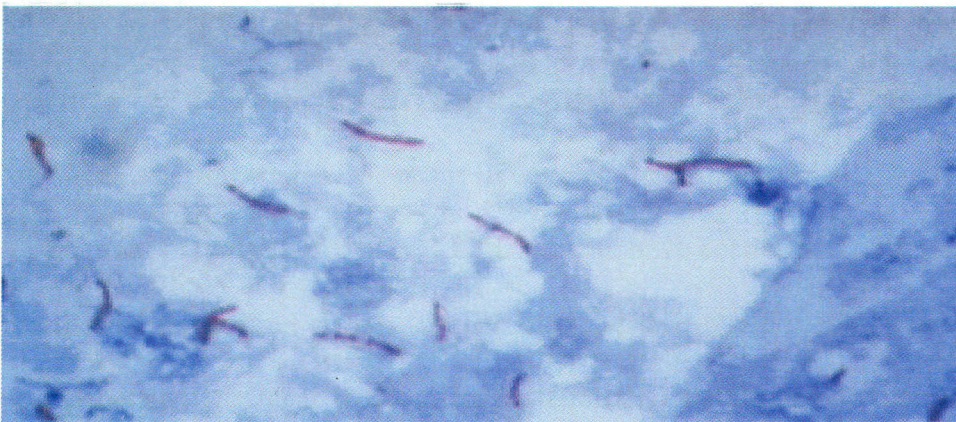
#### **2.13.2.1 Ziehl Neelson Stain**

The cell wall of acid fast bacilli contains fatty acids known as mycolic acids, which makes them resistant to the action of many chemicals. Because of this, the bacilli cannot be stained easily like in Gram stain. Strong dye concentration, application of heat, addition of phenol and longer staining time are required to stain the bacilli. Once stained it is difficult to de-stain them. This property is used to differentiate the AFB from all other materials like bacteria, cells and mucus will get decolorized by the action of strong acid or acid-alcohol, leaving the acid fast bacilli stained with primary stain, which is basic fuchsin in case of ZN staining or the fluorescent dye like auramine in case of fluorescent staining method. (Wolinsky, 1994)



**Plate 2.3: *Mycobacterium tuberculosis* ZN stain before treatment with 4% NaOH.**

Good stained smear by ZN method shows strong red AFB, against weak blue background. Contact time of carbol fuchsin is very important during staining. A minimum five minutes are required for good quality stains, but 10 minutes are preferred to get a strong AFB staining. The color and intensity of background is important so that it does not mask the AFB.



**Plate 2.4: *Mycobacterium tuberculosis* ZN stain after treatment with 4% NaOH.**

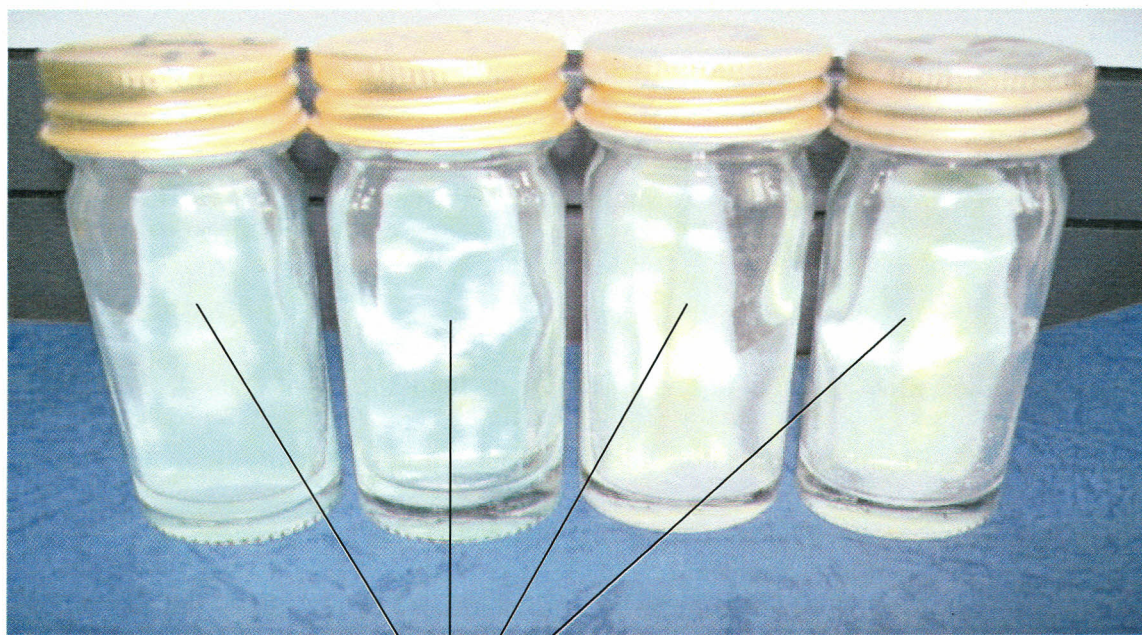
### 2.13.2.2 Florescent Microscopy

The technique of examining AFB by Fluorescence microscopy (FM) on the basis of auramine staining became available in the mid 1940s. Its sensitivity has been shown to be higher than that of ZN, (Githui *et al.*, 1993; Kubica 1980) with similar high specificity .It is widely used in industrialized countries but its use in developing countries is limited due to high investment and maintenance costs. Primarily due to perceived economic reasons, FM is mainly used in those settings where more than 50 specimens are examined per day. (Truant *et al.*, 1962; Tomon 1979) Fluorescence microscopy has been shown in numerous studies to be at least 10% more sensitive than traditional light microscopy (Ebersole, 1992; Steingart *et al.*, 2006). Thus, fluorescent stains are of paramount importance, not only in confirming the presence of *Mycobacterium* in a given specimen, but also in providing an estimated quantification of organisms.

### 2.14 Culture

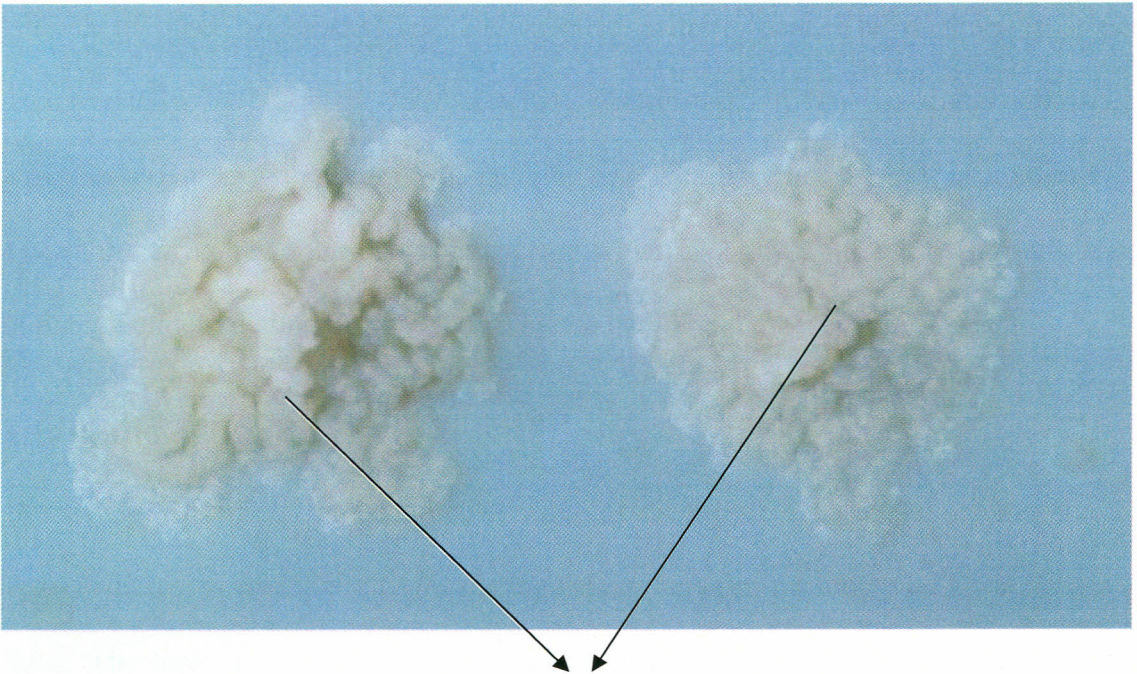
Culture of MTB using the conventional Lowenstein-Jensen (L-J) media is the “gold standard” in the diagnosis of TB (Aber ,1980).The definitive diagnosis of tuberculosis demands that *M. tuberculosis* is recovered on culture media and identified using differential *in vitro* tests. Many different media have been devised for cultivating tubercle bacilli and three main groups can be identified, egg-based media, agar-based media and liquid media. The ideal medium for isolation of tubercle bacilli should; Be economical and simple to prepare from readily available ingredients, Inhibit the growth of contaminants and Support luxuriant growth of small numbers of bacilli and Permit preliminary differentiation of isolates on the basis of colony morphology. The culture of sputum specimens, egg-based media should be the first choice. There is increasing

evidence that liquid media may give better results with other specimens. It is recommended that all sputum specimens submitted for culture also undergo microscopic examination. Lowenstein-Jensen (LJ) medium is most widely used for tuberculosis culture. LJ medium containing glycerol favors the growth of *M. tuberculosis* while LJ medium without glycerol but containing pyruvate encourages the growth of *M. bovis*. Both should be used in countries or regions where patients may be infected with either organism. **(Plate 2.5:** These 4 slants (tubes) shows growth on the Lowenstein Jensen medium (LJ) from the initial smear negative samples.



**Colonies on LJ Medium**

**Plate 2.5:** *Mycobacterium* isolates grown on LJ medium in Universal culture bottles.



*MTB colonies are small and buffy colored when grown on egg medium. Both types of media contain inhibitors to keep contaminants from out-growing MTB.*

**Plate 2.6: Colonies of *Mycobacterium tuberculosis* on LJ medium observed in the TB lab from the culture slants**

**2.15 Biochemical Tests**

The most studied methods involve the treatment of sputum with sodium hydroxide (NaOH) or Sodium Hypochlorite (NaOCL) and the centrifugation or the Sedimentation Method's recent meta-analysis concluded that these methods are average more sensitive than the direct smear (where sputum is directly examined without processing ) and retain the same degree of specificity.(Steingart *et al.*, 2006).

**2.16 Drug Sensitivity Test**

Recent development in the diagnosis of tuberculosis includes an automated system for detecting early growth of mycobacterium by a radiometric method (BACTEC 960: Becton Dickinson). Sputum or other homogenates are decontaminated as necessary and added to vials containing Middle brook.7H12 medium, an antibiotic mixture (to avoid the

growth of other organisms) and 14C-labelled palmitic acid. The medium is prepared commercially (BACTEC 960: Becton Dickinson) in rubber-sealed bottles. If *mycobacterium* growth occurs, 14C palmitic acid is utilized and 14 CO<sub>2</sub> is produced.

Growth of mycobacterium may be detected within 5-7 days, but positive results require further testing to distinguish between tubercle bacilli and other mycobacterium.

### **2.17 Sensitivity, Specificity**

Sensitivity, Specificity and Predictive value of diagnostic tests to the actual value of a diagnostic test is its reliability for distinguishing between persons who have disease and those who have not, depends on mainly two characteristics: The first is the Sensitivity which is the capacity to correctly identify diseased individuals in a population, or “true positives”. The greater the sensitivity, the smaller the number of unidentified case (false negatives) (DeReimer et al., 2000).

The second value is the Specificity, which is the capacity to correctly exclude individuals who are free of the disease, or” true negatives”. The greater the specificity, fewer false positives will be included. Sensitivity and specificity are attributes proper to each diagnostic method. However, when these methods are used in the field, the certainty of results is affected by the frequency of the phenomenon being measured or prevalence.

Other diagnostic values include:

#### **2.17.1 Positive predictive value (PPV)**

This is the probability of the disease being present, among those with positive diagnostic test results. The PPV of smear microscopy will decrease as prevalence drops.

### 2.17.2 Negative predictive value (NPV)

This is the probability that the disease was absent, among those whose diagnostic test results were negative.

### 2.18 Limitations of diagnosis by smear and Culture

Although microscopy is of intermediate complexity and gives results within a few hours, it fails to detect low numbers of *mycobacterium* present in a specimen and it's not specific to MTB complex (Githui, 2007). On the other hand, culturing which allows confirmation of cases and facilitates species identification requires skilled personnel and additional equipment. It also takes 2 to 8 weeks before results are available (Githui, 2007). However, shortcomings of AFB microscopy seriously limit both the extent and quality of its application and ultimately, its impact on TB control.

First, it requires equipment that is difficult to maintain in field setting, yields results that depend upon the studious attention of a trained and motivated technician, and is notoriously insensitive, especially in program conditions (Gebre *et al.*, 1995; Wilkinson 1997). This problem is made more critical by rising incidence of smear negative disease in countries where HIV infection is prevalent such as in sub Sahara Africa. Reflecting this lack of sensitivity along with deficiency in case reporting currently less than 20% of roughly 8million predicted annual cases of tuberculosis are identified as smear positive (WHO , 2000).

Control of tuberculosis is ultimately affected by halting disease transmission. It is known that by the time smear positive cases are detected they have already infected many of their close contacts. Lastly, the dependence of Direct Observation Therapy (DOTS) TB

control strategy on case detection tool with the equipment, training and quality control requirements of smear microscopy may ultimately limit its expansion. As for laboratory services beyond microscopy, little is available in most disease endemic countries outside referral centers



The subjects were either sero-positive or sero-negative for HIV and AIDS study population who were referred to the Central TB reference laboratory. The study area covers the whole 12 TB control zones in Kenya

### **3.2 The study design**

This study was a prospective, cross-sectional descriptive study which was undertaken at TB reference laboratory as routine activity (laboratory based,) TB register was used to identify patients who were sputum smear negative tuberculosis relapse.

#### **3.2.1 The study samples**

The selection of the specimens included in the diagnostic process strictly followed routine diagnostic procedures (direct smear microscopy and culture) at their facility. The study included specimens from all over Kenya. Relapse smear negative pulmonary tuberculosis cases at the laboratory registry were eligible for recruitment of the study.

#### **3.2.2 Inclusion criteria**

Sputum samples referred from other facilities, defined as sputum smear negative relapse. The intake period was a period of three months where all the sputum smear negative specimens referred to the central reference laboratory were included in the study.

#### **3.2.3 Exclusion**

Sputum samples which are referred at the Central reference Laboratory with other relapses were not included in the study.

### 3.3. Sampling technique and Sample, Size determination

#### 3.3.1 Sampling technique

This included negative relapse sputum smear specimens which were referred at the reference laboratory from their initial facility which were selected from the laboratory register.

#### 3.3.2 Sample size determination

The sample size calculation was done as used by Fisher *et al.* (1998) formula to determine the minimum size for negative sputum samples. Thus, TB proportion of all retreatment cases sent to the central reference Laboratory for culture is 23% in Kenya. (Annual NLTP report 2006. Republic of Kenya).

$$N = \frac{Z^2 * P * Q * D}{d^2}$$

Where:

N=the desired sample size

Z=the standard normal deviation (1.96) or 96% confidence interval

P=the proportion of the target population with the desired characteristics being measured (patients previously treated with anti-TB drugs)

Q=1-p

d=degree of accuracy

D=design effect =1

$$N = \frac{1.96^2 * 0.23 * 0.77 * 1}{0.05^2} = 272$$

Thus a minimum sample size of 272 specimens was required for the study.

### **3.4 Data collection methods**

#### **3.4.1 Data collection forms**

Data was collected using laboratory request forms taking in to consideration parameters of interest i.e. Name, age, gender previous treatment history ,relapse status ; smear, negative sputum. After which data was entered into a database using epi info (App. 9)

#### **3.4.2 Validation of Smear Negative and Sputum culture**

Validity was the extent to which a measurement or the study result correctly represented a characteristic or relationship of interest. In low setting resource country, clinical diagnosis was based mainly on patient sputum smear microscopy and sputum culture findings which can raise pre-test for TB to 50% or more. This was achieved through inoculating in three slopes while performing culture on the samples, identification and speciation of the isolates for *Mycobacterium* species from the initial smear negative samples.

#### **3.4.3 Laboratory procedures**

##### **3.4.3.1 Sputum culture**

Sputum was collected in sterilized containers and brought in to the laboratory. Sputum Samples were initially digested and decontaminated by the use of the modified Petroffs Sodium hydroxide(NaOH) method.(Somoskovi *et al.*,2003). After decontamination by use of 40% Sodium hydroxide the concertrated sediment was (neutralized) suspended in sterile phosphate buffered saline ( PH 6.8 ) or distilled water, centrifuged at 3000xg. Smears were prepared with 0.1ml of sediment using Ziehl Neelsen acid fast staining

method (Kent *et al.*, 1985). Microscopy scoring was done according to the WHO standard, with the scale defined as follows: negative no acid fast bacilli (AFB) observed; scanty 1-9 AFB in 100 fields; +, 10 to 99 AFB in 100 fields; ++, 1 to 10 AFB per field in at least 10 fields; + + +, >10 AFB per field in at least 10 fields.

#### **3.4.3.2 P-nitro benzoic acid inhibition test (PNB)**

Identification of these isolates was done on a few colonies, emulsified in a one flask containing glass beads and 2ml of sterile distilled water to obtain turbidity greater than McFarland 1 standard. PNB was incorporated into LJ medium at a final concentration of 500ug/ml, growth control was on LJ medium without inhibitory substances. The final concentration of PNB inhibition test using LJ medium as proposed by Tsukamura & Tsukamura (1964). The inoculum was prepared by scraping freshly grown colonies from the surface of the LJ medium. The slopes were observed on the 4<sup>th</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup> and 28<sup>th</sup> days for visible growth of colonies or otherwise. No growth observed in any PNB containing tubes after 28 days of incubation was indicative of the members of *M.tuberculosis* complex

#### **3.4.3.3 Bactec MGIT 960**

Drug susceptibility was done on MGIT for the drug profile from the initial samples. Inoculation was done using 500ul of 1:5 dilutions of the working suspension plus the growth supplements and the anti TB drugs for drug sensitivity testing (DST). Bactec MGIT 960 tubes were loaded into the Bactec MGIT 960 instrument within 2 hours of inoculation. Before loading the tubes, their barcodes were scanned and the instrument assigned them to their stations through the tube entry operation. Cultures are monitored

continuously for *Mycobacterium* growth by the use of oxygen-sensitive fluorescent sensors embedded in a silicon base. Manual reading can also be done using a semi-quantitative reader. The MGIT system has proved to be a rapid, easy-to-use technique with a high sensitivity for detection of *Mycobacterium* directly from clinical specimens (Hanna et al., 1999)

#### **3.4.3.4 Genotype MTBDR assay**

The Genotype MTBDR *plus* probe assay (Hain life science) was carried out according to manufactures instructions. Drug resistance mutation profile for Rifampicin (RIF) and Isoniazid (INH) for rapid diagnostic tool to detect *rpoB* and *Kat G* encoding RNA was typed by the use of Multi Drug Resistance TB *plus* kit (*MDRTB plus*) which is based on straight forward amplification and reverse hybridization technique.

DNA from solid cultures (LJ slant) was extracted by 3-5 colonies were suspended in 300ml Taq water (double distilled water), *Mycobacterium* were lysed by incubation at 95°C for 30 minutes (thermal lysis) and sonication for 15 minutes. Tubes were centrifuged at 13000rpm for 10 minutes, and 5ul of the lysate was used for amplification with the provided bio-tinylated primers. The amplification was performed in a (gene-Amp system) thermal cycler. Hybridization and washing was done by manually using the twin incubator hybridization tray. Stripes were for susceptibility or resistance to RIF and INH according to the manufactures instructions (Hillemann *et al.*, 2005)

#### **3.5 Quality Control**

Known positive and negative sputum specimens were included in every batch of specimens processed. All reagents and media were prepared in accordance with standard

operating- procedures SOPs used at the Central Reference laboratory (CRL-TB) in collaboration with the Supra reference lab (SRL) Australia. NaOH was prepared on weekly basis.

### **3.6 Data Management**

Data was processed using Statistical Package for Social Sciences (SPSS) version 14.2, epi info, A P-value of less than 0.05 was considered as significant. Other measures calculated were sensitivity, specificity, Positive and Negative Predictive values (PPV and NPV).

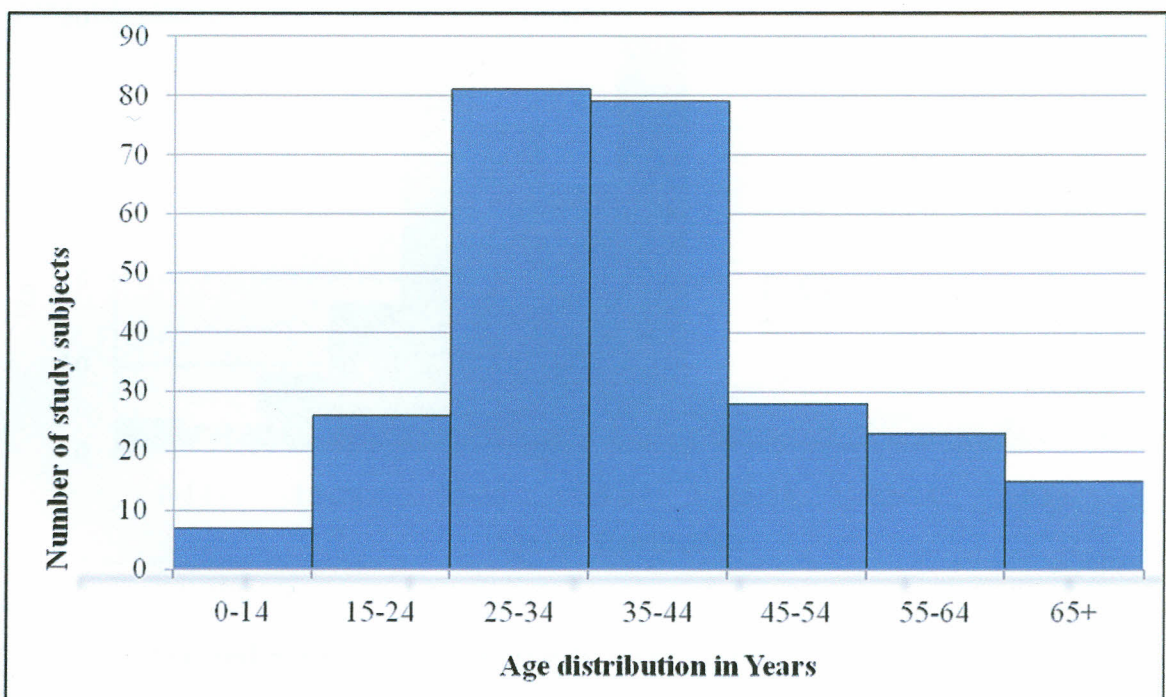
### **3.7 Ethical considerations**

Research clearance was sought from Kenyatta University, Kenyatta National Hospital (KNH) and Ministry of Science and Technology. The study was highly confidential, no risk involved in the study.

## CHAPTER FOUR: RESULTS

### 4.1 Age of the study subjects

The study subjects had an average mean for both sexes as 40 years, Male (1-71) years while the Females aged (6.8-80) years. From the data the mean age of the female's was 37 years and for the males was 41 years. The affected ages reflects that the productive age is prone to the infection between the ages of 15-54 years in both males and females in the study as shown in figure 4.1. Y-axis of figure 4.1 represents number of study subjects while the X-axis presents the age distribution.

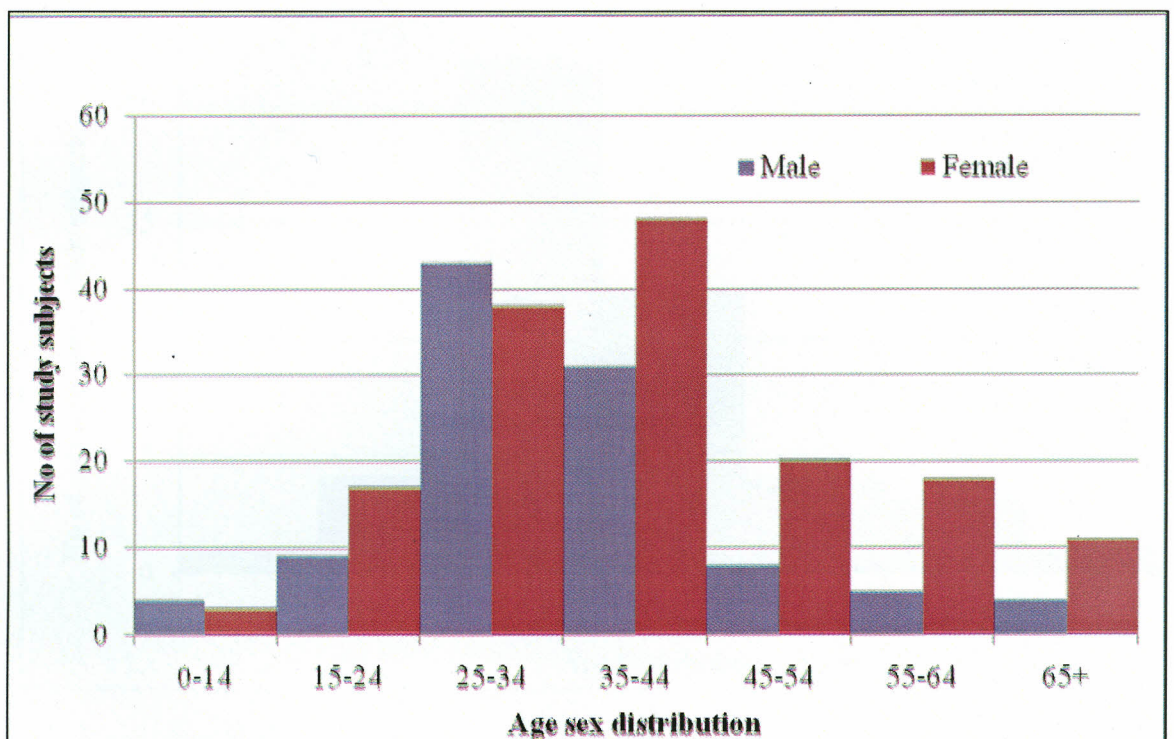


**Figure 4.1: Age distribution of the study subjects**

### 4.2 Age and Sex of the culture positive TB subjects

The age distribution of the study subjects which turned positive after culture was from both Male 13-65 years while the Females aged 6.8yrs -60 years. From the data the mean

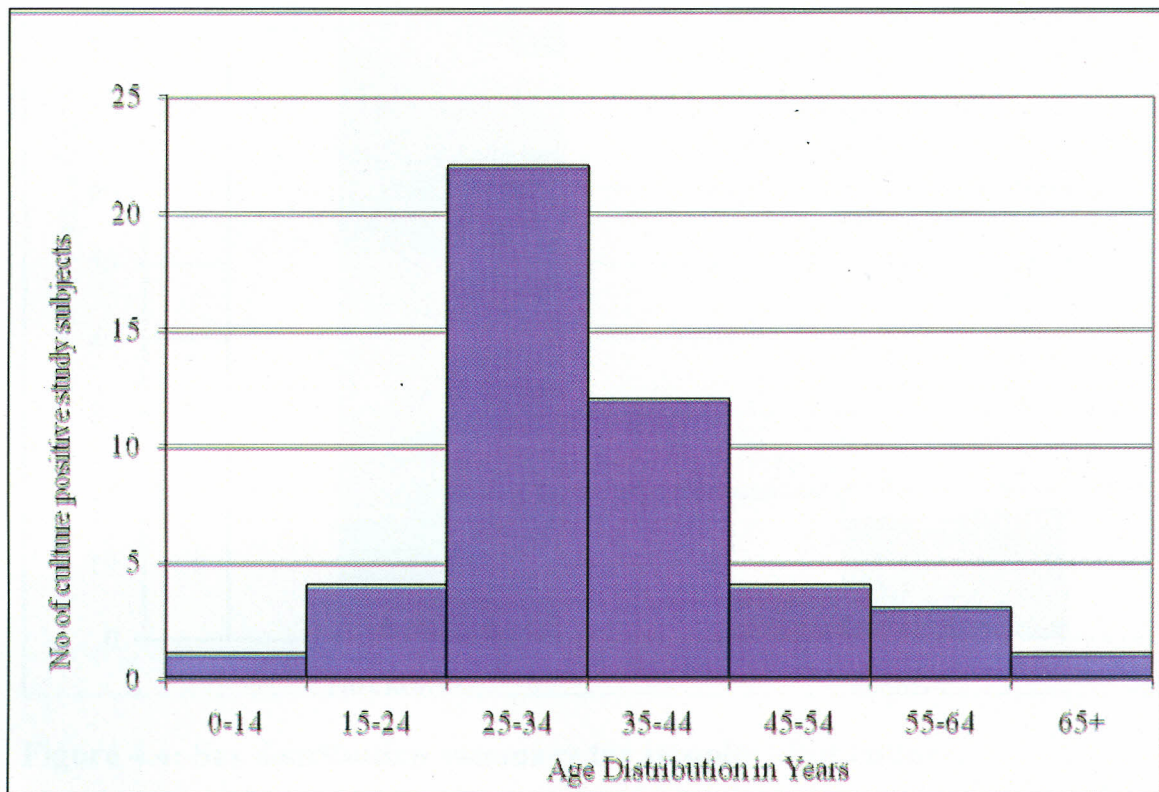
age for females was 33years and for the males was 37years and the average means age for both the sex of the study subjects was 35 years. The affected ages reflects that the productive age is prone to the infection between the ages of 15-54 years in both males and females in the study as represented in figure 4.2 below. Y-axis of 4.2 represents number of study subjects while the X-axis presents age sex distribution among the smear negative smear negative initial samples referred at the TB laboratory.



**Figure 4.2: Age and Sex of the culture positive TB subjects**

Figure 4.3 shows the summary of the samples used in the study showing that a total of 47 samples of the total 273 specimens considered in the study which were earlier diagnosed as smear negative were confirmed to be smear positive for MTB. When culture was done It also showed that there were more males than female turning out to be culture positive

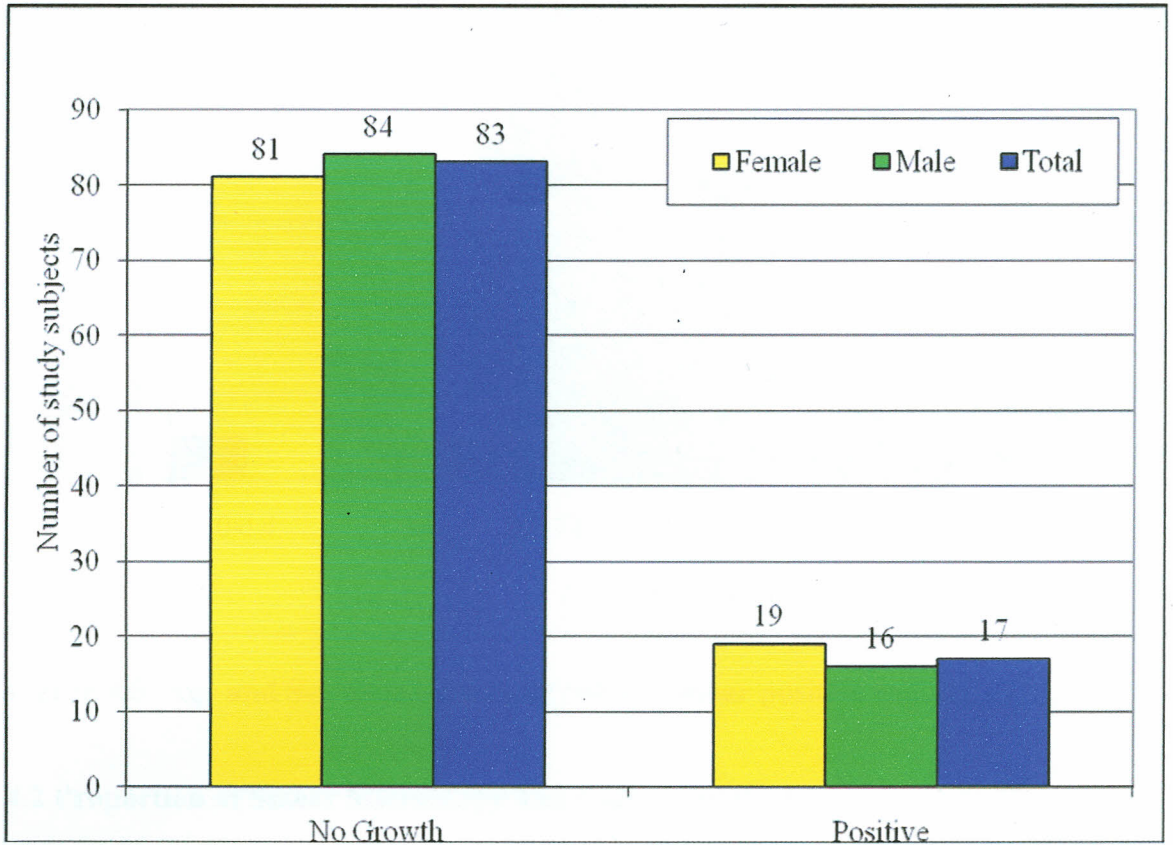
than female. (Odds ratio =84, <.43-168 >, CI-95%,  $\chi^2$  -27,  $P =0.6048$  with the two categories with significant difference) figure 4.3 with Y –axis representing number of culture positive study subjects with the X-axis presenting age distribution.



**Figure 4.3: Age distribution of culture positive study samples**

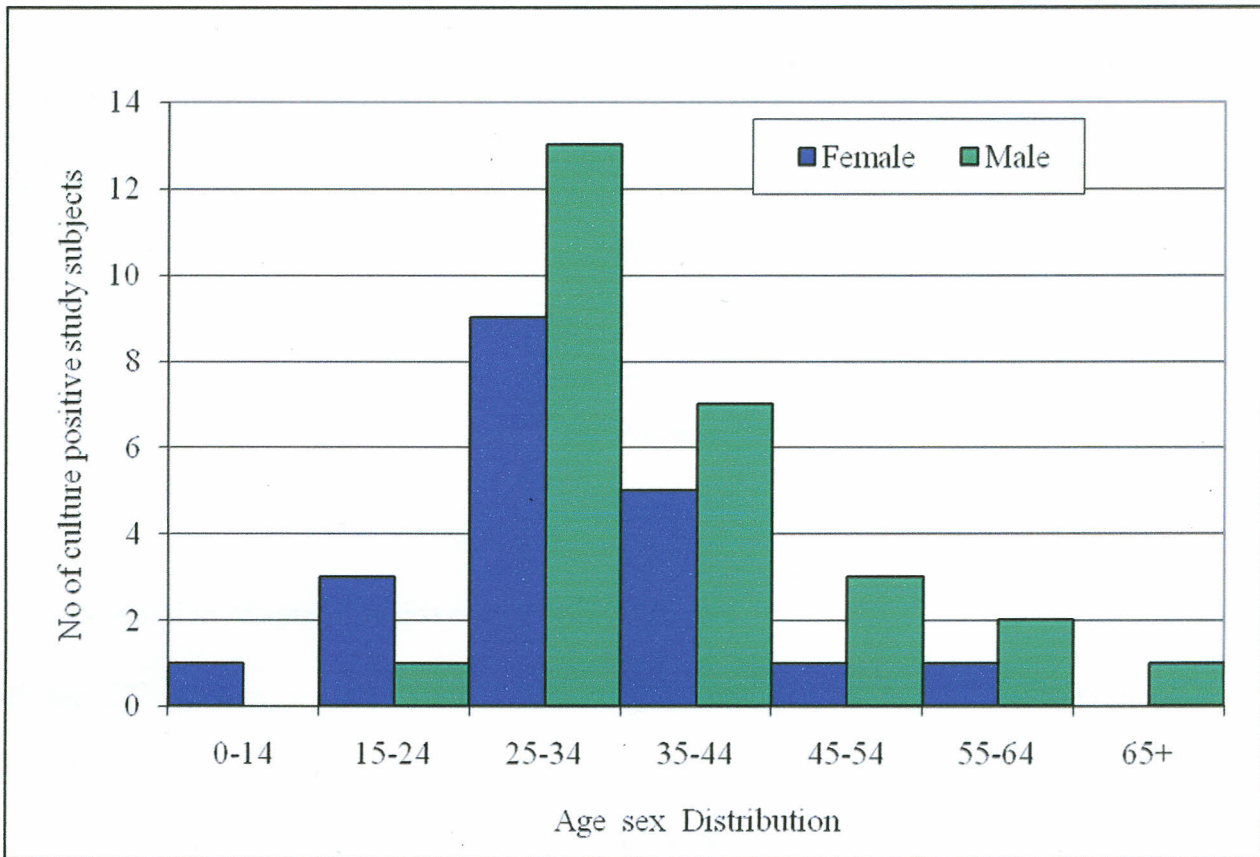
Figure 4.4 and shows that the sputum samples referred at the laboratory for the study had more males than females with the males 61% (166/273) and females being 39% (107/273). The male positivity rate was 16% while the females was 19% and both male and females having an average of 17 % among all the study subjects who were referred at the Central TB laboratory with the Y-axis being presented by number of study subjects

while the X-axis presents no growth and growth (positive) amongst the Male Female distribution amongst the study subjects.



**Figure 4.4: Sex distribution summary for samples after culture.**

Figure 4.5 shows the distribution of positive samples by their sex and age from the study samples, among the 273 samples which were analyzed in the laboratory showing more males than females.



**Figure 4.5: Age and Sex distributions of culture smear positive sample**

#### 4.2 Proportion of Smear Microscopy and Culture method

Of the 273 initially confirmed direct Ziehl Neelson smear negative sputum specimens 47 (17%) were culture positive. (Table 4.1)

**Table 4.1: Summary of the Smear/Culture results**

Direct Smear	Culture on LJ Pulmonary TB		
	+Ve	-Ve	Total
+ve	28	19	47
-ve	19	207	226
Total	47	226	273

**Table 4.2: Sensitivity, specificity, PPV and NPV of ZN staining method**

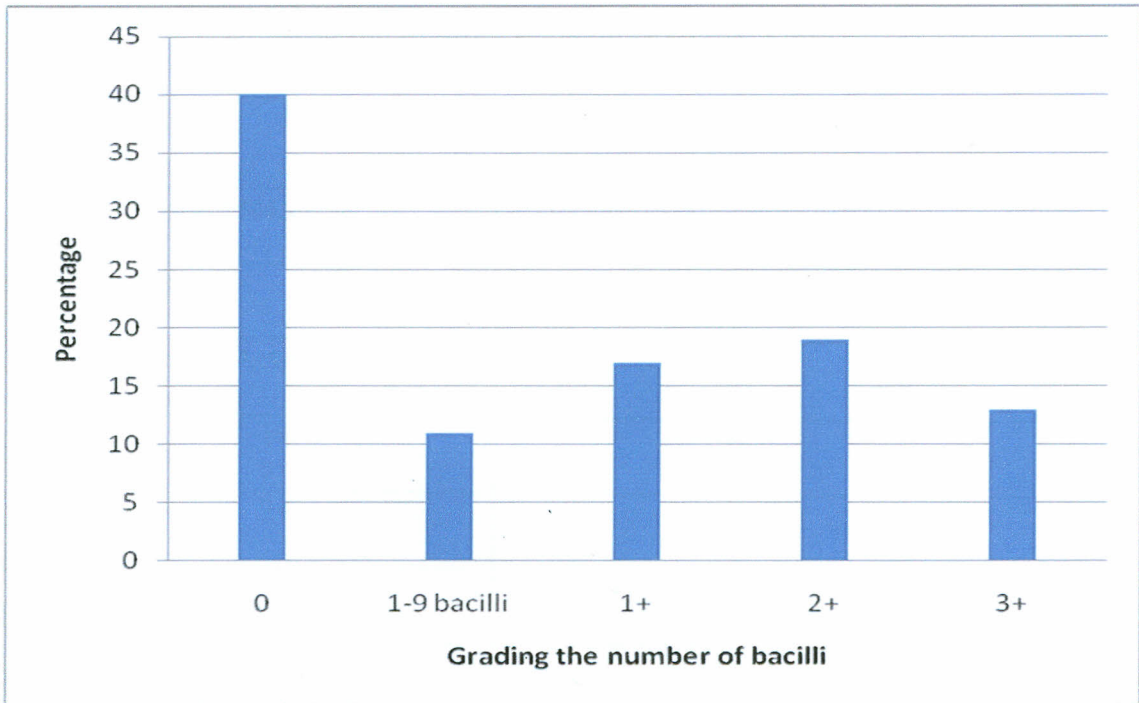
Method	%NaOH Concentration	%Sensitivity (95%CI)	% Specificity (95%CI)	%PPV (95%CI)	%NPV (95%CI)
Centrifugation	4.0%	60 %(47-70)	92%(89-94)	60%(47-71)	91%(89-94)

Key: *PPV-Positive Predictive values NPV-Negative Predictive value, and 95% confidence intervals.*

#### 4.2.1 Proportion of smear positive from study samples

Of the 273 sputum samples which were initially reported as negative smears for *Mycobacterium tuberculosis* 47/273 (17%) turned out to be positive after treatment with 4% Sodium hydroxide followed by Ziehl Neelson method .

Figure 4.6, illustrates the percentage distribution of the positivity rate of the acid fast bacilli, grading was done according to the recommended WHO grading. It further confirms the distribution of the positivity rate when percentages of the positivity rate were obtained.



Key: 1AFB=actual number seen, b=bacilli, 1+ bacilli more than 10 -99 per 100 oil fields, 2+ bacilli 1-10 bacilli in at least 50 oil fields and 3+ more than 10 AFB per oil field in at least 20 field

**Figure 4.6: Distribution of smear positivity of the culture positive study samples**

Table 4.3 shows the distribution of positivity after AFB microscopy was done and graded according to the WHO/IUALTD guidelines. There is a clear indication that 19 samples turned not to have any bacilli but when culture was done it turned out to be positive, this is an important observation which needs further investigation to deduce the reasons for such a behavior

**Table 4.3: showing the grading scale by WHO/IUALTD**

Grading Scale (WHO /IUALTD)	
Reporting scale	Acid Fast bacilli seen (AFB)
Negative	19
1- 9 bacilli (Actual number)	5
1+	8
2+	9
3+	6

### 4.3 Speciation of *Mycobacterium tuberculosis*

#### 4.3.1 P-nitro benzoic acid inhibition tests (PNB)

All the 47 clinical Isolates which initially grown on primary culture on LJ medium were further identified by using Lowenstein-Jensen (L-J) containing PNB reagent which showed 46 were susceptible and had no growth in PNB while only 1 showed growth in the media which indicates that it was not *Mycobacterium Tuberculosis*.

#### 4.3.2 MTBDR *plus* assay

Table 4.4 shows the mutation pattern from the study samples submitted for the study with most of the samples showing normal bands ,species identification (speciation) using DNA strip method ; wild type mutations ,the control band ,TUB .band. From the 47 isolates, 46 of the isolates which turned culture positive were due to true *Mycobacterium Tuberculosis* isolates strains while 1 was isolated as *Mycobacterium absseccus* which is a sub species of *Mycobacterium chelonoe* which showed no band (*water mycobacterium*)

and one multidrug resistant strain which had mutation on Rifampicin (*ropB*) and Isoniazid high levels (*katG*) and *inhA* (Isoniazid low levels). The interpretation was made from the mutation pattern chart shown in Figure 4.7

**Table 4.4: Mutation Pattern**

Mutation Pattern	<i>ropB</i>	<i>Kat G</i>	<i>InhA</i>
mut-1	1	2	0
mut-2	0	2	0
mut-3	1	0	0
<b>no bands</b>	<b>1</b>	<b>1</b>	<b>1</b>
Wt	44	42	46
Grand Total	47	47	47

Key:

<i>rpoB</i>	Rifampicin
<i>kat G</i>	high level Isoniazid
<i>inhA</i>	low level Isoniazid
mut1	Mutant1
mut2	Mutant2
mut3	Mutant3
Wt	Wild type
CC	Conjugate control
AC	Amplification control
TUB	<i>tuberculosis</i>

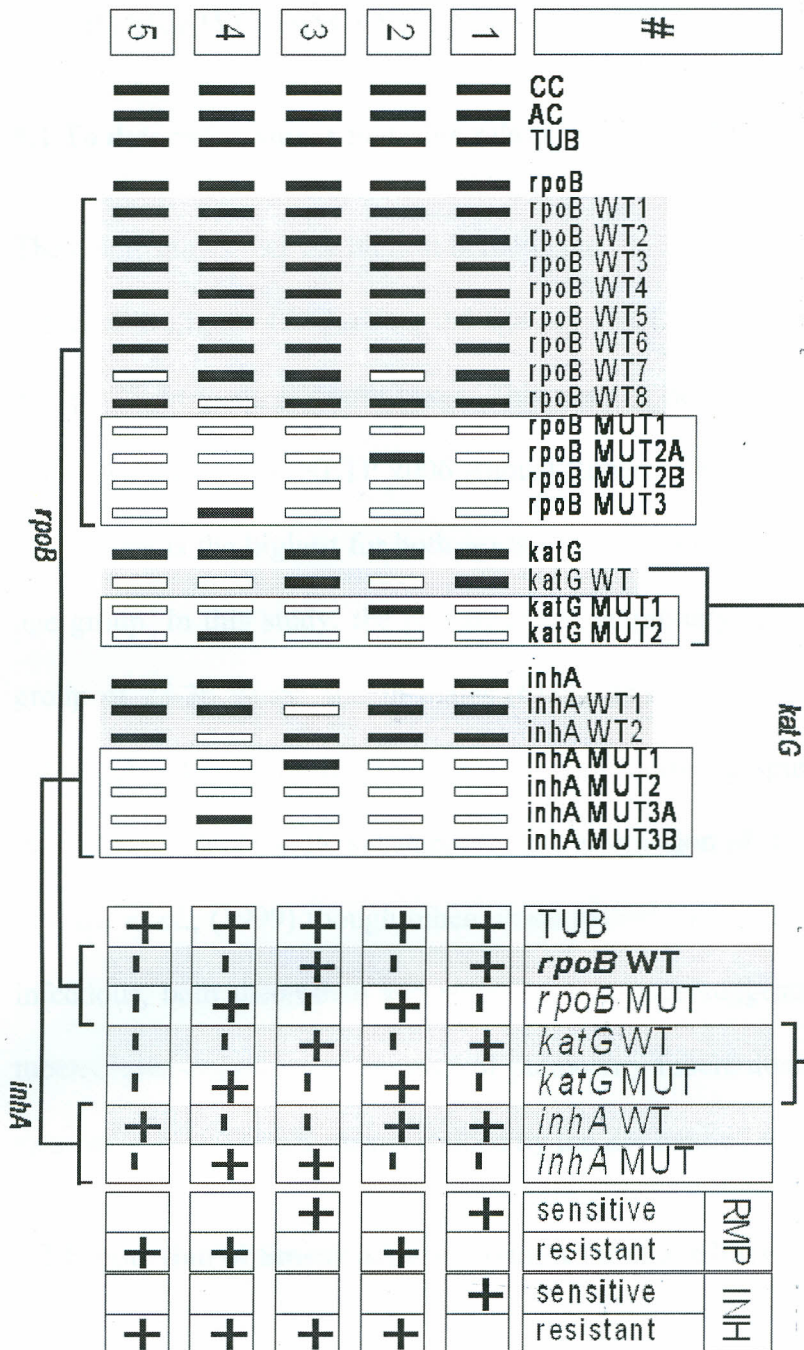


Figure 4.7: MTB Pattern *rpoB* and *katG* mutation table

## CHAPTER FIVE: DISCUSSION

### 5.1 To determine smear negative relapse cases if influenced by Age and Sex

The result shows that the highest burden is in the age group 25-44 years same behavior is depicted in Figure 4.3 showing the age sex distribution. The burden is higher in the age groups 25-44 years for both sexes. This trend is not surprising since it conforms to the national data for TB (NLTP 2006 Annual Report) showing that the productive age group 25-44 years is the highest for both sexes and this represents the country's high productive age group. In this study, the prevalence of pulmonary tuberculosis was high in the age group of 25-36 years; and in males (61%), as compared to females (39%) among the entire subjects. According to Banda *et al.*, (2000) the sputum positive cases are most infectious and contribute substantially to transmission of disease, but as per observations of Behr *et al.*, (1999) though tuberculosis patients with sputum smears negative are less infectious, both theoretical and empirical evidence suggests that they can still transmit tuberculosis. Laboratory diagnosis of pulmonary tuberculosis rests on the bacteriological examination of sputum smears stained by the ZN method for AFB.

### 5.2 Proportion of Smear negative samples that are bacteriological culture positive.

Ziehl Neelson (ZN) method of staining is the most commonly employed technique used. Unfortunately the sensitivity of Z.N. staining compared to culture is less than 50 % (Sonal *et al.*, 2001). The main objective of Tuberculosis control is to identify and treat patients with infections of pulmonary tuberculosis, the diagnosis of which relies on bacterial examination of sputum. The microscopic detection of Acid fast bacilli (ZN)

staining has been used as an aid in tuberculosis diagnosis for many years. Specimen were digested and concentrated by the use of sodium hydroxide alkali procedure and smears were made, stained by ZN method. The smears were graded according to IULTD/WHO criteria: (i) a true positive smear showed the presence of acid fast bacilli (ii) a true negative smear was one in which no bacilli was seen on smear microscopy.

The current study was the first to use 4% NaOH (Modified Petroffs method) centrifugation specifically on ZN negative smear specimens from peripheral at the Central Reference Laboratory. In this study 4% of NaOH was the concentration of choice which is recommended by WHO. From the two hundred and seventy three (273) sputum samples which had been confirmed as direct ZN smear negative, had an increase of 17% smear positivity and culture positive after analysis is an encouraging findings. In addition of the entire positive cultures 19 specimen which were smear negative were confirmed as culture positive results. NaOH culture positive samples further proves the reliability of the method which, suggest that it digests sputum which when followed by centrifugation greatly increases the number of bacilli and also the sensitivity (Miorner *et al.*, 1996).

By far smear microscopy is the most popular amongst all methods currently employed worldwide in the laboratory diagnosis of tuberculosis on account of its simplicity, speed, low cost and minimal requirement for equipment and technical skill. However it suffers from lack of sensitivity since a load of 5000-10,000 organisms/ml of specimen is required to give a positive report by Ziehl Neelsen staining from concentrated samples (Kent and Kubica, 1995). A more sensitive smear microscopy method that is also simple would be highly useful in diagnostic laboratories.

### **5.3 To compare the sensitivity and specificity of ZN with Culture method using concentrated sputum treated with 4%NaOH.**

The sensitivity of ZN direct method has ranged from 40 to 85 % in different laboratory settings. Involvement of a concentration step increased the sensitivity of the direct smear method from 54-57 % to 63-80% (Burchfield *et al.*, 2000; Garay *et al.*, 2000). The CDC method has shown sensitivity levels around 66 to 83% (Scott *et al.*, 2002). This study has shown similar level of sensitivity. Inclusion of a centrifugation step (3000 xg for 15 min) was reported to detect 500 and 5000 organisms/ml by smear microscopy and culture, respectively (Perera *et al.*, 1999). However other reports have suggested that the overall diagnostic sensitivity of smear microscopy was not increased by sputum liquefaction and concentration (Wilkinson *et al.*, 1997). It has been proposed that the use of fluorescence microscopy greatly improves the diagnostic value of the sputum smear especially in samples with a low density of bacilli that are likely to be missed on ZN stained smears which from this study has shown the same similar results both in sensitivity and sensitivity. (Githui *et al.*, 1993). The results indicate that the concentration method adds substantially to the sensitivity of direct microscopy, without much extra input. Although it slightly increases the time of the investigation, the procedure is simple and can be applied easily in a district laboratory with basic equipment and staff training.

The advantages of this centrifugation and sedimentation of sputum method for low-income countries have been described elsewhere: in three studies performed in Ethiopia and India, the use of the concentration method increased the number of samples positive for AFB by more than 100%. In Zambia, concentration method increased the sensitivity of the ZN stain from 43.4% to 76.3% (Burchfield *et al.*, 2000) and stratified their results

according to the HIV status of the patients and concluded that among HIV-positive patients the concentration method increased the sensitivity of the sputum smear from 38.5% (conventional direct microscopy) to 50%. This was compared to overall sensitivity, which increased from 54.2% to 63.1%. The results confirmed that the sensitivity of direct microscopy was lower however; there was greater sensitivity in the concentration method.

The higher sensitivity of the concentration method: sputum specimens that were only positive by the concentration method took longer to give a positive signal than specimens that were positive by both methods. If used routinely for diagnostic purposes, the turnaround time would considerably reduce the period of uncertainty for the clinician. At present, presumed sputum-negative PTB patients are put on a 'trial of treatment', which, according to national guidelines, should last for a minimum of 4 weeks before the patient is reassessed and a management decision is taken on the basis of the clinical response. Besides inducing psychological stress in the patient, this 'trial of treatment' is not cost-effective and warrants on its own an intensive search for alternative diagnostic methods. We conclude that in a high HIV prevalence area, direct microscopy lacks the required sensitivity to be considered the pillar of the diagnosis of pulmonary tuberculosis. We recommend that the concentration method be more widely used in settings such as, where the diagnosis of pulmonary tuberculosis still relies on bacteriological examination.

The Culture technique has considerable advantages, but its relatively high cost make it a less recommendable option for wide spread routine use in district laboratories. There is a significant reduction in time for diagnosis of ZN smears negative TB that usually takes

long process too. This concentration/sedimentation method will lead to early diagnosis of smear negative TB for prompt treatment thereby lowering the rate of transmission within the community and eventually reducing mortality and morbidity due to TB. Taking into consideration those with less than 1000-5000ml bacilli per ml of sputum is required for ZN positive smear (Tomon *et al.*, 2004) findings from this study shows that those which were initially negative turned out 47 / 273 were positive specimens. Clearly this indicates that 4%NaOH centrifugation method has potential to improve TB diagnosis. This approach also confirms that smear positivity is not just a boost of specimens which are already scanty into positives but indeed actual detection of AFBs not detected by direct ZN microscopy.

The current study shows that there are at least 17% of sputum smear positive TB patients who are diagnosed as being sputum smear negative .Based on this study, it can be concluded that large number of patients are diagnosed smear negative TB patients in the TB programme (2006 Annual report).This actually poses serious management challenges because smear positive TB patients are continually still spreading TB in the community coupled with the fact the WHO estimates that only 50% of TB patients are brought to treatment. The patients with smear negative pulmonary tuberculosis appear responsible for about 17% of TB transmission. This is because of atypical manifestations such as non cavitory lesions with dissemination of disease that are encountered smear negative patients. More importantly, in many low-income countries, cure is defined routinely by a single negative sputum smear at treatment completion plus a negative smear on at least one previous occasion (WHO, 2001).

#### **5.4 To identify recurrent or re-infection is due to *Mycobacterium tuberculosis* or other *Mycobacterium* species.**

Presumptive differentiation between *Mycobacterium tuberculosis* complexes (MTC) and Non- *Mycobacterium tuberculosis* (NTM) can be made by growth characteristics (rough and creamy colonies) and by microscopic observation of cording formation on Ziehl – Neelsen stain of a positive culture. (Monetrio *et al.*, 2003). The aim of the present study was to evaluate genotype Multi drug resistance TB *plus* kit (MDR TB *plus* kit) as a rapid diagnostic tool to detect rpoB and Kat G mutation that are associated with Rifampicin and Isoniazid respectively in Tuberculosis isolates. It is important to underline the capability of the MDRTB *plus* test to identify mutations conferring resistance to Rifampicin / Isoniazid in strains with a low level of resistance as reported by Sarvastava *et al.*, (2004) by routine sensitivity testing performed on Lowenstein Jensen medium. In laboratory testing, the most important contribution to the final diagnosis is made by identification of the bacillus, either through direct examination (sputum smear microscopy) or through its isolation in culture (culture medium). The average time to formation of *M. tuberculosis* colonies on LJ medium is four weeks, this being its greatest disadvantage.

The inhibition test results of 47 strains using L-J, MGIT 960 and MDRTB *plus* were in agreement with results of conventional tests performed on L-J and with results previously reported in the literature (Kobayashi *et al.*, 1999). All MTC strains including one standard strain from ATCC (ATCC-27294) and 46 clinical isolates were susceptible to PNB, 1 was resistant in L-J medium. *M. absseccus* was considered with both L-J and in MGIT 960. The key findings was the possibility of providing rapid and reliable results in

setting where a high prevalence of TB demands rapid procedures for identification and susceptibility testing against anti-tuberculosis drugs.

A nucleic acid amplification technique was championed as providing alternative sensitive, specific and rapid approaches to tuberculosis diagnosis. In recent years, nucleic acid-based approaches, PCR in particular, are also being employed as an adjunct test to arrive at a definitive diagnosis on a reasonable period of time. A variety of diagnostics have capitalized on the recent elucidation of molecular mechanisms of drug resistance, especially to Rifampicin and Isoniazid, which in most settings is a surrogate marker for MDR and which is almost always caused by a limited number of mutations in a single gene, *rpoB*, *KatG*, *InhA* (Ramaswamy, 1998). More than a dozen techniques have been described to detect relevant *rpoB*, *KatG*, *InhA* mutations, from simple electrophoresis techniques, to molecular beacons, to sequencing. The line probe assay, a straightforward amplification and reverse hybridization technique, has been available as a commercial assay for several years. Good correlation with standard drug susceptibility testing in isolates from a variety of countries suggests that it could have wide applicability, although it is too expensive for most settings. The line probe assay is usually used on culture isolates, but has been used on direct patient specimens which were smear positive.

The sensitivity of Genotype MDRTB *plus* assay for *rpoB*, *Kat G*, and *InhA*, of the 47 isolates which were culture positive, 46 (98%) were correctly identified by MTBC specific TUB capture probe. With respect to the assay for the resistance associated genes, amplification of *katG* provided a conclusive of results which other study has shown the

same results which allows the rapid and specific detection of most frequent mutations leading to Isoniazid and Rifampicin resistance in clinical *M. tuberculosis* Isolates (Cengiz *et al.*, 2006)

## 5.5 Conclusion

1. Laboratory plays a critical role in identification, rapid detection and treatment monitoring on TB patients in TB control.
2. Culture allows confirmation of cases and facilitates species identification which presents the true magnitude of the disease.
3. Quality control and quality assurance program is extremely important in laboratory testing and it should be an integral part of good laboratory practice
4. Smear Microscopy fails to detect low numbers of *Mycobacterium* presents in a specimen and is not specific for MTB neither complex nor drug resistance.
5. Genotypic assays for rapid detection of mutations within the *rpoB* and *katG* gene encoding the RNA polymerase associated with Rifampicin/Isoniazid resistant hold promise as a rapid and simple test for identification in this era of drug resistant.

## 5.6 Recommendation

1. Laboratory diagnosis of Pulmonary Tuberculosis rests on bacteriological examination of Smear and culture methods thus need to strengthen laboratory services in Kenya.
2. A more sensitive Smear Microscopy method that is simple would be highly useful in diagnostic laboratory especially Fluorescence Microscopy (FM).
3. Culture services should be introduced at regional levels to provide the true picture of the magnitude of the disease.
4. The application of this assay will be feasible when implemented at all busy facility and the regional levels by the TB control program to improve case detection which has championed as providing alternative sensitive, specific and rapid approach in TB diagnoses.
5. Quality control and Assurance aspects need to be appropriately addressed and monitored at every level of TB diagnoses.

## 5.7 Suggestion for Further Research work

1. To find out whether this is a social demographic profile because it may influence some of this outcome.
2. To evaluate the current laboratory status to find out the infrastructure, level of trainings of the staffs and their capacity.

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## **APPENDICES:**

### **Appendix 1**

#### **PREPARATION OF SPUTUM SMEARS**

Materials needed:

- New clean frosted slides
- Ordinary lead pencil for frosted end slides or diamond pencil non -frosted.
- Spirit lamp or Bunsen burner
- Applicator sticks or bamboo sticks.
- Disinfectant container with 5% industrial phenol
- Sputum specimens

#### **Smear preparation process**

Select new, clean, grease-free, unscratched slides which are free from fingerprints. Using a pencil, record the laboratory register, serial number and order number of the sputum specimen on the frosted end of the slide. If plain unfrosted slides have to be used, labeling is best done using a diamond pencil. Ensure that the number on each slide corresponds to the number on the specimen container.

#### **2. Sputum smearing**

Using the end of an applicator stick select and pick up the yellowish purulent particles of sputum. Prepare the smear in an oval shape in the center of the slide. The smear size should be 2–3 cm in length x 1–2 cm wide, which will allow 100–150 fields to be

counted in one length. For good spreading of sputum, firmly press the stick perpendicularly to the slide and move in small concentric circles or coil-like patterns.

Place the used stick into a discard container with 5% phenol. Use a separate stick for each specimen. Thorough spreading of the sputum is very important; it should be neither too thick nor too thin. Prior to staining, hold the smear about 4-5 cm over a piece of printed paper. If letters cannot be read, it is too thick.

### **3. Air drying of smear**

Allow the smear to air dry completely at room temperature.

Do not dry smears in direct sunlight or over a flame.

### **4. Fixing of the smear**

After the slide is completely dry, use forceps to hold the slide upwards. Pass the slide over the flame 2-3 times for about 2-3 seconds each time. Do not heat the slide for too long or keep it stationary over the flame, or else the slide will be scorched.

## Appendix 2

### ZIEHL-NEELEN CARBOL FUCHSIN SOLUTION

#### Reagents and Preparation

##### CARBOLFUCHSIN Stain

- |                             |       |
|-----------------------------|-------|
| ▪ Basic Fuchsin powder      | 3.0g  |
| ▪ Phenol crystals           | 50.0g |
| ▪ Methylated spirit/Ethanol | 100ml |
| ▪ Distilled water           | 900ml |

Dissolve basic fuchsin powder either in the liquefied phenol (melted at about 50°C), either in a mixture of the Methylated spirit and about 300ml of water, by swirling in a conical flask. Add the remainder of the reagents after complete dissolution of fuchsin powder, mix well.

##### 25% Sulphuric Acid

- |   |       |
|---|-------|
| ▪ Concentrated Sulphuric Acid (technical grade) | 250ml |
| ▪ Sterile distilled water                       | 750ml |

Carefully add the concentrated Sulphuric Acid to water slowly as the mixture generates heat. Cool off under a stream of tap water after adding half the amount the amount required as needed. .

##### Methylene blue

- |                         |        |
|-------------------------|--------|
| ▪ Methylene blue powder | 1.000g |
| ▪ Distilled water       | 1000ml |

Dissolve Methylene blue chloride in distilled water.

1. Stains are preferably stored in an amber bottle with leak proof cap;
2. Label bottles with the name of reagent and date of preparation.
3. Store at room temperature for six to twelve months.

Filtration of newly prepared stains is not needed if carbol fuchsin is filtered at time of staining. Before putting them into use, stains should undergo Quality control in the laboratory which has prepared them. This requires low positive (i.e.1+) and negative control smears (smear and fixed but unstained), which the lab has to prepare and store using suitable routine specimens. Each time a new lot of stains is prepared, 2 positive and 2 negative controls must be tested, using the routine staining procedure as described above. Positive controls should yield the expected number of AFB, stained strongly red, on a blue background, after a single cycle. Negative controls should have a clear blue background as well, and should not contain any AFB even after repeated staining cycles. These controls must be recorded in a logbook kept by any lab preparing stains. As a minimum it will show:

- Name of the stain
- Date of preparation (which can also be considered as the identification of the lot)
- Positive controls: ID number, result
- Negative controls: ID number, result
- Conclusion: accept or reject.

**Ziehl-Neelsen staining procedure:**

- Arrange slides in serial order on staining bridge, with smear side up
- Flood slides with filtered carbol fuchsin stain
- Gently heat to steam
- Keep the staining reagent for at least 10 minutes
- Rinse with water and drain
- Apply decolorizing solution for 3 minutes
- Rinse with water and drain
- Apply Methylene blue counter stain for 1 minute
- Rinse with water and drain
- Air dry on a slide rack

**Quality control on stains**

Quality of stains is of utmost importance and should be checked weekly, using a low positive (1+) control smear

**WHO/IUATLD GRADING SCALE**

Follow the scale when reporting smears:

- If no AFB is seen in at least 100 fields, report as negative for AFB.
- If 1–9 AFB are seen in 100 fields, report actual number of AFB seen.
- If 10–99 AFB are seen in 100 fields, report as (1+).
- If 1–10 AFB/field in at least 50 fields report as (2+).
- If more than 10 AFB/field in at least 20 fields, report as (3+).

Reporting scale	AFB seen
Negative	No AFB seen in at least 100 fields recorded as "0"
Actual number	1-9 AFB per 100 fields
(1+)	10-99 AFB per 100 fields
(2+)	1-10 AFB per field in at least 50 fields
(3+)	More than 10 AFB per field in at least 20 fields

## Appendix 3

### Sodium hydroxide (Modified Petroff) method

This method is used widely because of its relative simplicity and the fact that the reagents are easy to obtain. NaOH is toxic, both for contaminants and for tubercle bacilli; therefore, strict adherence to the indicated timing is required

### Reagents

#### Preparation of 4% sodium hydroxide (NaOH) solution

- Sodium hydroxide pellets (analytical grade) 4g
- Distilled water 100ml
- Dissolve NaOH in distilled water and sterilize by autoclaving at 121°C for 15 minutes.
- Sterile saline
- Sodium chloride pellets (analytical grade) 0.85g
- Distilled water 100ml
- Dissolve NaCl in distilled water and sterilize by autoclaving at 121°C for 15 minutes.
- An alternative method for preparing 4% NaOH is as follows:
- Add the contents of a 250g bottle NaOH pellets to 500ml distilled water and fill
- Water to the 625ml mark. Be careful since heat is released in this reaction.
- When needed, prepare a fresh 4% NaOH solution by adding 40% NaOH to
- Sterile distilled water in the proportion 1:10.

## Appendix 4

### CULTURE MEDIA

#### Precautions during media preparation

For media of the best quality, chemicals of certified purity, clean glassware and freshly distilled and sterilized water should be used. Directions for preparing media must be followed precisely and without modification. A few general points to obtain good quality media and avoid contamination of reagents and media are as follows:

- Keep the environment as clean as possible. Swab the work surface with a suitable disinfectant (e.g. 5% Methylated spirits) before dispensing sterile reagents and media.

Clean the floor with a wet mop to limit dust

- Use sterile glassware and equipment
- Use reagent grade chemicals and reagents unless otherwise specified
- Check the temperature of inspissators and hot air ovens
- Follow strict aseptic techniques when preparing media, e.g. Flaming flasks and tubes
- When preparing egg-based media, carefully clean egg shells before breaking
- Do not overheat medium during inspissation
- Do not leave prepared media exposed to light (including ultra-violet light), but store in the refrigerator in the dark when not in use
- Do not skimp on the volume of medium.

### LOWENSTEIN-JENSEN MEDIUM

#### Ingredients

Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH<sub>2</sub>PO<sub>4</sub>) 2.4g

Magnesium sulphate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ) 0.24g

Magnesium citrate 0.6g

Asparagine 3.6g

Glycerol (reagent grade) 12ml

Distilled water 600ml

Dissolve the ingredients *in order* in the distilled water by heating. Autoclave at  $121^\circ\text{C}$  for 30 minutes to sterilize. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

#### **Malachite green solution, 2%**

Malachite green dye 2.0g

Sterile distilled water 100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours.

#### **Homogenized whole eggs**

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

#### **Preparation of complete medium**

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution 600ml

Malachite green solution 20ml

Homogenized eggs (20-25 eggs, depending on size) 1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes and the tops are securely fastened. Inspissator the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

### **Coagulation of medium**

Before loading, heat the inspissator to 85°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 1 hour at 80°-85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilize it).

The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discolorations of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded.

### **Sterility check**

After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35°-37°C for 24 hours as a check of sterility.

### **Storage**

The LJ medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than 4 weeks.

**Appendix 5:****INOCULATION AND INCUBATION PROCEDURES****Inoculation procedures**

Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted. loops (wire or disposable) or pipettes can be used for primary cultivation, although plastic Pasteur pipettes are recommended. Each slope should be inoculated with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface. Fluid media can accommodate up to 1ml used for each specimen. Two slopes of LJ medium should be inoculated per specimen. In areas where *M. bovis* may be a problem, an additional slope containing pyruvate should be added.

**Incubation of cultures**

All cultures should be incubated at 35°-37°C until growth is observed or discarded as negative after eight weeks. Inoculated media should preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Thereafter, if incubator space is needed, bottles could be placed upright. Tops should be tightened to minimize evaporation and drying of media.

## Appendix 6:

### CULTURE EXAMINATION AND IDENTIFICATION

#### Examination schedule

All cultures should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are examined weekly, or if this is not operationally feasible, on at least three occasions, *viz*

- one week to detect rapidly growing *mycobacterium* which may be mistaken for *M. tuberculosis*
- three to four weeks to detect positive cultures of *M. tuberculosis* as well as other slow-growing mycobacterium which may be either harmless saprophytes or potential pathogens
- after eight weeks to detect very slow-growing *Mycobacterium*, including *M. tuberculosis*, before judging the culture to be negative. It is useful to label containers with cultures with the dates necessary for examination and to place containers in the incubator in chronological order.

Should contaminated cultures be found during the examination, those where the surface has been completely contaminated or where medium has been liquefied or discoloured them should be sterilized and discarded.

Cultures with partial contamination should be retained until the eighth week. Late contamination does not exclude the presence of *M. tuberculosis*; it is therefore advisable to prepare a smear from the surface of the medium. Should microscopy indicate the

presence of acid-fast bacilli, an attempt could be made to re-decontaminate and re-inoculate the culture.

### **Reading of cultures**

Typical colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigmented (cream coloured) and slow-growers, i.e. only appearing three weeks after inoculation. With doubtful cultures or when less experienced staff read cultures, the acid fastness should be confirmed by Ziehl-Neelsen (ZN) staining. For preliminary identification of tubercle bacilli the following characteristics apply:

- Tubercle bacilli do not grow in primary culture in less than one week and usually take three to four weeks to give visible growth
- The colonies are buff coloured (never yellow) and rough, having the appearance of bread crumbs or cauliflower
- They do not emulsify in the saline used for making smears but give a granular suspension
- Microscopically they are frequently arranged in serpentine cords of varying length or show distinct linear clumping. Individual cells are between 3µm and 4µm in length

### **Differentiation of *M. tuberculosis***

Presumptive diagnosis of tuberculosis should be made by an experienced laboratory technologist on the basis of the characteristics of tubercle bacilli described before, it is best to do confirmatory tests.

**PROCEDURE**

1. Add 1ml of sterile saline to the culture slant. If growth is confluent, puncture the medium with a Pasteur pipette to allow contact of the saline with the medium
2. Place the tube horizontally so the fluid covers the entire surface of the medium
3. Allow 30 minutes for the extraction of niacin. The extraction time may be longer if the culture has few colonies
4. Raise the slant upright for 5 minutes to allow the fluid to drain to the bottom.
5. Remove 0.5ml of the fluid extract to a clean screw cap tube
6. Insert the strip with the identification end up (an arrow may indicate which end to insert first)
7. Immediately seal the tube.
8. Do not let the middle of the strip get wet
9. Leave at room temperature for 15-20 minutes.
10. Occasionally agitate the tube without tilting it
11. Observe the colour of the liquid in the bottom of the tube against a white background (yellow = positive).
12. Discard any colour on the strip itself; this may occur because of oxidation of chemicals, especially at the top of the strip
13. Neutralize the strips with 10% sodium hydroxide or discard them into alkaline disinfectant.

## Appendix 7:

### Growth on Medium containing P-nitro benzoic acid (PNB)

LJ medium and growth containing p-nitro benzoic acid at 37°C. Problems with incubation at 25°C may be encountered in tropical regions. A refrigerated incubator should be used where available; as alternative, water bath within a refrigerator or cold room should be used.

### Procedure

Inoculate two slopes of LJ medium containing glycerol and one tube of LJ medium containing P-nitro benzoic acid (PNB) at a concentration of 500mg/liter. Incubate one LJ slope and the PNB slope at 37°C in an internally illuminated incubator and examine at 3, 7, 14 and 21 days. When growth is evident on the LJ slope examine it for pigment, remove slopes from the dark incubator as soon as growth is evident, loosen the caps to admit some oxygen and expose them to daylight (but not direct sunlight) or place 1m from a laboratory bench lamp for 1 hour. Re-incubate and examine for pigment the following day • Incubate the other LJ slope at 25°C and examine at 3, 7, 14 and 21 days

### Results and interpretation

*M. tuberculosis* does not grow within three days at 37°C and does not grow at all at 25°C or on PNB medium. It also does not produce yellow or orange pigment in the dark or after exposure to light.

### Identification *M. tuberculosis*

- Growth rate slow
- Growth temperature 35°-37°C only

- No pigmentation
- Niacin positive
- Nitrate positive
- Catalase negative at 68°C
- No growth on LJ medium containing p-nitro benzoic acid

## **RECORDING AND REPORTING OF LABORATORY RESULTS**

The laboratory findings are to useful, they must be communicated in ways that make sense to the different authorities: Health care workers use the findings for the diagnosis and management of tuberculosis; public health authorities use them for statistical and epidemiological purposes, while tuberculosis managers use the information to ensure that bacteriological proven patients are receiving appropriate chemotherapy. Culture procedures for tuberculosis bacteriology are notoriously time-consuming, often taking weeks or months to complete. For this reason, interim reports should be issued. The following schedule is recommended:

- If the cultures have been contaminated, a report should be sent out *immediately* and a repeat specimen requested
- If cultures are positive and growth has been identified as *M. tuberculosis* a report should be sent out *immediately*
- At *four weeks* an interim report (optional) could be sent out on all negative specimens, stating that another report will be issued in the event of the specimen becoming positive later on
- At *eight weeks* a final report should be issued containing all the data previously reported so that earlier interim reports can be destroyed and only the final report retained in the

patients' file Culture reports should be qualitative (i.e. positive or negative) as well as quantitative (i.e. number of colonies isolated). The *average number* of colonies on all the bottles/tubes per specimen should be reported.

### Reading

No growth

1-19 colonies

20-100 colonies

100-200 colonies

200-500 colonies

Confluent growth

Contaminated

### Report

Negative

Positive (number of colonies)

Positive (1+)                  Positive (2 +)

Positive (3 +)                  Positive (4 +)                  Contaminated

When reading cultures the following should be recorded in the laboratory register:

- Growth rate (slow / rapid)
- Number of colonies isolated
- Pigment production in the colonies (none / present and colour)
- Colony morphology (rough / smooth / shiny / flat)
- Results of differential tests

## QUALITY CONTROL

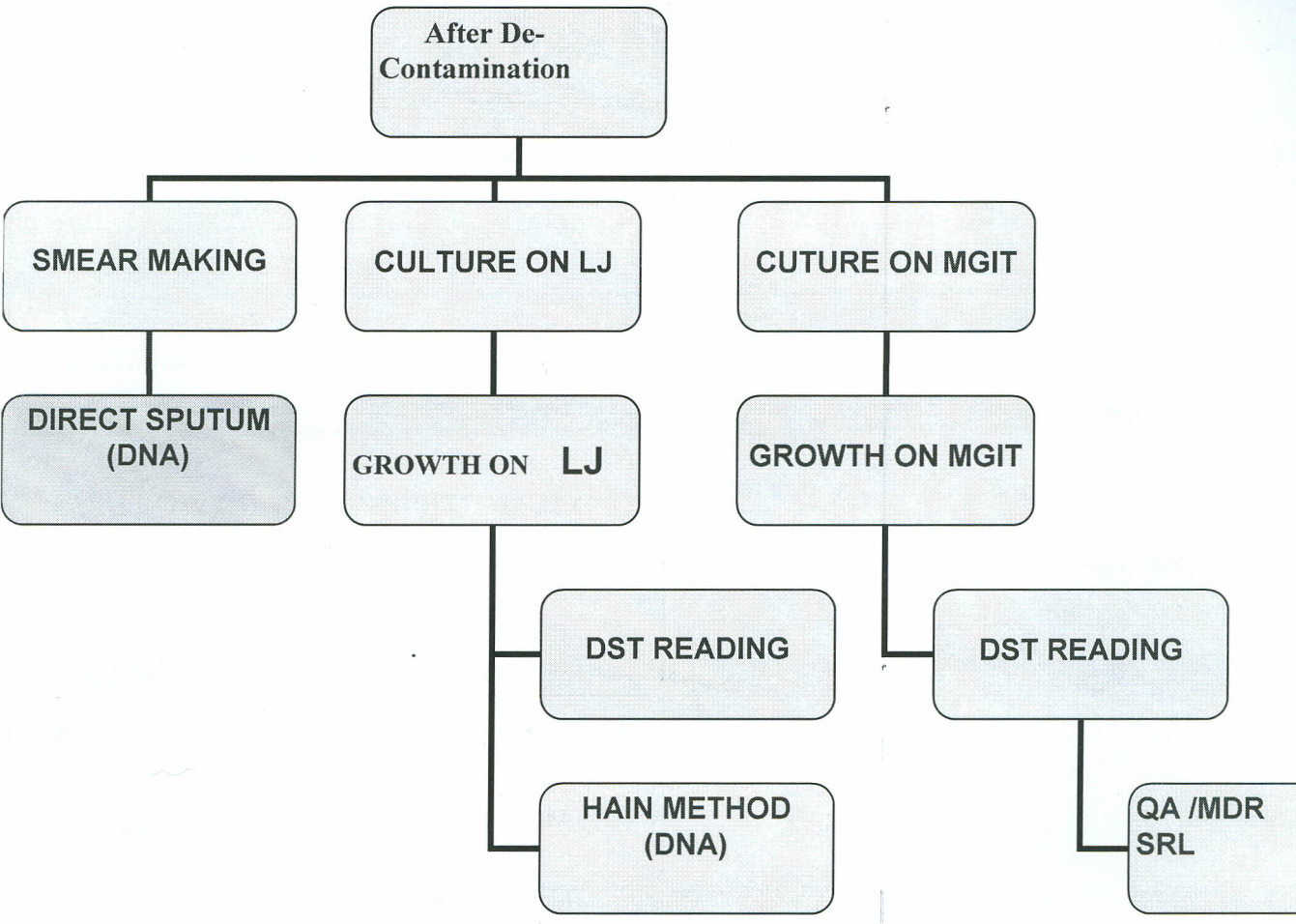
*Quality assurance* with regard to tuberculosis culture is a system designed to continuously improve the reliability, efficiency and use of culture as diagnostic and monitoring option. The purpose of a quality assurance programme is to improve the efficiency and reliability of culture services.

Quality control of culture is a process of effective and systematic *internal* monitoring of the performance of bench work in the culture laboratory. Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. This is accomplished by assessing - against acceptable established limits - the quality of specimens, the performance of decontamination,

Digestion and culture procedures, the quality of reagents, media and equipment, by reviewing culture results and by documenting the validity of culture methods.

Appendix 8 :

FLOW CHART ON SPUTUM SPECIMEN



Ministry of Health  
National Leprosy & Tuberculosis Programme

### CULTURE REQUEST FORM

Name:..... Registration No..... OP/IP No.....

Address:..... Age:..... Sex:.....

..... Clinic:..... Ward:.....

Facility:.....

Clinician name:..... Signature:..... Date:.....

Specimen of:..... Date of collection:.....

Examination required: Smear  Culture  Sensitivity

Type of patient: Sputum smear-positive relapse

Sputum smear-negative relapse

Returned after defaulting

Failure 1-st line treatment

Failure re-treatment

**Previous treatment:**

Isoniazid from: date ..... to.....

Streptomycin from: date ..... to.....

Rifampicin from: date ..... to.....

Pyrazinamide from: date ..... to.....

Ethambutol from: date ..... to.....

Other (specify) from: date ..... to.....

## Appendix

## REPUBLIC OF KENYA



## MINISTRY OF SCIENCE AND TECHNOLOGY

Telephone: +254020318581  
 Facsimile: +254020251991  
 Email: @scienceandtechnology.go.ke  
 Website: www.scienceandtechnology.go.ke

OFFICE OF THE PERMANENT SECRETARY  
 JOGOO HOUSE "B"  
 HARAMBEE AVENUE  
 P.O. Box 9583 00200  
 NAIROBI

REF: MOST13/001/37C792/2

21<sup>ST</sup> November 2007

JOSPHINE WAHOGO MBURU  
 KENYATTA UNIVERSITY  
 P.O BOX 43844  
 NAIROBI

Dear Madam,

**RE: RESEARCH AUTHORIZATION**

Please, refer to your application for authority to conduct research on "Isolation of Recurrent Tuberculosis Isolates from sputum smear negative relapse at CRL, Kenya". This is to inform you that you have been authorized to conduct research at the Kenyatta National Hospital for a period ending 28<sup>th</sup> February, 2008.

You are further advised to report to the Director, Kenyatta National Hospital before commencing your study.

On completion of your research, you are expected to submit two copies of your research report to this office.

Yours faithfully

A handwritten signature in black ink, appearing to read 'M. Ondieki'.

**M.O. ONDIEKI**  
**FOR: PERMANENT SECRETARY**

CC:

THE DIRECTOR  
 KENYATTA NATIONAL HOSPITAL  
 NAIROBI



Ref: KNH-ERC/ 01/ 206

**KENYATTA NATIONAL HOSPITAL**

Hospital Rd. along, Ngong Rd.

P.O. Box 20723, Nairobi.

Tel: 726300-9

Fax: 725272

Telegrams: MEDSUP", Nairobi.

Email: [KNHplan@Ken.Healthnet.org](mailto:KNHplan@Ken.Healthnet.org)

27<sup>th</sup> February 2008

Josephine N. Wahogo  
Dept. of Public Health  
Kenyatta University

Dear Josephine

RESEARCH PROPOSAL: "ISOLATION OF RECURRENT TUBERCULOSIS ISOLATES FROM SPUTUM SMEAR NEGATIVE RELAPSE AT THE CENTRAL REFERENCE TUBERCULOSIS LABORATORY, NAIROBI, KENYA"  
(P328/11/2007)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and **approved** your above revised research proposal for the period 27<sup>th</sup> February 2008 – 26<sup>th</sup> February 2009.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimen must also be obtained from KNH-ERC for each batch.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

**PROF A N GUANTAI**  
**SECRETARY, KNH-ERC**

c.c. Prof. K.M.Bhatt, Chairperson, KNH-ERC  
The Deputy Director CS, KNH  
Supervisors: Prof. A.S.S. Orago, National AIDS Control Council  
Prof. E.W. Kabiru, Dept. of Pathology, Kenyatta University