VALIDATION OF GENE XPERT AND LAM USING URINE FROM HIV PATIENTS WITH CLINICAL SIGNS FOR DIAGNOSIS OF TUBERCULOSIS ATTENDING THE MOI TEACHING AND REFERRAL HOSPITAL ELDORET KENYAN

BY

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A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD) IN BIOMEDICAL SCIENCE AND TECHNOLOGY (MEDICAL BIOTECHNOLOGY) IN THE DEPARTMENT OF BIOMEDICAL SCIENCES AND TECHNOLOGY

MASENO UNIVERSITY

DECLARATION

DECLARATION BY THE CANDIDATE

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DEDICATION

This work is dedicated to my family.

Abstract

Tuberculosis (TB) and HIV co-infections have a global prevalence with high morbidity and mortality and Africa is the worst hit. The HIV and AIDS has profound impact on the TB epidemic in Kenya, where up to 60% of TB patients are likely to be HIV co-infected and the mortality rate attributed to TB. Lack of diagnostic capacity has been a major barrier preventing an effective management of HIV-associated and drug resistant tuberculosis (TB). Microscopy is the gold standard for TB diagnosis and have various limitations. Molecular diagnostic methods are more specific and sensitive and have been applied in TB diagnosis. The *Mycobacterium tuberculosis*/Rifampin (Xpert MTB/RIF) assay is a rapid diagnostic tool that can simultaneously identify *M. tuberculosis* DNA and resistance to rifampin by nucleic acid amplification technique (NAAT). Urine lipoarabinomannan (LAM) antigen strip test is a rapid diagnostic tool that can detect LAM in antigenic form in the urine in patients with active TB. There is need to generate data to support recommendations for use of the assays for testing non-sputum clinical samples in African population especially in Western Kenya where currently there is a big problem in TB management. Urine as a biological sample for diagnostic testing is easy to collect, readily available and has a low infection risk to staff during collection. This study determined the optimal parameters (Zn, CD4+, Hb, Creatinine, proteinuria, hematuria), specificity and sensitivity for urine Xpert MTB/RIF assay and LAM strip test for diagnosis of tuberculosis using urine from HIV patients with active TB. This was a cross-sectional study in which 157 study participants were prospectively recruited. Normality tests were conducted using Shapiro and Wilks methods. The test for association between categorical variables was conducted using Pearson's Chi Square test. For skewed variables, a two sample Wilcoxon rank sum test was used. Cohen's kappa statistic (k) test was used to measure the level of agreement between the Xpert MTB/RIF and LAM determine strip test. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Expert MTB/RIF and LAM strip test were evaluated against the sputum microscopy method as a gold standard. About 55% were TB positive by sputum microscopy, Gene Xpert had 17(11%) of the patients positive with 94% and 6% being MTB detected low and medium, respectively. LAM strip test showed that 28% of the patients were TB positive. Radiology had 45% of the patients with infiltrates. Protein or blood in urine was significantly associated with TB positivity based on LAM strip determine test (48% vs 24%; p=0.021). The weighted kappa coefficient was 0.48 (95% CI=0.32-0.63; p<0.0001). Sensitivity of urine Xpert MTB/RIF against Sputum microscopy was 17%, with a positive predictive value of 29%. While the specificity of urine Xpert MTB/RIF against Sputum Microscopy was 91%, with a negative predictive value of 83%. Stratified by CD4 categories, the test was more sensitive (30% vs. 11%) and specific (92% vs. 89%) to CD4≤100 cells/mm^3 compared to CD4>100 cells/mm^3, respectively. Sensitivity and specificity of urine Gene Xpert MTB/RIF test compared to LAM strip test increased to 39% and 100%, respectively. The sensitivity of LAM determine strip test against Sputum Microscopy was 38%, with a positive predictive value of 25% while the specificity of LAM determine strip test against Sputum Microscopy was 74%, with a negative predictive value of 84%. Sensitivity increased to 60% for those with CD4≤100 cells/mm^3 whereas the specificity slightly increased to 76% for those with CD4>100 cells/mm^3. The comparison of urine LAM determine strip test against urine Xpert MTB/RIF and urine culture were similar and it increased the sensitivity and specificity to 100% and 81%, respectively. The plotted receiver operating characteristic (ROC) curve yielded an area under the curve (AUC) of 0.56 (95% CI=0.46-0.66) indicating that urine MTB/RIF® is not accurate when used alone. This study recommends that urine Xpert MTB/RIF® is a sensitive and specific tool for diagnosis of TB taking Zn, CD4 count, Proteinuria, and Hematuria as optimal parameters. Both Xpert MTB/RIF® and LAM can be used as an adjunct test for diagnosis of active TB using urine samples in combination with other tests in the diagnosis platform.

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LIST OF ABBREVIATIONS & ACRONYMS

AFB	Acid-Fast Bacilli
AG	Antigen
AMPATH	Academic Model Providing Access to Healthcare
ART	Antiretroviral Treatment
BCG	Bacillus of Calmette and Guerin
CD	Cluster of Differentiation
CI	Confidence Interval
CXR	Chest X-ray
DR	Drug-Resistant
DST	Drug Susceptibility testing
EPTB	Extra Pulmonary Tuberculosis
HIV	Human Immunodeficiency Virus
IREC	Institutional Research and Ethics Committee
LAM	Lipoarabinomannan
LTBI	Latent Tuberculosis Infection
MDR TB	Multidrug Resistant Tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
MHC	Major Histocompatibility Complex
MODS	Microscopic Observation Drug Susceptibility Assay
MTB	*Mycobacterium tuberculosis*
MTRH	Moi Teaching and Referral Hospital
NAATs	Nucleic Acid Amplification Tests
NPV	Negative predictive value
NTM	Non-tuberculosis mycobacterium

OD	Optical density
PCR	Polymerase Chain Reaction
PPD	Purified Protein Derivative
PPV	Positive predictive value
POC	Point-of-care
PTB	Pulmonary tuberculosis
RIF	Rifampicin
ROC	Receiver Operating Characteristic
RpoB	RNA polymerase ß
rRNA	ribosomal ribonucleic acid
SPC	Sample processing control
SOP	Standard operating procedures
SN	Sputum negative
SS	Sputum scarce
TB	Tuberculosis
TNF	Tumour necrosis factor
TST	Tuberculin Skin Test
WHO	World Health Organization
XDR	TB Extremely Drug Resistant tuberculosis
ZN	Ziehl-Neelsen

DEFINITIONS OF TERMS

Positive predictive value (PPV): The probability that the disease is present when the test is positive.

Negative predictive value (NPV): The probability that the disease is not present when the test is negative.

Sensitivity: The probability that a test result will be positive when the disease is present (true positive rate).

Specificity: The probability that a test result will be negative when the disease is not present (true negative rate).

Multidrug resistance: Multiple drug resistance (MDR), multidrug resistance or multiresistance is antimicrobial resistance shown by a species of microorganism to multiple antimicrobial drugs. The types most threatening to public health are MDR bacteria that resist multiple antibiotics; other types include MDR viruses, fungi, and parasites (resistant to multiple antifungal, antiviral, and antiparasitic drugs of a wide chemical variety).

Gene Xpert MTB/RIF: *Mycobacterium tuberculosis*/Rifampinin assay.

Nucleic acid amplification:nucleic acid amplification test (NAAT) is a molecular technique used to detect a particular pathogen (virus or bacterium) in a specimen of blood or other tissue or body fluid. It does so by detecting and amplifying the RNA or DNA of the pathogen, that is, making extra copies of its nucleic acids.

Sputum: Sputum is a mucousy substance (consisting of cells and other matter) that is secreted into the airways of the respiratory tract. Sputum is not the same as saliva, a substance secreted in the mouth to help with digestion. The terms sputum and phlegm are used interchangeably. The term mucus may sometimes be used, but sputum refers to that mucus specifically secreted in the respiratory tract, whereas mucus may also be produced in the gastrointestinal tract, urological tract, and genital tract.

Microscopy: Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye). There are three well-known branches of microscopy: optical, electron, and scanning probe microscopy. Optical and electron microscopy involve the diffraction, reflection, or refraction of electromagnetic radiation/electron beams interacting with the specimen, and the collection of the scattered radiation or another signal in order to create an image. This process may be carried out by wide-field irradiation of the sample (for example standard light microscopy and transmission electron microscopy) or by scanning of a fine beam over the sample (for example confocal laser scanning microscopy and scanning electron microscopy).

Urine: Urine is a liquid by-product of metabolism in the bodies of many animals, including humans. It is expelled from the kidneys and flows through the ureters to the urinary bladder, from which it is soon excreted from the body through the urethra during urination. Cellular metabolism generates numerous by-products, many nitrogenous (rich in nitrogen), that require clearance from the bloodstream. These by-products are eventually expelled from the body during urination, the primary method for excreting water-soluble chemicals from the body. These chemicals can be detected and analyzed by urinalysis.

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Co-infection of human immunodeficiency virus (HIV) and tuberculosis (TB) is the leading cause of death in patients infected with HIV living in resource-limited countries accessing antiretroviral treatment (ART) programs. In2010, there were 350,000 TB-related deaths in HIV–infected people, most of them in developing countries, and 22.5 million people were estimated to be living with HIV in sub-Saharan Africa (WHO, 2010). Globally, 2.8 million new cases of tuberculosis patients with HIV were reported as 30% in Africa, the majority in the sub-Saharan region; and 37% and 48% in Kenya (WHO, 2010).

Human immunodeficiency virus infection increases the risk of developing TB and also are twice as likely to experience sputum smear–negative pulmonary tuberculosis (PTB) than HIV-uninfected patients due to the fact that human immunodeficiency virus modifies the clinical presentation of the disease (Corbett, 2003; Moore, 2007). Extra pulmonary tuberculosis (EPTB) is also more common in HIV-positive patients (Bassett, 2010), which contributes to delayed TB diagnosis, leading to high mortality, and represents an important burden for health systems. The problem is increased in resource-limited settings that do not have other highly sensitive diagnostic tests (Cohen, 2010; Getahun, 2011; Lawn *et al*, 2005).

Alteration in the clinical and radiographic presentation of PTB among HIV-infected persons has long been recognized but effort to improve the diagnosis of TB in HIV-infected persons have not been easy due to lack of diagnostic tools with high sensitivity and easy to use (Dawson, 2010; Den Boon, 2005;Lawn, Kranzer *et al* 2011).

Direct smears can be used but they are often negative and do not differentiate *Mycobacterium tuberculosis* from non-tuberculous mycobacterium (Lawn *et al*, 2011). Culture, which is more sensitive, may take 2 to 8 weeks due to the slow growth rate of *Mycobacterium* while liquid culture may take 7-10 days (Monkongdee, 2009). Therefore there is a great need for implementing new diagnostic methods for tuberculosis to increase the sensitivity and speed of diagnosis in these patient groups especially in view of their high mortality and the risk of nosocomial transmission (Reid, 2009).

A range of new diagnostics for TB, employing different technologies (Gopinath, 2009) are now emerging. One area of renewed interest is analysis of urine samples (Green, 2009; Lawn *et al*, 2011; Lawn *et al*, 2009; Pai, 2009). Urine has many characteristics, which make it a potentially useful specimen for TB diagnosis. It is simple to obtain, even from very ill patients who may not be able to produce sputum. Urine sampling does not generate hazardous infectious aerosols and is relatively 'clean' and easy to handle in the laboratory. Urine may be cultured, tested by polymerase chain reaction (PCR) for mycobacterium transrenal DNA or tested for specific mycobacterial antigens such as lipoarabinomannan (LAM) (Boehme *et al*., 2010). Recent studies have shown that urine LAM may have diagnostic value in HIV-infected patients with low $CD4^+$ counts (Lawn *et al.*, 2012) but there is no data on the diagnostic utility where a sputum sample is compared to urine (Hillemann, 2011; Lawn *et al*., 2011;WHO, 2011). Sensitivity and specificity of urinary LAM have not be extensively been evaluated especially in sub-Saharan Africa where the burden of TB in immuno-compromised patients is high.

Recently, the Gene Xpert MTB/RIF rapid molecular assay (Boehme *et al*., 2011; Lawn *et al*., 2011) has been endorsed by the Scientific and Technical Advisory Board of the

World Health Organization (WHO) as the most sensitive fast test for TB diagnosis in paucibacillary respiratory (sputum samples) but there is paucity of data in the utilization of non-sputum samples in TB diagnosis (WHO, 2011). However, there are also reports of this diagnostic platform being used to diagnose TB by testing a range of extrapulmonary samples, including urine (Hillemann, 2011; Lawn, 2012).

The current study aimed at finding diagnostic sensitivity of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis using urine specimen to validate the work of Wood and Lawn since Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis using urine specimen need to be validated at different population (Lawn *et al*., 2012; Wood, 2012). Diagnostic accuracy was assessed by comparison with mycobacterial urine culture results. Sputum microscopy, which offered the reference test and compared it with lipoarabinomannan (LAM) determine strip test results and a reference standard consisting of combination of culture, sputum microscopy and clinical diagnosis of TB. The findings of this current study implies that Xpert MTB/RIF technology is a promising tool for rapid detection of tuberculosis in urine samples in individuals with *Mycobacterium tuberculosis* (active TB) prior to starting antiretroviral therapy (ART).

1.2 Problem Statement

Tuberculosis remains a key challenge to global public health. Efforts to tackle this disease have been severely hampered by the need for point-of-care (POC) techniques that use samples that are easy to obtain. In addition to what can help in detecting extra pulmonary TB Diagnosis of extrapulmonary TB (EPTB).

The detection of EPTB remains challenging since MTB bacilli present in tissue at sites of disease is often low and clinical specimens from deep-seated organs are difficult to

obtain (Green, 2009). Histochemical techniques are generally time-consuming to undertake, and establishing a diagnosis of TB with high specificity remains difficult. Tissue microscopy after special staining is often negative and when mycobacteria are seen, it is impossible to distinguish MTB from non-tuberculous mycobacterial disease. This often leads to delays in initiating treatment thus compromising patient care. The United State (US) Centre for disease control (CDC) recommends that nucleic acid amplification tests be performed on at least one respiratory specimen from each patient with signs and symptoms of pulmonary TB. However, no recommendation exists for their use in the investigation of patients suspected of having EPTB as evidence base is limited. This study was carried out in western Kenya where mortality due to HIV/TB co-infection is high as a result of delayed diagnosis. The aim of the current study was in an attempt to identify a diagnostic tool which is rapid and capable of detecting both *M. tuberculosis* complex and rifampin resistance and which is easy to use in the population.

1.3 The objectives of the Study

1.3.1 Broad objective

To validate Gene Xpert and LAM strip using urine from HIV patients with clinical signs diagnostic of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenyan.

1.3.2 Specific objectives

1. To determine the optimal parameters (Zn, CD4+, Hb, Creatinine, proteinuria, hematuria) for Gene Xpert MTB/RIF and LAM strip test for diagnosis of tuberculosis using urine from HIV patients at the Moi Teaching and Referral Hospital (MTRH) in western Kenya.

2. To establish specificity and sensitivity of Gene Xpert MTB/RIF technique using urine from HIV positive patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenya.
3. To determine the specificity and sensitivity of LAM strip test technique using urine from HIV positive patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenya.

1.4 Research Questions

1. What are the optimal parameters for Gene Xpert MTB/RIF and LAM strip test techniques for diagnosis of tuberculosis using urine from HIV patients at the Moi Teaching and Referral Hospital (MTRH) in western Kenyan?
2. What is the specificity and sensitivity of Gene Xpert MTB/RIF and LAM strip test techniques using urine from HIV patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenyan?
3. What is the specificity and sensitivity of LAM strip test techniques using urine from HIV patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenyan?

1.5 Significance of the Study

The findings of the present research fill a critical gap in the existing knowledge of the use of Gene Xpert in diagnosis of extrapulmonary tuberculosis in HIV patients using urine sample. Present study findings, indicate that urine Xpert MTB/RIF® is a sensitive and specific tool for diagnosis of tuberculosis using urine samples and that Zn, CD4 count, Proteinuria, and Hematuria are the optimal parameters for its validation and should be included in the routine monitoring of TB in HIV infected patients. The study findings especially on the urinary LAM are already being implemented by AMPATH

to diagnose patients who report with signs of tuberculosis. The study finding also indicated that there is improved performance in TB diagnosis when urine Xpert MTB/RIF®is combined with urine LAM.

Given the heavy burden TB places on our health care systems, better tests are needed to improve the detection of TB among asymptomatic individuals since early and accurate diagnosis leads to better prognosis.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiological Burden of Tuberculosis

Tuberculosis (TB) is an infectious disease caused by different kind organism, which belong to the genus *Mycobacteria*. Together with human immunodeficiency virus infection (HIV), Acquired Immunodeficiency Syndrome (AIDS) and malaria, Human TB is one of the biggest killers in the Word (Rodríguez-Morales, 2008).

TB is a disease that usually affects both humans and animals. It is caused by *Mycobacterium tuberculosis*, which is also known as the Koch's bacilli. *Mycobacterium tuberculosis* can affect any organ or tissue in the body. In some cases due to *M. bovis*, pulmonary disease can occur, with the hallmarks being cough with expectoration lasting more than 15 days (Castaneda- Hernandez, 2012a; Rodríguez-Morales, 2008). In such cases, previous to a microbiological diagnosis, individuals in such state are so-called respiratory symptomatic.

Mycobacterium tuberculosis belongs to the genus *Mycobacterium* that includes more than 50 other (Castañeda-Hernández, 2012a) species, often collectively referred to as non-tuberculous mycobacteria. Human TB is defined as a disease caused by members of the *M. tuberculosis* complex, which includes the tubercle bacillus: *M. tuberculosis*, *M. bovis*, *M.africanum*, *M. microti*, *M. canetti*, *M.caprae*, and *M. pinnipedii* (Kassim, 2004).

One distinguishing feature of the organisms belonging to the genus *Mycobacterium* is their cell envelope. Unlike gram-negative bacteria, there is no true outer membrane in *Mycobacterium*. The mycobacterial cell envelope is composed of a core of three macromolecules covalently linked to each other (peptidoglycan, arabinogalactan, and mycolic acids) and a lipopolysaccharide, lipoarabinomannan (LAM), which is thought

to be anchored to the plasma membrane (Camus, 2002). Mycolic acid, which is a beta-hydroxy fatty acid, is the major constituent of the cell envelope, accounting for more than 50 percent by weight; this structure defines the genus (Murray, 2005). Glycolipids are attached to the outside of the envelope layer through a connection to the mycolic acid layer; proteins are also embedded in this cell wall complex (Chany, 2003). Glycolipid components are implicated in "cord formation", whereby the bacilli clump together forming a serpiginous structure seen on microscopy (Wooldridge, 2009). These cell wall components give *Mycobacterium* its characteristic staining properties. The organism stains positive with Gram's stain. The mycolic acid structure confers the ability to resist distaining by acid alcohol after being stained by certain aniline dyes, leading to the term acid-fast bacillus (AFB).

Worldwide, microscopy to detect AFB (using Ziehl-Neelsen or Kinyoun stain) is the most commonly used procedure to diagnose TB (Getahun, 2007). However, a specimen must contain at least 10^4 colony forming units (CFU)/mL to yield a positive smear.

Microscopy of specimens stained with a fluorochrome dye, such as auramine O provides an easier, more efficient, and therefore more sensitive alternative. However, microscopic detection of mycobacteria does not distinguish *M. tuberculosis* from nontuberculous mycobacteria. Tuberculosis disease can also be manifested with hemoptysis, fever, night sweating general malaise, thoracic pain, anorexia and weight lost. This disease is still a significant public health problem due to highly transmissibility, but is highly potentially preventable and treatable condition (Curto, 2010; Dim, 2011; Orcau, 2011; Marais, 2010; Glaziou, 2009).

Tuberculosis is a human threat and still is a significant public health problem in the world, particularly in developing countries. Together with the burden of infection due to HIV, this coinfection drives most of the TB morbidity and mortality in many regions

(e.g. Africa) and makes more complicated its control and reduction in many terms. According to the World Health Organization (WHO) Global Tuberculosis Control Report 2011 (TB worldwide), in 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35million (range, 0.32–0.39 million) deaths from HIV-associated TB (World Health Organization, 2011).

2.2 Tuberculosis in Developing Countries

Tuberculosis is a disease present due to the increase of HIV co-infection in most countries in the world (WHO, 2012). Until two to three decades ago, TB was decreasing in importance in the world and was predominantly endemic in developing countries. However, after the origin of the AIDS pandemic in June 1981, TB has been increasing again in importance not only in these countries but also in developed countries such as North America and Europe. Besides this, the multiple problems that countries with a high burden of TB have to face are major in developing countries such as in Africa, Asia and Latin America. This is so especially when TB is also associated to other conditions and diseases, such as HIV, comorbidities, poverty and when living in resource-constrained areas where diagnosis and treatments not prompt as in other areas (WHO, 2012).

Global burden of TB in the world is estimated at 8.8 million (range, 8.5–9.2 million)

Incident cases of TB as shown in table below (Table 2.1) for rates ranging 123.7 to 133.9 cases/100,000 population; however, this can be very different among the WHO

Table 2.1:Estimated epidemiological burden of incidence TB, according to regions by the WHO in 2010 (World Health Organization, 2011).
298.7 cases/100,000population.

Incidence (cases)					Incidence rates (cases/ 100,000pop)		
Region	**Population**	**Best**	**Low**	**High**	**Best**	**Low**	**High**
Global burden	6,869,573,000	8,800,000	8,500,000	9,200,000	128.1	123.7	133.9
WHO African Region	836,970,000	2,300,000	2,100,000	2,500,000	274.8	250.9	298.7
WHO South-East Asia Region	1,807,594,000	3,500,000	3,200,000	3,700,000	193.6	177.0	204.7
WHO Eastern Mediterranean Region	596,747,000	650,000	580,000	730,000	108.9	97.2	122.3
WHO Western Pacific Region	1,798,335,000	1,700,000	1,500,000	1,800,000	94.5	83.4	100.1
WHO European Region	896,480,000	420,000	390,000	450,000	46.8	43.5	50.2
WHO Region of the Americas	933,447,000	270,000	250,000	280,000	28.9	26.8	30.0

Rates of incidence are important because they can show the real population problem of the disease. For example in South East Asia there are more crude number of cases of TB, estimated at 3.5 million (range, 3.2–3.7 million) incident cases. However, the incidence rates are quite lower than in Africa, at 177.0 to 204.7 cases/100,000 population.

The prevalence of TB is still difficult to estimate in some countries and in many cases are not reported. According to WHO, for 2010 the prevalence was estimated at 12.0 million (range, 11.0–14.0 million) cases (Table 2.2) for rates ranging 160.1 to 203.8 cases/100,000 population (Table 2.2). However this can be very different among the WHO regions in the world, being higher in Africa where those estimates can reach 274.8 to 394.3 cases/100,000 population (Table 2.2).

Table 2.2: Estimated epidemiological burden of prevalent of TB, according to regions by the WHO in 2010

	Prevalence (cases)				Prevalence rates (cases/ 100,000pop)		
Region	**Population**	**Best**	**Low**	**High**	**Best**	**Low**	**High**
Global burden	6,869,573,000	12,000,000	11,000,000	14,000,000	174.7	160.1	203.8
WHO African Region	836,970,000	2,800,000	2,300,000	3,300,000	334.5	274.8	394.3
WHO European Region	896,480,000	2,500,000	2,200,000	2,800,000	278.9	245.4	312.3
WHO Western Pacific Region	1,798,335,000	5,000,000	3,700,000	6,500,000	278.0	205.7	361.4
WHO Eastern Mediterranean Region	596,747,000	1,000,000	670,000	1,500,000	167.6	112.3	251.4
WHO Region of the Americas	933,447,000	330,000	260,000	410,000	35.4	27.9	43.9
WHO South-East Asia Region	1,807,594,000	560,000	430,000	720,000	31.0	23.8	39.8

In Africa, up to 65% of active tuberculosis (TB) cases are co-infected with HIV (WHO, 2011). Tuberculosis related mortality is highest in this patient sub-group, and district-level hospitals are inundated with patients with advanced immunosuppressant. With advancing HIV-related immunosuppression, the frequency of extra-pulmonary (EPTB) and disseminated forms of TB disease increase (Reid, 2009), sputum smear microscopy performance is reduced, and up to a third of patients are unable to produce sputum for diagnostic testing (Peter, 2012). Diagnosis is therefore challenging and often delayed, and post-mortem studies reveal a large burden of undiagnosed TB in HIV-infected hospitalized patients (Cohen, 2010; Cox, 2010). Recent studies have indicated that the rapid initiation of anti-TB treatment may reduce mortality (Holtz, 2011). There is a clear need for new, accurate, and rapid TB diagnostics that have utility in patients who cannot produce sputum.

In Kenya, from the TB Epidemiological and Impact Analysis Report of 2014, various trends were noted. Analysis of the trends in estimates of TB incidence suggests a consistent decline in new TB cases over time. The decline in TB cases started in 2005

following the decline in TB/HIV cases, which started in 2004. Furthermore, after a peak in 2006, the TB prevalence declined and thereafter plateaued from 2009 (NTLD Program Annual Report 2013). Tuberculosis mortality estimates suggest an increase in TB deaths in 2011-2012.

Data on TB prevalence and mortality are sparse in Kenya. Kenya has not conducted a national TB prevalence survey in the recent past (the last TB prevalence survey was conducted in 1956), but the NTLD Program was planning to carry out a prevalence survey in 2015 (NTLD Program Annual Report 2013).

There is currently no national level vital registration system with current status of the TB burden and the effectiveness of TB control interventions. Less than half of deaths are recorded. Results from a prevalence survey and vital registration systems if were available would provide data on the current status of the TB burden and the effectiveness of TB control interventions.

The TB case notification showed an upward trend from 2003 to 2007, when it peaked with 116,000 cases in Kenya. Thereafter, it has steadily declined, reaching an all-time low of 89,000 in 2013. This may be explained by better TB and HIV control efforts, as well as the recent introduction of the electronic case based surveillance. An inventory study would, however, be necessary to confirm this sharp decline.

2.3 Tuberculosis Control

2.3.1 Tuberculosis control at global level

Tuberculosis was recognized as a major global public health problem in the early 1990s, when the World Health Assembly adopted a resolution calling for increased efforts to control the diseases. The resolution set two major targets for global action against TB: the detection of 70% of new smear-positive cases, and cure of 85% of such cases. In 1994, the internationally recommended control strategy, directly observed

therapy short-course (DOTS), was launched, followed in 1998 by the Stop TB Initiative. In 2000, this became the Stop TB partnership, a global network of international organizations, countries, sponsors, non-governmental organizations and other interested parties committed to controlling and eventually eliminating TB. The first Stop TB partners' Forum, held in Washington in 2001, launched a Global plan to Stop TB for the period 2001-05. Subsequently, a plan for 2006-15 was launched in January 2006, and more recently an updated version for the period 2011-15 has been developed. These global plans form the overarching framework for the Stop TB partnership's combined action (NTLD Program Annual Report 2013).

2.3.2 The Millennium development goals and stop TB strategy

The adoption of the Millennium development goals (MDGs) by the global community in 2000 provided renewed impetus for TB control efforts (WHO, 2008). It calls for action to combat HIV and AIDS, malaria and other diseases, including TB (WHO, 2008). Widespread implementation of the DOTS strategy led to significant progress in control of the disease. For example, by 2007 the treatment success rate was among new smear-positive cases had reached 86%, while in 2008, the global case detection rate was 61% (UN, 2008). However, by 2005 it had become clear that DOTS alone would not be sufficient to achieve global TB elimination. In 2006, therefore, the Stop TB strategy was launched, and it was designed to meet both the TB-related MDGs targets and the Stop TB partnership targets for 2015 (WHO, 2006). The Stop TB strategy underpins the Global plan Stop TB 2006-2015 (WHO, 2008).

2.3.3 The current status of tuberculosis in Kenya

Kenya is one of the 22 high burden TB countries that together account for more than 80% of the world's TB cases. World health organization (WHO) estimated that there

were 120,000 new cases of TB in Kenya in 2012. The estimated 9,500 (5,400-15,000) deaths due to TB make it the fourth leading cause of mortality in the country. Since 2006, a gradual decline in case notification has persisted, suggesting that incidence may be declining following years of high treatment success, currently at over 88% (NTLD Program Annual Report 2013). Case detection has been enhanced through community engagement, inclusion of the private sector, intensified case finding, pro-poor enablers such as nutritional support, TB and HIV collaborative activities, and an increased focus on identifying TB in children (NTLD Program Annual Report 2013).

Human immunodeficiency virus and Acquired immunodeficiency syndrome continues to be an important driver of the TB epidemic in Kenya, with approximately 37% of patients with TB also living with HIV. Tuberculosis related deaths among people living with HIV have declined from a high of 12% in 2004 to 5% in 2012, as access to anti-retroviral therapy (ART) and cotrimoxazole preventive therapy (CPT) have increased. Approximately 74% of TB patients co-infected with HIV were initiated on HAART in 2012. Nearly all (98%) HIV infected TB patients were initiated on CPT in the same year (NTLD Program Annual Report 2013). Programmatic Management of Drug-Resistant TB (PMDT) was initiated in 2007. In 2013, 254 cases of multi-drug resistant TB (MDR-TB) were identified and started on treatment compared to 60 in 2007. Twenty eight percent of notified MDR-TB cases occurred among refugees residing in Kenya. The Kenyan government made an important humanitarian and public health decision to manage these cases with the resources and infrastructure of the Ministry of Health (NTLD Program Annual Report 2013). The WHO currently estimates that there are 2,750 cases of MDR-TB in the country (WHO, 2008. A drug-resistance survey is currently ongoing to define the estimates of prevalence of DR-TB in the country (NTLD Program Annual Report 2013).

Sustained political commitment for TB has been fundamental to the success of the NTLD Program.

Kenya is the first country in Africa, region to reach WHO targets for both TB case detection and treatment success. The NTLD Program has successfully managed a high quality program through a cascade of TB coordinators at decentralized levels (NTLD Program Annual Report 2013). A network of trained and skilled health workers has consistently enabled the rapid uptake of new policies and technologies, while also providing the platform for supportive supervision to address operational challenges on a systematic basis. The full integration of TB service provision into the primary care system has enabled mentorship and decentralized touch points for coordination with community based organizations and care providers (NTLD Program Annual Report 2013).

Kenya maintains a policy of evidence-based strategy development and program implementation. Having the first real-time electronic case-based surveillance system is evidence of the desire for data for action. New approaches, such as the engagement of all care providers and collaborative TB and HIV activities have been successfully scaled up rapidly in Kenya due to the cascade system and the use of evidence of their effectiveness to achieve buy-in at all levels (NTLD Program Annual Report 2013).

2.3.4 Trends in notified TB cases in Kenya (2003-2013)

The TB case notification patterns vary across reporting zones. A majority of zones experienced gradual decline in notified cases from 2007-2012 (e.g. Western, Nyanza South, Nairobi South) while on the contrary, a few zones showed an increase. They included South Rift Valley and North Eastern, especially those harboring refugee groups. Some zones like Nyanza North and Nairobi North had variable case

notification reporting (NTLD, 2013). These stark differences are likely evidence of data management problems. Males had higher TB case notification rates than females among all age groups, except for children (0-15 years) and young adults (15-24 years), (NTLD, 2013).

The NTLD Program has continued to successfully screen about 93% of all notified TB cases for HIV. The current prevalence of HIV among notified TB cases is about 37% (NTLD, 2013). The HIV prevalence among notified TB cases is also 37% but varies by region. Zones with higher HIV prevalence are associated with higher co-infection rates. Prisons contributed to about 1% of the notified TB cases in 2013. Surveillance for DR-TB among retreatment cases has led to increased reporting with 290 cases notified in 2013. Refugees constituted 28% of the MDR-TB cases detected in 2013 (NTLD, 2013). The relative numbers of new bacteriologic ally confirmed (smear positive) cases and extra pulmonary TB cases have remained fairly consistent over time (NTLD, 2013). From 2003 to 2012 the proportion of new cases that were bacteriologic ally confirmed ranged from 37.3 – 43.0%, while the proportion of new extra pulmonary cases increased gradually since 2003, but maintained a narrower range: 15.1% to 18.2%. For the past five years, the proportion of retreatment cases has remained just below 10% of all notified TB cases (NTLD, 2013). Data on TB in other high-risk populations and in patients with underlying comorbidities is not readily available (NTLD, 2013).

2.3.5 Framework action plan to fight TB in Kenya

Within the context of a newly devolved health system, the goal of the 2015-2018 national strategic plan (NSP) is to accelerate the reduction of TB disease burden through provision of people-centered, universally accessible, acceptable and affordable quality services in Kenya (NTLD, 2013).

The NSP seeks to achieve the following by 2018:Reduce the incidence of TB by 5% by 2018, compared to 2014 by Increase case notification of new cases to 85% of estimated prevalence, Increase case notification of MDR-TB to at least 75% of estimated prevalence (Baseline TBD: DR Survey), Increase treatment success rate to 85% among all HIV-infected TB patients, Ensure treatment success of at least 90% among all drug – susceptible forms of TB, Increase treatment success rate to at least 80% among all cases of DR-TB and Reduce case fatality among HIV-infected TB patients to <5% (NTLD, 2013).

2.4 Challenges of TB diagnosis

Tuberculosis can occur at any stage of HIV disease, and its manifestations depend largely on the level of immunosuppression. Early during HIV disease, symptoms and signs are similar to those in HIV-uninfected persons: the lungs are most commonly affected, with cough, fever, and respiratory signs along with radiographic lesions, often with cavitations. On the other hand, extra pulmonary sites are more often involved among patients with immunosuppression, and pulmonary TB resembles primary disease (lymph node enlargement, military disease, and minimal parenchymal lesions). Common extra pulmonary sites include lymph nodes (superficial) and pleura; less commonly, the brain, pericardium, meninges, and abdomen are affected. In general pulmonary TB in HIV-infected patients bears many similarities to childhood TB; both are paucibacilliary, involve hilar and mediastinal lymph nodes, lack cavitation, and are smear negative. Diagnostic tests for TB in this population need to be not only more sensitive but also applicable to sites other than pulmonary sites.

Furthermore, physicians caring for HIV-infected need to consider tuberculosis in the differential diagnosis of many different symptom complexes and also screen for

tuberculosis regularly. In fact, active case findings give high yields when implemented in clinics that treat HIV-infected persons, including antenatal women (Gupta *et al.,* 2007; Sutton *et al.,* 2009; Shah *et al.,* 2009). Clinical algorithms often have high sensitivity but poor specificity, and the WHO has recommended using an algorithm that emphasizes the use of chest x-ray examination and sputum culture early in the evaluation (Were *et al.,* 2009). None response to a course of broad-spectrum antibiotics, such as amoxicillin or the combination of sulfamethoxazole and trimethoprim, could be used as supportive evidence, but this is often complicated because partial response of cough or fever is common even with underlying TB. Efforts should be made to confirm the diagnosis. The following investigations may be performed depending on the indication, cost, and availability:

2.4.1 Direct smear microscopy detection

Direct smear microscopy also known as Acid-fast microscopy is still a major tool of TB diagnosis in the majority of high burden settings and in mycobacteriology. Microscopy has the advantage of being quick, inexpensive, and specific method for TB diagnosis in most setting with a sensitivity of up to 80% in immunocompetent patients (Steingart *et al.,* 2007). It has a specificity of over 99% for mycobacterium spp. It requires standard reagents with long shelf-lives and it can be applied successfully in any laboratory. However, it has several drawbacks including: i) declined sensitivity (as low as 20%) in HIV co-infected patients, hence providing false negative in 25%-50% of patients with active TB (Kim *et al.,* 1984; Gordin and Slutkin, 1990). ii) very low performance in paucibacillary EPTB (<5% sensitivity in pleural, CSF and pericardial fluids), and iii) does not allow for drug susceptibility testing (Steingart *et al.,* 2007; Kwan, 2011).

Despite extensive efforts to optimize smear microscopy, including the use of concentration, bleaching, fluorescent staining and LED microscopy, performance remains sub-optimal in high HIV prevalent settings (Wilson *et al.,* 2006). Some studies have shown that HIV-infected patients are more likely to have smear-negative pulmonary or extrapulmonary disease (Reid, 2009; Harries, 2006). A wide range of acid-fast smear positivity has been reported (31 to 81 percent) (Steingart *et al.,* 2006). In a study from South Africa among 584 HIV-infected patients, only one-third of the 116 positive cultures were smear-positive (Hassim *et al.,* 2010). In a study from Tanzania, a minority of patients who required treatment for suspected TB had positive microbiology (AFB smear or culture) (Bakari *et al.,* 2008). Smear-negative pulmonary TB occurs more commonly in HIV-infected patients because of their lower prevalence of pulmonary cavities (Garay, 1995). The yield on sputum culture is substantially higher (85 to 100 percent), since culture can detect as few as ten bacteria per mL of sputum (Reid, 2009).

In patients infected with HIV, a positive smear for acid-fast bacilli (AFB) is very specific for Mycobacterium TB, even in a setting with a high incidence of *Mycobacterium avium* complex (MAC), which will stain similarly. At San Francisco General Hospital, for example, 248 of 271 (92 percent) expectorated sputum samples that were positive for AFB grew Mycobacterium TB on culture (Yajko *et al.,* 1994). This value is comparable to that found in HIV-negative patients. Hence it is not an adequate criterion.

2.4.2 Chest radiography

Chest X-ray (CXR) also known as chest radiography, is mostly used as the diagnostic tool for TB and is available to clinicians at hospitals in high and low burden settings

(Wilson *et al.,* 2006). However, the sensitivity and specificity of CXR for TB diagnosis in different settings is highly variable especially when used alone in region with high HIV prevalence.

About 10%-71%of HIV/TB co-infected patients have normal CXR results despite the fact that their culture results are positive (Wilson *et al.,* 2006). Several factors such as HIV status, primary clinic or hospital setting, experience of CXR reader and even the sex of the patient contribute to the variability of the CXR performance (van Cleeff, 2005). In classic pulmonary TB, upper lung fields are typically involved and pulmonary cavitation is very suggestive of the diagnosis. However, as immunity declines, patients are more likely to have atypical radiographic findings, including non-cavitary pulmonary infiltrates with no particular preference for the upper lung fields (Greenberg *et al.,* 1994).

Notably, in HIV co-infected patients who are severely immune-compromised the radiographic appearances of TB can be atypical or absent, with one study showing up to 32% of active TB cases with a normal CXR (Wilson *et al.,* 2006). One study evaluated 133 AIDS patients with TB at a single institution in New York City in which the chest radiograph showed patterns typically of primary TB (36%) (Greenberg *et al.,* 1994). These findings included pleural effusion, intrathoracic lymphadenopathy (mediastinum and hilum), or middle or lower lobe consolidation without cavitation. Patterns compatible with post-primary (reactivation) TB—29 %(Jones *et al.,* 1993; Keiper*et al.,* 1995; Lessnau *et al.,* 1994; Perlman *et al.,* 1997).

Chest radiographs cannot distinguish between current active TB and previously occurred infection. Furthermore, the poor specificity of CXR means that a number of patients are inappropriately treated with anti-TB treatment. The need for a definitive

microbiological diagnosis remains high. Laboratory results must not only be accurate but also available in the shortest time possible.

2.4.3 Mycobacteria tuberculosis culture

Mycobacteria tuberculosis culture is considered the reference standard diagnostic tool for TB (Katoch, 2004). Some studies across Africa showed that culture can detect active pulmonary tuberculosis (PTB) and extra-pulmonary TB that was shown to be negative by smear microscopy (Hepple *et al.,* 2011). However, the culture method is limited by the extremely slow growth rate of the *M. tuberculosis,* taking 4-6 weeks for results to be available, thereby limiting its utility for clinical decision-making and/or delaying the commencement of treatment (Drobniewski *et al.,* 2003). Additionally, in HIV-infected patients often presenting with paucibacillary disease, the diagnostic yield of a single culture is reduced (Katoch, 2004).

Furthermore, the performance of culture requires laboratory infrastructure and trained microbiologist to limit contamination rates (Aziz *et al.,* 2007). Laboratory infrastructure and human resources remain limited in resource poor and high TB burden settings (Peter *et al.,* 2012).

2.4.4 Novel molecular diagnostic tools for TB

Polymerase chain reaction (PCR) methods that identify specific sequences of *M. tuberculosis* DNA or rRNA are exciting new tests for TB diagnosis and are known as nucleic acid amplification tests (NAATs). A number of commercial NAATs are widely available and utilized for TB diagnosis. Polymerase chain reaction has been, very useful in amplifying and detecting DNA fragments of *M. tuberculosis* from uncultured clinical samples such as cerebral spinal fluid (CSF) and urine (Del Portillo *et al.,* 1991) usually in amounts too small to be seen by routine staining techniques. A positive

nucleic acid amplification assay in a patient with smear-positive AFB likely represents TB (Kaplan et al., 2009). A study by Partolli *et al*., (1991), demonstrated that PCR had much higher sensitivity than any other method, such as microscopic visualization or DNA hybridization that were used in the direct detection of *M. tuberculosis* (Del Portillo *et al.,* 1991). However, PCR involves the use of robust infrastructure that requires highly sterile environments, highly skilled personnel and also highly bio-hazard material. Nevertheless, another study by Orallo *et al.,* (2008) also demonstrated the useful and potential role of PCR in the detection of *M. tuberculosis* using EPTB samples such as CSF, pleural fluid, synovial fluid, pericardial fluid, urine, bone marrow aspirate, tissue biopsies etc (Orallo, 2008). In this study, they showed that although PCR had higher sensitivity than most of the methods in the diagnosis of TB, the method had low specificity that was likely to be attributed to false results from non-tuberculous infections and other contaminants (Orallo, 2008). A more rapid user-friendly real-time PCR that can be used with little skill and reduced bio-hazard is currently available yet producing results with higher sensitivity and specificity (Orallo, 2008).

2.4.5 Xpert MTB/RIF

Xpert MTB/RIF assay is a real-time PCR platform with excellent sensitivity, specificity and low indeterminate rate, which can provide a result in less than two hours. The Xpert MTB/RIF assay integrates DNA extraction, genomic amplification, and semi-quantitative detection of *M. tuberculosis* complex and rifampicin (RIF) resistance into a fully automated system (Moure, 2011; Soini *et al.,* 1996). Based on this data, the World Health Organization (WHO) endorsed the Xpert MTB/RIF assay for use in the investigation of pulmonary TB and MDR-TB. The WHO made strong

recommendations that Xpert MTB/RIF should be used for frontline TB diagnosis in HIV-infected patients and MDR-TB suspects (WHO, 2011).

2.4.6 Validating the Xpert MTB/RIF assay for the diagnosis of extrapulmonary Tuberculosis (EPTB)

The World Health Organization (WHO) recently endorsed the implementation of Gene Xpert MTB/RIF assay for national TB programs in developing countries (WHO, 2011). The Xpert MTB/RIF assay is a new fully automated diagnostic molecular test with analytical sensitivity of genome copies of purified DNA and 131cfc/ml of sputum and is also able to detect more than 99.5% rifampicin resistance mutations, an indicator of multidrug resistant TB in less than two hours (WHO, 2011). The Xpert MTB/RIF assay requires minimal biosafety infrastructure and training, and data from controlled clinical validation studies has shown a sensitivity of 92%compared to culture utilizing a single specimen (WHO, 2011). However, these data come from clinical trials, and information about the performance Xpert MTB/RIF assay is desirable before worldwide implementation.

Although PTB is more common than EPTB, extrapulmonary tuberculosis can be presented in up to 40% of HIV-infected patients with tuberculosis (Kingkaew, 2009). Although Xpert MTB/RIF assay was initially validated only for pulmonary specimens, studies have shown that the Xpert MTB/RIF assay can increase more than three times the rapid diagnosis of EPTB compared to microscopy (Kaplan, 2009). Extrapulmonary TB is more common in HIV-infected patients than in the general population regardless of the CD4 lymphocytes count and has been associated with high morbidity and mortality (Kaplan, 2009). This study showed the experience of using Xpert MTB/RIF

assay in Moi Teaching and Referral Hospital, Western Kenya, for the diagnosis of EPTB in a cross-sectional study of HIV-infected patients with low $CD4^+$count.

The Clear view TB LAM ELISA (Alere Medical Innovations, USA) detects LAM antigen in the urine and has recently evolved into a new point-of-care lateral flow test (Alere Determine-TB LAM Ag strip test) (Peter, 2010). Recently, it was found that this assay offered the greatest benefit in hospitalized HIV co-infected patients with advanced immunosuppression (Peter, 2012). The Clear view TB LAM ELISA has been endorsed by the World Health Organization and is being rolled out in South Africa as a frontline test for individuals with suspected TB (WHO, 2010; WHO, 2011). Given the high accuracy of this test in sputum samples (sensitivity and specificity of 90% and 99%) (Boehme *et al.,* 2011), it represents a considerable advance over smear microscopy for the diagnosis of pulmonary-TB. However, acquiring a diagnostic sample remains a major hurdle in HIV-infected sputum scarce patients suspected of having active TB. Sputum induction, using ultrasonic nebulization, may facilitate obtaining sputum, but this is often unavailable in hospitals in resource-poor settings and infection control is a concern. Tissue biopsies and aspirated samples may be obtained from extra-pulmonary disease foci (e.g. bone marrow and liver, pleural and pericardial fluid) but specialized skill and equipment requirements limit the availability and affordability in resource-poor settings. Urine is easily obtainable from sputum scarce patients but there are few data about the performance of newer diagnostic tests using urine (Flores, 2011).

2.4.7 Sensitivity of urine based Xpert MTB/RIF and LAM determine for TB diagnosis

There are limited published data about the performance of MTB/RIF using urine samples. In a selected laboratory cohort interrogating extra-pulmonary samples found that MTB/RIF sensitivity was 100% in 6 culture-positive urine samples with unknown HIV status (Hillemann, 2011), while in HIV-infected outpatients pre-ARV initiation, report the overall sensitivity of urine MTB/RIF was 19% (Lawn *et al*., 2012). It is likely that HIV-infected patients with more advanced immunosuppression accounted for the higher urine MTB/RIF sensitivity. Studies have found a strong association between declining CD4 cell count, LAM in the urine, proteinuria, and increasing urine MTB/RIF positivity (Peter, 2012). This may reflect renal TB as part of disseminated TB, increased bacillary burden in those with the most advanced immunosuppression, a 'leaky' filtration mechanism or a combination of these (Peter, 2012).

The sensitivity of urine MTB/RIF was markedly improved by the centrifugation and pelleting of ~2–10 mls urine (Peter, 2012). Indeed, the concentration of a number of biological samples, such as cerebrospinal and pleural fluid, has improved the performance of traditional TB diagnostics (Stewart, 1953). However, concentration is also believed to increase PCR inhibition and potentially the rate of indeterminate test results. With only a single indeterminate test result (on a 1 ml urine sample), and no change in the mean internal positive control in centrifuged samples, 10mls of centrifuged and pelleted urine is optimal when using MTB/RIF. The incremental yield of using volumes greater than 10 mls will require further evaluation (Peter, 2012).

2.5 The Relevance of MTB DNA in Urine to TB Disease

The Xpert MTB/RIF assay detects intact *tuberculosis* bacilli. This is because the cartridge-based processing entails lysis, washing and deposition of whole mycobacteria on a filter membrane prior to ultrasonic disruption real-time PCR amplification and detection (Lawn *et al*., 2011). Thus detection of *M. tuberculosis* in urine using Xpert indicates renal tract involvement with TB as the bacilli would otherwise be unable to enter the urine. Also it reflects lack of disease anatomic compartmentalization in patients with advanced immunodeficiency. Assessment of urine samples using Xpert provides a means of rapid assessment of disseminated TB.

2.5.1 Extrapulmonary Tuberculosis (EPTB) and HIV-coinfection

Extrapulmonary tuberculosis (EPTB) is TB disease affecting organs other than the lungs, and the most common forms include body cavity (pleural, pericardial and ascitic), lymph node, and meningeal (Sharma, 2004). Extrapulmonary tuberculosis is responsible for 10-20% of global TB cases and has increased substantial in areas of high HIV prevalence as the incidence of EPTB and disseminated forms of TB increase with worsening immunosuppression (Harries, 2001; Hsu *et al.*, 2011).The diagnosis of EPTB is particularly difficult and is the most important obstacle to improved management. Both conventional and novel sputum-based diagnostic tools such as smear microscopy, Xpert MTB/RIF and *M. tuberculosis* culture have reduced diagnostic accuracy (Hsu *et al.*, 2011). Consequently, diagnosis often requires invasive, expensive tissue sampling for histologically diagnosis in order to improve the likelihood of microbiological confirmation (Peter *et al.*, 2012). Empiric treatment based on clinical and radiological screening is a commonplace in the management of EPTB, and novel non-sputum based diagnostics and diagnostic strategies are urgently required

(Peter *et al.*, 2012). Therefore this study was able to validate Xpert MTB/RIF for urine use hence contributing to the improvement of diagnosis of non-sputum sample hence early detection of EPTB.

2.5.2 Urine for TB diagnosis

Urine as a biological sample for diagnostic testing is appealing. Urine is easy to collect, readily available and has a low infection risk to staff during collection (Torrea *et al.*, 2005). Urine antigen detection is the most common diagnostic technique employed for infectious diseases, and TB diagnosis is no exception (Flores *et al.,* 2011). A number of *M. tuberculosis* antigens have been evaluated in urine for the TB diagnosis (Flores *et al.,* 2011). Of these 12 evaluated TB antigens, lipoarabinomannan (LAM) is the most extensively evaluated and promising (Flores *et al.,* 2011). Therefore if urinary LAM is used in the diagnosis of urinary tuberculosis in this EPTB, there will be high chance of detecting TB hence early treatment.

2.5.3 Urine lipoarabinomannan (LAM) for TB diagnosis

Lipoarabinomannan (LAM) can be detected in the urine of patients with active TB. This heat-stable glycolipid travels in the bloodstream and passes through the renal filtration barrier without major changes and is thus detectable in an antigenically intact form in urine (Boehme *et al.*, 2005; Reither, 2009;Hamasur, 2001) (see Figure 2.).

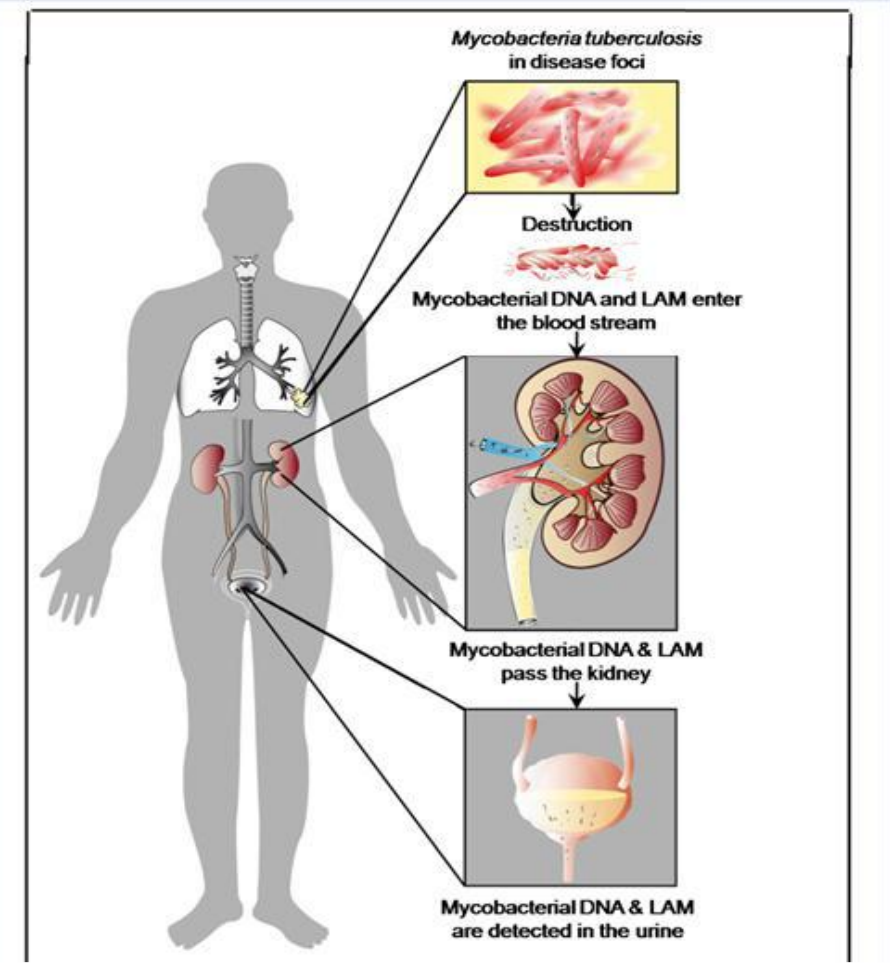

Figure 2: Annotated diagram illustrating the passage of mycobacterial DNA and lipoarabinomannan antigen from infection site to urine LAM.

Lipoarabinomannan (LAM) is a heat-stable 17.5kD glycolipid that forms one of the main components of the outer cell wall of mycobacterial species, and is a heterogeneous immune-reactive glycoconjugate (Reither, 2009). It accounts for up to 15% of the total bacterial weight and is an important virulence factor of *M. tuberculosis* (Briken, 2004; Hunter, 1986; Minion, 2011). Lipoarabinomannan (LAM) consists of three distinct structural domains, including a phosphatidylinositol (PI) anchor, a branched mannan, and a branched arabinan (Dinadayala, 2006).

There are three types of LAM namely mannose-capped LAM (ManLAM), phospho-myo-inositol capped LAM (PILAM) and non-capped arabinose LAM (AraLAM) (Nigou, 2003). The different types of LAM capping determine the ability of LAM to modulate immune responses; ManLAM (simply referred to as LAM) is the commonest form in M. tuberculosis and is a very potent anti-inflammatory molecule and virulence factor (Nigou, 2003). The mannose caps, as recently discovered, may be involved not only in attenuating host immune response, but also in mediating the binding of mycobacteria to and subsequent entry into macrophages (Chatterjee, 1998). ManLAM is postulated to modulate a number of host responses including: cytotoxic oxygen free radical scavenging; inhibition protein kinase C activity and; prevent the interferon gamma transcription in macrophages and T-cells. The inhibition of macrophage activation, abrogation of T-cell activation and blockage of the cytocidal activities contributes immensely to the persistence of M. tuberculosis within the mononuclear phagocytes and their dissemination to other parts of the body. This shows that ManLAM, which also possesses much less potency in evoking TNF-alpha and other responses (compared to other types of LAM) is an immunogenic virulence factor of much clinical and diagnostic significance (Chan, 1991; Chatterjee, 1997).

2.5.4 The specificity urinary LAM for *M. tuberculosis*

Notwithstanding that the urinary LAM is the most promising point-of-care (POC) diagnostic test; there still remains the major setback of cross reactivity with other microorganisms that have LAM-like glycolipids, thereby increasing the possibility of false positive LAM results. Although LAM is a lip polysaccharide specific to the genus Mycobacteria, it is interesting that LAMlike glycolipids are also found in several

species of fungi and bacteria and these are likely to cross-react with LAM antibodies (Moreira *et al.,* 2008).

2.5.4.1 Use of a standardized urine sample collection methodology

Since false-positive urine LAM test is likely to be caused by environmental contamination or unrelated infections (colonization or disease by NTM mycobacterial or fungi), there is a need to establish a standardized urine sample collection methodology (Dheda *et al.,* 2010). This is important, and is done in order to maintain sample sterility, thus it will likely significantly decrease the rate of false positive urine LAM ELISA/lateral flow tests. The importance of preventing contamination by normal vaginal, perineal and anterior urethral flora cannot be overemphasized and remains the responsibility of the laboratory to provide the patient with sterile, wide-mouthed glass or plastic, jars, beakers or other suitable receptacles which should have tight-fitting lids (WHO, 2011). Suprapubic aspiration urine collection method is the gold standard; unfortunately the method is not practical in many cultural settings. However, whenever possible, urine specimen should be collected in the morning, before the patient has voided urine and ensure that mid-stream urine or a clean-catch urine is collected (WHO, 2011).

2.5.4.2 The LAM lateral flow strip test

The Alere Medical innovations have recently developed a simple lateral flow format of the Clearview TB LAM ELISA using the same polyclonal antibody coupled to gold nanoparticles (Peter *et al.,* 2012; Lawn, 2012). The Determine-TB LAM Ag strip test (Alere, USA), requires just 60.l of unprocessed urine, can be performed at the patient bedside without the need for laboratory skills or electricity and cheaper (Peter *et al.,* 2012). Urine is placed on to the loading platform, and after just 25mins the test is read

using the manufacturer's provided reference card to interpret band intensity. The presence of a band in the control window indicates a valid test, and then the color intensity of the band in the patient window is matched to the reference card and graded intensity 0-5. Thereafter, the manufacturer and reference card categorizes patients with grade 0 bands as test 'negative' and a grade-1 or higher band as test 'positive'. This simple test offers great potential for resource-limited high TB HIV burden settings.

2.5.4.3 Urine LAM strip test initial diagnostic accuracy evaluation

To date, the urine LAM strip test has only been evaluated in two studies. The first in an out-patient antiretroviral clinic setting where the test was used for TB screening patients prior to the initiation of antiretroviral therapy irrespective of symptoms, while the second was a cohort of hospitalized patients with suspected TB HIV co-infection (Peter *et al.*, 2012).

The evaluation of the Determine TB-LAM test strips by Lawn *et al.*, (2012) in outpatients that were being screened for antiretroviral therapy at a primary care setting in South Africa showed very promising results (Lawn, 2012; Lawn, 2009).Overall and using the manufacturer-recommended suggested grade-1 cut-point; they found a sensitivity and specificity of 28.2% and 98.6% respectively. Sensitivity consistently improved with advancing immune-suppression, increasing to 39% and 51.7% in patients with CD4<200 and CD4<100 cells/ml respectively (Lawn, 2012). As expected, urine LAM strip test diagnostic accuracy was found to be similar to that of the urine LAM ELISA assay performance (Lawn, 2009, Shah, 2009).

Furthermore, the inter-reader reliability between two readers was found to be excellent (K=0.97), but it was noted that the median LAM ELISA optical density of urine LAM strip positive samples was 0.681 (IQR 0.164–2.431; range 0.102–3.291) meaning that few grade-1 positive samples were present in the patient cohort (Lawn, 2009).

The effectiveness and utility of the LAM strip test is dependent on its high specificity, which other studies working with the ELISA version showed (Dheda, 2010; Gounder *et al.,* 2010). The performance and utility of the LAM strip test was also evaluated by other studies and was found to be promising.

Urine LAM strip test in hospitalized TB HIV co-infection Peter *et al.,* (2012) recently evaluated the diagnostic accuracy of the urine LAM strip test for diagnosis of TB detection in hospitalized patients with suspected HIV confection. Overall using the manufacturer's suggested grade-1 cut-point and a composite reference standard, urine LAM strip sensitivity and specificity was 66% and 96%, respectively. However, inter-reader reliability was found to be only moderate using the grade-1 cut-point. In addition, amongst a non-TB control group, test specificity and positive predictive value (important for rule-in test utility) was reduced using the grade-1 versus the grade-2 cut point (Peter, 2012). Hence, this study recommended the use of a grade-2 and not the grade-1 cut-point to optimize the rule-in utility of the test (Peter, 2012). Sub-group analysis indicated that the urine LAM strip test performs best in patients with lower CD4 cell counts and significantly outperformed smear microscopy in the diagnosis of EPTB (Peter, 2012). Furthermore, in the clinically important smear-negative or sputum scarce (SN/SS) HIV-coinfected patient sub-group, the urine LAM strip test sensitivity was 38% increasing to 49% in patients with CD4= 100 cell/ml (Peter, 2012).

Both the outpatient and in-patient studies of the urine LAM strip test showed promising results, but important differences were found. Most notably, the optimal urine LAM strip test cut-point requires further clarification. Possible explanations for the differences in study findings include study populations differences, batch variability, reader experience and/or blinding procedures, and duration and stability of LAM strip tests during storage (Peter, 2012).

2.5.4.4 LAM strip test as adjunctive diagnostic test

Urine LAM strip testing is unable to offer microbiological confirmation and/or drug susceptibility testing. Furthermore, studies of urinary LAM show only modest to good overall sensitivity even amongst HIV-infected patients with advanced immunosuppression (Peter *et al.,* 2012; Lawn *et al.,* 2012). Thus, urine LAM strip testing will likely function as a diagnostic adjunct to existing, largely sputum-based conventional and novel rapid TB diagnostics such as smear-microscopy or Xpert MTB/RIF (Peter, 2012). In line with this rationale, both Peter *et al* (2012) and Lawn *et a.,l* (2012) evaluated the combined performance of urine LAM and either sputum smear-microscopy or Xpert MTB/RIF (Peter, 2012; Lawn *et al.,* 2012). Interestingly, in both outpatient and in-patient cohorts the combined sensitivity of sputum smear-microscopy and urine LAM strip testing was significantly higher than the use of each test alone (Peter, 2012; Lawn *et al.,* 2012). In addition, Lawn *et al.,* (2012) found that combined sputum-based Xpert MTB/RIF and urine LAM strip testing was not significantly better than sputum Xpert MTB/RIF alone this means the performance is increased when sputum Xpert is used alone compared to LAM (Lawn *et al.,* 2012). However, the sub-group of sputum scarce patients, in whom sputum-based diagnosis is unhelpful, was reduced by the use of sputum induction to aid sample acquisition. Sputum induction is not readily available in the majority of primary care clinic settings (Peter, 2012; Lawn *et al.,* 2012). The use of urine based Xpert MTB/RIF in sputum scarce patients was not evaluated in either study.

2.6 Prevalence of multidrug resistance among patients diagnosed with TB by Gene Xpert using urine

Drug resistance is defined as a decrease in the *in-vitro* susceptibility of *M. tuberculosis* of a sufficient degree to be reasonably certain that the strain concerned is different from

a wild strain that has never come into contact with the drug. Multi-drug resistant TB (MDR-TB) is caused by a strain of *M. tuberculosis* that is resistant to two or more anti-TB drugs (Curry, 2008). Multidrug resistant (MDR) TB is a public health concern (Boehme *et al*., 2011). Its burden particularly in developing countries is a relevant issue to be addressed. In some countries, proportions as high as 26% of the new TB cases can be MDR-TB. MDR strains arose over the past 30 years as a variety of anti-tuberculosis drugs were introduced in medicine, and they largely discount the results of chemotherapy for tuberculosis (Prozorov, 2012).

The most dangerous of them are strains with extensive drug resistance (XDR), which are resistant to four or five different drugs on average. The molecular mechanisms that make a strain resistant are considered XDR and MDR strains result from successive and usually independent resistance mutations, which arise in various regions of the mycobacterial genome (Prozorov, 2012)**.**

In addition, the formation of resistant strain is affected by the phenomenon of tolerance and mycobacterial latency in infected tissues (Prozorov, 2012). The increasing emergence of MDR and XDR TB in the era of HIV infection presents a major threat to effective control of TB. Clinical MTB isolates were considered to be drug-resistant if the number of colonies that grew from a specimen cultured on a drug containing plate was >1% of the number that grew on a drug free plate by the proportion method (Curry, 2008).

A drug resistant isolate can be categorized as single drug resistant or MDR. It can be further categorized as exhibiting primary or secondary (acquired) resistance. In primary resistance, the patient from whom the isolate is obtained has never received any of the anti-TB drugs. In secondary resistance, the patient has been previously treated for TB with the specific drug(s) to which the isolate is resistant (Curry, 2008).

Acquired resistance may thus be found in culture positive cases in the following categories: patients with treatment failure, patients who relapse after successful completion of treatment and those who return after treatment interruption (Bassett, 2010). The relationship between drug resistance in *M. tuberculosis* strains and their virulence/transmissibility needs to be further investigated. Understanding the mechanisms of drug resistance in *M. tuberculosis* would enable the development of rapid molecular diagnostic tools and furnish possible insights into new drug development for the treatment of TB (Zhang, 2009).

Xpert has been found to have very high but sub-optimal specificity for rifampicin resistance. Rifampicin (RIF) resistance rarely occurs in isolation and usually indicates resistance to a number of anti-TB drugs (WHO, 2008). Rifampicin resistance is normally seen in MDR-TB and has a reported frequency of greater than 95% in such isolates (Curry, 2008).

Use of Xpert MTB/RIF assay has been endorsed by the WHO as a rapid method for simultaneous detection of MTB and rifampicin resistance (as a surrogate marker for MDR-TB. Current recommendations suggest that TB patients infected with rifampicin susceptible MTB diagnosed on the basis Xpert MTB/RIF assay be prescribed first line anti-tuberculosis therapy (ATT) (isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E)/streptomycin (S) (WHO, 2008). This recommendation however raises a concern that isolates susceptible to rifampicin but resistant to other first line ATT especially isoniazid will not be detected by Xpert MTB/RIF. These concerns are supported by recent studies suggesting poor outcome in isoniazid mono-resistant TB cases treated with standard first line therapy (WHO, 2011).

Globally, isoniazid resistance alone or in combination with other drugs has an estimated prevalence of 10.3% amongst new cases, 27.7% amongst previously treated

patients and 13.3% for combined (new and retreated) cases. Isoniazid resistance amongst new and previously treated and combined cases within South-East Asian region is estimated at 10.3%, 36.8% and 15.7% and in Pakistan at 8.9%, 28.5% and 18.7% respectively (Francis, 2008). Among rifampicin susceptible TB isolates, retrospective analysis of aggregated data documents isoniazid resistance rate (alone and in combination with other drugs) of 7.2%, 15.3% and 7.7% in new, retreated and combined cases respectively (Francis, 2008). For all patients, testing for susceptibility to first-line agents should be performed if this is affordable to optimize efficacy of the therapeutic regimen and to decrease transmission of drug-resistant TB (Hassim *et al.*, 2010).

CHAPTER THREE

METHODOLOGY

3.1 The Study Area

The study was carried out at the Moi teaching and referral hospital l (MTRH) clinics laboratory. This is a hospital that serves clients from all over North-Rift, parts of western and Nyanza province in Western Kenya. Moi teaching and referral hospital has been providing care to HIV patients in Western Kenya since 2001.As of January 2013, MTRH had enrolled over 150,000 individuals and had almost 80,000 patients active in care. An average of 300 new adult patients are enrolled every month at the MTRH site where this study was carried out. A number of TB patients coinfected with HIV are seeking treatment at MTRH.

Moi Teaching and Referral Hospital is situated at an altitude of 2104m (6903 feet) and 0°31′N 35°17′E of the prime meridian (Appendix I and figure 3).

The climate of western Kenya and North-Rift is characterized by an average annual rainfall of about 2000mm. Rainfall is heaviest in March, April and May, with a slight drier June, and a second peak roughly in August to September. Temperature is fairly constant throughout the year, with mean daily minimums of about 11^0C and a mean daily maximum of about 26^0C (Ojany and Ogendo, 1988).

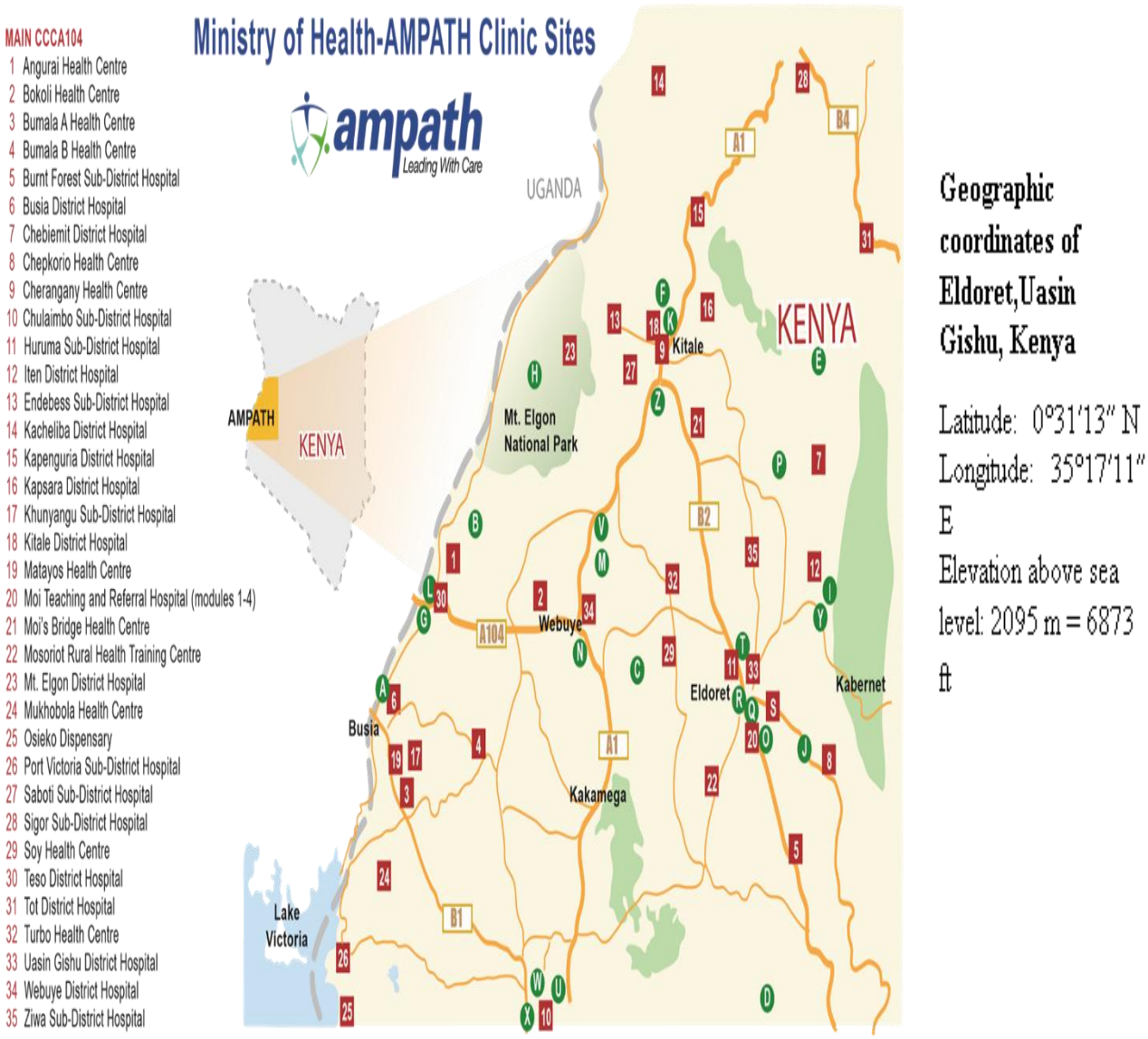

Figure 3:Geographic coordinates of Eldoret, Uasin Gishu, Kenya.

3.2 The Study Sample Size Determination

Presence of the *tuberculosis* bacilli in the body constitutes an infection but only 5% of infected individuals will develop active disease upon initial infection with risk of progressing to active TB being highest in the first two years. Another 5% will develop active disease in the course of their lifetime if they do not receive treatment. The chances of developing TB are much higher if the individual is HIV-infected. Ten percent (10%) of all HIV-positive individuals harbor MTB. About 9% of newly

diagnosed TB patients globally (30% in Africa, 48% in Kenya) are HIV-infected. 8-10% of newly enrolled HIV patients in MTRH are diagnosed with TB.

In order to be 95% confident that the sensitivity of the kit is within plus or minus 5% of the population-estimated sensitivity, the present study adopted the Buderer's formula (Buderer, 1996) for diagnostic studies:

$$\text{Sample size } (n) \text{ based on sensitivity} = \frac{Z^2_{1-\alpha/2} * S_N * (1-S_N)}{L^2 * \text{Prevalence}}$$

$$\text{Sample size } (n) \text{ based on specificity} = \frac{Z^2_{1-\alpha/2} * S_P * (1-S_P)}{L^2 * (1-\text{Prevalence})}$$

Where;

n = required sample size,

S_N= anticipated sensitivity,

S_P= anticipated specificity,

α = size of the critical region (1 − α is the confidence level),

$Z_{1-\alpha/2}$ = standard normal deviate corresponding to the specified size of the critical region (α),

L = absolute precision desired i.e. level of accuracy

The prevalence of the condition in the population of interest is 37% whereas the anticipated sensitivity and specificity of the test is are 96.1% and 97.9% respectively. The standard normal deviate corresponding to 95% confidence interval is 1.96 while the absolute precision desired, L, is 0.05.

Substituting this in the formula yields;

$$n \text{ (Sensitivity)} = 1.96 \times \frac{0.961 * (1-0.961)}{0.05 * 0.37} = 157$$

$$n \text{ (Specificity)} = 1.96 \times \frac{0.979 * (1-0.979)}{0.05 * (1-0.37)} = 51$$

The minimum sample size 157, given that it will give the precision of 0.05 or less for both sensitivity and specificity. Hence, to achieve the desired precision of 0.05 or less for both sensitivity and specificity, we needed a total of 157 study participants.

3.3 The study design

This was a cross-sectional study in which 158 study participants were prospectively recruited. Participants included adults 18 years of age or older with HIV and CD4+ count of less than 200 cells/mm3. Other laboratory values (urinalysis, complete blood count, biochemical analysis) were also collected. Morning urine samples was collected from patients with suspected TB infection and analyzed for the presence of *M.tuberculosis* DNA in urine. Acid fast-staining, culture, clinical symptoms and laboratory and radiological findings were also evaluated. Also information on host factors (age, gender, patient category, site of TB, HIV status, CD^{+} count) was obtained from the hospital patient records.

3.4 Inclusion and Exclusion Criteria

3.4.1 Inclusion criteria

i. Male and female aged 18 years old and above.
ii. HIV positive patients with $CD4^{+}$ less than 200 cells/mm3
iii. Untreated TB suspect
iv. Both smear positive and smear negative TB patients
v. Provide signed Informed consent

3.4.2 Exclusion criteria

Treated TB cases more than 90 days

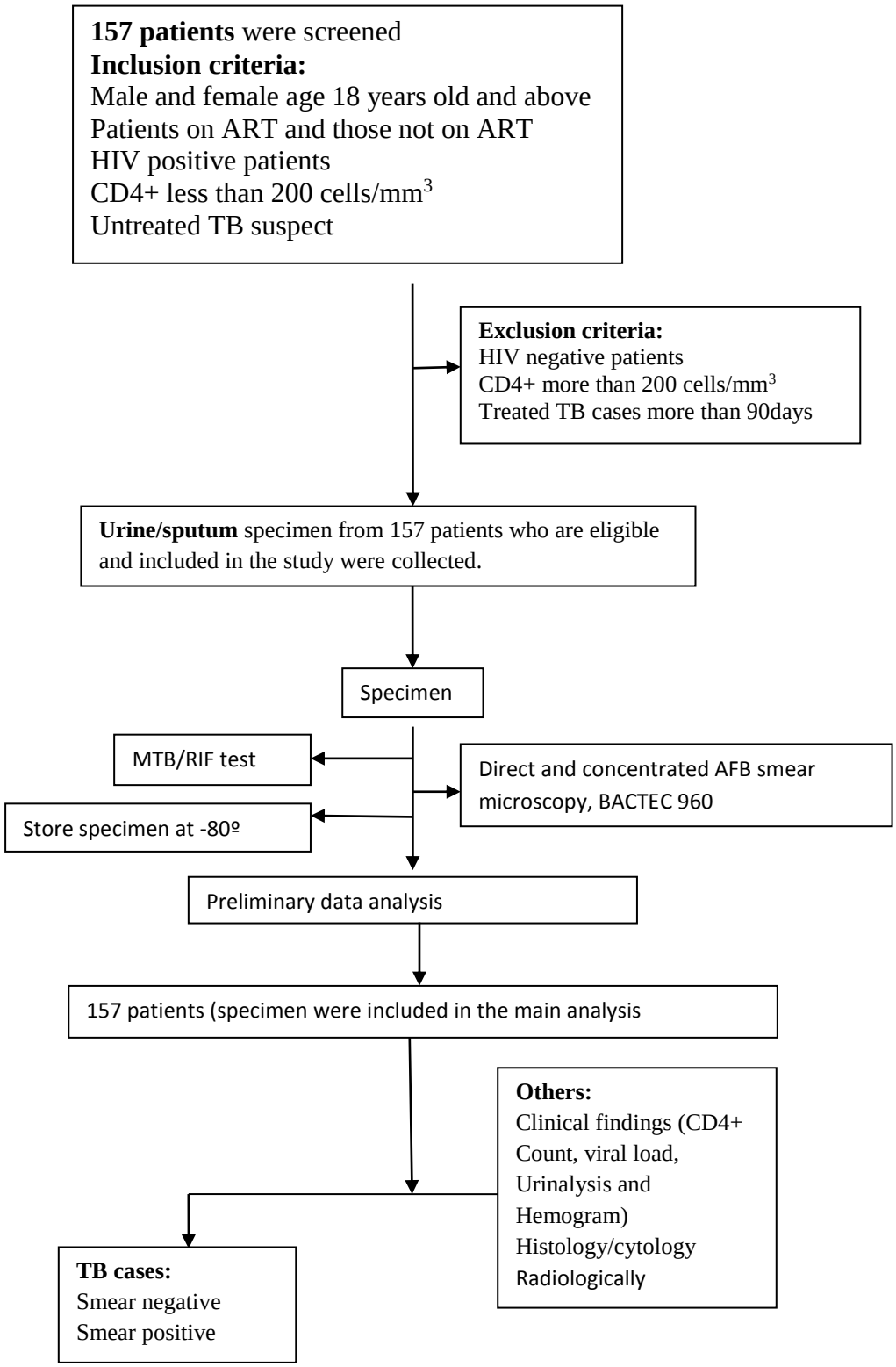

Figure 3.1 :*Patients Flowchart Explaining the Patient sample Flow in this Study.*

3.5 The study population

The study population consisted of 157adult participants who were prospectively recruited. Participants from both in-patients and out-patients clinic at MTRH were recruited between October 2013 and December 2014. Patients were referred for study inclusion by attending clinicians if the patient was suspected to have HIV-TB co-infection. Only patients, who refused to consent, HIV negative patients, $CD4^{+}$ more than 200 cells/mm3 and treated TB cases more than 90dayswereexcluded from study enrolment. All participants provided written informed consent.

3.6 Diagnostic Sample Collection and Handling

Attending clinicians with no association to the study team decided on the timing and extent of diagnostic work-up, commencement of anti-TB treatment, and final discharge from hospital. TB diagnostic work-up was standardized, but routine local hospital practice included the collection of two sputum samples in patients able to expectorate and, if EPTB was suspected, the collection of 1–2 non-sputum samples from clinically involved sites (e.g. urine). Further details of biological samples were collected for TB culture. The local reference laboratory processed all clinical specimens collected for TB diagnosis. Smear microscopy was performed on processed sputum, which was also cultured using the MGIT 960 liquid culture system (BD Diagnostics, USA). The reference standard for definite-TB was liquid culture positivity for *M. tuberculosis.*

3.6.1 Urine Sampling and LAM Methodology

All study patients were required to give 10–30 ml midstream urine in a fresh standard, sterile container after recruitment. Prior to urine collection, patients were asked to clean the urogenital area with a clean wipe. A urine dipstick test (*Uri*CHECK 9, RapiMed Diagnostics) was immediately performed after collection to assess for protein, blood and leucocytes. Fresh urine sample was used if the sample was kept within 8 hours at

room temperature. If the test sample was to be run within 3 days of collection, urine sample was stored at 2-8^{0}C and if the testing was delayed for more than 3 days, urine was frozen and stored at −20°C for later batched testing. Alere Determine ^{TM} TB LAM Ag was performed on thawed urine. All thawed samples were centrifuged at 10,000g for 5 minutes at room temperature and 60uL test sample was carefully collected from the supernatant.

3.6.2 Interpretation of LAM Strip Results

Reading and interpretation of results was made possible through the use of Reference Scale Card, which was provided in the test kit by holding it alongside the patient window. Lipoarabinomannan (LAM) antigen positive was characterized by appearances of two bars i.e control and patient bars. Purple/gray bar appear in both the control window and the patient window of the strip. The color intensity of the patient bar should be equal to or stronger than any of the colored bars in the positive range on the Reference Scale Card. The test results were still positive even if the patient bar appears lighter or darker than the control bar (Peter *et al.,* 2012).

Lipoarabinomannan (LAM) antigen negative was characterized by the appearance of one Purple/gray bar in the control window of the strip and no Purple/gray appears in the patient's window of the strip (Peter *et al.,* 2012). Invalid results were characterized by no appearances of Purple/gray bar in the control window of the strip even if a Purple/gray bar appeared in the patient's window and the results were repeated.

Indefinite LAM Antigen results characterized by the appearance of unclear or incomplete Purple/gray bar Purple/gray bar in the control window or in the patient window of the strip despite the color intensity of the bars.

3.6.3 Urine Xpert MTB/RIF methodology

All HIV-infected patients with culture positive TB had MTB/RIF performed using 1 ml of unprocessed, thawed urine according to the manufacturers' suggested procedure for sputum samples. In addition, a random sample of ~50% (78/157) of culture negative non-TB patients had urine MTB/RIF performed. The MTB/RIF operator was blinded to the clinical status of these patients. Briefly, the sample reagent was mixed at a 2:1 ratio with ~1 ml of urine. Two milliliters of the reagent sample mix was transferred into an MTB/RIF assay cartridge and inserted into the Gene Xpert instrument (Theron *et al.*, 2011). Additionally, if the MTB/RIF negative using a 1 ml urine sample, a second pelleted urine was performed, where possible, using a median of 10 (IQR 5–10) ml urine (see Appendices 7 and 8).

3.6.3.1 Principle of the Procedure

The Xpert MTB/RIF assay (Cepheid Gene Xpert® System) is a hemi-nested real-time PCR diagnostic test that is capable of detecting *M. tuberculosis* complex DNA in a sample while simultaneously detecting rifampicin resistance including a semi quantitative assessment of bacillary load. The 15 minute-long step of mixing the bactericidal buffer with the sample is the only hands-on step of the whole process; this pre-amplification phase renders the *M. tuberculosis* non-viable and harmless. All these qualities of the Xpert MTB/RIF assay makes it a choice diagnostic tool for near patient usage especially in settings with poor bio-containment facilities.

This process makes use of five overlapping molecular probes complementary to the whole *M. tuberculosis* rpoB gene containing the 81 base pair rifampicin resistance-determining region (RRDR) and they cover all the mutations found in >99.5% of all rifampicin resistant strains ensuring that there no cross-reactivity with NTM occurs. If

there are at least two of these rpoB probes that are positive within two cycles of each other, then the result will be *M. tuberculosis* positive; also if at least a single rpoB probe does not result in a measurable signal and/or a presence of a 3.5 cycle or a significant deviation in the cycle threshold (Ct) value between the earliest and latest rpoB signals then the system regards that as a RIF resistance result.

An estimation of 95% of RIF resistant cases arise as a result of mutations found in the RRDR. This hemi-nested PCR amplification assay, integrated into a single disposable cartridge, depends on six color fluorescent molecular beacons to detect the presence of any amplified target. The Xpert MTB/RIF has a robust and full process control that acts as a quality check for bacterial trapping, bacterial lysis, DNA extraction, amplification, and probe detection; this process makes use of *Bacillus globigii*, a spore-forming soil organism.

The Xpert MTB/RIF assay has been found to have a limit of detection (LOD) of 131 CFU/ml of sputum. The Xpert LODs for urine and other extrapulmonary specimens have not been established yet. This could have been a vital piece of information especially in the optimization phase of these extrapulmonary specimens for use in Xpert MTB/RIF studies. However the performance of Xpert MTB/RIF in sputum as reported by the World Health Organization (WHO) is worth investigating as this gives a good forecast of how it is likely to perform in extra pulmonary specimens like urine.

3.6.3.2 Interpretation of Urine MTB/RIF Results

Results were displayed in the "View Window" of the Xpert MTB/RIF as a result of fluorescent signals that are quantitated and processed through an embedded calculation algorithm in the Xpert MTB/RIF software. Final results come out as MTB NEGATIVE or MTB POSITIVE, with either RIF resistance DETECTED, RIF resistance NOT

DETECTED or INDETERMINATE. In the event that the M. tuberculosis positive result is displays RIF resistance DETECTED, or INDETERMINATE, the amplicon would be extracted under sterile conditions on ice using an insulin syringe and transferred into a PCR appendorf tube and stored at -80^{o}C for later sequencing. Repetition of tests were only necessary in the event of results that were displayed as "INVALID", "INDETERMINATE", "ERROR" or "NO RESULT" all these could mean that there was a problem or faulty in the way the sample preparation was done or the volume transferred into the cartridge might have been too small for processing.

Cycle threshold (Ct) values were also displayed in the event of a positive result. Cycle threshold values represented a number of cycles needed for the fluorescent signal to cross a given background level or threshold. The Ct values are known to be inversely proportional to the amount of target M. tuberculosis DNA in the urine sample, hence a lower Ct value are representative of a higher initial concentration of the *M. tuberculosis* in the urine and higher Ct values reflect a lower initial concentration in the urine sample.

3.6.4 Sample culture and Drug Susceptibility Testing

Mycobacterium strains cultured was sub-cultured on Lowenstein-Jensen slants (Munsiff, 2006). Drug susceptibility testing (DST) was performed at MTRH reference laboratory using the indirect proportion method with middle brook 7H10 medium for the following anti-TB drugs at the indicated concentrations; isoniazid (1.0µg/ml), rifampicin (1.0µg/ml), streptomycin (1.0µg/ml), and ethanbutol (1.0µg/ml), see appendices 12 and 13 for detailed information.

3.7 Data Collection

Information on host factors (age, gender, patient category, site of TB, HIV status,) was obtained from the hospital patient records containing clinical and laboratory data registered for all adults attending MTRH clinics, Eldoret, Kenya. When patients are seen at the MTRH clinics for the first time, information on host and clinical factors are obtained from the patient and stored in the MTRH clinics database for the purpose of monitoring patient's prognosis. This data can be retrieved whenever the patients revisit the clinic.

3.8 Data Analysis and Presentation

Demographic, clinical and microbiological characteristics of the study participants were summarized using descriptive statistics. Categorical variables were summarized as frequencies and percentages while the continuous variables were summarized as mean and SEM or median and the corresponding inter quartile range. The test for association between categorical variables was conducted using Pearson's Chi Square test while the test of association between continuous variables were conducted using a two-sample t-test if they are normally distributed. If the continuous variables were skewed then a two sample Wilcox on rank sum test was used. Normality tests were conducted using Shapiro and Wilks normality test.

The study adopted the Cohen's kappa statistic (k) test to measure the level of agreement between the Gene Xpert MTB/RIF and LAM determines strip techniques in detecting TB. The kappa measure of agreement is stated as:

$$\kappa = \frac{P(A) - P(E)}{1 - P(E)}$$

Where P (A) is the proportion of times the two techniques are in agreement, and P (E) is the proportion of times the two techniques are expected to agree by chance alone. Complete agreement corresponds to a κ value of 1, complete disagreement (i.e. purely

random coincidences of rates) corresponds to aκ value of 0. A negative value of kappa would mean negative agreement. The current study used the following kappa (κ) scale to rate the strength of agreement: a κ< 0.21 was considered poor, aκ between 0.21 and 0.40 fair, a κ between 0.41 and 0.60 moderate, aκ between 0.61 and 0.80 good, and a κ> 0.80 very good.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) with their 95% confidence intervals (CI) was computed for Gene Xpert MTB/RIF and LAM determine against the culture method. The validity of a method is considered to be adequate if the sensitivity and specificity was 50% and 70% respectively as used in other studies with similar settings.

Validity of the results was ensured by both external quality control (EQC) and internal quality control (IQC) systems for the sample analysis and instruments used in this study.

Statistical analysis was performed using SAS version 9.3 (SAS Institute, Cary, North Carolina, USA) and p-value of less than 0.05 was used to define statistical significance.

3.9 Ethical Considerations

Ethical clearance for the present study was obtained from the Institutional Review Ethics Committee (IREC) at the MTRH (see appendix 3 and 4). All patients involved in the study were asked for written consent before samples were collected (see appendix 5 and 6). There was no additional risk to the study participants and the study is expected to provide valuable information towards improvement of diagnosis of both active and latent TB particularly in HIV-infected patients. Findings were discussed with the relevant health provider. Information was provided in appropriately accessible language. Data collected was regarded as confidential. Data collected was entered into the electronic database.

CHAPTER FOUR

RESULTS

4.1Optimal parameters (Zn, CD4 count, Hb, Creatinine, Proteinuria, and Hematuria) for Gene Xpert MTB/RIF and LAM Strip determine Test for diagnosis of tuberculosis using urine from HIV patients at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya

The primary outcome was Tuberculosis (TB) positivity based on Gene Xpert MTB/RIF and LAM Strip Test. The explanatory variables included, demographic variables i.e., age, gender; clinical variables (e.g., $CD4^+$ count, WHO staging, HB level); microbiological variables including urinalysis, acid fast, rifampicin resistance); and radiological findings). Demographic, clinical and microbiological characteristics of the study participants were summarized using descriptive statistics (Table 4.1).

This study recruited 157 HIV positive participants to the study in the period 2011-2013. The overall mean age in years was 39 (9.1) and female participants comprised of 54% of the participants. About a third of the participants (31%) had CD4 count $\leq$ 100 cells/mm^3. The WHO disease progressions in two-thirds of the participants were either in the 3^{rd} or 4^{th} stage. The median hemoglobin level was 12.0 (IQR, 10.1-13.6) implying that more than half of the participants were not anemic. All these results are summarized in Table 4.1.

Table 4.1: Demographic and clinical characteristics of the study participants

Characteristics	**N=157 n, (%)**
Gender (Female)	85 (53.8)
Age (years), mean	38.7 (9.1)
CD4 count, cell/mm^3	
≤ 100	49 (31.0)
> 100	106 (67.1)
Missing	3 (1.9)
WHO stage	
Stage I & II	25 (15.8)
Stage III & IV	105 (66.5)
Missing	28 (17.7)
Hemoglobin, median (IQR)	12.0 (10.1-13.6)

Table legend. 157 HIV positive participants were recruited to the study between 2011-2013. Overall mean age in years was 39 (SD, 9.1) with female participants comprised of 54% with a third of the participants (31%) having CD4 count ≤ 100 cells/mm^3 in 3rd or 4thWHO disease stage. The median hemoglobin level was 12.0 (IQR, 10.1-13.6) implying that more than half of the participants were not anemic.

Among participants with clinical symptoms, those with weight loss, TB, Pneumonia, Urethral tract infection (URTI) and those with a combination of TB or Pneumonia or Meningitis formed a cumulative of 73% (61/84) of all diagnosed symptoms (results shown in table 4.2).

Table 4.2: Clinical diagnosis among the study participants

Clinical symptom	n (%)
Adenitis, TB	1 (0.6)
Allergy	1 (0.6)
Anemia	3 (1.9)
Asymptomatic HIV	3 (1.9)
Bronchospasm	1 (0.6)
Conjunctivitis	1 (0.6)
Dehydration	1 (0.6)
Gastroenteritis	2 (1.3)
Herpes zoster	3 (1.9)
Hypertension	2 (1.3)
Meningitis	1 (0.6)
Migraine	1 (0.6)
Milliary Tuberculosis	5 (3.2)
Pneumonia	8 (5.1)
Pneumonia, Tuberculosis	7 (4.4)
Pneumonia, Tuberculosis	1 (0.6)
Pericardial effusion	1 (0.6)
RTI, Cough	10 (6.3)
Tuberculosis	15 (9.5)
Tuberculosis meningitis	1 (0.6)
URTI	9 (5.7)
Weight loss	7 (4.4)
None	74 (46.8)

Table legend. Clinical diagnosis of tuberculosis in study participants included weight loss, TB, Pneumonia, URTI and those with a combination of TB or Pneumonia or Meningitis.

The median urine culture was 125 (IQR, 110-272). Majority had no results on urinalysis (85%), however, '+Blood' and '+Protein' was reported in 65% and 26%, respectively, among those with non-nil result. Approximately a fifth (28%) had positive acid-fast result of which slightly more than half (55%) of this being positive. Based on Gene Xpert test, 17 (11%) of the participants were identified as TB positive of which 94% were MTB detected low and 6% were MTB detected medium. Rifampicin was not

detected in 88% of the TB Gene Xpert positive participants with the remaining (12%) being indeterminate, whereas all of them were sensitive to drugs. When tested using the LAM test, 28% of the participants tested positive for TB. Radiological findings revealed that majority of participants had infiltrates (45%), normal (19%), milliary TB (15%) and opacities (11%). The median creatinine was 62.3 (IQR 49.1-75.9). All these results are shown in Table 4.3.

Table 4.3: Microbiological and laboratory characteristics of the study participants

Characteristics	**n, (%)**
Urinalysis	
+ BLOOD	15 (9.5)
+ BLOOD, + PROTEIN	1 (0.6)
+ BLOOD, ++ PROTEIN	1 (0.6)
+ PROTEIN	6 (3.8)
NIL	135 (85.4)
Acid Fast (ZN)	
+	16 (10.1)
++	5 (3.2)
+++	8 (5.1)
NEG	129 (81.7)
TB status (Gene Xpert)	
MTB detected low	16 (10.1)
MTB detected medium	1 (0.6)
MTB not detected	141 (89.2)
Rifampicin (RIF) resistant	
RIF indeterminate	2 (11.8)
RIF not detected	15 (88.2)
Drug sensitivity	
Streptomycin, Isoniazid, RIF, Ethambutol	17 (100.0)
None	0 (0.0)
LAM	
Negative	114 (72.2)
Positive	44 (27.8)
Urine culture	125 (110-272)
Radiological findings	
Cavitation	2 (1.3)
Adenitis	3 (1.9)
Infiltrates	71 (44.9)
Lung collapse	1 (0.6)
Milliary TB	23 (14.6)
Normal	30 (19.0)
Opacities	18 (11.4)
Pericarditis	1 (0.6)
Pleural effusion	3 (1.9)
Suggestive	1 (0.6)
Tb meningitis	4 (2.5)
Tb spine	1 (0.6)
Creatinine, median (IQR)	62.3 (49.1-75.9)

Table legend????

As shown in Table 4.4, presence of protein or blood in urine was significantly associated with TB positivity based on LAM strip determine test (48% vs 24%; p =0.021) based on Pearson's Chi Square test.

Table 4.4: Association between blood and protein urinalysis result and TB results based on GeneXpert result and LAM determine strip techniques

	TB Testing Technique					
	GeneXpert result			**LAM determine result**		
Urinalysis (blood and protein) result	**Negative n (%)**	**Positive n (%)**	**P-value**	**Negative n (%)**	**Positive n (%)**	**P-value**
Negative	122 (90)	13 (10)	0.277	102 (76)	33 (24)	**0.021***
Positive	19 (83)	4 (17)		12 (52)	11 (48)	

Table legend. *significant at α=0.05Table 4.4presence of protein or blood in urine was significantly associated with TB positivity based on LAM strip determine test (48% vs 24%; p-value=0.021).

The weighted kappa coefficient was 0.48 (95% CI=0.32-0.63; exact p<0.0001). Hence, the amount of agreement between the Gene Xpert and LAM was modest. This is summarized in Table 4.5.

Table 4.5:Cohen's kappa statistic (k) test for the agreement between the Gene Xpert MTB/RIF and LAM determine strip test techniques in detecting TB

LAM determine strip test technique	**GeneXpert technique**			**Kappa (k)**	
	Positive	**Negative**	**Total**	**(95% CI)**	**P-value**
Positive	17	27	44	0.48 (0.32-0.63)	**<0.001**
Negative	0	114	114		
Total	**17**	**141**	**158**		

Table legend. The proportions for categorical variables are presented as frequencies and percentages whereas continuous variables are presented as mean with SEM or median with corresponding interquartile range. The Cohen's kappa statistic (k) test was used to measure the level of agreement between the Gene Xpert MTB/RIF and LAM strip determine techniques in detecting TB. The Kappa statistic with its 95% confidence interval is reported.

4.2 Specificity and Sensitivity of Gene Xpert MTB/RIF technique using urine from HIV positive patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in western Kenya.

The study recruited 157 HIV positive patients between the period 2011-2013. The median age was similar for those with lower CD4 cells (≤100) compared to those with CD4>100 [38 (IQR 34-43) vs 39 (IQR 33-46)]. The median hemoglobin levels for those who tested positive using Gene Xpert MTB/RIF and LAM determine strip test as well as those with CD4>100 was normal (Hb>11.0 g/mm^3). However, patients with CD4≤100 had median Hb levels less than normal (Hb=10.7, IQR 10.0-12.9). Among the female11% and 15% were positive based on Gene Xpert MTB/RIF and LAM determine strip test respectively with a majority (71%) having CD4 >100 cells/mm. Among patients who were in WHO stage III and IV, about a sixth had CD4 >100 cells/mm^3. All these results are summarized in Table 4.6.

Table 4.6: Demographic and clinical characteristics among HIV-infected patients in MTRH, 2011-2013

	Gene Xpert		**LAM determine**		**CD4 count**	
Characteristics	**Positive**	**Negative**	**Positive**	**Negative**	**≤ 100 cell/mm3**	**> 100 cell/mm3**
Gender (Female)	9 (10.6)	76 (89.4)	13(15.3)	72 (84.7)	24 (29.3)	58 (70.7)
Age (years), median (IQR)	38 (32-45)	39 (33-45)	40 (34-45)	38 (33-45)	38 (34-43)	39 (33-46)
WHO stage						
Stage I & II	3 (12.0)	22 (88.0)	5 (20.0)	20 (80.0)	4 (16.0)	21 (84.0)
Stage III & IV	11 (10.5)	94 (89.5)	16(15.2)	89 (84.8)	39 (37.9)	64 (62.1)
Hemoglobin, median (IQR)	12.6 (10.6-14.1)	11.8 (10.0-13.6)	11.2 (9.9-13.9)	12.0 (10.1-13.6)	10.7 (10.0-12.9)	12.5 (10.2-14.1)

Of the 157 patients; 17 (11%), 29 (18%), 44 (28%) and 17 (11%) were identified as positive for TB based on Gene Xpert MTB/RIF, Sputum Microscopy, LAM determine strip test and Urine Culture, respectively (Table 4.7).

Table 4.7: Urine Gene Xpert MTB/RIF results versus Sputum Microscopy from HIV patients with signs of Tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya

Characteristics	Sputum microscopy		LAM determine		Urine culture		Total
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	
Gene Xpert MTB/RIF							
Positive	5(17.2)	12(9.3)	17(38.6)	0 (0.0)	17(100)	0 (0.0)	17(11%)
Negative	24(82.8)	117(90.7)	27(61.4)	114(100.0)	0 (0.0)	141(100.0)	141(89%)
Total	**29(18%)**	**129(82%)**	**44(28%)**	**114 (72%)**	**17 (11%)**	**141 (89%)**	

The sensitivity of Gene Xpert MTB/RIF using urine against Sputum Microscopy was 17%, with a positive predictive value of 29%. On the other hand, the specificity of Gene Xpert MTB/RIF using urine against Sputum Microscopy was 91%, with a negative predictive value of 83%. When stratified by CD4 categories, the test showed to be more sensitive (30% vs 11%) and specific (92% vs 89%) to CD4≤100 cells/mm^3 compared to CD4>100 cells/mm^3, respectively. On the other hand, when urine Gene Xpert MTB/RIF test was compared with LAM determine strip test, the sensitivity and specificity increased to 39% and 100%, respectively. Similar increase was realized within the CD4 cell categories. Gene Xpert MTB/RIF test and Urine culture had a perfect agreement leading to a sensitivity and specificity of 100% each.

The negative and positive likelihood ratios for urine Gene Xpert MTB/RIF against Sputum Microscopy were 0.9 and 1.9, respectively. The positive likelihood ratio implies that a person with TB is 1.9 times more likely to have a positive result based on Gene Xpert MTB/RIF test than a person without TB. Conversely, the negative likelihood ratio shows that a person without TB is 0.9 times more likely to have a negative test based on Gene Xpert MTB/RIF test compared with a person with TB. When stratified by CD4 categories, the negative and positive likelihood ratios for urine Gene Xpert MTB/RIF against sputum microscopy were 0.8 and 3.9, respectively for CD4≤100 cells/mm^3 and 1.0 and 1.1, respectively, for CD4>100 cells/mm^3. These results are shown in Table 4.8.

Table 4.8: Sensitivity and specificity of Gene Xpert MTB/RIF using urine from HIV patients with signs of Tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya

Indicator	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Likelihood Ratio*	
					Negative	Positive
Gene Xpert MTB/RIF vs Sputum microscopy						
Overall	17.2	90.7	29.4	83.4	0.91	1.85
CD4 ≤100	30.0	92.3	50.0	83.7	0.76	3.90
CD4 >100	11.1	89.8	18.2	83.2	0.99	1.09
Gene Xpert MTB/RIF vs LAM						
Overall	38.6	100.0	100.0	80.9	0.61	—
CD4 ≤100	33.3	100.0	100.0	72.1	0.67	—
CD4 >100	42.3	100.0	100.0	84.2	0.58	—
Gene Xpert MTB/RIF vs Urine culture						
Overall	100.0	100.0	100.0	100.0	0.00	—
CD4 ≤100	100.0	100.0	100.0	100.0	0.00	—
CD4 >100	100.0	100.0	100.0	100.0	0.00	—

Table legend. * The negative likelihood ratio is calculated as (1-sensitivity) ÷ specificity, and the positive likelihood ratio as sensitivity ÷ (1-specificity).[1]

The plotted receiver operating characteristic (ROC) curve yielded an area under the curve (AUC) of 0.54 (95% CI=0.47-0.61) as shown in figure 4.1. Evidently, the curve comes closer to the 45-degree diagonal hence the urine Gene Xpert MTB/RIF is not accurate when used alone for identifying TB positive persons.

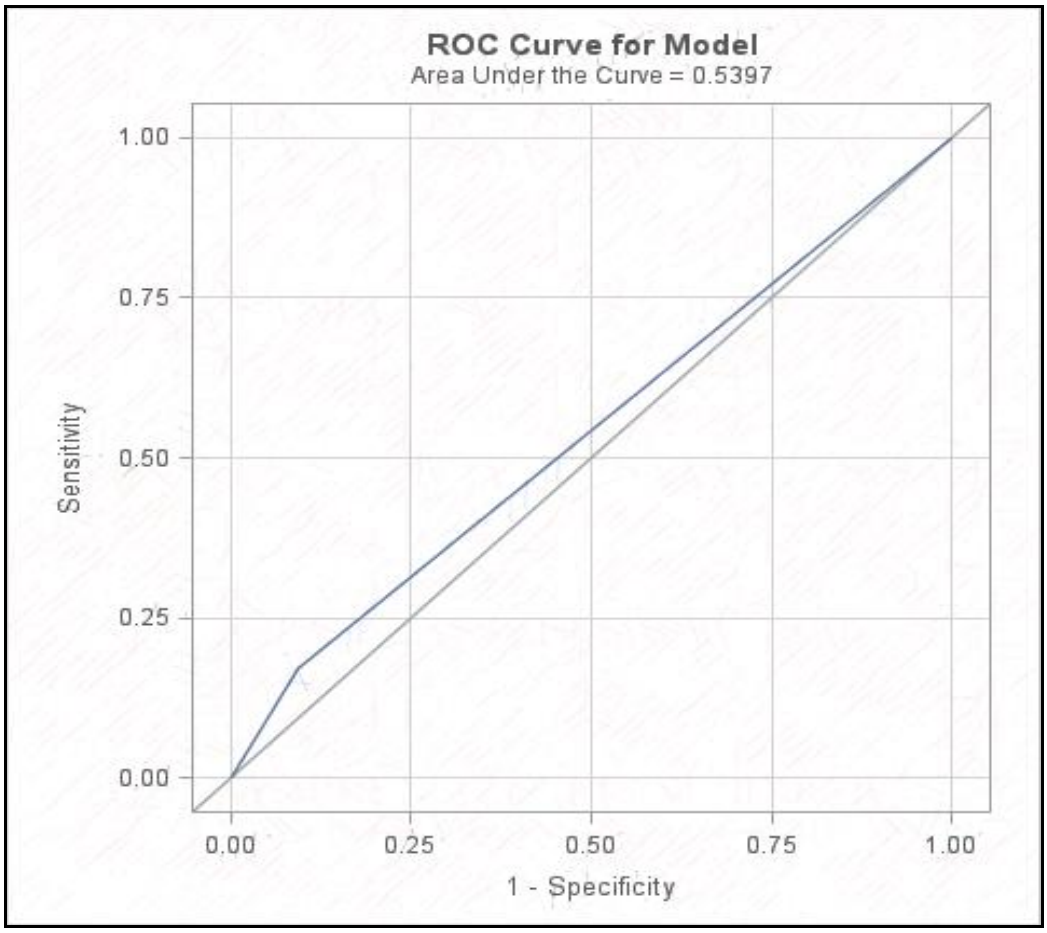

Figure 4.1: Receiver operating characteristic (ROC) curve of Urine Gene xpert MTB/RIF test against Sputum Microscopy for identifying TB among HIV-infected patients with signs of Tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya. The area under the receiver operating characteristic curve was 0.54 (95% CI=0.47-0.61).

Table 4.9:Gamma statistics for measuring the correlation between the GeneXpert results and CD4 cell cut-off points

CD4 cell cut-off points	Gene Xpert results		Total	Gamma coefficient (95% CI)
	Positive	**Negative**		
CD4 ≤100	6 (12%)	43 (88%)	49	0.09 (-0.43;0.62)
CD4 >100	11 (10%)	95 (90%)	106	
Total	**17**	**138**	**155**	

Table legend. The Gamma correlation coefficient (0.09) revealed a weak correlation between CD4 cells cut-off point (CD4≤100/CD4>100) and Gene Xpert MTB/RIF results. The asymptotic 95% CI for the gamma coefficient contains zero implying that the correlation was not statistically significant (Table 4.9).

4.3.Specificity and sensitivity of LAM determine strip test using urine from HIV patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya.

Table 4.10: Demographic and clinical characteristics among HIV-infected patients in MTRH, 2011-2013

Characteristics	LAM determine		Gene Xpert MTB/RIF		CD4 count, cell/mm3	
	Positive	Negative	Positive	Negative	≤ 100 cell/mm3	> 100 cell/mm3
Gender (Female)	13 (15.3)	72 (84.7)	9 (10.6)	76 (89.4)	24 (29.3)	58 (70.7)
Age (years), median (IQR)	40 (34-45)	38 (33-45)	38 (32-45)	39 (33-45)	38 (34-43)	39 (33-46)
WHO stage						
Stage I & II	5 (20.0)	20 (80.0)	3 (12.0)	22 (88.0)	4 (16.0)	21 (84.0)
Stage III & IV	16 (15.2)	89 (84.8)	11 (10.5)	94 (89.5)	39 (37.9)	64 (62.1)
Hemoglobin, median (IQR)	11.2 (9.9-13.9)	12.0 (10.1-13.6)	12.6 (10.6-14.1)	11.8 (10.0-13.6)	10.7 (10.0-12.9)	12.5 (10.2-14.1)

Of the 157 patients; 44 (28%), 17 (11%), 29 (18%), and 17 (11%) were identified as positive for tuberculosis based on, LAM determine Strip Test, Gene Xpert MTB/RIF, Sputum Microscopy and Urine Culture, respectively (Table 4.11).

Table 4.11: Urine Gene Xpert MTB/RIF results versus Sputum Microscopy from HIV patients with signs of Tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya

Characteristics	Sputum microscopy		GeneXpert MTB/RIF		Urine culture		Total
	Positive	Negative	Positive	Negative	Positive	Negative	
LAM determine							
Positive	11 (37.9)	33 (25.6)	17(100.0)	27 (19.2)	17(100.0)	27 (19.2)	44 (28%)
Negative	18 (62.1)	96 (74.4)	0 (0.0)	114 (80.8)	0 (0.0)	114 (80.8)	114(72%)
Total	**29(18%)**	**129(82%)**	**17(11%)**	**141(89%)**	**17(11%)**	**141(89%)**	

The sensitivity of LAM determines Strip Test against sputum microscopy was 38%, with a positive predictive value of 25%. On the other hand, the specificity of LAM determines Strip Test against Sputum Microscopy was 74%, with a negative predictive value of 84%. Putting CD4 stratification into consideration, sensitivity increased to 60% for those with CD4≤100 cells/mm^3 whereas the specificity slightly increased to 76% for those with CD4>100 cells/mm^3. The comparison of LAM determines Strip Test against Gene Xpert MTB/RIF and Urine culture were similar and it increased the sensitivity and specificity to 100% and 81%, respectively. When stratified by CD4 categories, the comparison of LAM determine Strip Test against Gene Xpert MTB/RIF and Urine culture increased the sensitivity to 100% but only increased specificity to 84% for persons with CD4>100 cells/mm^3.

The negative and positive likelihood ratios for LAM determine Strip Test against Sputum Microscopy were 0.8 and 1.5, respectively. When stratified by CD4 categories, the negative and positive likelihood ratios for LAM determine Strip Test against Sputum Microscopy were 0.6 and 2.0, respectively, for CD4≤100 cells/mm^3 and 1.0 and 1.2, respectively, for CD4>100 cells/mm^3. These results are shown in Table 4.12.

Table 4.12: Sensitivity and specificity of LAM determine strip test using urine from HIV patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya

Indicator	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Likelihood Ratio*	
					Negative	Positive
LAM vs Sputum microscopy						
Overall	37.9	74.4	25.0	84.2	0.83	1.48
CD4 ≤100	60.0	69.2	33.3	87.1	0.58	1.95
CD4 >100	27.8	76.1	19.2	83.8	0.95	1.16
LAM vs GeneXpert MTB/RIF						
Overall	100.0	80.9	38.6	100.0	0.00	5.24
CD4 ≤100	100.0	33.3	72.1	100.0	0.00	1.50
CD4 >100	100.0	84.2	42.3	100.0	0.00	6.33
LAM vs Urine culture						
Overall	100.0	80.9	38.6	100.0	0.00	5.24
CD4 ≤100	100.0	33.3	72.1	100.0	0.00	1.50
CD4 >100	100.0	84.2	42.3	100.0	0.00	6.33

Table legend. * The negative likelihood ratio is calculated as (1-sensitivity) ÷ specificity, and the positive likelihood ratio as sensitivity ÷ (1-specificity).[1]

The plotted receiver operating characteristic (ROC) curve yielded an area under the curve (AUC) of 0.56 (95% CI=0.46-0.66) as shown in figure 5. Evidently, from Figure 4.2, the curve comes closer to the 45-degree diagonal hence the LAM determine strip test is not accurate when used alone for identifying TB positive persons.

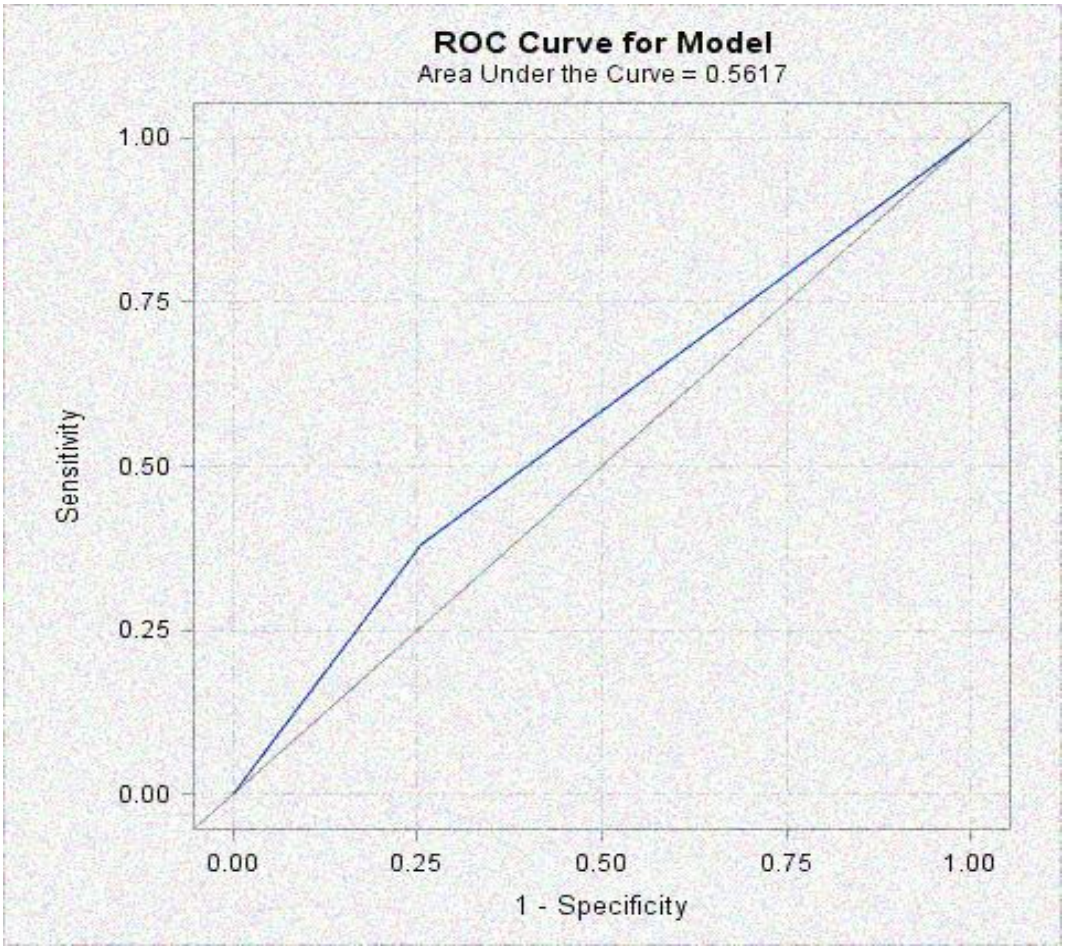

Figure 4.2: Receiver operating characteristic (ROC) curve of LAM determine Strip Test against Sputum Microscopy for identifying TB among HIV-infected patients with signs of tuberculosis at the Moi Teaching and Referral Hospital (MTRH) in Western Kenya. The area under the receiver operating characteristic curve was 0.56 (95% CI,=0.46-0.66).

Table 4.13: Gamma statistics for measuring the correlation between the LAM strip results and CD4 cell cut-off points

CD4 cell cut-off points	LAM strip results		Total	Gamma coefficient (95% CI)
	Positive	**Negative**		
CD4 ≤100	18 (37%)	31 (63%)	49	0.28 (-0.05;0.62)
CD4 >100	26 (25%)	80 (75%)	106	
Total	**44**	**111**	**155**	

Table legend. Shows the gamma statistics for measuring the correlation between LAM strip results and CD4 cell cut-off points. The results show no correlation between between LAM strip results and CD4 cellcut-offpoints.

CHAPTER FIVE

DISCUSSION

5.1. Optimal parameters (Zn, CD4 count, Hb, Creatinine, Proteinuria, and Hematuria) for Gene Xpert MTB/RIF and LAM Strip determine Test for Diagnosis of Tuberculosis using urine from HIV patients

Extra pulmonary tuberculosis among patients suspected of having TB in this study is high and a major problem in the diagnosis of TB when co-infected with HIV. This has led to poor diagnosis and delay in treatment of patients with TB. These findings however are in agreement with other studies recently reported from South Africa which found high TB prevalence using urine from immunosuppressed persons (Peter *et al.*, 2012). About a third of the participants had CD4 count ≤ 100 cells/mm^3. This is in agreement with the study done in South Africa, which reported highest urine, MTB/RIF sensitivity of 61% in CD4 count ≤ 100 cells/ml. This implies that the lower the CD4 count the higher chances of developing TB and the case of this study participants the higher chance of detecting TB in urine (EPTB). According to the WHO disease progressions, two-thirds of the participants were either in the 3^{rd} or 4^{th} stage. This means that participants were severely immunocompromised. The hemoglobin level for these particular groups of individuals was 12.0, which is within the normal ranges implying that more than half of the participants were not anemic.

This study was also able to observe that among participants with clinical symptoms, those with weight loss, TB, Pneumonia, URTI and those with a combination of TB or Pneumonia or Meningitis formed a cumulative of 74% of all diagnosed symptoms. Approximately 28% had positive acid-fast result of which slightly more than half (55%) of this being positive.

Tuberculosis should be suspected in patients with fever, cough, night sweats, and weight loss, regardless of chest roentgenogram findings (Leung *et al.*, 1996). In

patients with residual immune function, with a CD4 count >200/μL, MTB resembles reactivation tuberculosis, with cavitation and upper lobe infiltrates on chest roentgenography, and tuberculin skin tests are often positive. With severe immunosuppression and CD4 counts below 200/μL, hilar adenopathy, pleural effusions, lack of cavitation or consolidation but presence of a miliary pattern more typical of primary MTB infection appear (Havlir and Barnes, 1999; Leung *et al.,* 1996).

Based on Gene Xpert test, 17 (11%) of the participants were identified as TB positive of which 94% were MTB detected low and 6% were MTB detected medium. The study has shown that urine Xpert is a useful tool in detecting TB in HIV patience and offer incremental yield when it comes to diagnosis of tuberculosis using urine as a testing sample. Rifampicin was not detected in 88% of the TB Gene Xpert positive participants with the remaining (12%) being indeterminate, whereas all of them were sensitive to drugs. This is an indication that, in this particular population there is no drug resistance and the diagnosed cases can be treated with the drugs of choice (isoniazid (H), rifampicin (R), pyrazinamide (Z), ethambutol (E)/streptomycin (S)). When tested using the LAM test, 28% of the participants tested positive for TB. The presence of LAM in urine could indicate the presence of disseminated tuberculosis i.e. tuberculosis other than pulmonary tuberculosis, principally pertaining to the kidney.

The organ distribution of MTB in AIDS is widespread. Extrapulmonary MTB is found in70% of patients with a CD4 lymphocyte count less than 100/μL and in 28% of those with a CD4 lymphocyte count greater than 300/μL. This could be explained by the fact that when CD4 lymphocyte count are less than 100/μL its an indication of severe immunosuppression hence high chance of developing EPTB. This is also evident in autopsy done in other studies where it was seen that the respiratory tract was involved

most frequently, followed by spleen, lymph node, liver and genitourinary tract. Bone marrow, gastrointestinal tract, and adrenal are less common sites of involvement with *Mycobacterium tuberculosis* uncommonly identified in central nervous system, heart, and skin (Klatt *et al.,* 1994; Waxman *et al.,* 1995). The findings indicate renal abnormalities and a failure in glomerular filtration mechanisms.

This study also found that presence of protein (proteinuria) or blood (hematuria) in urine was significantly associated with TB positivity based on LAM determine strip test. Human immunodeficiency virus infected patients who have severe immunosuppression have high bacillary burden, which could be the cause of this situation. Also can be as a result of HIV associated nephropathy (HIVAN), a nephrotic syndrome. This can be explained by the fact that HIVAN is characterized by marked proteinuria and a rapid progression to renal failure and end stage renal disease (ERSD) (Schwartz *et al.,* 1998; D'Agati and Appel, 1998; Herman and Klotman, 2003). Immune complex mediated glomerulonephritis may also occur in HIV-infected patients. The antigen that forms the basis for the immune complex formation is p24. A proliferative glomerulonephritis ensues and patients can present with proteinuria and renal failure (Kimmel *et al.,* 1993). More studies are needed to explain more on the mechanisms involved.

Radiological findings revealed in this study are that majority had infiltrate, milliary TB and opacities respectively. Chest x-ray (CXR) is mostly used as the diagnostic tool for TB available to clinicians at hospitals in high and low burden settings (Wilson *et al.,* 2006). However, the sensitivity and specificity of CXR for TB diagnosis in different settings is highly variable especially when used alone in region with high HIV

prevalence. This means that if chest X-ray is to be used alone, majority of TB cases would be left out leading to wrong diagnosis.

About 10%-71% of HIV/TB co-infected patients have normal CXR results despite the fact that their culture results are positive (Wilson *et al.,* 2006). Several factors such as HIV status, primary clinic or hospital setting, experience of CXR reader and even the sex of the patient contribute to the variability of the CXR performance (van Cleeff *et al.,* 2005). Notably, in HIV co-infected patients who are severely immune-compromised the radiographic appearances of TB can be atypical or absent, with one study showing up to 32% of active TB cases with a normal CXR (Wilson *et al.,* 2006; van Cleeff *et al.,* 2005). Chest radiographs cannot distinguish between current active TB and previously occurred infection. Furthermore, the poor specificity of CXR means that a number of patients are inappropriately treated with anti-TB treatment with intendant morbidity. The need for a definitive microbiological diagnosis remains high. Laboratory results must not only be accurate but also available in the shortest time possible.

5.2 Specificity and sensitivity of Gene Xpert MTB/RIF using urine from HIV patients with signs of tuberculosis

Mortality due to co-infection with HIV/TB specifically in sub-Saharan Africa is high. This is due to delayed diagnosis and treatment as a result a diagnostic tool with poor sensitivity. For us to address these concerns, this study determined the specificity and sensitivity of Gene Xpert MTB/RIF using urine from HIV patients. Gene Xpert MTB/RIF test has a probability of 0.17 to positively diagnose a person who has TB and a likelihood of 0.83 to correctly identify those who do not have TB. The urine Gene Xpert MTB/RIF may not be accurate when used alone for identifying TB positive persons.

The findings of this study are in agreement with previous study findings reported by Hsu *et al*., (2011) which have revealed improved sensitivity with an increase in immunosuppression (Hsu *et al*., (2011)). Based on the current study findings, it is evident that urine Gene Xpert MTB/RIF has a good sensitivity in patients with low $CD4^{+}$ count hence it can be used in HIV positive patients with difficulties in producing sputum as a rapid tool to make fast diagnosis. Also when urine Gene Xpert MTB/RIF is combined with other tests, its sensitivity increases. These study findings are in agreement with other studies (Hsu *et al*., 2011). Thus urine Gene Xpert MTB/RIF is useful in patients with extrapulmonary tuberculosis (EPTB) and can be used alone or in combination. The fact that urine Gene Xpert MTB/RIF offers a rapid diagnosis within 12 hours and detection of early TB. It can also detect rifampicin susceptibility test, which is an added advantage over the other urine-based diagnostics. This makes it appropriate tool for hospital use where resources are inadequate.

One of the study limitations is that sputum Gene Xpert MTB/RIF was not done. If sputum Gene Xpert MTB/RIF results were available, the performance of Xpert MTB/RIF would have been compared using urine and sputum samples. Since sputum Gene Xpert MTB/RIF detects tuberculosis in patients with pulmonary tuberculosis only, the presence of MTBDNA in urine leading to Xpert positivity is highly suggestive of genital-urinary tuberculosis due to the fact that it most often asymptomatic (Hsu *et al.,* 2011). Clinical manifestations of urogenital tuberculosis (UTB) are not specific leading to delayed diagnosis and treatment which can cause kidney dysfunction, ureteral stricture, and shrunken bladder. Since diagnosis of UTB is made very late, there is need for a more sensitive diagnostic tool. In this case urine Xpert MTB/RIF that is a real time PCR assay would be very important. The sensitivity of PCR methods on urine sample was previously reported to be between 60% and 100% for diagnosis of

UTB (Hemal *et al*., 2000). Since urine Xpert MTB/RIF is a PCR method, the results may be affected by presence of metabolites, drugs or any other biological materials in the body fluids (Manjunath *et al*., 1991). False negative results may be due to the fact that the distribution of bacteria is not homogeneous. Therefore it would be very important to test several specimens from a patient and select qualified specimens with good concentrations for analysis to be done. Low sensitivity might be as a result of lack of bacilli in the urine sample, which could be due to the fact that bacilli are eliminated as waste from time to time.

No correlation was seen between urine Xpert MTB/RIF and CD4 cell cut-off points. The absence of correlation between Xpert MTB/RIF and CD4 cell cut-off points could reflect inadequacy of samples in terms of amount, kidney defects and complete body bacillary burden.

In conclusion, Xpert MTB/RIF is a novel sputum based diagnostic that may be applied to urine as a sample. Also the combination of Xpert MTB/RIF and other urine-based methods could improve the TB diagnosis in HIV patients who are severely immunosuppressed. Urogenital tuberculosis should be highly suspected whenever urine Xpert MTB/RIF is positive. Urine Xpert MTB/RIF can be considered as an instant diagnostic tool before the other tests in the diagnostic platform. Therefore, urine Xpert MTB/RIF can meet the needs of the health care scenery since it has the potential to strengthen the diagnosis of TB in urine hence an increase in case findings. In addition, due to the fact that urine Xpert MTB/RIF is fast in producing results and requires no special training, it gives room for speedy enactment of treatment.

5.3. Specificity and sensitivity of LAM strip test using urine from HIV patients with signs of tuberculosis

The study findings are in agreement with previous study findings, which have revealed improved sensitivity with an increase in immunosuppression (Lawn, 2012, Shah et al., 2010; Shah *et al.,* 2009). Work done by Lawn *et al*., (2012) on pathogen and host factor potentially impacting the detection of LAM in urine while Shah *et al*. (2010) reported on HIV-infection, mycobacteria, and positive sputum smear were risk factors for a positive LAM test. Work done by Shah *et al.*,(2010) further suggested that HIV-infected TB suspects with advanced immunosuppression, a group in which sputum microscopy is of low yield, may be a target population for whom the urine LAM test would be particularly useful as they found that among HIV-infected patients, individuals with CD4counts of <50 had an average OD that was 1.05 optical density units higher than that for individuals with CD4 counts of >150($P < 0.0001$) (Shah *et al.,* 2009). Also in another study reported by Lawn (2011) which indicated that this assay has sensitivity which is convenient for the diagnosis of HIV-associated TB in patients with advanced immune-deficiency (Lawn *et al.,* 2009). In another study by Wood *et al*., (2011) specified that urinary LAM is associated to host immune factors (Wood *et al.,* 2011). This implies that the host immune system plays a major role as seen in this study that the lower the CD4counts, the higher chances of detecting LAM in urine.

When stratified by CD4 categories, the negative and positive likelihood ratios for LAM determine strip test against sputum microscopy were 0.6 and 2.0, respectively, for CD4≤100 cells/mm^3 and 1.0 and 1.2, respectively, for CD4>100 cells/mm^3. The current findings are in agreement with those described in a systematic review of seven studies that evaluated test precision using only microbiologically confirmed cases sensitivity

was 13%–93% and specificity 87–99% (Talbot *et al.*, 2012; Boehme *et al.*, 2005; Shah *et al.*, 2009; Manjunath *et al*., 1991; Cleeff *et al.*, 2005; Hemal *et al*., 2000; Wood *et al.*, 2011). In addition, Boehme and colleagues (Boehme *et al.*, 2005) indicated that using an earlier version of the present urine LAM assay (Chemogen, South Portland, Maine) to evaluate 231 TB suspects (69%HIV-positive) and 103 healthy controls in Tanzania. Another study (Talbot *et al.*, 2012) also maintained that the role of urine LAM in the diagnosis of tuberculosis is reliable as they found among 69 cases of sputum or blood culture confirmed tuberculosis, LAM sensitivity was 65% and specificity 86% as compared to 36% and 98% for the sputum smear.

Dissimilarities, which were seen in test features, might possibly be as a result of diverse LAM testing approaches and different population. Urine collection and processing may influence the test precision, although analysis of sub-groups in which the urine used in the assay was either fresh or previously frozen found no statistically significant differences between these groups.

Based on the current study findings, it is evident that LAM strip test has a good sensitivity in patients with low CD4 + count hence it can be used in these particular group of patients as a rapid tool to make fast diagnosis. In addition, when LAM strip test is combined with other tests, its sensitivity increases. Thus LAM strip test is useful in patients with extrapulmonary tuberculosis (EPTB). The fact that LAM strip test is easy and rapid to use, makes it the most appropriate diagnostic tool in poor-resource settings.

5.4. Study Limitations

Potential limitations noted in this study were:

a). LAM strip test results were not used to make clinical decisions.

b). Additional samples may be required since LAM strip test does not have information on the susceptibility test.

c). For the LAM evaluation, this study did not use fresh urine samples but instead used frozen urine sample, which may possibly have interfered with the performance of the test.

d). The diagnostic performance parameter used in the current study was based on the sensitivity of the test only. If urine Xpert MTB/RIF specificity were used to determine whether the test is useful, it would have helped in decision-making.

f). No sputum Xpert MTB/RIF was done. If sputum Gene Xpert MTB/RIF results was available, the performance of Xpert MTB/RIF would have been compared using urine and sputum samples.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1. Summary of Findings

The results showed that urine Xpert MTB/RIF® is a sensitive and specific tool for diagnosis of tuberculosis using urine samples and that Zn, CD4 count, Proteinuria, and Hematuria are the optimal parameters for its validation. Patients with CD4≤100 had HB levels less than normal implying that this particular group of patients was anemic. The sensitivity of combined urine Xpert MTB/RIF® urinary LAM determine strip increases as the immunity of the patients decreases. Both urine Xpert MTB/RIF® and Urinary LAM can be used as an adjunct test for diagnosis of active TB and in combination with other tests in the diagnosis platform since the sensitivity is improved when combined with other tests than when alone. Therefore, urine Xpert MTB/RIF® and LAM determine strip test are not accurate when used alone for identifying TB positive persons.

6.2 Conclusions

i) Renal function and glomerular dysfunction may be as a result of renal tuberculosis as suggested by the strong association between urine Xpert MTB/RIF® positivity and presence of protein or blood in urine.

ii) Xpert MTB/RIF is a novel sputum based diagnostic that may be applied to urine as a sample. Also the combination of Xpert MTB/RIF and other urine-based methods could improve the diagnosis in HIV patients who are severely immunosuppressed. Urine Xpert MTB/RIF® is a highly sensitive and specific tool for diagnosis of TB using urine in immunosuppressed individual where sputum diagnosis is not feasible. Urine Xpert MTB/RIF® can offer rifampicin drug susceptibility of MTB/RIF. On the other hand urinary LAM strip test is only highly sensitive (but not specific) in diagnosis of tuberculosis in HIV patients particularly in immunosuppressed individual.

iii) The LAM determines strip test is not accurate when used alone for identifying TB positive persons.

iv) It can be used as an adjunct test for diagnosis of TB and in combination with other tests in the diagnosis platform.

6.4. Recommendations from the current study

a) This study recommends that presence of protein or blood in urine should be used as optimal parameters when determining tuberculosis using Xpert MTB/RIF® assay.

b) This study recommends the use of urine Xpert MTB/RIF® assay in HIV-infected patients with advanced immunosuppression in combination or alone.

c) This study recommends the use of LAM strip test in HIV-infected patients with advanced immunosuppression in combination with other tests in the diagnostic platform.

6.5. Suggestions for Future Research

a) Further researches are needed in renal biopsy in HIV-infected patients with advanced immunosuppression to confirm the current findings.

b) There is need for larger studies, which uses bigger population. In such settings, the assay would need to be tested in different populations using different non-sputum sample (CSF, pleural fluid, pus etc.) in Kenya.

c) There is need for larger studies, which uses fresh urine samples in HIV-infected patients with advanced immunosuppression to confirm the current findings. This is because it is not clear whether fresh samples could have yielded better results than frozen samples.

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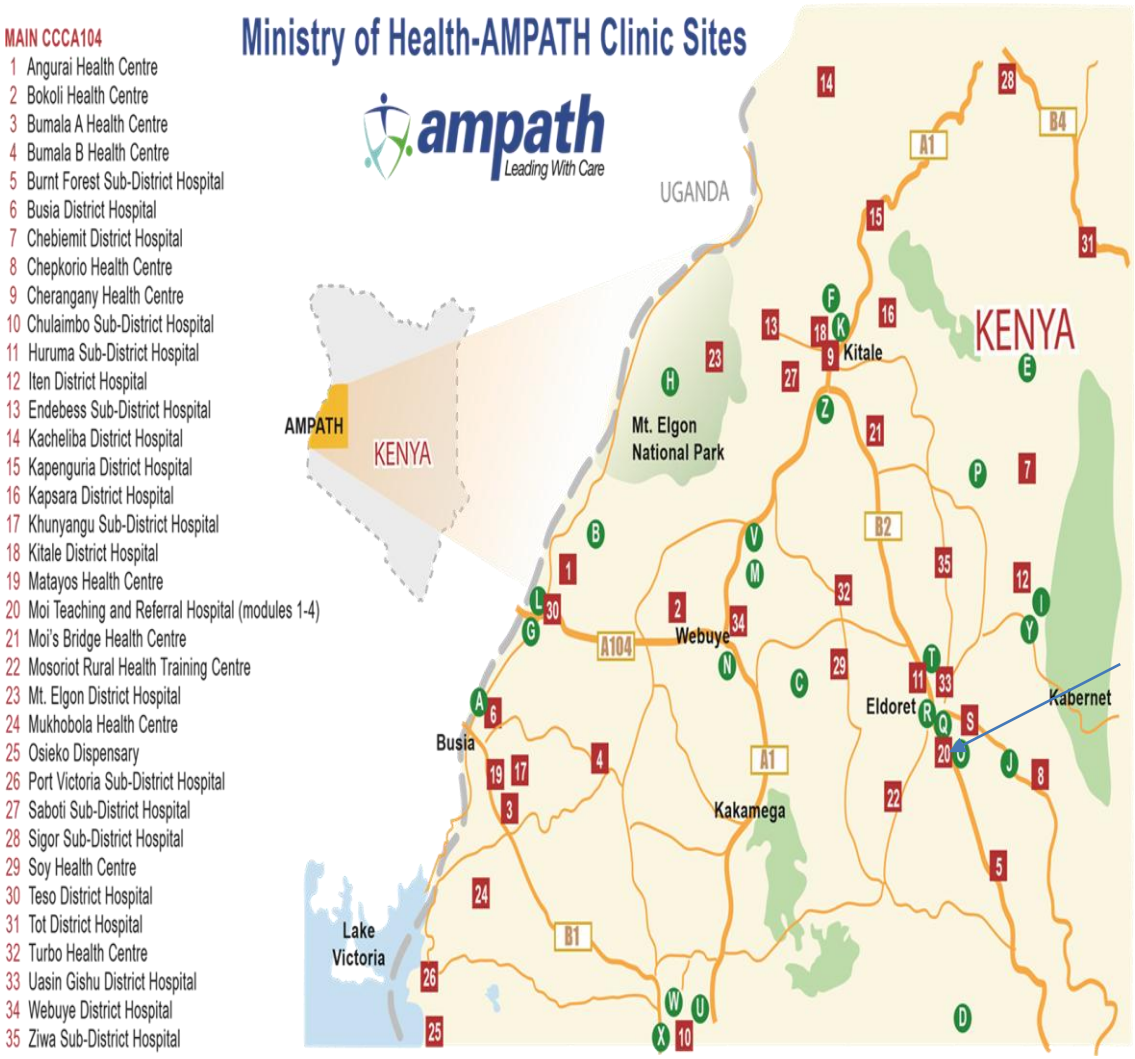
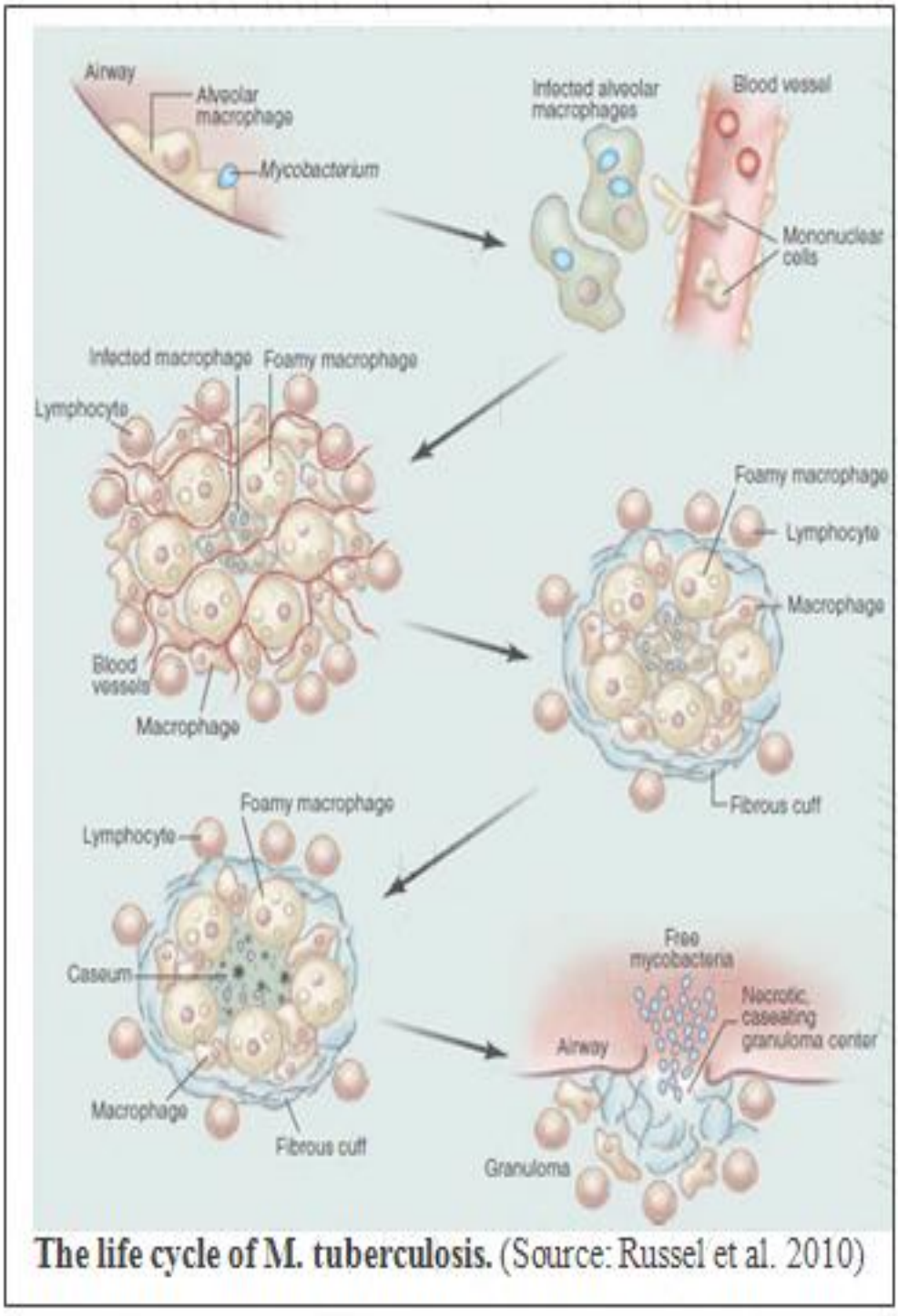
APPENDICES

APPENDIX 1: MAP OF KENYA SHOWING ELDORET



The map shows the Location of Moi Teaching and Referral Hospital (arrowed) (Adapted from Google – Map data ©2011).

APPENDIX 2: LIFE CYCLE OF MYCOBACTERIUM SPP.



The life cycle of M. tuberculosis. (Source: Russel et al. 2010)

APPENDIX 3: IREC APPROVAL

INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE (IREC)

MOI TEACHING AND REFERRAL HOSPITAL
P.O. BOX 3
ELDORET
Tel: 33471//2/3

MOI UNIVERSITY
SCHOOL OF MEDICINE
P.O. BOX 4606
ELDORET

Reference: IREC/2013/141
Approval Number: 0001084

4th October, 2013

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE APPROVED 04 OCT 2013 P.O. Box 4606 ELDORET

Iddah Maulid Ali,
Moi Teaching and Referral Hospital,
P.O. Box 03-30100,
ELDORET-KENYA.

Dear Ms. Maulid,

RE: FORMAL APPROVAL

The Institutional Research and Ethics Committee have reviewed your research proposal titled:-

"Detection of Multidrug Resistant Tuberculosis in Urine and Sputum Samples of HIV –Infected Patients Using Xpert MTB/RIF and LAM Elisa Technique".

Your proposal has been granted a Formal Approval Number: ***FAN: IREC 1084*** on 4th October, 2013. You are therefore permitted to begin your investigations.

Note that this approval is for 1 year; it will thus expire on 3rd October, 2014. If it is necessary to continue with this research beyond the expiry date, a request for continuation should be made in writing to IREC Secretariat two months prior to the expiry date.

You are required to submit progress report(s) regularly as dictated by your proposal. Furthermore, you must notify the Committee of any proposal change (s) or amendment (s), serious or unexpected outcomes related to the conduct of the study, or study termination for any reason. The Committee expects to receive a final report at the end of the study.

Sincerely,

PROF. E. WERE
CHAIRMAN
INSTITUTIONAL RESEARCH AND ETHICS COMMITTEE

cc	Director	-	MTRH	Dean	-	SOM	Dean	- SON
	Principal	-	CHS	Dean	-	SPH	Dean	- SOD

APPENDIX 4: MOI TEACHING AND REFERRAL APPROVAL

MOI TEACHING AND REFERRAL HOSPITAL

Telephone: 2033471/2/3/4
Fax: 61749
Email: director@mtrh.or.ke
Ref: ELD/MTRH/R.6/VOL.II/2008

P. O. Box 3
ELDORET

18th February, 2014

Iddah Maulid Ali,
Moi Teaching and Referral Hospital,
P.O. Box 03-30100,
ELDORET-KENYA.

RE: APPROVAL TO CONDUCT RESEARCH AT MTRH

Upon obtaining approval from the Institutional Research and Ethics Committee (IREC) to conduct your research proposal titled:-

"Detection of Multidrug Resistant Tuberculosis in Urine and Sputum Samples of HIV-Infected Patients Using Xpert MTB/RIF and LAM Elisa Technique".

You are hereby permitted to commence your investigation at Moi Teaching and Referral Hospital.

11/02/2014
DR. W. ARUASA
AG. DIRECTOR
MOI TEACHING AND REFERRAL HOSPITAL

INSTITUTIONAL RESEARCH & ETHICS COMMITTEE
18 FEB 2014
APPROVED
P. O. Box 4606-30100 ELDORET

CC - Deputy Director (CS)
- Chief Nurse
- HOD, HRISM

APPENDIX 5 :INFORMED CONSENT (ENGLISH VERSION)

You are invited to take part in this research study called **DETECTION OF MULTIDRUG RESISTANT TUBERCULOSIS IN URINE AND SPUTUM SAMPLES OF HIV INFECTED PATIENTS USING XPERT MTB/RIF AND LAM ELISA TECHNIQUE**. It is being carried out in Ampath/MTRH clinic Western Kenya. Before you decide if you want to take part this study, it is important for you to understand why the research is being done and what it will involve. Please take time to read this information sheet carefully or ask someone to read it to you.

This is a consent form. It gives you information about this study. The study staff will talk with you about this information. You are free to ask questions about this study at any time. We will give you a copy to keep. Please ask the clinic nurses or counsellors if there is anything that is not clear or if you would like more information.

Your participation in this research study is entirely voluntary. This means that you may decide that you do not wish to take part now or you may wish to withdraw from the study later. These decisions will not influence the care you receive now or in future. We hope that if you decide to join the study but withdraw later, you would give a reason for your decision. More importantly we hope that you would continue to allow us to provide follow-up care, which involves continued regular medical checkups, even if you are no longer taking the study medicines. Take time to decide whether or not you wish to take part.

Purpose of study:
HIV is the virus that causes AIDS. At the moment there is no cure for HIV. However there are anti-HIV medicines, called 'anti-retrovirals' or ARVs, which can control HIV and keep people well for a long time. Unfortunately in many African countries patients often are only able to come for treatment when they are very sick and HIV has already had a big effect on their bodies. For example they may often have malnutrition, diarrhoea, infections such as **tuberculosis,** serious lung infections (pneumonia) and other severe infections. This means that more people die sometime during the first three months after starting ARVs; this is quite different compared to patients in rich countries. This study intends to assess the sensitivity and clinical utility of Xpert MTB/RIF assay and LAM ELISA for rapid diagnosis of tuberculosis in HIV-infected patients with low CD4+ levels using urine versus sputum.

Number of people taking part in the study:
If you consent, you will be one of approximately 157 participants in this research and subsequent follow up will be done.
Procedure for the study:
If you agree to be in this study, you will be doing the following:
1). Consent to a TB diagnosis being done on your urine to determine the presence or absence of TB in urine.
2). Participate in an interview about your state of health before visiting TB clinic. This will include the problem you are facing and what treatments you used.
It is estimated that the both the consenting and verbal interview will take between 45 minutes to one hour to complete.
NB: Urine will be collected as soon as consent is granted and verbal interview is taken.

Risks and Benefits:
The risks of taking part in this study include emotional distress while answering the questions in the interview. Some information discovered during the interview may be upsetting to you. However, you have a choice to have that information relayed to you or not.
The immediate direct benefits to as an individual for participating in the study include identifying the TB infection and fighting it hence reducing the risk of dying from this infection. It will also contribute to the knowledge hence improved standards of care and management of HIV and AIDS patients in our care, in Kenya and the rest of the world.

Confidentiality:
All efforts will be made to keep personal information confidential. However, we cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You identity will be held in confidence in reports in which study may be published and database in which results may be stored.

Costs:
Taking part in this study will not lead to added costs to you. This study will pay for the laboratory processing of urine.

Payment:
You will not receive any payment, gifts or inducements for participating in this study.

Compensations for injury:
There are no anticipated injuries that could arise from participating in this study.

Contacts for questions or problems:
For questions about the study, please contact the research team (iddah M.Ali on 0722400223).

Participant's Consent
In consideration of all of the above, I give my consent to participate in this research study.
Participant's Signature: _____ Date: _____
(Must be dated by the subject)(put your mark if unable to write)
Name of the Participants _____

Signature of the witness: _____ Date: _____
(if unable to write)
Name of the Witness _____
Signature and Name of the Person obtaining Consent: _____
Date: _____
Name: _____

APPENDIX 6:INFORMED CONSENT (SWAHILI VERSION)

Wewe niwalioalikwa kushirikikatikautafiti huuutafitiunaoitwa **DETECTION OF MULTIDRUG RESISTANT TUBERCULOSIS IN URINE AND SPUTUM SAMPLES OF HIV INFECTED PATIENTS USING XPERT MTB/RIF AND LAM ELISA TECHNIQUE**. NiunaendeshwakatikaAmpath/MTRHklinikiMagharibi mwa Kenya. Kabla ya kuamuakama unatakakuchukua sehemuya utafiti huu, ni muhimu kwa wewe kuelewani utafiti utafiti gani unaofanywana utahusu nini.Tafadhalichukua muda wako kusomakaratasi hii yahabarikwa makini aukuuliza mtu akusomee ili uweze kuelewa zaidi.

Hii nifomu ya ridhaa.Inakupataarifa kuhusuutafiti huu.Wafanyakazi wa utafiti watazungumzana wewe kuhusuhabari hii.Kuuliza maswali kuhusuutafiti huuwakati wowote ni bure na hakuna malipo yatakao tozwa.Sisi tutakupanakalaya kutunza.Tafadhali muulizemuuguzikliniki auwashaurikama kuna kitu chochote am ambacho si wazi au ungependa kufahamishwa zaidi.

Ushiriki wakokatika utafiti huu nihiari kabisa. Hii ina maana kwambaunaweza kuamuakwamba wewe unatakakuchukua sehemu ya utafiti huu auunaweza kuamua unatakakuondoka kutokautafiti wabaadaye.Maamuzi hayahayataweza kushawishihudumaya kupokeasasa hivi au katikasiku zijazo.Ni matumaini yetukwamba kama weweutaamua kujiunga nautafitilakinikuondoabaadaye, wewebila kutoasababu ya uamuziwako.Muhimu zaidini matumaini yetukwamba ungependakuendeleakuruhusu sisi kutoakufuatiliahudumaambayo inahusishailiiendeleamara kwa mara na matibabu,hata kama wewe kutumia dawa kwenye utafiti huu.Kuchukua muda wakuamua kamaunataka kuchukuasehemu hii.

Madhumuni ya utafiti:

HIV ni virusi vinavyosababisha UKIMWI. Kwa sasa hakuna tiba ya ugonjwa huu. Hata hivyo kuna kupambana na ugonjwa huu kwa kutumia madawa, yanayoitwayo 'anti-retrovirals' au ARVs, ambayo yanaweza kudhibiti ugonjwa huu na kuweka watu pamoja na hai kwa muda mrefu. Kwa bahati mbaya katika nchi nyingi za Afrika wagonjwa mara nyingi hawana uwezo wa kuja kwa matibabu na wanapata matibabu wakati wao ni wagonjwa sana na tayari alikuwa na athari kubwa kwenye miili yao. Kwa mfano wanaweza mara nyingi kuwa na utapiamlo, kuhara, magonjwa kama vile kifua kikuu, maambukizi makubwa ya mapafu (pneumonia) na magonjwa mengine makali. Hii ina maana kwamba zaidi ya watu hufa wakati mwingine wakati wa miezi mitatu ya kwanza baada ya kuanza ARVs, hii ni tofauti kabisa ikilinganishwa na wagonjwa katika nchi tajiri. Utafiti huu unatarajiwa kutathmini unyeti na matumizi ya kliniki ya Xpert MTB / Rif assay na LAM ELISA kwa ajili ya utambuzi wa haraka wa kifua kikuu kwa wagonjwa walioambukizwa na virusi vya ukimwi na CD4+ chini ya kiwango kinacho hitajika kutumia mkojo dhidi ya makohozi.

Idadi ya watu kushiriki katika utafiti:

Kama wewe ridhaa, utakuwa mmoja wa washiriki takriban 157 katika utafiti huu na baadae kufuatilia itafanyika.

Utaratibu kwa ajili ya utafiti:

Kama unakubali kuwa katika somo hili, utakuwa kufanya yafuatayo:

1). Idhini ya utambuzi wa TB kuwa kufanyika kwenye mkojo wako kuamua kuwepo au kutokuwepo kwa TB katika mkojo.

2). Kushiriki katika mahojiano kuhusu hali yako ya afya kabla ya kutembelea TB kliniki. Hii ni pamoja na tatizo wewe ni inakabiliwa na nini matibabu unaweza kutumika.
Inakadiriwa kwamba wote waliokubaliana na mahojiano matusi itachukua kati ya dakika 45 hadi saa moja ili kukamilisha.

NB: Mkojo utakusanywa haraka kama ridhaa imetolewa na mahojiano matusi ni kuchukuliwa.

Hatari na Faida:
Hatari ya kushiriki katika utafiti huu ni pamoja na dhiki hisia wakati wa kujibu maswali katika mahojiano. Baadhi ya taarifa aligundua wakati wa mahojiano inaweza kuudhika. Hata hivyo, unaweza kuchaguwa taarifa ambayo ungependa kuambiwa au la.
Haraka moja kwa moja na kama mtu binafsi faida kwa ajili ya kushiriki katika utafiti ni pamoja na kubaini maambukizi ya TB na mapigano hayo hivyo kupunguza hatari ya kufa kutokana na ugonjwa huu. Ni pia kuchangia maarifa na hivyo kuboresha viwango vya huduma na usimamizi wa wagonjwa wa UKIMWI katika uangalizi wetu, nchini Kenya na wengine wa dunia

Usiri:
Jitihada zote zitafanywa kuweka taarifa binafsi za siri. Hata hivyo, hatukuhakikishii usiri kabisa. Habari yako binafsi inaweza kuwa wazi kama inavyotakiwa na sheria. Wewe utambulisho utafanyika katika kujiamini katika ripoti ambayo utafiti inaweza kuwa na kuchapishwa na database ambayo matokeo inaweza kuhifadhiwa.

Gharama:
Kushiriki katika utafiti huu si kusababisha kuongezeka kwa gharama kwako. Utafiti huu utajitahidi kulipa malipo ya usindikaji wa maabara ya mkojo.

Malipo:
Huwezi kupokea malipo yoyote kama zawadi, au vishawishi kwa ajili ya kushiriki katika utafiti huu.

Compensations kwa kuumia:
Hakuna majeruhi kutarajia yanayoweza kutokea kutokana na kushiriki katika utafiti huu.

Mawasiliano kwa ajili ya maswali au matatizo:
Kwa maswali kuhusu utafiti, tafadhali wasiliana na timu ya utafiti (iddah M.Ali juu 0722400223).

Mshiriki Ridhaa
Katika mawazo ya yote ya juu, natoa idhini yangu kushiriki katika utafiti huu utafiti.
Sahihi ya mshiriki: _____ Tarehe: _____
(Lazima kuwa na tarehe kwa mada) (kuweka alama yako kama hawawezi kuandika)
Jina la Washiriki _____
Saini ya Shahidi: _____ Tarehe: _____
(kama hawawezi kuandika)
Jina la Shahidi _____
Sahihi na Jina la Mtu kupata Ridhaa: _____
Tarehe: _____
Jina: _____

APPENDIX 7. SAMPLE PROCESSING PROCEDURE

1. Briefly, the sample reagent is mixed at a 2:1 ratio with 1 ml of urine.

2. Two millilitres of the reagent sample mix is transferred into an MTB/RIF assay cartridge and inserted into the GeneXpert instrument.

3. Additionally, if the MTB/RIF is negative using a 1 ml urine sample, a second pelleted urine MTB/RIF was performed, where possible, using a median (IQR) of 10 (5–10) ml urine.

4. Urine is centrifuged at 3000 g for 15 minutes and the pellet re-suspended in 1 ml of sterile phosphate-buffered saline.

5. In culture-negative non-TB urine samples used for MTB/RIF, pelleting of up to 10 mls was performed where possible.

6. Cartridges are inserted into the GeneXpert device, and then automatically generated results which are read after 90 min.

Reference:

Kent P.T., Kubica G.P. (1985). Public Health Mycobacteriology—A Guide for Level III Laboratory, Centers of Disease Control, Atlanta, Publication no. PB 86-216546.

APPENDIX 8. URINE XPERT MTB/RIF ASSAY

Principle: The Xpert MTB/RIF assay (Cepheid GeneXpert® System) , shown in figure 1.4, is a hemi-nested real-time PCR in-vitro diagnostic test that is capable of detecting M. tuberculosis complex DNA in a sample while simultaneously detecting rifampicin resistance including a semiquantitative assessment of bacillary load. The 15 minute-long step of mixing the bactericidal buffer with the sample is the only hands-on step of the whole process; this pre-amplification phase renders the M. tuberculosis non-viable and harmless as shown in figure 2.5 [78]. All these qualities of the Xpert MTB/RIF assay makes it a choice diagnostic tool for near patient usage especially in settings with poor bio-containment facilities [78, 79].

This process makes use of five overlapping molecular probes complementary to the whole M. tuberculosis rpoB gene containing the 81 base pair rifampicin resistance-determining region (RRDR) and they coverall the mutations found in >99.5% of all rifampicin resistant strains ensuring that there no cross-reactivity with non-tuberculous mycobacteria occurs [32] . If there are at least two of these rpoB probes that are positive within two cycles of each other, then the result will be M. tuberculosis positive; also if at least a single rpoB probe does not result in a measurable signal and/or a presence of a 3.5 cycle or a significant deviation in the Cycle threshold (Ct) value between the earliest and latest rpoB signals then the system regards that as a RIF resistance result [80, 81].

An estimation of 95% of RIF resistant cases arise as a result of mutations found in the RRDR [36, 39]. This hemi-nested PCR amplification assay,integrated into a single disposable cartridge, depends on six colour fluorescent molecular beacons to detect the presence of any amplified target [36, 80]. The Xpert MTB/RIF has a robust and full process control, that acts as a quality check for bacterial trapping, bacterial lysis, DNA extraction, amplification, and probe detection; this process makes use of Bacillus globigii, a spore-forming soil organism [82].

The Xpert MTB/RIF assay has been found to have a limit of detection (LOD) of 131 CFU/ml of sputum [80]. The Xpert LODs for urine and other extrapulmonary specimens have not been established yet. This could have been a vital piece of information especially in the optimization phase of these extrapulmonary specimens for use in Xpert MTB/RIF studies. However the performance of Xpert MTB/RIF in sputum as reported by the World Health Organisation (WHO) is worth investigating as this gives a good forecast of how it is likely to perform in extrapulmonary specimens like urine.

Procedure: The Xpert MTB/RIF assay procedure consists of two stages that involve the manual preparation of reagents and specimen and an automated stage. The manual phase is characterized by a 15-minute treatment of the urine samples with a NaOH and isopropanol-containing reagent which decontaminates the sample thereby significantlyeradicating any possibility of biohazard due to its ability to reduce the

viability of M. tuberculosis [79, 81]. The urine sample: reagent buffer ratio is 1:2. The mixture is transferred manually into a disposable plastic cartridge that is preloaded with liquid reagents lyophilized reagent beads after which the cartridge is carefully inserted into the Xpert MTB/RIF assay machine. The rest of the procedures are automated [36]. Figure 2.5 summarizes all the steps and procedures of the Xpert MTB/RIF assay that should be followed. Sample extraction, amplification and detection of M. tuberculosis and RIF resistance is entirely automated and done in a single cartridge; and this substantially reduces cross contamination [36].

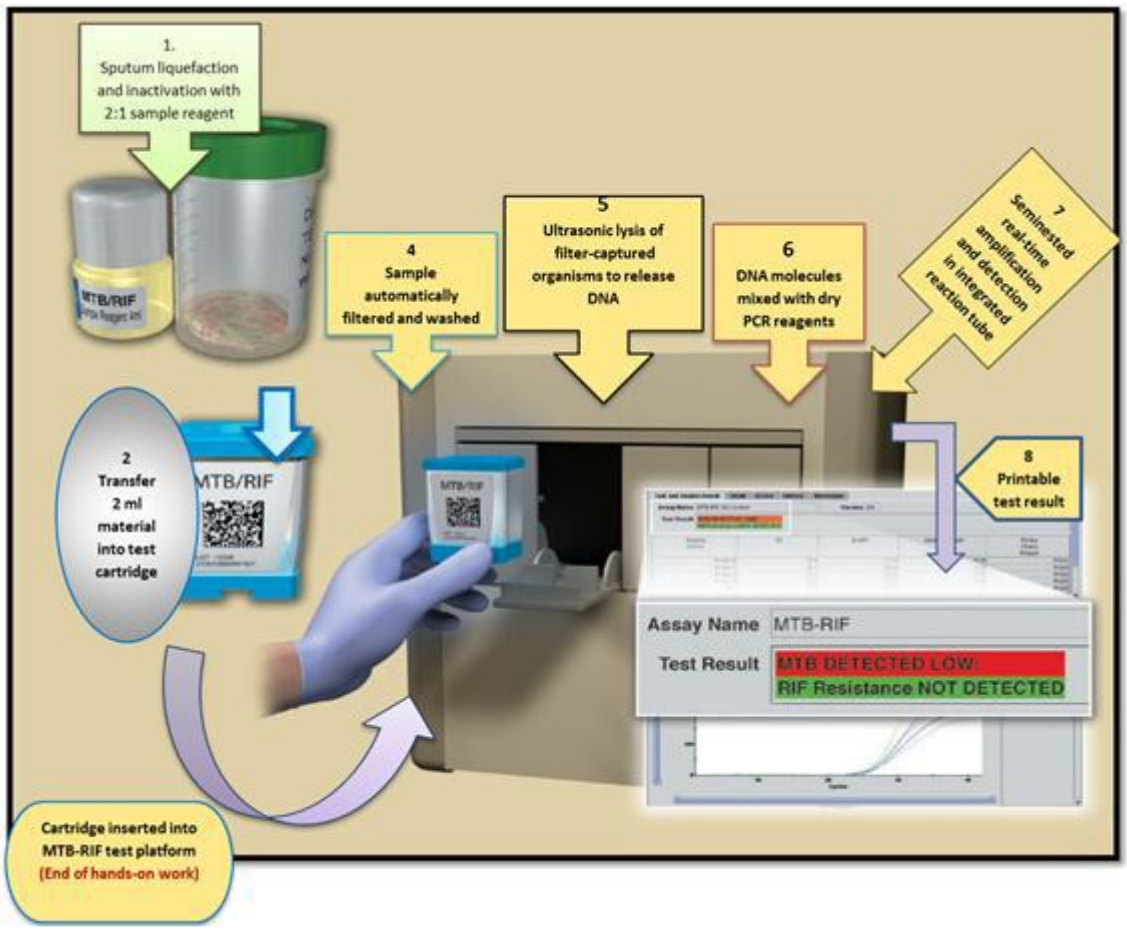


Figure 5. Assay Procedure for the MTB/RIF Test. 2 ml of bactericidal buffer is added to 1 ml of well-mixed urine. The resultant mixture is shaken for about 15 seconds, incubated at room temperature for 7 minutes, and shaken again and further incubated again for 8 minutes (a total of 15 minutes). 2 ml of the inactivated sample reagent: sample mixture is then taken out and transferred to the disposable plastic MTB/RIF cartridge and loaded into the GeneXpert device. The remaining steps that follow are automated. The automatically generated results are ready after 2 hours and are shown on the monitor screen reflecting whether MTB or RIF resistance have been detected

[7, 8].

Xpert MTB/RIF results interpretation and evaluation (adapted from the manufacturer's instructions) :

Results were displayed in the "View Window" of the Xpert MTB/RIF as a result of fluorescent signals that are quantitated and processed through an embedded calculation algorithm in the Xpert MTB/RIF software. Final results come out as MTB NEGATIVE or MTB POSITIVE, with either RIF resistance DETECTED, RIF resistance NOT DETECTED or INDETERMINATE. In the event that the M. tuberculosis positive result is displays RIF resistance DETECTED, or INDETERMINATE, the amplicon would be extracted under sterile conditions on ice using an insulin syringe and transferred into a PCR appendorf tube and stored at -80oC for later sequencing. Repetition of tests were only necessary in the event of results that were displayed as "INVALID", "INDETERMINATE", "ERROR" or "NO RESULT" as these could mean that there was a problem or faulty in the way the sample preparation was done or the volume transferred into the cartridge might have been too small for processing. Cycle threshold

(Ct) values were also displayed in the event of a positive result. Ct values represented a number of cycles needed for the fluorescent signal to cross a given background level or threshold. The Ct values are known to be inversely proportional to the amount of target M. tuberculosis DNA in the urine sample, hence a lower Ct value are representative of a higher initial concentration of the M. tuberculosis in the urine and higher Ct values reflect a lower initial concentration in the urine sample. Depending on the Ct value of the target M. tuberculosis DNA, positive results are shown as a semi-quantitation and displayed as HIGH, MEDIUM, LOW or VERY LOW as shown in table 2.2 below.

Table 2.2.XpertMTB/RIF result name and Ct value range

MTB result	**Ct range**
High	<16
Medium	16-22
Low	22-28
Very Low	>28

Appendix 9. DIAGNOSTIC TEST PARAMETERS

Sensitivity: The probability that a test result will be positive when the disease is present (true positive rate).

Specificity: The probability that a test result will be negative when the disease is not present (true negative rate).

Positive predictive value (PPV): The probability that the disease is present when the test is positive.

Negative predictive value (NPV): The probability that the disease is not present when the test is negative.

Calculation of the diagnostic test parameters

The starting point for the calculation of the sensitivity, specificity, PPV and NPV was the construction of a 2x2 table with the index test results on one side of the table and the reference standard (culture) results on the other as shown in Appendix (Florkowski, 2008).

Table ???? shows the 2x2 table format that was used to calculate* the sensitivity, specificity, PPV and NPV

REFERENCE STANDARDS			
	Disease Present**tandard**	Disease absent	Total
Index test positive	True positive (TP)	False positive (FP)	TP+FP
Index Test negative	False negative (FN)	True negative (TN)	TN+FN
Total	TP+FN	TN+FP	

**Calculations using the 2x2 table*

Sensitivity = TP/(TP+FN)

Specificity = TN/(TN+FP)

Positive Predictive Value (PPV) = TP/(TP+FP

Negative Predictive Value (NPV) = TN/(TN+FN)

APPENDIX 10. STANDARD OPERATING PROCEDURE FOR BACTE MGIT 960 TB SYSTEM

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

Purpose:

The purpose of this guideline is to describe specific laboratory test procedures to follow for BACTEC MGIT 960 TB system.

Scope:

Applies to the processing of MTRH/AMPATH TB Reference Laboratory sputum specimens.

PROCEDURE FOR PRIMARY ISOLATION OF MYCOBACTERIA FROM SPUTUM

A. Introduction

MGIT contain modified middle broke 7H9 broth base. When supplemented with MGIT growth supplement and PANTA it provides an optimum medium for growth of majority of mycobacterial species. Specimens that are mucoid are expected to contain contaminating bacteria as normal flora need to be digested and decontaminated before inoculation. After decontamination the specimen should be centrifuged and the sediment is used for preparation of smear and inoculation for culture.

B: PRINCIPLE OF THE BACTEC ™ MGIT ™ 960 SYSTEMS

1) MGIT medium

The MGIT (mycobacterium Growth Indicator Tube) consist of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contain 7.0 ml of modified middle brook 7H9 broth base. This medium is terminally sterilized by autoclaving. An enrichment, MGIT OADC (oleic acid, albumin dextrose and catalase) or MGIT 960 growth supplement is added to make the medium complete. This growth supplement is essential for growth of many mycobacteria especially those belonging to *M tuberculosis* complex .Addition of MGIT PANTA is necessary to suppress contamination. This liquid medium in

conjunction with oxygen sensor at the bottom of the MGIT tube, help in detection of mycobacteria growth more rapidly than solid media.

Bactec MGIT960 Equipment Operation and Maintenance
See Appendix 1

1. Principle of detection

A fluorescence compound Tris 4,7- diphenyl -1,10- phenonthroline ruthenium chloride pentahydrate is embedded in silicon on the bottom of the 16×100mm round bottom tube. The special fluorescence compound is sensitive to the presence of oxygen dissolved in broth medium and remain very weakly fluorescent when uninoculated MGIT tube is observed under UV light. When bacteria grow in the medium they consume oxygen present in the medium. The depletion of oxygen from the medium triggers emission of fluorescence from the sensor which is detected by the BACTEC MGIT 960 instrument with UV light source. The more oxygen depleted the higher the fluorescence. The inoculated tubes entered into the instrument are continuously incubated at 37°C and monitored at every 60min, for increasing fluorescence. The intensity of fluorescence is directly proportional to the extent of oxygen depletion. The instrument declares a tube negative if it remains negative for six weeks (42days). The detection of growth can be visually observed by the presence of turbidity.

B. Important safety precautions.

Perform all procedures e.g. processing of specimen, smear preparation, inoculum preparation, making dilution, inoculation of media, subculture, etc. in a suitable biological safety cabinet in room dedicated for mycobacterial work.

- Use proper protective gowns, gloves and respirator masks.
- Use appropriate mycobacterial disinfectants especially of phenol based
- Prior to use examine all MGIT tubes for evidence of damage. Do not use any tube that exhibits crack(s) or defect.

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

C) Specimen handling.

1. Collection

Specimens should be collected in clear preferably sterile containers with tight fitted lids or caps.Three negative specimens collected at different times should be processed for each new case. At least one morning and two spot specimens of coughed out sputum (not saliva) 2-10 ml each should be collected.

2. Transportation

Specimens should be transported to the lab as soon as possible. Delay in transportation especially in hot weather, result in an increase in contaminating bacteria. Transport specimens in a container in which temperature could be maintained as low as possible.

3. Storage

Upon receipt, store specimens in cool condition, preferably in the refrigerator.

C. DIGESTION DECONTAMINATION AND CONCENTRATION

Material/ Equipments required

-Disposable 50ml plastic tubes (falcon tubes)

-Phosphate buffer PH 6.8(0.067M). Commercially prepared (myco Prep) or lab prepared and sterilized.

-Sterile NaoH – NACL citrate solution (myco prep)

-Centrifuge with a minimum 3000-3500xg force and safety shield (refrigerated centrifuge is preferred)

-Vortex mixer

-Timer

-Pipettes/transfer pipettes or pipettor with cotton plugged pipette tips

Procedure

- If specimen is not collected in 50ml centrifuge tube, transfer 3 to 5 into 50 ml centrifuge tube with a screw cap.

- For autopsy study samples, macerate the tissue in 4% NAOH in a Petri dish using two needles and transfer into a 50ml falcon tube.
- Add NaOH-NACL sodium citrate solution in volume twice to the quantity of sputum. Tighten the cap.
- Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so that the whole tube is exposed to NaOH-NACL solution
- Wait a total of 20 to 25 minutes (up to 25 minutes maximum) after the addition of NaoH-NACL solution. Vortex lightly and hand mix /invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.

NB Myco prep once opened should be consumed within 24 hrs.

- Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NACL powder (30-35 Grams) directly into the specimen. Mix well.
- After 25 minutes are over, add phosphate buffer (pH 6.8) up to the ring on the centrifuge tube (plastic has a ring for 50ml mark). Mix well (lightly vortex or invert a few times). Addition of sterile water is not suitable alternative for phosphate buffer.
- Centrifuge the specimen at a speed of 3000g or more for 15 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria
- After centrifugation, carefully decant the supernatant as much as possible into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid.
- Add a small quantity (1 to 2 ml) phosphate buffers (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.
- Use the re-suspended pellet for smears and for inoculation of MGIT and other media e.g L.J.

1. SMEARS FOR ACID-FAST BACTERIA (AFB)

Smear preparation

Prepare smear from all processed specimens before inoculation into medium.

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

- After digestion/decontamination and re-suspension of the pallet, mix the specimen well with pipette and place about one drop or 2-3 loopfuls on a clean microscope slide
- Spread the smear about 1 ½ cm x 1 cm
- Allow the smear to air dry completely.
- Heat fix the smear either by passing over the flame three to four times or by heating on a slide warmer at 65-75 for 2-3 hours or overnight. Do not overheat or expose smear to UV light.
- Perform all the above procedures in a biological safety cabinet, handle the smear carefully since mycobacteria will still be viable.
- Perform Z-N staining and examine.

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

F. PREPARATION AND INOCULATION FOR CULTURE

2. Reagents

(a.) MGIT tube- contains 7.0ml of modified 7H9-broth base. There is a fluorescent sensor embedded with silicone on the bottom of the tube. Keep the caps closed until you are ready to make any addition to the medium. Open the cap for as little as possible.

(b) MGIT Growth supplement (enrichment)- provided for BACTEC MGIT 960 7ML tube. Must be added to MGIT medium prior to inoculation of a specimen.

Contains 15ml of the following approximate formula.

Bovine albumin	50.0gm
Dextrose	20.0gm
Catalase	0.03gm
Oleic acid	0.1gm
Polyoxyethyline state (POES)	1.1gm

It is sterile product handle aseptically do no use if turbid or looks contaminated. Do not leave MGIT tube caps open during the addition of OADC. Add while in biological safety cabinet, to avoid environmental contamination of the medium.

C. MGIT PANTA

- An anti-microbial added prior to the inoculation of the specimen. Each vial of MGIT PANTA contains a lyophilized mixture of the antimicrobials with the concentrations at the time of production as follows;

Polymyxin B	6,000 unit.
Amphotericin	600 ug
Nalidixic Acid	2,400 ug
Trimethoprim	600 ug
Azlocillin	600ug

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

3.Procedure.

a) Reconstitution of PANTA-;

Reconstitute MGIT PANTA with 15.0ml MGIT Growth supplement by-pippetting 15ml-s of growth supplement into PANTA. Mix until completely dissolved. Add 0.8ml of this enrichment to each MGIT tube.

The enrichment with reconstituted PANTA should be added to MGIT medium prior to inoculation of specimen in MGIT tube. Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/PANTA.

NB-Reconstituted PANTA should be stored at 2-8°c and consumed within 5days

b) **Inoculation of MGIT Medium;**

-Label MGIT tube with specimen number

-Unscrew the cap and asceptically add 0.8ml of MGIT growth supplement/ PANTA to each MGIT tube use adjustable pipettor.

-Using a sterile pipette transfer 0.5ml of well mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette tip for each specimen.

- Immediately recap the tube tightly and mix by inverting the tube several times.
- Wipe tube and caps with a mycobactericidal disinfectant.
- Work under the hood for the specimen inoculation

.C) Inoculation of Additional Media;

Solid medium most commonly an egg based medium, e.g L.J is used. Inoculate 0.1ml to 0.25ml of processed /concentrated specimen onto the solid medium (two drops).

D) Precautions-

Open MGIT tubes for as short period of time as possible.

- Make all addition inside the hood
- Do not open several tubes at a time.

- Recap tube tightly. Loose cap may affect the detection of fluorescence.
- Volume greater than 0.5ml of decontaminated specimen may disturb the pH of the medium and cause false fluorescence. It may also increase the contamination or otherwise adversely affect performance of the MGIT medium.

e) Incubation

Incubation temperature: - the instrument maintains 37° ±1°c since the optimum tempersture for growth of *m.tuberculosis* is 37°C, the temperature has to be close to 37°c.

NB-All inoculated tubes should be entered in the BACTEC MGIT 960instrument after scanning each tube.

- Keep the cap tightly and not to shake the tube. This helps in maintaining the O2 gradient in the medium.
- If specimen is suspected of containing mycobacterium which require temperature other than 37°c, eg M.*haemophilum* M. *marinum*, M *chelonae* and *m. ulcerans* ,two sets of media should be inoculated one at 37^0C and the other at 30^0C in an on side incubator.
- Length of incubation: - tubes should be incubated until they become positive by instrument. After maximum of six weeks the instrument flags the tube negative if there is no growth. Some species e.g *m.ulcerans* and *m. genevence* may require extended period of incubation

f) **Detection of positive growth**

The instrument indicates when a tube becomes positive for growth. An indicator green light shows exact location of the positive tube in the drawer of the instrument. The tubes should be removed and scanned out of the machine. After scanning, the tubes should be visually observed. Mycobacterial growth appears

not very turbid, granular while contaminating growth appears very turbid. Growth of *M. tuberculosis* complex settles at the bottom.

Time to detection of positive growth depends on several functions.

- Number of viable AFB inoculated into MGIT tube.
- Type of mycobacterial growth e.g. *M tuberculosis M bovis* grows slower than NTM such as *M avim*
- Type of specimen
- Treatment status of patients eg specimen from chronic treated patient who often have drug resistance take longer to grow.
- Processing of specimen:- High or low Ph causes injury or death to mycobacteria during processing, thus take longer for revival and growth of viable mycobacteria. In some instances, as much as 60 – 70% of mycobacteria are killed during processing.
- Loss of mycobacteria during centrifugation
- NB:- in some instance especially if mycobacterial growth is extremely slow or there is less oxygen consumption during mycobacterial growth, there may be growth in the MGIT broth without the presence of fluorescence. It is recommended that the time of incubation protocol should be increased and all negative tubes should be visually observed for turbidly and growth before discarding. If there is any suspicion of presence of growth, an AFB smear and subculture should be done.

WORK- UP OF POSITIVE CULTURES

1. **AFB smears from positive MGIT tube**

Once a MGIT tube is positive by flourescence or visual observation, prepare a smear and stain with carbol fuchsin stain (ZN stain).

Procedure

- Use a clean slide
- Mix the broth by vortexing and then by using a sterile pipette, place 1-2 drops of this on the slide and spread it on a small area (1.5 cm x 1 cm)
- Let it dry
- Heat- fixes the smear by passing it on the flame a few times. Do not leave the smear openly exposed to UV light of the safety cabinet.
- Stain the smear with Z-N
- Place a drop of oil immersion on stained and completely dried smear and screen under a low power objective to locate stained bacteria switch to oil immersion objective lens for detailed observation.
- If the broth appears turbid or contaminated irrespective of AFB smear results, subculture on a blood Agar CBA or TSI to rule out the presence of contaminating bacteria.
- If the smear is negative for AFB and the tube does not appear to be contaminated, (broth clear) re-enter the tube into the instrument for further monitoring. Repeat AFB smear after 1-3 days.

2. **Dealing with contamination**

a). **Bacterial decontamination**

For liquid media slightly higher contamination of 7-8% is accepted. Low rate may be due to harsh decontamination process which will also affect growth of mycrobacteria hence reduce positivety rate and increase time to detection of positive mycobacterial culture. --Higher contamination i.e >7% may be due to;

- Improper or under decontamination

- Very mucoid specimen which are hard to liquefy and may result in high contamination.
- Long storage and transportation of the specimen after collection.
- Use of non-sterile materials such as pippetes, tubes, etc.
- Reagents prepared, stored in bulk and used for long period of time.

b) Detection of contamination

- Make a smear and stain with Ziehl - Neellsen stain. Presence of non acid fast bacteria on smear confirms contamination.
- Sub-culture a loopful on B.A, CBA, or brain hearts infusion (BHI) agar plate .Divide the plate and identify specimen by a marker. Incubate this subculture at 35+_ 1°C and observe after 24 to 48 hrs. If contaminating growth appears, confirm by gram and ZN stain..
- If contamination is confirmed with negative AFB smear from the broth, discard the specimen and report as contaminated. If contamination is confirmed with positive AFB from the broth, follow the isolation procedure.

c) How to control high contamination Rate

- Review all procedures and make sure all recommended steps are followed closely. If contamination persist,
- Increase NaoH concentration (not more than 1.5to final concentration in the specimen.. Do not increase exposure time.
- Increase the concentration of PNTA by reconstituting with smaller volume of growth supplement. Instead of 15.0ml use 10ml to reconstitute PANTA. Add regular 0.8ml volume in the MGIT tube.
- Do not change the NaoH concentration and PANTA at the same time. Try one procedure at a time and document improvement of result.
- If there seems to be a common source of contaminating bacteria, check sterility of all reagents and only disperse small quantities of all reagents and use only one at a time. Left overs should be discarded.
- Try to reduce time between collection of specimen and processing and if specimens need to be stored, use refrigeration.

- Transport specimens with ice and in an insulated chest especially in hot weather.
- Inverting the tube during decontamination processing helps decontamination of the inside surface of the top of the tube.

Isolation of mycobacteria from contamination or mixed culture

Decontamination of contaminated culture.

- Transfer whole MGIT broth into 50ml centrifuge tube.
- Add an equal quantity of 4%sterile NaoH solution
- Mix well and let stand for 15-20 min, mixing and inverting the tube periodically.
- Add phosphate buffer pH 6.8 after 15-20minutes upto 40ml mark. Mix well.
- Centrifuge at 3000xg for 15-20 minutes.
- Pour off the supernatant fluid.
- Re-suspend the sediment in 0.5 ml buffer and mix well.
- Inoculate 0.5ml into a fresh MGIT tube with MGIT growth/PANTA supplement.
- Enter in the instrument and follow for observation of growth.

b) Isolation of mixed mycobacterial culture in middle brook Agar plate.

Agar plate -:

-AFB positive culture with more than one mycobacterial species, may be separated by striking a small culture of positive broth on a middle brook 7H10 or 7H11 agar plate. Spread the loopful on the full surface of the plate to achieve isolation colonies. Incubate the inoculated plate at 37°C+-I°C in a plastic bag. Observe for growth periodically up to six weeks.

c) Cross contamination

This happens during the processing of specimens especially at the time when NaoH-NACL solution is added to the specimen or when buffer is added to the tubes.

- Aerosol generation or splashing during the addition causes cross-contamination by contaminating the next tube or contaminating the reagent stock solution.

- Touching the lip of specimen tube by the reagents container during pouring, may also lead to high contarmination.
- Stock solution of reagents get contaminated with mycobacteria commonly found in water. Aliquoting small quantities reduce the chances of cross contamination. In the event of a cross contamination episode all reagents, equipment and biosafety cabinets must be thoroughly checked.

3 Sub culturing a positive MGIT tube

Incase a visual growth is not sufficient, incubate for an additional 1-3 days. If growth on solid medium is required for biochemical testing and speciation, inoculate approximately 0.25ml of a well mixed positive MGIT broth on solid medium eg LJ. If MGIT is used as stand-alone system, it is recommended that positive MGIT broth be subcultured on a solid medium to achieve isolated colonies and for storage. Incubate at 37^0 C ±1°C and examine periodically.

4. Identification of isolated mycobacteria.

Tentative differentiation may be made by the following observations.

a) Rate of growth-:*M.bovis, M. turberculosis , M.Kansisii* are slow growers taking longer time to turn positive in MGIT tube compared to other mycobacteria (NTM)

b) In liquid media, *M. turberculosis*, appear granular except *M. Kansisii*, while growth of NTM does not show flakes or granules rather it forms uniform slight turbidity.

c) Smear from a positive MGIT broth culture, belonging to TB complex form a typical clumps and serpentine cords, while other mycobacteria appear loose, smaller clumps, and cording or single cells.

d) Other methods used are:-

- Molecular method e.g accuProbe
- Lateral flow immunochromatography test (capilia TB test)
- Inoculation (subculture) on LJ slants may be used for biochemical testing for speciation.

H) RESULTS/ REPORTING.

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

Report results only when a MGIT tube is positive by the instrument and a smear made from the positive broth is also positive for AFB. Do not report positive culture unless smear made from the positive tube is definitely positive for AFB. In rare cases, MGIT tube may be negative in the instrument, but will be positive by AFB smear and /or subculture. In such a case report as positive. In case the identification requires additional time, result may be reported as culture positive for AFB, identification pending.

- Negative culture should be reported after completion of the incubation protocol of the instrument and visual observation of the negative tubes.
- Contaminated culture must be reported as contaminated after confirmation by smear or subculture on bacterial medium.

Conventionally, reports are sent at the following points

a) Smear from specimen ZN- report positive or negative with the staining method used.
b) Culture:- positive (with confirmation by AFB smear) preferably after completion of identification
c) Culture negative upon completion of the incubation protocol (42 days).

LIMITATION OF THE PROCEDURE

a) Colony morphology and pigmentation cannot be observed in liquid media.
b) Even if single viable contaminating bacterium survives decontamination and PANTA inhibition, it may contaminate the entire medium.

c) A positive culture from a clinical specimen cannot be correlated with colony forming unit (CFU) present in the specimen which sometimes is used to establish important NTM infection.

d) MGIT tube that appears positive may contain a mixed growth of more than one type of mycobacteria.

e) Sometimes excessive carryover of reducing agent or alkali may cause false fluorescence of the sensor for a short time.

f) Use of PANTA antibiotic mixture although necessary for suppression of contaminating bacteria may have some inhibitory effect on some mycobacteria other than *M tuberculosis* complex.

K. QUALITY CONTROL

1. Quality control of AFB staining

It is recommended to include a positive control and a negative control with each batch of slides for staining. Prepare smear from positive. *M. tuberculosis and,* MOTT (NTM).

A suspension is used to make a positive smear and also from solid medium.

Bacterial suspension such as E.coli can be used for the negative control.

All control should be examined before clinical specimen slides.

To monitor the quality of microscopy techniques,

have a second person to look at selected number of positive and negative smears and those that have very few AFB on smear. Compare the results of the two technicians.

- For external quality control (EQC) follow standard procedure established in the lab.

2 Quality control testing of MGIT medium

Every new lot of MGIT medium or every new lot of the enrichment should be quality control tested by the user upon receipt and before it is used routinely.

STANDARD OPERATING PROCEDURE FOR BACTEC MGIT 960 TB SYSTEM.

a) Qc strains:-

The following 3mycobacterial culture are recommended for quality control testing

- *M tuberculosis*
- *M Kansasii*
- *M. fortuitum*

b) preparation of culture suspension

- Subculture the above mycobacteria on several LJ slants
- Incubate at 37+ 1^0 C
- Observe growth visually
- If there is good confluent ,and pure growth used this for making suspension.
- Growth should appear within 10-15 days of sub culturing and should be used within this period.
- Remove the growth from the slant by carefully scrapping the colonies, off the slant with a sterile spatula made form wooden applicator sticks. Care should be taken not to scrap culture medium which gives false turbidity measurement.
- Transfer growth into a screw cap tube containing 4ml of sterile 7H9 broth and glass beads to break the clumps i.e tube A
- Vortex the tube for 1-2 minutes. Make sure the suspension is well dispensed and very turbid
- Let the suspension stand undisturbed for 20 min.
- Using a transfer pipette, careful transfer the supernatant to another sterile screw cap glass tube i.e tube B. Avoid picking up any sediment. Let it stand for 15min.
- Carefully transfer the supernatant into screw cap glass tube i.e. tube C. without taking any sediment.
- Adjust the turbidity of suspension in tube C to McFarland # 0.5 turbidity standard by adding more 7H9 broth or sterile saline/. Deionized water (H_2O) and mix well. If the suspension is too turbid, transfer some of the suspension to another sterile tube and adjust the turbidity to McFarland #0.5 standard

This is a working suspension for QC test. It may be frozen in small Aliquot (1-2) in appropriate tube/vials at –70oC + 10oC. May be used up to 6 months. Once thawed do not re-freeze.

c) preparation of dilution-:

Dilute the working suspension McFarland 0.5, freshly prepared or (frozen) 1.5 by taking 1.0ml of suspension and adding into 4.0ml of sterile water or saline. Mix well.

(tube 1).

- Dilute two more times 1:10 by adding 0.5 ml of suspension tube 1 into sterile water or saline (tube 2). Mix well and then again add 0.5ml from tube 2 to 4.5 ml of sterile saline or distilled dionized water (tube 3). Mix well. Final dilution 1:500. Stop here for *M tuberculosis* and use tube 3 for Qc testing.
- For *M fortuimtum*, further dullute tube 3,1:10. Take 0.5ml of suspension from tube C and add to 4.5ml of sterile water or saline and mix well. Final dilution 1:4 1:10 take 0.5ml from tube 4 to 4.5ml of sterile water or saline and mix well. Final dilute 1:500 (tube 4). Use tube 4 for QC testing.
- For *M Kansasii* dilute tube 4 once again 1:10 by adding 0.5ml from tube 4 to 4.5ml of sterile saline/water, mix well. Final dilute 1:50,000(Tube 5). Use tube 5 for QC testing.

d) Inoculation/ incubation

- Supplement MGIT medium with Growth supplement and PANTA
- Inoculate 0.5ml from tube 3 to each of two MGIT tube for M tuberculosis. Similarly inoculate 0.5ml from tube 4 for *M. fortuitum* and tube 5 for *M.kansisii* into each of the two labeled MGIT tubes. Mix.
- Enter the inoculated tubes in the MGIT 960 instruments. Take the tubes out when indicated positive by the instrument. Retrieve data for time to positive.

e) Expected result

- *M tuberculosis* tube fluorescence positive 6-10days
- *M kansisii* tube fluorescence positive 7-11 days
- *M fortuitum* tube fluorescence positive 1-3 days

APPENDIX 11: SUSCEPTIBILITY

TESTING

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

PURPOSE:

To describe the procedure for susceptibility testing of isolates of MTBC, using BACTEC 960/MGIT system.

Introduction:

Antimicrobial susceptibility testing is critical in prescribing an effective drug regime for a tuberculosis patient, especially in areas where drug resistance incidence is high. It is also important in the follow-up of patients who are on antimicrobial therapy but are not responding to therapy.

The BACTEC MGIT 960 susceptibility testing for streptomycin (S),isoniazid(I),Rifampin(R), and ethambutol (E),called SIRE and PZA, is a rapid and qualitative procedure for susceptibility of M. tuberculosis for the four drugs using critical concentrations .in addition ,high test concentrations for S,I and E are also available in case of testing against higher concentrations is indicated.
Multi-drug resistant MTB, (MDRTB),is when one is resistant to any of the primary drugs :isoniazid(INH),Rifampin (R),ethambutol (E) Pyrazinamide (PZA),makes the disease more difficult and expensive to treat.

Principle

Isolated cultures from TB patients are subjected to growth in the presence of a known concentration of a test drug .A control is also included with no addition of the drug. if the patient 's isolate grows in the control but does not grow in presence of the drug , it is considered susceptible .on the other hand ,if it grows in both the tubes ,then it is considered to be resistant to that drug.
Method of proportion (MOP); resistance to a drug is detected when 1% or more of the bacterial population is resistant to the drug at the critical concentration.
MTB has a low level of intrinsic resistance to anti-tuberculous drugs .by using the MOP, this low level resistance is eliminated and only significant (>1%) resistance is detected.
The critical concentration is defined as the lowest concentration of drug that inhibits 95% of wild strains of MTB that have never been exposed to drugs, while at the same time allowing growth of MTB that have been isolated from patients who are not responding to therapy (resistant)

THE BACTEC 960/MGIT AST SYSTEM

The BBL MGIT 7ml mycobacteria growth indicator tube is a tube containing modified middlebrook 7H9 broth which supports the growth and detection of mycobacteria .The MGIT tube contains a fluorescent compound embedded in silicone at the bottom of a round-bottom tube. The fluorescent compound

is sensitive to the presence of oxygen dissolved in the broth .actively growing microorganisms consumes the oxygen which allows the compound to fluoresce. The AST test is based on growth of the mycobacterium tuberculosis isolate in a drug containing tube compared to a drug free tube (growth control).
The BACTEC MGIT 960 instrument monitors hourly the tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the growth control tube is used by the instrument to determine susceptibility results.

The BT 960 instrument automatically interprets these results and reports a susceptible or resistant result.

Equipment

- Biosafety cabinet (certified annually)
- BACTEC 960 instrument (with AST feature enabled)
- Printer attached to BACTEC 960
- Refrigerator
- Freezer (-70^0 C)
- Incubator (+37^0 C)
- Autoclave
- Vortex

Supplies

- Repeater Pipetter
- AST Carriers (2, 3,4,5,8 Tubes capacity)
- AST racks
- Sterile pipette tips, 100Ul (Plugged for culture and unplugged for drugs)
- Combitips, 10ml
- Screw capped glass tubes, 16×125 mm with caps
- Nalgene tubes 2ml
- Glass beads
- Microscope slides
- Plastic graduated, disposable pipettes, 1ml
- Phenolic detergents S7 disinfectant (S7)
- Sharps discard containers
- Autoclave bags, red (sharps), clear (other waste)
- Benchpad
- Slant for holding LJs
- Lysol spray
- Ethanol, 70%

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

Reagents

1. BD SIRE AST Kit:
   - Streptomycin (S)
   - Isoniazid (INH)
   - Rifampin (RIF)
   - Ethambutol (EMB)
   - SIRE Supplement
2. BD PZA AST kit:
   - Pyrazinamide (PZA)
   - PZA Supplement
3. BD INH 0.4mg/L kit:
   - INH 0.4mg/L kit
   - INH 0.4mg/L
   - SIRE Supplement
4. PZA test media
   - MGIT tubes
   - OADC/PANTA MGIT supplement
5. Sterile normal saline in 4, 4.5.10Ml aliquots
6. MGIT media, aliquoted 4Ml IN 16×125 MM Tubes with beads (autoclaved)
7. Blood agar plates (BAPS) in canisters:
8. LJ slants
9. Cryovials containing 1Ml OF 10% nutrient glycerol for freezing

AST Test using S,I, R, and E (as needed)

I. Drug Preparation (I,R,E,10.4)

BD BT 960 SIRE Drug Kit-On receipt, store the lyophilized drug vials at 2-8 deg c.

Note: The test kit includes streptomycin (STR), however as per the CLSI, the OPHL considers STR to be a second line drug and therefore it is not tested as part of the first line panel

Reconstitute S,I, R, E, and PZA drugs with 4ml and 2.5ml of sterile distilled water respectively

Final Concentrations:

STR 1.0ug/ml of the medium

INH 0.1ug/mL of the medium

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

RIF 1.0 ug/mL of the medium
EMB 5.0ug/mL of the medium
PZA 100.0µG/ml. of the medium

High level INH: recostitute with 2MI OF sterile distilled water

Final concentration 0.4mg/L

Quality Control: See 13.B New Drug Lot#s, when reconstituted, must meet QC requirements before being released for use

Once reconstituted, the antibiotic solutions are aliquoted,0.25 MI Per labeled cryovial, frozen and stored at -70deg,c for up to six months ,not to exceed the original expiration date

Once thawed use immediately. Discard unused portions

SPECIMEN PREPARATION

All preparations detailed below are from pure cultures of M.tuberculosis complex isolated from patient specimens
Preparation of the isolate from solid media growth must be <14 days old:

1. Label dilution Tubes (5), A-E

- Tube A-8-10 Glass beads & 4MI 7h9 Broth
- Tube B-Empty to use for supernatant of tube a
- Tube C –Empty to use for supernatant of tube b
- Tube D-4.0 MI sterile saline
- Tube E-10.0 MI Sterile saline
- 1 Solid media slant

2. Prepare Suspension

- Harvest growth, place in tube A(Suspension should be >1.0McFarland Standard)
- Vortex Tube(2-3 minutes)
- Allow tube A to stand for 20 minutes for clumps to settle
- Transfer Supernatant from tube A to Tube B

- Allow Tube B to stand for 15 minutes
- Transfer supernatant from Tube B to C
- Adjust Tube C to a 0.5 McFarland Standard
- Transfer 1Ml of Tube C to Tube D. This is the Inoculum source for the drug containing tubes (1:5)

- Transfer 0.1Ml of Tube D to Tube E. This is the inoculum for the growth control tube only (1:100)
- Preparation of inoculum from a positive MGIT tube

N/B: It is important to prepare the inoculum using the following tine references to obtain the appropriate organism concentration for then susceptibility test

1. The first day of an instrument positive MGIT tube is considered Day 0
2. For the preparation of the test inoculum, a positive MGIT tube should be used the day after it first becomes positive on the BT 960 instrument(Day 1),up to and including the fifth day(Day 5)after instrument positivity. A tube which has been positive longer than 5 days must be sub cultured to a fresh MGIT tube. To subculture, add 0.8Ml MGIT supplement to the MGIT. Add 0.5Ml OF A 1:100 dilution of the old culture to the new MGIT tube. Test in the BT 960 instrument until positive, and use from one to five days following positivity

3. Of the tube is a Day 1 or Day 2 positive, mix well and proceed to "inoculation procedure for susceptibility test", without dilution

4. If the tube is a Day 3, Day 4, Or Day 5 positive mix well then dilute 1Ml of positive broth in 4 ml of sterile saline (1:5 dilution).Use the diluted suspension for the inoculation procedures. Proceed to "Inoculation procedure for susceptibility test"

Inoculation Procedure for MGIT 960 SIRE (And 14 as needed) Susceptibility Test:

1. Label four 7ml MGIT and PZA tubes of each test isolate

2. Label as: C (Growth Control), S, I (INH), R (RIF), E (EMB) and PZA

3. Aseptically add 0.8Ml of MGIT SIRE and PZA supplement to each tube

NOTE: It is important to use the supplement supplied with the kit
4. Aseptically pipette, using micropipette, 100Ul working drug concentrations to each of the appropriately labeled MGIT Tubes. No antibiotics are added to the MGIT control tube

5. If using a MGIT Day 3-5 dilute 1:5 using 1.0ml of well mixed MGIT culture and 4.0 ML of sterile saline. This is now the growth suspension. MGIT Tubes Day 1 and 2 are used undiluted as the growth suspension

6. **Growth Control tube Preparation and inoculation:**
Aseptically pipette 0.1ml of the organism suspension into 10ml of sterile saline to prepare the 1:100 growth control suspension. Mix the growth Control Suspension thoroughly Inoculate 0.5ml into the MGIT tube labeled "C".

7. **Drug Containing tube Inoculation:**
Aseptically pipette 0.5ml of the organism suspension into each of the four remaining drug tubes (strepto. INH, RIF, EMB and PZA)

8. Tightly recap the tubes. Mix tubes thoroughly by gentle inversion 3 to 4 times

9. Spray all tubes with Lysol spray

10.Load tubes into the appropriate AST carrier(5 and2 Place).Enter the AST set into the BT 960 using the AST set entry feature(refer to MGIT 960 Users manual AST Instructions).Ensure that the order of the tubes in the AST set Carrier is C,s,I,R,E and PZA

11. Streak 0.1ml of the organism suspension to a BAP. Seal with par film and place in canister Incubate at 35-37 C for 4 days

12. Check and record the blood agar plate daily for 4 days, for bacterial contamination. If the blood agar plate shows no growth, then allow AST testing to proceed. If the blood agar plate shows growth, discard the AST set (refer to the BACTEC MGIT 960 Users manual, AST Instructions) and repeat testing with pure culture

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

1) PZA Test
   I. Reagents

   BT MGIT 960 PZA Kit:

   PZA Drug
   PZA Supplement

   PZA MGIT Media
   (pH is 5.9 as PZA is active at this reduced pH)

   II. Reconstitution of PZA drug
   Each PZA drug vial is reconstituted with 2.5 mL sterile distilled water

   A liquots of 0.25ml diluted PZA are frozen in crovials at -70 deg C for 6months
   For QC see 13

   Drug vials are thawed for use. Discard any unused portion of drug

2) **Test Preparation**
   Label 2 PZA test media tubes C and P
   Add 0.8 ml PZA supplement to each tube
   Add 0.1ml PZA drug to the P tube (no drug is added to the control tube)

   I. Preparation of specimen inoculum and inoculation of PZA tubes
   See SIRE specimen preparation 6

   1) Inoculate the drug tube labeled P with 0.5ml of the same organism suspension that used for the SIRE test
   2) Grow control , prepare a 1:10 dilution from the organism suspension (0.5 in 4.5ml sterile saline)
   3) Inoculate 0.5mL of this dilution into the PZA control
   4) Tightly recap the tubes. Mix well by inversion 3 or 4 times
   5) Spray outer tubes with Lysol
   6) Load tubes to a 2 tube AST carrier
   7) Ensure that the order of tubes is C.P
   8) Enter into the BT 960 (see 8)

3) **Entry of all AST tubes to BT 960**

- Scan AST set carrier into the BACTEC MGIT 960 instrument
- Scan subculture tube in to the 960 instrument independently
- A drawer is indicated to AST tests only in one of the BT 960s

4) Monitoring of tests in progress
All AST tests in progress in the BT 960 instruments are monitored daily by checking for growth units (GU's) in the Epicenter. See Appendix

A rise in GU will give an early indication of any developing resistance or contamination so that intervention or repeat testing can be initiated.

5) Interpretation of results

- Instrument will interpret results between 4-13 days for SIRE tests and days 4-21 for PZA tests
- Records daily and final results on log sheet.
- Susceptible results: when the test strain is susceptible to all drugs tested. This will occur when on the day that control tube reaches a GU of 400 the drug tubes have GUs of <100, and the time is within the acceptable parameters for the test
- Drug resistance: this will occur when there is a rise in GU =/> 100, in a drug test tubes(s), with no growth on the BAP, and growth in the control tube, GU=400, within the acceptable time parameters.
- Print the growth chart from the Epicenter on any resistant isolates

In the case of drug resistance a repeat test is set up for those drugs which show resistance in the initial test. This is to confirm resistance and to confirm that drugs were in fact added to the initial test.
In the case of resistance to PZA, testing to determine whether the strain is m.bovis or m.bovis BCG is initiated since these species are resistant to PZA.
In the case of resistance to INH at the critical concentration (0.1mg/l)a repeat test at that concentration PLUS a
Test using the high concentration (0.4mg/l) is set up .
Any test that show resistance to Rifampin or any two of the first line drugs is further tested for susceptibility to second line drugs .
Any test for drug resistance must be checked to eliminate the possibility of contamination .
Restant results that are inconsistent ,for example a different result between the initial and the repeat test again .investigate for contamination .the test may be repeated using the BT 460 system if drugs and reagents are available investigations for genetic mutations associated with resistance may be done if available.
10. Acceptable parameters for the test

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

Test completion within 4-13 days for SIRE test and 4-21 days for PZA tests
Over or under inoculation of test that result in completion of the test outside these limits, will be rejected by the instrument.
This test must be repeated
The test is complete on the day that the GU in the equals to 400
11. Limitations of the test
AST test can only be carried out on pure, viable cultures of MTBC
Contamination

1. Growth on the BAP from the original inoculum; culture is contaminated. Check to see if alternative culture is available for the same patient .decontamination may be attempted by retreating the culture with 3%NAOH as per MGIT contaminated cultures.
   If the is no other culture and decontaminating is not successful, issue a report ; unable to perform susceptibility testing due to culture contamination.
2. A rise in GU in an individual test tube, with visual turbidity .subculture 0.1ml to a BAP and make a smear to stain with kinyoun . Check the BAP from the original inoculum .if contamination is only found on the BAP from the individual test tube, repeat the test from the original MGIT tube .in case contamination was introduced during the inoculation procedure, to one tube only.
3. Mixed cultures . Sometimes MTBC cultures also contain NTM.. This will give false resistant susceptibility results as most as most NTM are resistant to anti-TB drugs. Mixed cultures can be detected by unusual rapid growth, morphology on a smear which is not typical. Of MTBC and multi resistance to anti TB drugs. To investigate possible mixed cultures test the MGIT tube showing growth with the Genotype test, SOP-C-TB-071. if an MTBC culture contains NTM, susceptibility testing cannot be done. Check other cultures from the same patient for pure MTBC. Sometimes a single colony of MTBC can be picked from an LJ slant, even if NTM is present.

   If an NTM is detected in the drug tube, the original MGIT is also tested by the Genotype test to investigate the presence of the NTM there.

   When mixed cultures are detected a report is issued "MTB plus NTM (Name of Species) susceptibility testing of the MTB is not possible.

Repeated specimens should be requested so that a pure culture of MTBC might be isolated.

6) Quality control

- Media quality control

MGIT tubes are tested by QC during routine use (see SOP C TB 010)
Drug quality control

- Frequency

Each time new lot # of drugs is reconstituted
Once per week with routine tests
INH 0.4 only when used in testing, once per week.

- Quality control (QC) strain: M.tuberclosis ATCC™ 27294 (H37 Rv)

Use slant growth from LJ solid media <14 days old or growth in a MGIT tube, day 1-5
Label 7 ml MGIT TUBES (8)
H37 C, H37 R, H37 E, H37 1-4, H37C, H37 (subculture vial)

For inoculums, preparation, inoculation of the QC test, monitoring, reading and interpretation of results, see V1 (I, R, E) and V11D(PZA)

The QC test follows the same procedure and interpretation as used for patient tests except that the QC strain of MTB is used instead of the patient strain

- Expected results:

MTB QC strain is sensitive to all first line drugs.

- Discrepant QC results

If the QC fails, do not report patient results for the drugs that failed for that testing period. Repeat the QC for the drugs and patient isolates affected by the initial QC failure. If the repeat QC does not perform as expected, do not report patient results. Do not use the product. Contact BD technical services at (800) 638-8663

- Quality control for ZN staining

It is recommended that a positive control and a negative control be included for each batch of slides for staining. Prepare slides from positive M.tuberculosis and MOTT smears (NTM).
A positive smear can also be prepared from a suspension or solid medium.

SUSCEPTIBILITY TESTING OF *MYCOBACTERIA TUBERCULOSIS* BY MGIT 960 METHOD

All control should be examined before clinical specimen slide to monitor the quality of microscopy techniques, have a second person to look at a selected number of positive and negative smears and those with very few AFB on smear. Compare the results of the two technicians.

Reference:

- SOP-C-TB-010 culture for isolation of Mycobacteria, using the BACTEC MGIT system.

APPENDIX 12: PUBLICATION I

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Optimal parameters for Gene Xpert MTB/RIF and LAM Strip determine Test for Diagnosis of Tuberculosis using urine from HIV patients at Moi Teaching and Referral Hospital, Kenya

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ABSTRACT

The application of Gene Xpert MTB/RIF® assay for testing non-sputum clinical samples in Western Kenya has not been reported. We are reporting on use of Zn, CD4 count, Hb, Creatinine, Proteinuria, and Hematuria as optimal parameters for Gene Xpert MTB/RIF and LAM Strip determine Test for diagnosis of tuberculosis. Urine from HIV/TB co-infected adults (n=158) with CD4+ count <200 cells/mm^{3}was analyzed for mycobacteria tuberculosis DNA. Acid fast-staining, culture, clinical symptoms, laboratory and radiological findings were also assessed. Acid fast staining(55%) were TB positive, however, Gene Xpert test revealed 17(11%) of the patients were TB positive with 94% and 6% being MTB detected low and medium, respectively. Rifampicin resistance was not detected in 88% of the TB Gene Xpert positive patients with the remaining (12%) being indeterminate. LAM test revealed that 28% of the patients were TB positive. Radiology revealed that 45% of the patients had infiltrates. Presence of protein or blood in urine was significantly associated with TB positivity based on LAM strip determine test (48% vs 24%; p-value=0.021). The weighted kappa coefficient was 0.48 (95% CI 0.32-0.63; exact p-value <0.0001).The findings of this study show that urine Xpert MTB/RIF® can be used for diagnosis of TB using urine and taking Zn, CD4 count, Proteinuria, and Hematuria as optimal parameters.

Keywords: *Urine, mycobacterium, Proteinuria, Hematuria, HIV Infection*

INTRODUCTION

HIV and tuberculosis combined are cause of death in patients with HIV. In 2010, 2.8 million new cases of tuberculosis were reported in Africa, the majority in the sub-Saharan area; and 37% of tuberculosis episodes were diagnosed among HIV

APPENDIX 13:PUBLICATION II

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GENE XPERT MTB/RIF SPECIFICITY AND SENSITIVITY IN TUBERCULOSIS DIAGNOSIS USING URINE SAMPLES FROM HIV PATIENTS WITH SIGNS OF TUBERCULOSIS AT THE MOI TEACHING AND REFERRAL HOSPITAL IN WESTERN KENYA

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between all authors. Author IMA designed the study, wrote the protocol and interpreted the data. Authors IMA and KM anchored the field study, gathered the initial data and performed preliminary data analysis. Authors IMA, BG and WE managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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Original Research Article

ABSTRACT

The *Mycobacterium tuberculosis*/Rifampin (Xpert MTB/RIF) has been reported to be a fast molecular tool in the determination of the existence of mycobacterium tuberculosis DNA in mucoid (sputum) samples. However, the deployment of Gene Xpert for testing non-sputum clinical samples in the diagnosis of suspected tuberculosis in western Kenya population has not been evaluated. We are reporting on Specificity and sensitivity of urine Gene Xpert MTB/RIF from HIV patients clinically suspected of tuberculosis. Urine analysis from 158 HIV patients suspected of TB with CD4+ count <200 cells/mm^3 were done. Among the female 11% and 15% were positive based on urine Gene Xpert MTB/RIF and urine LAM respectively with a majority (71%) having CD4 >100 <200 cells/mm. Sensitivity and specificity of urine Xpert was equated to smear microscopy, at the same time with urine culture. Sensitivity of urine Xpert against sputum microscopy was 17%, with a positive predictive value of 29%. Specificity of urine Xpert against sputum microscopy was 91%, with a negative predictive value of 83%. Stratified by CD4 categories, the test showed to be more sensitive (30% vs 11%) and specific (92% vs 89%) in patients with CD4≤100<200 cells/mm^3 compared to those with CD4>100 <200 cells/mm^3 respectively. The data suggest that Gene Xpert assay is a sensitive and specific tool that rapidly identifies *Mycobacterium tuberculosis* using urine as a sample in immunocompromised patients.

Keywords: Urine; Xpert MTB/RIF; *Mycobacterium*; sensitivity; specificity.

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APPENDIX 14:PUBLICATION III

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Review Article

Tuberculosis in Immunocompromised Patients: Review Article

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Abstract: The purpose of these Studies have been published in the area of tuberculosis but much is not known in the area of interaction between HIV and tuberculosis, etiology, immunological mechanism involved, risk factors, diagnostic features, diagnostic tools, immunogenetics and complications involved. The recent results of Tuberculosis have been reported in people living with HIV indifferent parts of the world. Cases are unrecognized due inaccurate diagnosis and hence are treated as other diseases. However the most recent studies have shown that tuberculosis and HIV coexist together especially extrapulmonary tuberculosis and that there are other highly sensitive and quick to yield results. In conclusion The Tuberculosis in HIV is as a result of complex interactions between the host-pathogen and the immune mechanisms involved in the protection. Overall, this review has expanded our understanding of the mechanism involved in the pathogenesis and the relationship between tuberculosis and kidney disease. It has opened new line of investigations that will ultimately result in a better clinical practice.
Keywords: HIV, extrapulmonary tuberculosis.

INTRODUCTION

Co-infection between HIV and tuberculosis is the leading cause of death in patients infected with HIV living in resource limited countries accessing antiretroviral treatment (ART) programmes. In 2010, there were 350,000 tuberculosis-related deaths in HIV–infected people, most of them in developing countries, and 22.5 million people were estimated to be living with HIV in sub-Saharan Africa [1]. During the same year, 2.8 million new cases of tuberculosis were reported in Africa, the majority in the sub-Saharan area; and 37% of tuberculosis episodes were diagnosed among HIV infected patients. A major challenge to diagnosing pulmonary tuberculosis (PTB) is alteration of the presentation of PTB due to HIV infection [2]; [3]. HIV infection increases the risk of developing tuberculosis but also modifies the clinical presentation of the disease [4]. HIV-infected patients are twice as likely to experience sputum smear–negative pulmonary tuberculosis (PTB) than HIV-uninfected patients and extra pulmonary tuberculosis (EPTB) is also more common in HIV-positive patients [5] which contributes to delayed tuberculosis diagnosis, leading to high mortality, and represents an important burden for health systems. The problem is increased in resource-limited settings without routine access to mycobacterial culture or other highly sensitive diagnostic tests [6]; [7]. Alteration in the clinical and radiographic presentation of PTB among HIV-infected persons has long been recognized [8]; [9]. Direct smears can be used but they are often negative and do not differentiate mycobacterium tuberculosis from non-tuberculous mycobacterium [10, 11]. Culture, which is more sensitive, may take 2 to 8 weeks due to the slow growth rate of mycobacterium while liquid culture may take 7-10 days [12]. There is a great need for implementing new diagnostic methods for tuberculosis to increase the sensitivity and speed of diagnosis in these patient groups especially in view of their high mortality and the risk of nosocomial transmission [13]. A range of new diagnostics for TB is now emerging, employing various different technologies [14]. One area of renewed interest has focused on the potential for TB diagnosis to be made from analysis of urine samples [15]; [16]; [17]). Urine has many characteristics which make it a potentially useful specimen for TB diagnosis as it is simple to obtain, even from very ill patients who may not be able to produce sputum. Urine sampling does not generate hazardous infectious aerosols and is relatively clean and easy to handle in the laboratory. Urine may be cultured, tested by polymerase chain reaction (PCR) for mycobacterial transrenal DNA or tested for specific mycobacterial antigens such as lipo arabinomann an (LAM) [18]. Recent studies have shown that urine LAM may have diagnostic value in HIV-infected patients with low CD4+ counts [19]. Currently there is