

**Gene Xpert Testing Of Stool Samples for the Diagnosis of
Pulmonary Tuberculosis in Children Less Than 15 Years in
Hospitals In and Around Nairobi**

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

This work is dedicated to my beloved family: my Dad Mr. Haile Welday, my mom Mrs. Saba Afewerki, my husband Mr. Henok Kudus and my two sons Natnael and Krubiel who supported and encouraged me all through to this level. They are the cornerstones of all my achievements.

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LIST OF ABBREVIATIONS

AKUH	Aga Khan University Hospital
BCG	Bacille Calmette Guérin
CXR	Chest X-ray
DNA	Deoxyribonucleic Acid
DST	Drug susceptibility test
DLTLD	Division of Leprosy, Tuberculosis and Lung disease
GA	Gastric Aspirates
GCH	Gertrude's Children's Hospital
HIV	Human Immunodeficiency Virus
KDH	Kiambu District Hospital
MDH	Mbagathi District Hospital
MDR-TB	Multiple Drug Resistant Tuberculosis
MSF	Medecins Sans Frontieres
MTB	<i>Mycobacterium Tuberculosis</i>
NRTL	National Reference Tuberculosis Laboratory
PCR	Polymerase Chain Reaction
PTB	Pulmonary Tuberculosis
RIF	Rifampicin
TB	Tuberculosis
TST	TB Skin Testing
WHO	World Health Organization
XDR	Extensively drug-resistant

ABSTRACT

Globally Tuberculosis (TB) is a major infectious disease. In 2011, there were an estimated 8.7 million new cases with 6% childhood tuberculosis. The majority of the cases occurring in high TB burden countries like Kenya. Accurate pediatric pulmonary tuberculosis (PTB) diagnosis is a challenge. Symptoms are nonspecific, young children are unable to expectorate sputum samples and the procedures for obtaining respiratory samples are invasive. Thus *Mycobacterium tuberculosis* (*M.tuberculosis*) cultures and smears are often not performed. This study was done to assess stool samples as an alternative to respiratory samples for the diagnosis of pediatric PTB using stool Xpert MTB/RIF assay test and, to determine its sensitivity for detecting the DNA of MTB in stool. The study was a laboratory based cross-sectional prospective design. Children less than 15 years of age, who attended Gertrude's Children's Hospital Nairobi and Kiambu District Hospital and were suspected to have pulmonary tuberculosis on clinical grounds from September 2013 to April 2014 were included. Stool specimens were collected after consent was obtained, from their parents or legal guardians. Data of the clinical features and the results of the routinely done tests by the hospitals were obtained from the filed medical records. A total of 91 stool samples were collected from pediatric cases where the median age was 3. Stool for Xpert was processed in two ways, direct and prior extraction of DNA using QIAGEN stool DNA extraction kit. Out of this, 53(58.2%) had sputum ZN smear microscopy. Six (11.3%) of them were confirmed smear positive for PTB. Stool Gene Xpert tested positive in all the six smear positive children as well as four (7.5%) smear negative patients. Stool Gene Xpert with 100%

sensitivity and 89.36% specificity had significant association (P value = 0.000). Thus this study reports that *M. tuberculosis* DNA can be detected in stool using Xpert testing with a higher sensitivity than sputum smear microscopy. Therefore stool which can easily be obtained are an appropriate alternative sample for the diagnosis of PTB using Xpert assay for children unable to give respiratory samples. Furthermore Xpert turn round time is less than two hours.

CHAPTETER ONE

1.0 INTRODUCTION

1.1 Background of the study

Tuberculosis (TB) is a worldwide pandemic. In 2011, the estimation of new TB cases was about 8.7 million and 1.4 million people died from TB. These deaths involved almost one million HIV negative individuals and 430,000 people who were HIV-positive (WHO, 2011). Asia and Africa had the highest TB burden. India and China together account for almost 40% of the world's TB cases and 60% of these are in the South-East Asia and Western Pacific regions. The African Region has 24% of the world's cases and the highest rates of cases and deaths per capita (WHO, 2011). *M. tuberculosis* the causative agent of TB has developed strains that are multiple drug resistant (Githui *et al.*, 2008) and this is of serious concern in many countries, because it is making it the most prevalent and life threatening infectious disease (WHO, 2003). Worldwide, 3.7% of new cases and 20% of previously treated cases have been estimated to have MDR-TB. India, China, the Russian Federation and South Africa have almost 60% of the world's cases of MDR-TB. The highest proportions of TB patients with MDR-TB are in Eastern Europe and central Asia. Almost 80% of TB cases among people living with HIV reside in Africa. Though estimating the burden of TB in children (aged less than 15) is difficult, from the report done in 2011, When estimated for the first time there were 0.5 million cases and 64,000 deaths among children (WHO, 2011).

Today global tuberculosis control is facing major challenges. It still requires much effort to ensure quality care accessibly without barriers of gender, age, type of disease, social setting, and ability to pay (Swaminathan and Rekha, 2010). Co-infection of *M. tuberculosis* and HIV (TB/HIV), multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis in all regions, especially in Africa make control activities more complex and demanding (Singh, 2006). Inadequate case detection and diagnosis is also one of the main reasons for the global burden of TB (WHO, 2003).

1.1.2. The Burden of Tuberculosis in Africa

The African region witnessed profound increase in the incidence of TB which was attributed to the high HIV prevalence in the region in 2003. Though Africa is home of 11% of the world's population it reports more than a quarter of the global burden of TB. There are sub-regional differences in the burden of TB in Africa. Southern and eastern Africa has the highest per capita burden. Most eastern African countries report less than 200 cases per 100,000 populations with the exception of Kenya (AU, 2006).

1.1.3. The Burden of Tuberculosis in Kenya

Kenya has a large and rising TB disease burden and is ranked 13th among the 22 high burden countries that collectively contribute about 80% of the world's TB cases. The TB case notification rate (CNR) rose from 51 to 338 per 100,000 populations between 1987 and 2007(GOK, 2009).The epidemiology of tuberculosis in Kenya evolved due to economic, environmental and other several factors over years

(Sitienei *et al.*, 2013). As in the rest of Sub-Saharan Africa, the large increase of TB is attributed primarily to the Human Immunodeficiency Virus (HIV) (GOK, 2009). Apart from the HIV epidemic, poor socio-economic status leading to overcrowded slums in the peri-urban areas coupled with poor nutrition and limited access to health services have been identified as contributing factors to the high TB burden (GOK, 2013).

1.1.4. The Burden of Childhood TB

A child is defined as any individual who is under 15 years of age (WHO, 2012). TB is an important cause of childhood morbidity and mortality worldwide (Nelson and Wells, 2004). World Health Organization estimated the extent of childhood tuberculosis to constitute about 6% out of all incident cases, with the majority of cases occurring in high TB burden countries. Approximately 300,000 children less than 15 years of age die of tuberculosis per year worldwide (WHO, 2012). Seventy five percent (75%) of these childhood cases occur in the twenty two (22) high TB-burdened countries such as Kenya which is among the top 5 in the Sub-Saharan Africa (GOK, 2013). In Kenya, TB in children below the age of 15 years accounts for about 10% of all cases every year (GOK, 2009). This is a significant proportion which requires special attention (GOK, 2013).

Childhood tuberculosis (TB) is also a marker of TB transmission within a community (Schaaf *et al.*, 2014). Children can usually be infected with tuberculosis by an adult or an older child with sputum smear-positive PTB. They may also be infected by contact with smear-negative but culture-positive cases less commonly.

Children can present with TB at any age, but the most common age is between 1 and 4 years. The frequency of childhood TB depends on the intensity of the epidemic, the age structure of the population, the available diagnostic tools and whether contact tracing is routinely undertaken. The risk of infection in children depends on the extent of exposure to infectious droplet nuclei. For example, if a mother has sputum smear-positive PTB, her infant is more likely to become infected because of the very close contact and the higher risk of inhaling a large number of infectious droplets. The greater the exposure to infection, the greater the likelihood disease (WHO, 2003).

1.2 Statement of the problem

The burden of TB in children is difficult to establish because of lack of diagnostic tools. Symptoms are nonspecific, and are similar for other illnesses like malnutrition, malaria, HIV, or other viral or bacterial infections. Consequently, TB is usually hard to recognize based on symptoms alone. The standard diagnostic test for TB used in adults is not at all adapted for children. Usually the test for *M. tuberculosis* infection requires respiratory samples such as sputum which is very difficult to obtain in children. Young children tend to swallow sputum when they cough which makes respiratory samples like sputa not easily accessible. Gastric aspirate from a child's stomach, induced sputum from lungs and bronchoscopy can be done to obtain samples but it is traumatic for the child. These procedures also require well-trained staff, a good health facility and a good laboratory, all of which can be rare in resource-poor settings. Consequently *M. tuberculosis* cultures and smears are not easy to perform or are often negative even though the disease is present. This makes

the mortality rate of children with TB high due to difficulties in diagnosis resulting in delayed treatment or missed diagnosis. They can also be potential reservoirs for communication. This study was conducted to find a solution to the challenges of pediatric TB diagnosis using stool sample in Xpert MTB/RIF assay as an alternative to the respiratory samples.

1.3 Justification

Children and infants are unable to expectorate sputum samples. It is known that most sputum is swallowed, thus tuberculosis DNA may remain intact after intestinal transit. Therefore, testing stool for tuberculosis DNA from swallowed sputum may diagnose pulmonary tuberculosis. Stool can easily be collected in the field or in clinics and this can also be a solution to the invasive procedure for collecting respiratory samples or sputum from children.

1.4 Hypothesis

Stool sample cannot be used to diagnose pulmonary tuberculosis in children using Xpert MTB/RIF assay.

1.5 Objectives

1.5.1. General objective

To assess stool samples for the diagnosis of pulmonary tuberculosis (PTB) in children using the Xpert MTB/RIF assay.

1.5.2. Specific objectives

1. To determine the appropriateness of stool as a sample to diagnose PTB in children.
2. To determine sensitivity of the Xpert MTB/RIF assay in detecting *M. tuberculosis* in the stools of children.
3. To determine the efficacy of the QIAGEN stool DNA extraction kit in purifying *M. tuberculosis* DNA from stool sample.

1.6 Limitations of the study

The proposed sample size with a 5% ($d=0.05$) difference from the actual value was 138 and was not possible to attain within the given time frame of the study. Instead that was changed to 7% ($d=0.07$) with the addition of 20%. This is because there were not enough cases in the first selected site compared to the limited time of the study. Therefore extension of the study site was requested from the Kenyatta University Ethical Review Committee. Subsequently three different hospitals were added to the previously approved one which is Gertrude Children's Hospital. These were Kiambu District Hospital, Mbagathi District Hospital and St.Mary's missionary Hospital. Permission to carry out the study from the authorities of the administration of these hospitals was obtained from Mbagathi District Hospital and Kiambu District Hospital but not from St.Mary's missionary Hospital hence St.Mary Missionary Hospital was excluded. Very few, only two cases were reported from Mbagathi District Hospital and this made very little contribution to the study despite the great efforts made. There were also few occasions where by parents or guardians of the patients refused to consent.

The other limitation of this study was the dependence on the use of respiratory samples for diagnosis of PTB. In the public hospitals diagnosing of PTB using respiratory samples for children suspected of PTB is not in practice, due to the shortage of equipment to perform procedures for getting respiratory samples. This led the study to encounter inconsistency of the methods used for diagnosing pediatric PTB in these different hospitals.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tuberculosis (TB)

M. tuberculosis is the bacterium that causes most cases of tuberculosis. It was first described on March 24, 1882 by Robert Koch.. *M. tuberculosis* is an obligate aerobe (Gram positive mycobacterium) but has unusual, waxy coating on its cell surface called mycolic acid. This makes the cell impervious to Gram staining. Acid fast staining techniques are usually used instead. *M. tuberculosis* divides every 15 to 20 hours which is extremely slow compared to other bacteria, which tend to have division times measured in minutes. It is a small, rod-like bacillus that can withstand weak disinfectants and can survive in a dry state for weeks (Cole *et al.*, 1998).

There are different definitions for TB. Tuberculosis suspect is any person who presents with symptoms or signs suggestive of TB (WHO, 2009). Bacteriologically confirmed Tuberculosis case is when a biological specimen is positive by smear microscopy, culture or the WHO approved rapid diagnostics (WRD) techniques like Gene Xpert MTB/RIF assay, while clinically diagnosed TB case is one without the biological confirmation but who has been diagnosed with active TB by a health worker (clinician or other medical practitioner) and has decided to treat the patient with a full course of TB treatment (WHO, 2013). Furthermore both biologically confirmed TB and clinically diagnosed TB cases can also be classified in to different

types according to the anatomical site of the disease, previous history of treatment, drug resistance as well as HIV status (WHO, 2013).

According to anatomical site of the disease TB is divided into two as pulmonary tuberculosis (PTB) and extra pulmonary tuberculosis (EPTB). Pulmonary tuberculosis affects the lung and /or the tracheobronchial tree while EPTB is TB affecting organs other than the lung like lymph nodes, bone, joints, skin and meninges (GOK, 2013). Pulmonary tuberculosis is when *M. tuberculosis* is affecting the lungs and is the most common tuberculosis infection. It is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease. When people with lung TB cough, sneeze or spit, they propel the TB germs into the air. A person needs to inhale only a few of these germs to become infected (WHO, 2012).

Globally the human immunodeficiency virus (HIV) has greatly influenced the burden of TB (Swaminathan and Rekha 2010). Human immunodeficiency virus (HIV) infection increases the risk of developing TB after infection (Fauci *et al.*, 2008). Dually infected patients have a higher mortality rate than HIV negative patients due to immunosuppression (AU, 2006). In 2006 it was estimated that from the 1.7 million people who died as a result of TB infection about 200,000 individuals were with HIV /TB co-infection (Fauci *et al.*, 2008). Therefore TB infection is also explained in relation to the HIV status and is categorized as HIV positive, HIV negative or with unknown status (WHO, 2013).

Based on the drug susceptibility test (DST) TB patients can also be classified as nonresistant if not resistant to any drug as well as if the resistance is to one first-line anti-TB drug only. Multidrug resistant (MDR), when resistance is to at least both isoniazid and rifampicin (Marks *et al.*, 2009). Extensively drug-resistant TB (XDR TB) is a rare type of multidrug-resistant tuberculosis (MDR-TB) that is resistant to isoniazid and rifampin, plus any fluoroquinolone and at least one of the three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin) (Fauci *et al.*, 2008). The resistance to rifampicin is included when it is detected using phenotypic or genotypic methods, regardless of the resistance to other anti-TB drugs (WHO, 2013). As a result, this form of the disease is more difficult to treat than ordinary TB and requires up to 2 years of multidrug treatment (Fauci *et al.*, 2008). After the completion of the first course of treatment TB patients are usually tested for the outcome and are classified accordingly (WHO, 2013).

2.2 Epidemiology

Tuberculosis is a global health problem causing ill-health among millions of people each year and ranks second leading cause of death from all the infectious diseases in the world (WHO, 2013). From the latest report of 2012 there were about 8.6 million new TB cases and 1.3 million deaths worldwide (WHO, 2013). Previous studies showed that Africa and specifically Sub-Saharan Africa encounters the most TB epidemic (Esmael *et al.*, 2013). The highest TB incidence worldwide is contributed by the 22 high TB burden countries (WHO, 2010) Where Kenya is ranked 13th among them (GOK, 2009).

The figure below shows the list of the 22 high TB burden countries that account for 80% of the tuberculosis cases in the world (WHO, 2010).

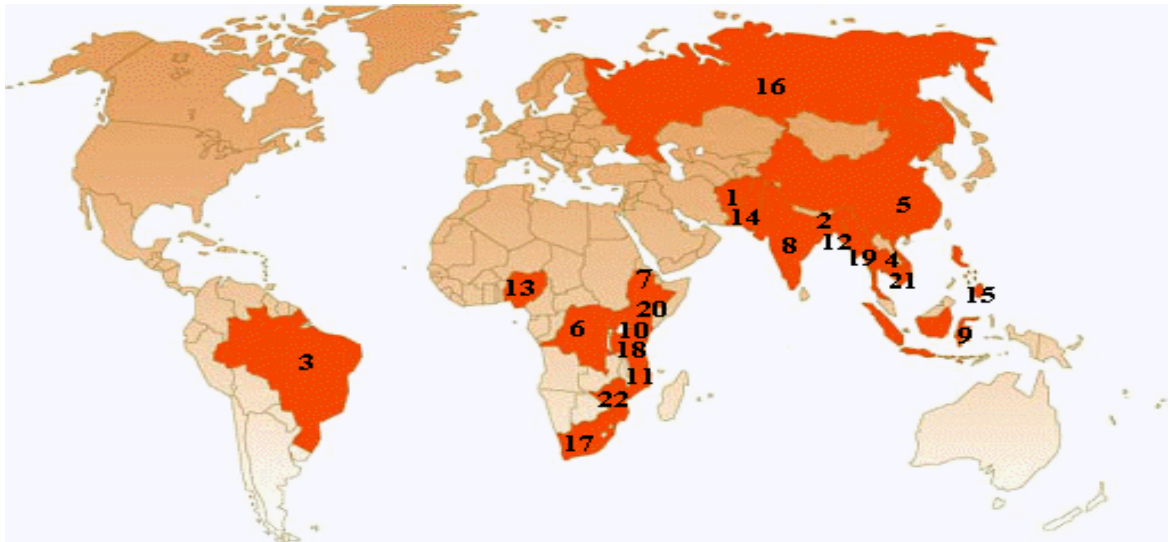


Figure 2.2 Country data by WHO region of estimated epidemiological burden of TB, 2010

1	Afghanistan	22	12	Myanmar	20
2	Bangladesh	6	13	Nigeria	4
3	Brazil	14	14	Pakistan	8
4	Cambodia	21	15	Philippines	9
5	China	2	16	Russian Federation	11
6	D.R.Congo	10	17	South Africa	5
7	Ethiopia	7	18	U.R of Tanzania	15
8	India	1	19	Thailand	18
9	Indonesia	3	20	Uganda	16
10	Kenya	13	21	Vietnam	12
11	Mozambique	19	22	Zimbabwe	17

The epidemiology of tuberculosis in Kenya evolved over years due to both economic and environmental factors. For example Nairobi the capital city of Kenya is currently showing 6.9% population growth rate; this has led to informal settlements like slums. More than half of the population lives in slums which cover just 5% of the city area and the poor life style as well as crowded environment (Sitienei *et al.*, 2013). In addition Kenya hosts about 630,000 refugees in camps and in urban Nairobi. Most of them are from Somalia whose TB incidence is believed to be high because of the weak TB control program (UNHCR, 2013). These all can potentially expose to the high risk of TB transmission.

There are about one million estimated cases of TB in children worldwide, where 80 % occur in the 22 high-burden countries. In low-burden countries, childhood TB constitutes about 5% of the TB caseload, compared with 20–40% in high-burden countries (Swaminathan and Rekha 2010). TB epidemic of children is usually under reported even though they carry huge tuberculosis burden particularly in endemic areas (Pastrana,2013), Regional data from the World Health Organization (WHO) in 2007 showed that smear-positive TB in children aged less than 15 years accounted for 0.6–3.6% of reported cases. However, because 95% of cases in children 12 years of age are smear negative, these data underestimate the true burden of TB in children (Swaminathan and Rekha 2010).

Children in developing countries are at high risk of getting TB infection because of the HIV infection prevalence. In 2007 the majority of children with smear positive

TB who were 15 years of age were in Africa and Southeast Asia. Childhood TB is also closely associated with poverty, crowding, and malnutrition, with consequently higher death and lower treatment success rates. The rapid progression from infection with *M. tuberculosis* to disease is a unique aspect of TB in children. Young age and HIV infection are the most important risk factors for severe or disseminated disease. This has a strong correlation with immunosuppression, underlying nutritional status and socioeconomic status (Swaminathan and Rekha 2010).

Tuberculosis (TB) follows HIV/AIDS as the greatest killer disease worldwide and is a leading killer of people living with HIV causing one quarter of all deaths (WHO, 2013). There is marked increase of TB infection in countries with a high prevalence of HIV infection. Thus, most cases occur in young adults, who are often parents of young children (Swaminathan and Rekha 2010). Multi-drug resistant TB (MDR-TB) is present in virtually all countries surveyed. About one-third of the world's population has latent TB, these are people who have been infected by TB bacteria but are not (yet) ill with disease and cannot transmit the disease (WHO, 2013).

TB prevalence is different among different sex and age groups (Wood *et al.*, 2010). Until adolescence the incidence of TB infection is similar in both males and females (Holmes *et al.*, 1998). Prevalence studies done using tuberculin skin test (TST) show that male prevalence exceed female after 16 years of age (Diwan and Thorson 1999). Some studies indicate that there is a difference in the response to TB infection and disease which could be due to the difference in the physiological development and immunity of the different sexes (Thorson *et al.*, 2007). There is also some evidence

that men have higher risk of developing active TB as they are more exposed to the risk factors like alcohol and smoking than women and they attend health services more symptomatic compared to women. On the other hand women produce less good quality of specimen in their sputum samples (Lawson *et al.*, 2010).

Previously it was noticed that globally the ratio of female to male tuberculosis cases was 1/1.5-2.1 (Diwan and Thorson 1999). This difference could be biological as sex is a key factor for modulating innate immunity, host response to infection and disease progression as well as socially constructed gender patterns (Lawson *et al.*, 2010). Yet though most TB cases occur among men the burden of TB in women is also high (WHO, 2013).

Disease diversity of tuberculosis as well as the mortality and morbidity varies substantially between different age groups. Disease risk after primary infection with *M. tuberculosis* is greatest in very young children less than 4 years. This slowly declines at age of 5 to 10 years then increases during adolescence at the age of 15 to 30 years. These differences in disease risk are accompanied by differences in the response to infection and clinical features of disease (Donald *et al*, 2010).

2.3 TB Transmission

Mycobacterium tuberculosis is transmitted by the spread of the small airborne nuclei droplets. These are generated when PTB infected individuals are coughing, sneezing, talking, or singing. These minuscule droplets can remain air-borne for minutes to hours after expectoration. Transmission of *the M. tuberculosis* can be influenced by

the number of bacilli in the droplets, the virulence of the bacilli, exposure of the bacilli to UV light, degree of ventilation, and occasions for aerosolization. Introduction of *M. tuberculosis* into the lungs leads to infection of the respiratory system and can cause PTB. The organisms can also spread to other organs once they join the blood stream, such as the lymphatics, pleura, bones/joints, or meninges, and cause EPTB (Knechel, 2009).

2.4 TB Risk Factors

Factors that determine the risk of TB infection in children are the same as in adults. The risk of infection is exposure and proximity and duration of contact depending on the severity of infectiousness of the source case (GOK, 2013). The risk of developing disease after infection is determined by various factors, including age at exposure, nutritional and immune status, genetic factors, virulence of the organism, and magnitude of initial infection (Swaminathan and Rekha 2010).

The risk of progress to disease after primary infection is highest in very young children who are less than 2 years (Kahn and Starke, 1995). The risk is lowest in immune competent children aged 3-10 years but increases again if children develop primary infection after 10 years of age. The use of immunosuppressive drugs, lack of BCG vaccination, severe malnutrition, overcrowding and poverty can also be important risk factors (Kahn and Starke, 2006). Poor treatment for previous TB disease, age, sex (males more than females), Diabetes, alcoholism and tobacco smoking are other major risk factors (GOK, 2013).

The life time risk of developing TB for people infected with MTB only is 10% while HIV/TB co-infected individuals have a much higher risk of falling ill since they are with compromised immune systems (Corbett *et al.*, 2003). People ill with TB can infect up to 10-15 other people through close contact in a year (Swaminathan and Rekha 2010).

2.5 Pathogenesis

TB infection and disease develop when a droplet of nuclei of the bacilli is inhaled into the lung and travel to the alveoli. Once inhaled, some of the infectious droplets of the bacilli settle throughout the air ways while the majorities are trapped in the upper parts of the airways where the mucus-secreting goblet cells exist. This physical defense provides the body with an initial protection from infection when exposed to tuberculosis (Knechel, 2009). Bacteria in droplets that bypass the mucociliary system and reach the alveoli multiply in the alveoli to join the blood stream and spread to the different parts of the body where the bacilli can cause infection. *M. tuberculosis* favors areas of the body with good oxygen concentration like the upper lung lobes, the brain, kidneys, and bone.

The immune system is triggered after infection, large macrophages start to engulf and stop or kill the bacilli. These macrophages are the most abundant immune effector cells that exist in alveolar spaces. They are part of the innate immune system that acts as the next line of host defense. These provide the body with an opportunity to destroy the invading mycobacteria and prevent infection (Knechel, 2009). Antibody responses contribute little to protection against TB. However,

mycobacterium-specific antibodies may be capable of enhancing both innate and cell-mediated immune responses (Harald, 2008).

After being ingested by macro phages, the mycobacteria continue to multiply slowly within the macrophage, with bacterial cell division occurring every 25 to 32 hours regardless of whether the infection becomes controlled or progresses (Knechel, 2009). This is because alveolar macrophages in the lungs are unable to digest *M. tuberculosis*. Its cell wall prevents the fusion of the phagosome with a lysosome. *M. tuberculosis* blocks the bridging molecule, early endosomal autoantigen 1 (EEA1) and this blockage does not prevent fusion of vesicles filled with nutrients. The bacteria also carry the UreC gene, which prevents acidification of the phagosome as well as evade macrophage-killing by neutralizing reactive nitrogen intermediates (Mann *et al.*, 2009).

In the majority of the hosts TB infections cell-mediated immunity is effective enough for the infection to subside to a state where tubercle bacilli remain dormant within the infectious foci and it is called as latent TB. In latent TB, viability of the bacilli is maintained and reactivation may occur later in life (Druszczyńska *et al.*, 2012). A small number of antigen-specific T cells that survive can then become long-lived memory T cells (Kaech *et al.*, 2002).

The outcome is determined by the quality of the host defenses and the balance that occurs between host defenses and the invading mycobacteria (Knechel, 2009). When the infection is not properly contained, bacilli may spread systemically to multiple

organs. In some individuals, proliferation of bacilli continues until the infection becomes severe enough to cause disease called primary TB (Harald, 2008).

There is a protective vaccination Bacille Calmette Guérin (BCG) against tuberculosis. Studies report that BCG induces antibodies by improving the phagocytosis action of the macrophages; it also increases the proliferation and IFN- γ production of mycobacterium-specific T-cells (Valliere *et al.*, 2005).

2.6 Symptoms of Tuberculosis

The symptoms of TB are not specific and can also be present in other illnesses like HIV, malnutrition, malaria or other viral or bacterial infections. These symptoms typically include weight loss, poor appetite, cough and fever that can last for many weeks. Consequently TB is usually hard to recognize based on symptoms alone (MSF, 2010). Symptoms of active lung TB can include cough with sputum and blood at times, chest pains, and night sweats. In children the symptoms of TB may include unintended weight loss, poor growth, swollen glands and chills (WHO, 2013).

2.7 Diagnosis of Tuberculosis

Early TB diagnosis and treatment to avoid progressing to disease are challenges that need to be overcome. The WHO expects to reduce the prevalence and the mortality rate of the disease by 50% by 2015, from the rates found in 1990 (Sztajn bok *et al.*, 2009). Various methods are used for testing PTB, such as Chest Radiography (X-ray), tuberculin skin test (TST), bacteriological tests like Sputum smear microscopy, culture, molecular tests based on DNA (PCR).

2.7.1 Chest Radiography (CXR)

Chest Radiography (CXR) is a commonly used diagnostic test to complement clinical evaluation for patients with suspected tuberculosis (TB) (Graham, 2014). According to earlier reports Chest Xray can suggest active and inactive TB. In active TB infiltrate or consolidations and / or cavities are often seen in the upper lungs with or without mediastinal or hilar lymphadenopathy, however lesions may appear anywhere in the lungs. Nodule with poorly defined margins and pleural effusion can also suggest active TB. Discrete fibrotic scar, discrete nodule(s) without calcifications also can be signs that suggest inactive TB (GOK, 2013). In the study done in Vietnam in patients with smear positive tuberculosis it was found that despite similar disease duration, men presented with advanced CXR findings at the time of diagnosis of TB more often than women and this correlated with what is known in clinical features (Thoron *et al.*, 2007).

For children with suspected TB a wide range of radiological abnormalities can be observed like Lymphadenopathy (right hilar, left hilar and bilateral), Mediastinal and Parenchymal consolidation. The most common radiological abnormalities in children are lymphadenopathy, isolated, or associated with parenchymal consolidation (Milkovic *et al.*, 2005). Specificity is lower in high TB endemic settings where severe pneumonia, malnutrition and human immunodeficiency virus (HIV) infection as well as co-infections of other respiratory pathogens in children are common. The sensitivity of chest radiography is also low, for detecting the enlargement of the regional lymph nodes, which is the most common pathological sign that follows

primary infection with *M. tuberculosis*, and this leads to over diagnosis of TB in children (Graham, 2014)

2.7.2 Tuberculin skin test (TST)

Tuberculin skin test is a skin test developed by Koch in 1890 but the current technique in use was described by Charles Mantoux in 1912 and is commonly known as Montoux test. The tuberculin most widely used is purified protein derivative (PPD), which was developed from *M. tuberculosis* culture (Nayak and Acharjya, 2012). TST is applied for both active and latent TB but its main utility is for latent TB infection (LTBI) though it does not distinguish between LTBI and the disease because it can only detect the presence or absence of the bacilli in the body. Difficulties in attaining microbiological confirmation increase the supportive role of the TST in the diagnosis of TB, especially in children and in patients with extra-pulmonary disease (Harald, 2008).

Tuberculin skin test needs to be read in 48 to 72 hours after the administration and good care should be taken in reading and interpreting the results. The person's medical risk factor can also determine the size of the induration for the result to be positive (Nayak and Acharjya, 2012). Besides BCG vaccination, HIV infection and severe malnutrition can affect the tuberculin skin test results especially in children (GOK, 2013). Tuberculin skin test can give false positive results for patients with infection by mycobacterium other than tuberculosis and previous BCG vaccination. Previous studies showed positive correlation between scar presence and TST reactivity in children as well as adults (Harald, 2008). It can also give false negative

results in weakened immune system, very young age children who are less than six months and to some viral illnesses like measles, chicken pox and HIV. Consequently these various factors lower its specificity and sensitivity which leads to a wide range of results from the presence of reaction in uninfected children to complete absence of the reaction in some children with confirmed TB disease (Nayak and Acharjya, 2012).

The study which was done to compare the performance of TST against interferon gamma release assay (IGRA) also recommends further investigation on the influence of BCG immunisation on TST specificity for all induration thresholds to determine which threshold optimises specificity without compromising sensitivity for all age groups (Michael and Fidler, 2014). While another study done to compare TST and IGRA evaluated both tests and showed a good agreement, the only difference was IGRA was highly specific compared to TST and was not being influenced by previous BCG vaccination. IGRA is more sensitive in very young children who are less than 48 months as well (Liela *et al.*, 2009). Interferon gamma is a promising new assay for the diagnosis of both the latent tuberculosis infection and active disease. It proved to have, better sensitivity and specificity than the TST, even in children and immunosuppressed patients. IGRAs are very expensive and need specialized laboratories and professionals trained to perform them within reasonable time (Sztajnbock *et al.*, 2009).

2.7.3 Sputum Smear Microscopy

Many countries still rely on a long-used method called sputum smear microscopy to diagnose TB (WHO, 2013). Especially for countries with low income and high tuberculosis prevalence, sputum smear microscopy is the only cost-effective tool for diagnosing patients with infectious tuberculosis and to monitor their progress in treatment. The World Health organization (WHO) and the International Union Against Tuberculosis and Lung Diseases (IUATLD) recommended a collection of two sputum samples for smear microscopy with at least one being an early morning (EM) sample (Ssengooba *et al.*, 2012). Sputum smear positive tuberculosis is usually defined as a person presenting with respiratory symptoms with at least two positive sputum smear microscopy examinations (IUATLD, 2000). It is simple relatively easy to perform and to read, inexpensive and uses appropriate technology method (IUATLD, 2000). With such tests, diagnosis can be made within a day (WHO, 2013).

However, sputum smear microscopy has significant limitations in its performance. The sensitivity is grossly compromised when the bacterial load is less than 10,000 organisms/ml sputum sample. The requirement of a series of examinations may lead to the missing of some of the results as well as treatment, if some of the patients don't appear for the next examination and/or for the result. Limited resources like electricity, competent operator, experience of observer combined with large numbers of samples can also reduce the observation time and the sensitivity as well. It also has a poor track record in extra-pulmonary tuberculosis, pediatric tuberculosis and in patients co-infected with HIV and tuberculosis (Desikan 2013). Because getting

sputum or other respiratory samples from children and HIV patients for smear microscopy is very difficult. This test is less applicable for pediatric TB diagnosis (MSF, 2010). This test does not also detect numerous cases of less infectious forms of TB (WHO, 2013). That is why there are significant efforts underway to optimize smear microscopy (Smart, 2012).

2.7.4 Gene Xpert MTB/RIF

Gene Xpert MTB/RIF is a new cartridge-based fully automated rapid molecular diagnostic test for TB and rifampicin resistance within 100 minutes (Smart, 2012). It was developed by FIND, Cepheid and the University of Medicine and Dentistry of New Jersey with the funding from NIH, and the Bill and Melinda Gates Foundation. Scientific evidence was reviewed by the WHO through an Expert Group and then WHO's Strategic and Technical Advisory Group for TB (WHO, 2013).

Xpert MTB/RIF assay provides results from unprocessed sputum samples. It purifies concentrates, amplifies using rapid, real-time PCR and identifies targeted nucleic acid sequences in the TB genome (Xpert®, 2012). Xper MTB/RIF assay targets the *rpoB* gene hot spot region. Any deviation from the wild type sequence resulting in a delay in the appearance of the signal exceeding a predetermined cycle threshold which is 3.5, between the earliest and latest (*CT*) values, is reported as RIF resistant (Ioannidis *et al.*, 2011). The sample is mixed with bactericidal buffer prior to the addition to the cartridge. This step reduces the viability of MTBC organisms, therefore making the use of the assay suitable for use near patients in settings with limited bio containment facilities (Banada *et al.*, 2010). Xpert reports a qualitative

result that is MTBC detected as very low, low, high and very high. Gene Xpert MTB/RIF assay is being rolled out by WHO from 2010 in a number of countries in order to improve the speed and accuracy of TB diagnosis. Gene Xpert MTB/RIF assay reduced the delays between presentations, diagnosis and starting TB treatment substantially (Smart, 2012).

Gene Xpert MTB/RIF assay was evaluated with microscopically negative and positive pulmonary specimens from patients with substantial clinical indications for tuberculosis. For microscopically positive pulmonary samples, the sensitivity and specificity were 90.6%, 94.3%, respectively. While for the negative specimens, the respective values were 86.3%, 93%. This indicated that GeneXpert assay was highly effective for tuberculosis diagnosis and identification of rifampin-resistant strains in smear-negative samples (Ioannidis *et al.*, 2011).

WHO strongly recommends the use of Gene Xpert MTB/RIF assay as the diagnosis for initial test for MDR TB and HIV co-infection TB patient with the expectation of an increased diagnosis of MDR TB and HIV/TB patients in areas of high prevalence (WHO, 2013). Even if MDR TB and HIV infection are of low prevalence it gives a conditional recommendation as the diagnostic test for follow on test.

Pilot studies done on diagnosis of pediatric intra-thoracic tuberculosis from stool samples using the Xpert MTB/RIF assay suggests that Xpert on stool might be useful for the diagnosis of pediatric intra-thoracic TB, as Xpert on stool and GA was equally sensitive and gave more rapid results than culture (Walters *et al.*, 2012).

Xpert testing of stool from children with suspected PTB detected 47% of the children with culture-confirmed tuberculosis and sputum Xpert detected 65% cases. The study showed that stool Xpert can be a promising test for diagnosis of PTB in children and recommended further studies, utilizing optimized specimen processing protocols are required (Nicoll *et al.*, 2013).

2.7.5 The QIA amp DNA Stool Mini Kit

The purity of DNA extracted from stool samples is a key issue in the sensitivity and usefulness of biological analyses such as PCR for infectious pathogens and non-pathogens (McOrist *et al.*, 2002,) QIAGEN is a kit that enables the isolation and detection of contents of any biological sample (QIAGEN , 2012). In a study done for the comparison of relative efficacy of extraction of bacterial DNA from stool , using four commercial kits (Fast DNA kit, Bio 101; Nucleospin C+T kit, Macherey-Nagal; Quantum Prep Aquapure Genomic DNA isolation kit, Bio-Rad; QIAamp DNA stool mini kit, Qiagen), the QIAamp kit was the most effective extraction method and led to the detection of bacterial DNA (McOrist *et al.*, 2002). QIAamp DNA Stool Mini Kit is optimized for use with up to 220 mg fresh or frozen stool, or larger amounts of stool. Starting with larger amounts increases the likelihood of purifying DNA from low-titer sources in stool samples.

The process includes lysis and adsorption of impurities to InhibitEX. Stool samples are lysed in Buffer ASL. Human cells lyse efficiently at room temperature. Bacterial cells and those of other pathogens in the stool are effectively lysed by incubating the stool homogenate at 70°C and if necessary to 95°C for detection of cells that are

difficult to lyse for example cells of *Mycobacterium tuberculosis*. After lysis, DNA-damaging substances and PCR inhibitors present in the stool sample are adsorbed to InhibitEX. After inhibitors and DNA-degrading substances have been adsorbed to InhibitEX, then the DNA in the supernatant is purified on QIAamp spin columns (QIAGEN , 2012).

2.8 Treatment and Control

The vast majority of TB cases is treatable with a standard six-month course of four antimicrobial drugs and can be cured when medicines are taken properly (WHO, 2011). The four first-line drugs are isoniazid, rifampicin, ethambutol and pyrazinamide. Treatment for multidrug resistant TB (MDR-TB), defined as resistance to isoniazid and rifampicin (the two most powerful anti-TB drugs) is longer, and requires more expensive and toxic drugs (WHO, 2013). The basic principles of treatment and recommended standard anti-TB regimens for children are similar to those for adults (Swaminathan and Rekha 2010).

The treatment of drug susceptible tuberculosis is cheap and highly effective, yet worldwide the disease remains a serious cause of illness and death, as serious as to have been declared a 'global emergency' in 1993. It is estimated that between 2000 and 2020, nearly one billion people will be newly infected, 200 million will get sick, and 35 million will die from TB if control measures are not significantly improved. (Grange and Zumela, 2002).

2.9 Diagnosis of Tuberculosis in Children

Tuberculosis is particularly difficult to diagnose in children. This is because all existing diagnostic tests for TB in children have short comings (WHO, 2006).

Advances have been made in diagnostic of adult TB, but development in children is lagging behind (Mukherjee *et al.*, 2012). The absence of accurate pediatric TB diagnosis has resulted in the underestimation of the burden of TB in children (Swaminathan and Rekha 2010).

Tuberculosis diagnosis in children relies on careful and thorough history and physical examination, chest Xray (CXR), Tuberculin skin test (TST) and sputum smear microscopy, (GOK, 2013). Although diagnosis of childhood PTB requires chest X-ray (CXR), findings are often not specific and certainly not diagnostic. However, radiographic and clinical findings suggestive of TB become more specific when it has been established that the child has been in close contact with a diagnosed case of PTB. A positive history increases the likelihood that the child does indeed have TB (GOK, 2013). A positive tuberculin test does not indicate the presence or extent of tuberculosis disease, it only indicates infection. In a child who has had BCG, an induration of 10-14 mm may be due to vaccination or TB infection. A negative tuberculin skin test does not exclude TB infection, if the clinical features and contact history are suggestive because the tuberculin test is less likely to be positive in a child with TB if the child also has severe malnutrition, HIV infection or disseminated TB such as TB meningitis (Harald, 2008).

Scoring systems have been produced for screening and diagnostic purposes (see appendix 5), these systems are one way of trying to improve the diagnosis of childhood TB. The basis of a score system is the careful and systematic collection of diagnostic information. A score below a certain threshold indicates a high likelihood

of TB. A score of 7 or more indicates a high likelihood of TB (Crofton, 1998) for helping to diagnose childhood TB. Still the evaluation is difficult in the absence of a “gold standard” diagnosis. They are likely to be even less accurate in regions where childhood malnutrition and HIV infection are common.

Bacteriological confirmation is usually not possible (WHO, 2003). Test requires a respiratory sample. Sputum is very difficult to obtain in children, especially young children that are unable to expectorate sputum samples when they cough (MSF, 2010). Other methods of obtaining material, such as gastric lavage, can be problematic to implement as routine diagnostic procedures, are less sensitive and generally not useful (WHO, 2003). Procedures used to obtain samples from a child’s lung or stomach can be traumatic for the child and they require well-trained staff, a good health facility and a good laboratory, all of which can be rare in resource-poor settings (MSF, 2010).

Culture which is a gold standard in bacteriologic tests and uses 100 cfu of sputum per ml as a sample for high specificity is not suitable in children as it is in adults .It is difficult to easily get the required amount of the sample in children and AIDS patients (Shingadia and Novelli, 2003). Consequently this lack of diagnostic method for TB is making it a central cause of child morbidity and mortality worldwide (Nellson and Wells, 2004). Hence what is needed is a non-sputum-based diagnostic test that gives a fast result so that children, who are at high risk of dying from TB, can be promptly treated (MSF, 2010).

It is known that children swallow most of the sputum since they cannot spit it out and tuberculosis DNA can survive intestinal transit (Cordova *et al.*, 2010). In a study done in Lema, Peru which evaluated pediatric TB diagnostic strategies, from 236 hospitalized children with suspected tuberculosis 16 patients confirmed pulmonary tuberculosis by nasopharyngeal, and gastric aspirate cultures. Stools from these 16 children were used to evaluate stool polymerase chain reaction (PCR) for tuberculosis diagnosis compared with 23 stool samples from healthy control children using two DNA extraction techniques (Fast-DNA mechanical homogenization and Chelex-resin chemical extraction). PCR after Fast-DNA processing was positive for 6/16 culture-proven tuberculosis patients versus 5/16 after Chelex extraction with the sensitivity of 38% and 31%, respectively. All controls were negative thus specificity was 100%. If sensitivity can be increased, stool PCR would be a rapid, non-invasive, and relatively bio-secure initial test for children with suspected pulmonary tuberculosis (Wolf *et al.*, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Site

The study was carried out in different hospitals in and around Nairobi. Patients were recruited from Gertrude's Children's Hospital and all its satellite clinics, Kiambu District Hospital and Mbagathi District Hospital. The laboratory analysis was done at the pathology department of the Aga Khan University Hospital, Nairobi and in the National Reference Tuberculosis Laboratory, as they have advanced laboratory equipment needed for this specific investigation.

Gertrude Children's Hospital is the only pediatric hospital in the region, therefore has wide catchment that gave wide representative of the needed population for the study. This hospital started 60 years ago and is now an ultramodern establishment with modern medical equipment. The main Hospital located in Muthaiga runs for 24 hours and has 80 pediatric beds, about two hundred qualified staff members a dental clinic, X-ray, ICU and 21 specialist clinics. The hospital has both in and outpatient services. The hospital runs a comprehensive care clinic where children are on follow up for antiretroviral treatment. The largest number of patients with suspected tuberculosis was seen in this clinic. Kiambu District Hospital is a public hospital located in Kiambu County, Kenya, while Mbagathi District Hospital is a public hospital located in Mbagathi, Nairobi. Both have chest X-ray clinics that host both adults and children TB patients for diagnosis and treatment.

3.2. Study Design

The study was a laboratory based cross-sectional prospective design. Consecutive children patients (age <15) with symptoms suggestive of pulmonary TB who were attending to the Gertrude children's hospital and other satellite clinics of the hospital, Nairobi, Kiambu district hospital and Mbagathi district hospital were enrolled . They were approached by the clinicians attending to them and referred to the investigator for the explanation about the study. Consent was sought from their parents or guardians or their legal representatives and participation was entirely voluntary. Procedures comprised of interviews regarding medical history and results of clinical examination and chest radiography were taken from the patient record files by the investigator. Data of Sputum analysis was obtained from the hospital laboratory. Those who consented and were willing to participate in the study were given plastic pots to put stool samples. Samples were carried to the laboratory using cooler boxes. The amount of stool collected was 2g of stool (the amount picked by a wooden stick commonly used in laboratory processes and only 0.15g and 0.18g were used for testing in the study. Then the results of sputum diagnosis were compared with the stool sample results which originated from the study.

3.3 Study Population

The study population comprised all the children under 15 years of age who were suspected of TB, attending at Gertrude children's hospital and all its satellite clinics in Nairobi, Kiambu district hospital and Mbagathi district hospital since September 2013 to April 2014. Sample collection included all children suspected for PTB who presented the clinical symptoms and /or from whom respiratory samples such as

sputum / induced sputum/ pharyngeal aspirate had been collected for smear microscopy or culture.

3.3.1. Inclusion Criteria

All children under 15 years presented to these hospitals and clinics with signs and symptoms suggestive of pulmonary tuberculosis, who were referred by the doctor for TB investigation, and whose guardian or parent consented, were included in the study.

3.3.2. Exclusion Criteria

Children above 15 years old, children whose parent or guardian refused consent were excluded from the study.

3.4. Sample size and Sampling

3.4.1 Sample size

Participants were picked consecutively till the obtained number was attained. The minimum sample size was determined by using the following Fisher's formula (Fosgate, 2009).

$$n = \frac{Z_{1-\alpha/2}^2 p(1-p)}{d^2}.$$

$Z_{1-\alpha/2}^2$, Z score at $1-\alpha/2$ confidence level, standard normal deviate that corresponds to 95% confidence interval (1.96).

P is the estimated proportion of PTB incidence in children which is 10% (GOK, 2009). Where $1-p$ is the estimated non prevalence proportion.

d is largest difference of the estimated proportion that could be accepted in the research. For this study it was 7% ($d=0.07$).

Since there is a report of 10% of PTB incidence in children under 15 years in Kenya, (GOK, 2009) prevalence rate was assumed as 10% at 95% confidence level, the minimum sample size that could be collected was 71 using the formula given above, for more accuracy 20% of this minimum sample size was added. Therefore 91 patients in total were recruited into the study.

3.4.2 Sampling

Stool samples were collected from each patient with symptoms suggestive of pulmonary TB who present to the Gertrude children's hospital and other satellite clinics of the hospital, Nairobi, Kiambu district hospital and Mbagathi district hospital. If stool samples were not available on the same day of recruitment, the subsequent stool samples were collected the next day. Stool specimens were stored at -20 and/or -80°C within 2 h. Xpert testing was performed within a maximum one week of storage. Laboratory work was done in the Pathology Laboratory of the Aga Khan University Hospital, Nairobi and in the national reference tuberculosis laboratory.

3.5. Laboratory Procedures

3.5.1 Direct Stool Test using Gene Xpert

Stool processing: Stool was taken out from the freezer and left at room temperature to melt for about 5 min. 0.15g of thawed stool (confirmed by weighing) was picked

by a wooden stick commonly used in laboratory processes. It was then placed in 2.4ml PBS and vortexed briefly before removing the swab. The sample was left undisturbed for 20 min at room temperature, to allow large particles to sediment before removing 2 aliquots of 1ml supernatant. One aliquot was tested immediately with Xpert and the other was stored at 4°C for later duplicate testing (within 1 week) as needed. One aliquate was centrifuged at 3,200g for 10 min. Xpert testing was then performed as per the manufacturer’s instructions (plate 3.1), using a 2:1 ratio of Xpert reagent to sample (Nicoll *et al.*, 2011).

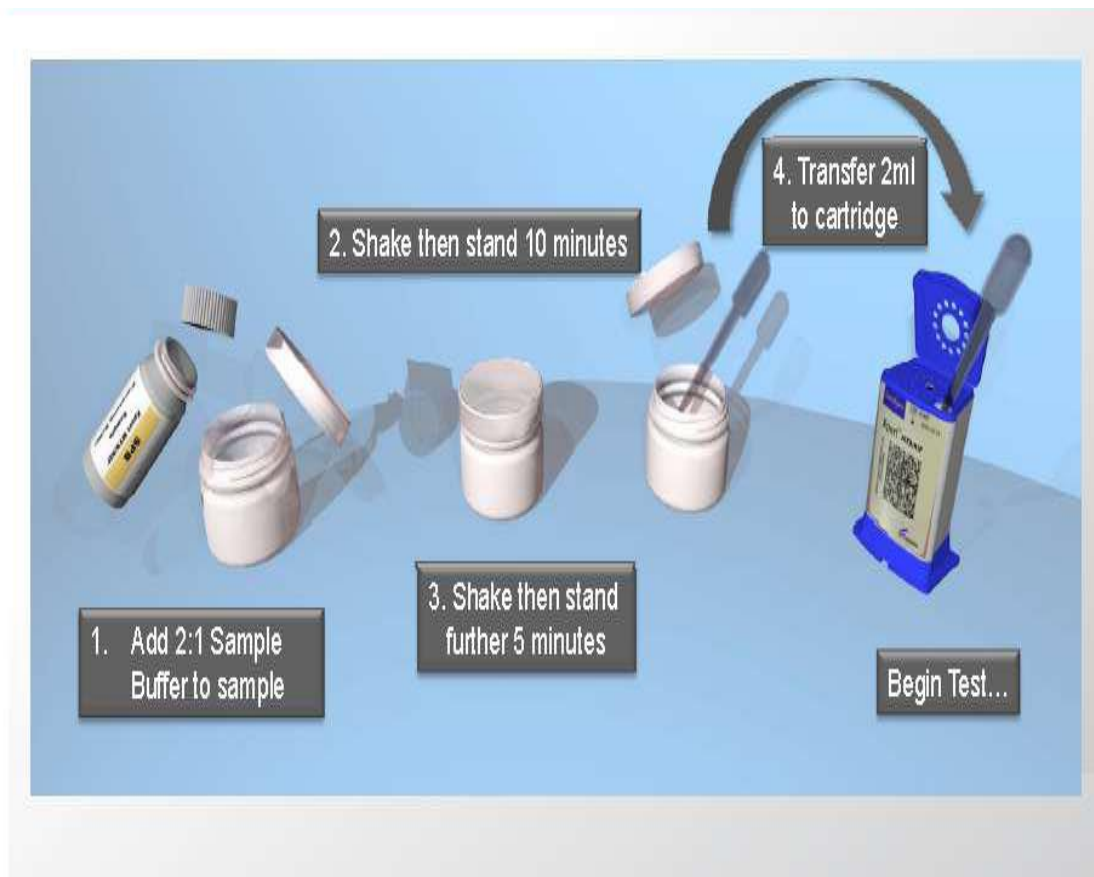


Plate 3.1 Preparing the 2:1 ratio Xpert reagent to sample

3.5.1 Stool Extracting DNA using QIAGEN stool DNA Extraction Kit prior to Xpert testing:

DNA was isolated from stool using the QIAGEN stool DNA extraction kit as per the manufacturer's instructions. The QIAamp DNA Stool Mini Kit was designed for rapid purification of total DNA from up to 220mg stool and is suitable for both fresh and frozen samples. The fast and easy procedure comprises the following steps (Figure 3.5.3)

- i) Lysis of stool samples in Buffer ASL
- ii) Adsorption of impurities to InhibitEX
- iii) Purification of DNA on QIAamp spins columns.

0.18–0.22 stool was weighed and put in a 2 ml microcentrifuge tube. The tube was then placed on ice. When the sample was frozen, a scalpel was used to scrape bits of stool into a 2 ml micro centrifuge tube on ice. All the following steps were performed at room temperature (15–25°C). 1.4 ml Buffer ASL was added to each stool sample, vortexed continuously for 1 min or until the stool sample was thoroughly homogenized. This helps ensure maximum DNA concentration in the final elute. The suspension was heated for 5 min at 95 °C in order to increase total DNA yield 3- to 5-fold and helps to lyse bacteria and other parasites. Sample was vortexed for 15s and centrifuged at full speed for 1 min to pellet stool particles. 1.2 ml of the supernatant was put into a new 2 ml micro centrifuge tube and the pellet was discarded.

InhibitEX Tablet was added to each sample, vortexed immediately and continuously for 1 min until the tablet was completely suspended. Suspension was incubated for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. Samples were centrifuged at full speed for 3 min to pellet inhibitors bound to InhibitEX matrix. Then all the supernatant was put into a new 1.5 ml micro centrifuge tube, while the pellet was discarded. The supernatant was centrifuged at full speed for 3 min again. 15 μ l proteinase K was put into a new 1.5 ml micro centrifuge tube. 200 μ l supernatant was pipetted into the 1.5 ml micro centrifuge tube containing proteinase K then 200 μ l Buffer AL was added and vortexed for 15 sec, in order that the sample and Buffer AL to be thoroughly mixed to form a homogeneous solution. The sample was incubated at 95°C for 10 min, centrifuged briefly to remove drops from the inside of the tube lid. 200 μ l of ethanol (96–100%) was added to the lysate, and mixed by vortexing.

The lid of a new QIAamp spin column was labeled and placed in a 2 ml collection tube. The complete lysate was then carefully applied to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 min. The QIAamp spin column was then placed in a new 2 ml collection tube, and discarded the tube containing the filtrate. Each spin column was closed in order to avoid aerosol formation during centrifugation. The QIAamp spin column was carefully opened and 500 μ l Buffer AW1 was added, closed and centrifuged at full speed for 1 min. Then the QIAamp spin column was placed in a 2 ml collection tube, and the collection tube containing the filtrate discarded. The QIAamp spin column

was opened carefully and 500 μ l Buffer AW2 was added. The cap was closed and centrifuged at full speed for 3 min. Then the collection tube containing the filtrate was discarded. The QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube was discarded with the filtrate. It was then centrifuged at full speed for 1 min to eliminate the chance of possible Buffer AW2 carryover. The QIAamp spin column was transferred into a new, labeled 1.5 ml micro tube and centrifuged. The QIAamp spin column was carefully opened and 200 μ l Buffer AE was pipetted directly onto the QIAamp membrane. The cap was closed then incubated for 1 min at room temperature, then centrifuged at full speed for 1 min to elute DNA. The Xpert testing was then performed as per the manufacturer's instructions, using a 2:1 ratio of Xpert reagent (dilution buffer) to sample. Since 2ml of sample is needed to be fed to the cartridge, the volume of the eluted DNA was made to volume by using molecular water.

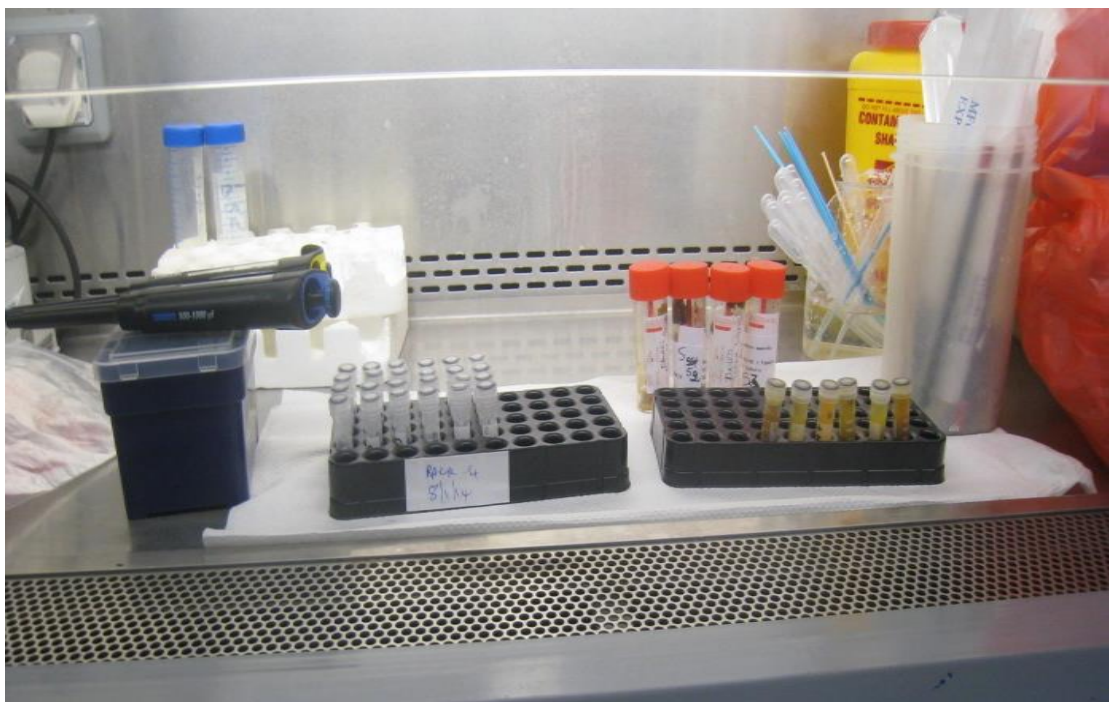


Plate 3.2 QIA amp stool processing

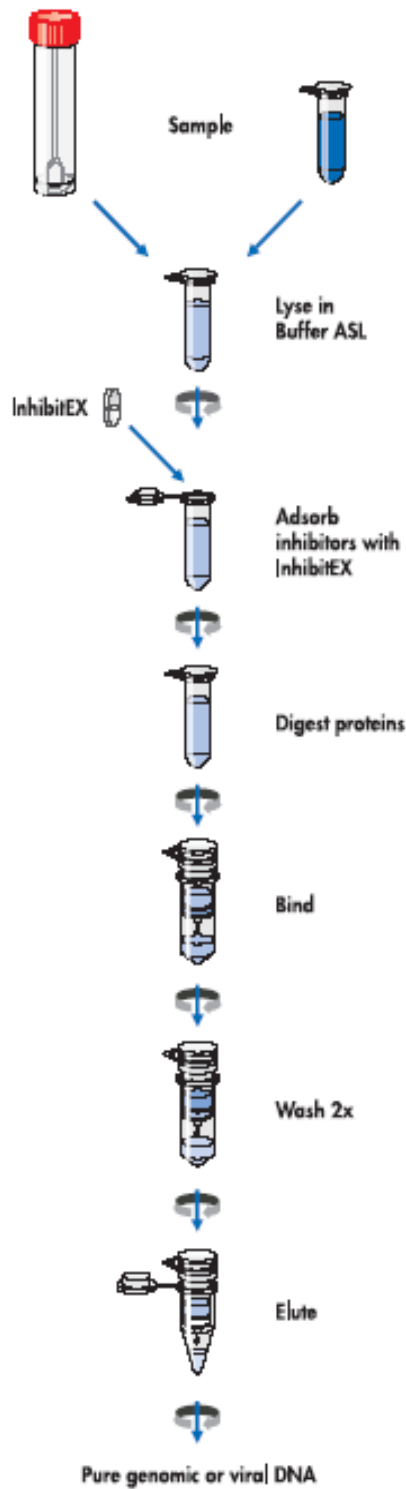


Plate 3.3 the QIA amp DNA Procedure (Source QIAGEN, 2012)

Procedure for Preparing the Cartridge: the Xpert MTB/RIF cartridge lid was opened; 2ml of the diluted sample was transferred using a sterile pipette, into the open port of the cartridge then Closed. Care was taken to make sure that the lid snaps firmly into place. Remaining liquefied sample was kept for up to 12 h at 2 – 8 °C to repeat test when required. For example in cases where there was power block outs. The test was started before 30 min before adding the sample to the cartridge (Plate 3.4) (Tenover , 2009).

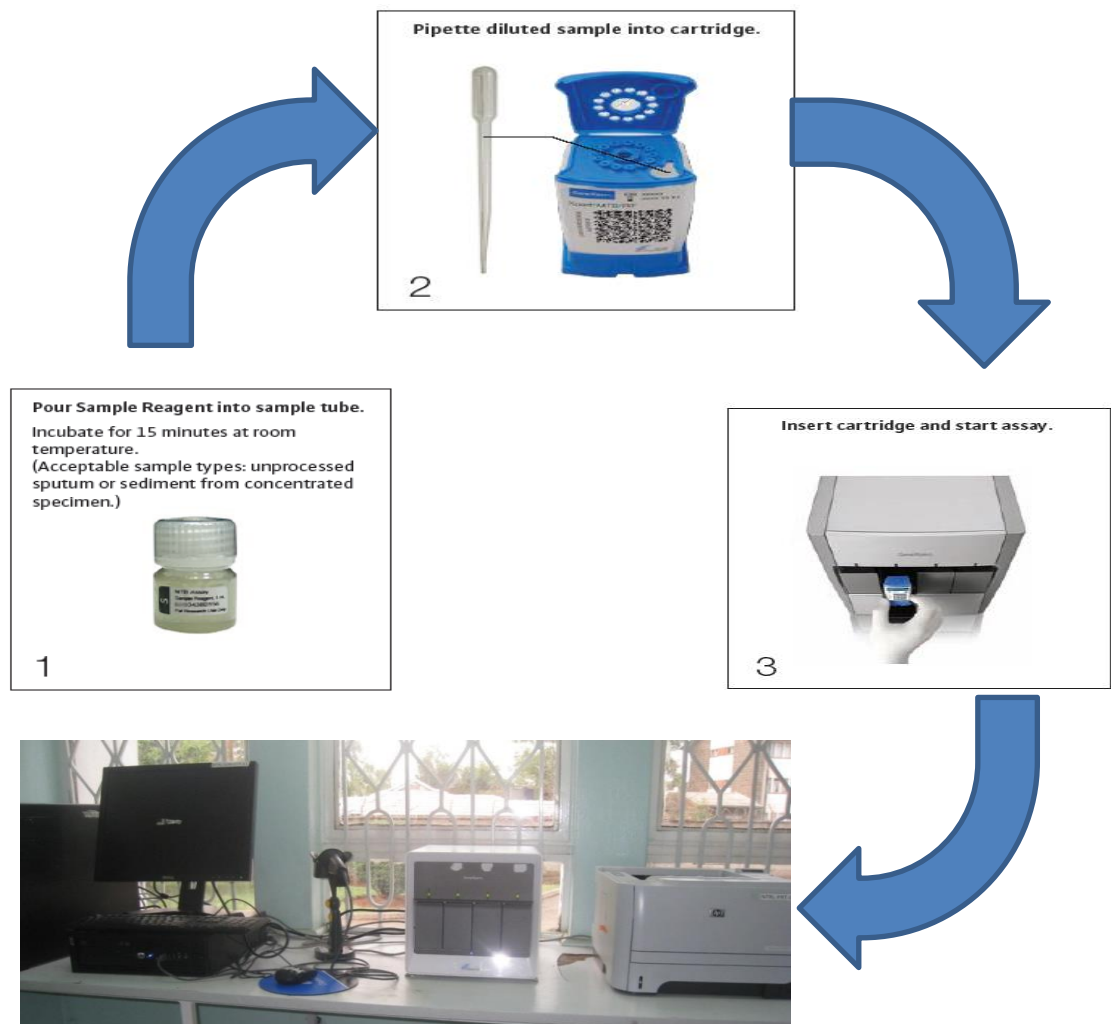


Plate 3.4: Illustration of work flow of processing a sample to be tested in the Xpert MTB/RIF

3.5.3. Analysis of the DNA using Xpert MTB/RIF

Gene Xpert MTB/RIF test is a molecular test. Cartridge with sample diluted by the reagent was fed to the machine for the detection of TB bacterium using biochemical reagents and followed by PCR (polymerase chain reaction) technique. The cartridge it uses contains all the elements necessary for the reaction, including lyophilized reagents, liquid buffers and wash solutions. Target detection and characterization were then performed in real time using a six-color laser detection device (Smart , 2012).

3.6. Ethical Clearance

Ethical clearance to carry out this study was obtained from Kenyatta University Ethical Review committee (PKU/127/E160) and Gertrude's Children's Hospital Ethical Review Board (Appendix 1 and 2). The permission to collect samples from patients was sought from the authorities of Gertrude Children's Hospital, Kiambu District Hospital and Mbagathi District Hospital. Written informed consent was obtained from all participants' parents, guardians or their legal representatives. The study was conducted according to the principles expressed in the Helsinki declaration as revised in 2000. Sample collection lasted for about 8 months.

3.7. Data collection method

Data was collected using questionnaire and from the results of the tests done by stool Gene Xpert in the laboratories. Using the questionnaire information regarding demographic characteristics and clinical features of the children suspected to have PTB was obtained. Age was used to filter the participants in the study against their

clinical profile. Results of the molecular examination for MTB from the stool test using Xpert MTB/RIF in the laboratory, was recorded both in soft copy in the computer as well as filled in the file box as hard copies. All these recorded results were securely kept. The data was then transferred to Microsoft office Excel 2010 and saved both in computer and flash disks. A password was used for the data in the computer and the file box was kept in a lockable cabinet.

3.8. Data Analysis

The collected data was imported from excel to the statistical package for social sciences (SPSS) Version 20 and the results analyzed for comparison and association of the different diagnosis methods and tests done for MTB, using the Chi-square test. Data was then presented in tables and graphs.

CHAPTER FOUR

4.0 RESULTS

A total of 91 children suspected of PTB were recruited during the study from one private and two public hospitals in Kenya. The demographic data of the children suspected for PTB that was obtained using questionnaires showed that 72, 17 and 2 patients were recruited from Gertrude's Children's Hospital, Kiambu District Hospital and Mbagathi District Hospital respectively. From these all 52 were male children while 39 were females with the median age of 3. These are represented in tables. These children were suspected of PTB as they showed three or more clinical features in the scoring system which is cough more than for two weeks, weight loss, fever or/and night sweat and history of contact (GOK,2009). Then they were subjected to tuberculin skin test (TST) and/or chest X-ray (CXR) for respiratory signs suggestive of TB, as well as sputum ZN microscopy where available, (GOK, 2013) see appendix 5.

The results of these clinical features which were taken from the patient's record files are summarized in the form of figure in percentage and Table in frequency respectively. Including the results of the sputum ZN smear microscopy that was taken from the routine test done for children suspected of PTB in the laboratory of the hospital as well as that of stool Gene Xpert which was done to 91 of the children suspected of having PTB. Tables and figures are also representing the comparison between these tests.

4.1 Distribution by hospitals, sex, age and body mass index

As it can be seen in Table 4.1 from the total 91 recruited patients in this study most of the participants were from Gertrud Children's Hospital accounting for 79.1%, where 44 were males and 28 were females. While 18.7% (6 were male and 11 females) are from Kiambu District Hospital. The least participants were from Mbagathi District Hospital accounting for 2.2% and where both males. The prevalence was higher in male children than in females covering 52 (51.1%) and 39 (42.9%) respectively.

Table 4.1 Distribution of study participants (recruited patients) by hospitals

Hospital	Frequency	Percentage (%)	Male	Female
GCH	72	79.1	44	28
KDH	17	18.7	6	11
MDH	2	2.2	2	0
Total	91	100	52	39

GCH = Gertrude's Children's Hospital, KDH = Kiambu District Hospital, and

MDH = Mbagathi District Hospital.

Table 4.2 shows that the average age of the children recruited in the study was 3 years, while the minimum age is 0.08 years (that is one month) and the maximum age is 14 years and 11 months. The average body mass index was 15.2, the minimum and maximum body mass index were 7.3 and 24.9 respectively.

Table 4.2 Distribution of study participants by age and body mass index

	Mean	P50	Min	Max	Std. Deviation
Age	4.8	3	0.08	15	4.3
BMI	15.2	15.2	7.3	24.9	3.5

Age = represented in years, BMI = Body Mass Index of the child, P50 = median age, Min = minimum age, Max = maximum age, and Std. Division = standard deviation

As shown in Table 4.3 the age group is represented in 3 categories. Those who are below 5 years of age were including the highest number 72.5%, 5-10 years were 15.4% and 11-15 years of age were 12.1%.

Table 4.3 Age group (in years) and sex specific frequency distribution

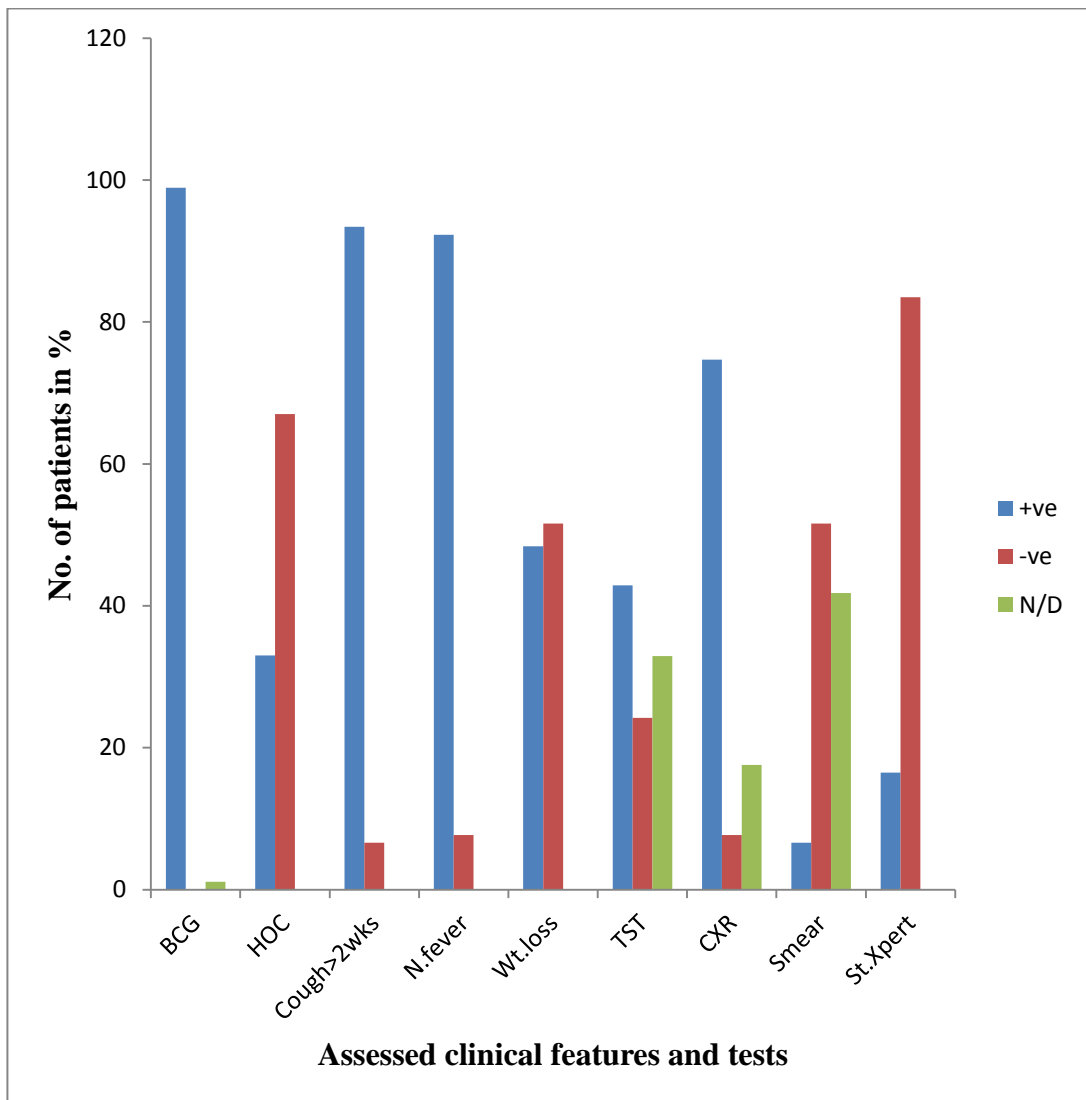
Age Group	Male	Female	Total	Percentage (%)
Below 5	37	29	66	72.5
5-10	11	3	14	15.4
11-15	4	7	11	12.1
Total	52	39	91	100

4.2 Most common clinical features and tests used for the diagnosis of PTB compared to stool Gene Xpert test.

In all the following Tables and figure **Yes (+ve)** stand for BCG vaccination done, clinical features suggestive for PTB present and *M. tuberculosis* detected by the tests

done. **No (-ve)** implies to clinical features absent and *M. tuberculosis* not detected by the tests done, and **N/D** = BCG and tests not done.

From Figure 4.1 (representing the data with percentage) and Table 4.4 (with the frequency) it can be observed that these children were showing not all but most of the clinical features and examinations as positive which made them to be suspected for PTB. The results obtained using the questionnaire from both the participants' answers and the record files of the participants are as indicated in the figure. The immunization for PTB (BCG vaccination) was done to all 98.9% except one (1.1%). 33% had a history of contact while 67 % did not. Cough greater than two weeks, night fever, weight loss were the most common symptoms in patients with PTB and they are accounting 92.9%, 92.3% and 48.4% respectively. Tuberculin skin test, chest X-ray and ZN-smear microscopy (where available) were the most common tests done for children suspected for PTB, and positively detected results are accounted for 42.9%, 74.7%, and 6.6% respectively. In some hospitals these tests were not available thus 33% for TST, 17.6% for CX-ray and 41.8% for ZN-smear microscopy were not done. The reason why this was not done was either it is because of the unavailability of the facilities or because of the severe illness of the patient, who couldn't undergo the procedures for the test. Stool gene Xpert test was done to all children suspected for PTB and who participated in the study. A total of 16.5 % were detected as positive for PTB.



BCG = BCG vaccination, Cough>2Wks = Cough greater than 2weeks, N.fever = night fever, Wt.loss = Weight loss, S.G.Xpert = Stool Gene Xpert, TST = Tuberculin Skin Test, CXR = Chest Xray, Smear = ZN smear microscopy

Figure 4.1 Most common clinical features and tests used for the diagnosis of PTB compared to stool Gene Xpert test (in percentage)

Table 4.4 Frequency distribution of the most commonly used diagnosis methods for PTB

	BCG	HOC	Cough>2wks	N.fever	Wt.loss	TST	CXR	Smear	St.Xpert
+Ve	90	30	85	84	47	39	68	6	15
-Ve	-	61	6	7	44	22	7	47	76
N/D	1	-	-	-	-	30	16	38	0
Total	91	91	91	91	91	91	91	91	91

BCG = BCG vaccination HOC= history of contact for TB, Cough>2Wks = Cough greater than 2weeks, N.fever = night fever, Wt.loss = Weight loss, TST = Tuberculin Skin Test, CXR = Chest Xray, Smear = ZN smear microscopy and St. Xpert = Stool Gene Xpert

Table 4.5 illustrates how history of contact for PTB can be compared with the results of the stool Gene Xpert assay. Results show that from the patients who had history of contact for PTB, 7 and from those who did not 8 were tested positive by the stool Gene Xpert assay test respectively. This is showing no significant association (P- value=0.1).

Table 4.5 Comparison and association between History of contact of TB and Stool Gene Xpert

HOC	Stool Gene Xpert			P-value=0.1
	+Ve	-Ve	Total	
+Ve	7	23	30	
-Ve	8	53	61	
Total	15	76	91	

Table 4.6 shows that from the 75 patients who did both CXR and stool Gene Xpert assay test, 68 patients showed signs suggestive for PTB. From them only 14 were confirmed to have TB by the stool Gene Xpert test while 54 tested negative. Seven showed neither signs suggestive of TB after the CXR test nor *M. tuberculosis* was detected by stool Gene Xpert test. This resulted no significant association P-value=0.1

Table 4.6 Comparison and association between CXR and Stool Gene Xpert

CXR	Stool Gene Xpert		Total	
	+Ve	-Ve		
+Ve	14	54	68	
-Ve	0	7	7	P-value=0.1
Total	14	61	75	

CXR=Chest X-ray

Table 4.7 illustrates the comparison between TST test and stool Xpert test. In total 61 patients did TST test where 39 were tested positive and 22 tested negative. Stool Gene Xpert tested positive 11 from the 39 who tested positive by TST and 2 more from the 22 who tested negative by TST. This result showed significant association (P=0.05).

Table 4.7 Comparison and association between TST and Stool Gene Xpert test

TST	Stool Gene Xpert		Total	
	+Ve	-Ve		
+Ve	11	28	39	
-Ve	2	20	22	p-value=0.05
Total	13	48	61	

TST= Tuberculin skin test

4.3 The Sensitivity comparison of Sputum ZN-smear microscopy VS stool Gene Xpert

Table 4.8 represents the comparison between the sputum ZN smear microscopy test and stool Gene Xpert test. From the 91 recruited patients who did stool Gene Xpert test, only 53 did sputum smear microscopy test (where available) and 38 did not. From these 53 patients who did both test (sputum ZN-smear Microscopy and stool Gene Xpert) 6 tested positive by both. In addition 5 who tested negative by sputum smear microscopy were tested positive by stool Gene Xpert. These means only 6 out of the 53 patients tested positive by sputum smear microscopy while 11 out of the 53 tested positive by stool Xpert. From the 38 patients who did not test by sputum ZN-smear Microscopy 4 where tested positive by stool Xpert test. All in all stool Gene Xpert tested positive for 15 out of the 91 patients without missing the 6 that tested positive by sputum ZN-smear Microscopy and showed 100% sensitivity and sensitivity is 89.36%.

Table 4.8 ZN-smear microscopy VS stool Gene Xpert to show sensitivity of stool Gene Xpert

Sputum smear	Stool Xpert		Total	Sensitivity =100% Specificity =89.36%
	-Ve	+Ve		
-Ve	42	5	47	
+Ve	0	6	6	
N/D	34	4	38	
Total	76	15	91	

+Ve = *Mycobacterium tuberculosis* detected, -Ve = *Mycobacterium tuberculosis* not detected. N/D = test not done

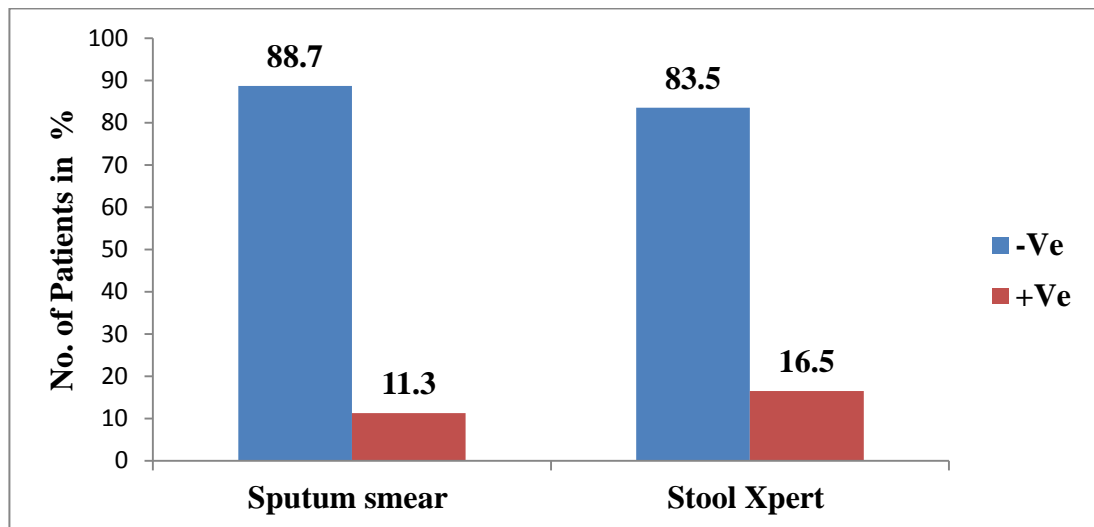


Figure 4.2 ZN-Sputum smears Microscopy vs. Stool Gene Xpert tests

In Figure 4.2 the first blocks show the percentages of positive and negative results after both ZN smear microscopy and stool gene Xpert tests were done for the 53 samples. While the second blocks show the percentages of the positive and negative results of the 91 patients who did stool Gene Xpert test. All in all stool Xpert test detected positive more than sputum smear microscopy test did, that is 6 out of 53 (11.3%), while stool gene Xpert tested positive for 15 out of the 91 (16.5%).

Table 4.9 illustrates the comparison of the results obtained by sputum ZN smear microscopy (where available) with the results of the stool Gene Xpert assay tests for the 53 patients who did both test. As it can be observed in this Table sputum smear microscopy and stool Gene Xpert test are significantly associated ($P=0.000$).

Table 4.9 Comparison and association of ZN-smear microscopy Vs Stool Gene Xpert tests.

Sputum smear microscopy	Stool Xpert		Total	P-value=0.0000
	-Ve	+Ve		
-Ve	42	5	47	
+Ve	0	6	6	
Total	42	11	53	

+Ve = *Mycobacterium tuberculosis* detected, -Ve = *Mycobacterium tuberculosis* not detected.

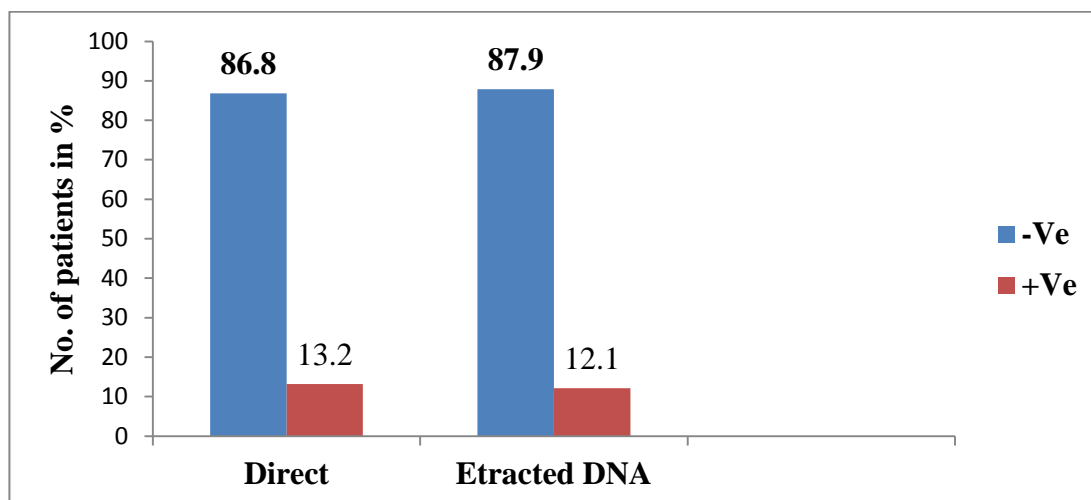
4.4. Direct stool Gene Xpert vs. DNA extraction stool Gene Xpert

Stool Gene Xpert test was done in two different methods (Direct and Prior DNA extraction using QIA Gen stool DNA extraction kit). These were done to all (91) patients. Table 4.10 and Figures 4.3 compare the results of both methods in frequency as well as in percentage respectively. From Table 4.10, it can be observed that from the 91 stool samples of the patients who did both the direct stool Gene Xpert test and prior DNA extraction Gene Xpert test, MTB was detected positive in 12 and 11 patients respectively.

Table 4.10 Results Direct stool Gene Xpert vs. DNA extraction stool Gene Xpert

	Direct	Ex. DNA
-Ve	79	80
+Ve	12	11
Total	91	91

+Ve = *Mycobacterium tuberculosis* detected, -Ve = *Mycobacterium tuberculosis* not detected.



Direct = Direct stool processing Xpert test, Extracted DNA = prior DNA extracted using QIAGEN stool DNA extraction kit for Xpert test

Figure 4.3 Direct stool Gene Xpert vs DNA extraction stool Gene Xperet

Figure 4.3 shows the percentage of positive and negative results after both direct stool and prior extraction of stool DNA was done for Gene Xpert testing of samples from the 91 children suspected for having PTB and it can be observed in this Figure that there is no much difference in performance of the two stool processing methods.

Table 4.11 indicates comparison and associations of the results of Gene Xpert assay tests, obtained by the direct processing of stool with the results of the prior extraction of the DNA using QIAGEN stool DNA extraction kit. MTB was detected (tested positive) in 8 out of the 91(all) stool samples of the patients who did stool Gene Xpert test using both stool processing methods. In addition direct stool processing method detected 4 which the prior DNA extraction stool processing method missed. On the other hand prior DNA extraction stool processing method detected 3 positives that were tested negative by direct stool processing method. In total 12 (8+4) and

11(8+3) were positive by each direct stool processing method and prior DNA extraction method respectively. All in all stool Gene Xpert assay tested 15 (7+8) positives and 76 negatives. Prior extraction of DNA from stool using QIAGEN stool DNA extraction kit showed no considerable difference in the outcome after the test was run by the Gene Xpert but with some degree of error (P=0.0001).

Table 4.11 Comparison and associations of Direct stool Xpert Vs DNA extracted stool

Direct	Extracted DNA		Total	
	-Ve	+Ve		
-Ve	76	3	79	
+Ve	4	8	12	P-value=0.0001
Total	80	11	91	

+Ve = *Mycobacterium tuberculosis* detected, -Ve = *Mycobacterium tuberculosis* not detected.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

The main purpose of this study was to assess the appropriateness of stool to be used as a sample for the diagnosis of PTB in children under 15 years of age using the Gene Xpert MTB/RIF assay, as a solution to the challenges in getting respiratory samples. The results given by the Gene Xpert MTB/RIF assay test were all interpretable for the presence or absence of MTB, with the information for rifampicin resistance or not. The study results show that stool, which can easily be obtained from clinics and the fields is an appropriate sample for the diagnosis of PTB in children. The DNA of *M. tuberculosis* in stool can be detected using Gene Xpert assay with 100% sensitivity and 89.36% specificity.

This study recruited 91 children under the age of 15 with median age of 3. These children were suspected to have PTB on clinical grounds or referred to TB tests by their clinicians. The number of children patients was greater in GCH compared to KDH illustrated in table 4.1 GCH deals specifically with children and has better facilities for the diagnosis of PTB.

Previous studies reveal that infections with *M. tuberculosis* is similar in males and females until adolescence (Holmes *et al.*, 1998) and higher in males after 16 years of age (Diwan and Thorson, 1999). As can be observed from Table 4.3 there were more

male patients than females which comprises of 57.1% and 42.9% respectively. In adults there is also some evidence that men have higher risk of developing active TB as they are more exposed to the risk factors like alcohol and smoking than women and they attend health services more symptomatic compared to women (Lawson *et al.*, 2010). However for children less than 15 years of age the risk factor related to sex is not fully understood, as researches within this field are insufficient, but can be suggested that it could be due to the differences in the immune response, which can play part in symptoms, signs and outcomes of tuberculosis and other bacterial infections (Diwan and Thorson, 1999).

Patient age and immune response can be factors that can determine progression of the disease after infection (Cruz and Starke, 2007). Comparing the age difference in children most of the children suspected for PTB (72.5%) are under 5 years. Very young children that are less than 5 years of age have a high risk of infection and disease (Rie *et al.*, 1999) while children from 5 to 10 years of age have lower risk (Cruz and Starke, 2007). This is likely due to the fundamental differences of the immune system among young children and adults (Lewinsohn, 2004).

TB diagnosis in children is a challenge (Triasih and Graham, 2011). This is because the sputum smear microscopy is not possible for the majority of children, who usually swallow their sputum; hence smear microscopy is of low sensitivity (Graham, 2014). In poor setting hospitals, obtaining respiratory samples like gastric lavage from young children can be problematic. Procedures are traumatic as well as demand sophisticated facilities and trained personnel. This means that

bacteriological confirmation is usually not possible and that the diagnosis of PTB in children is often presumptive (WHO, 2003). Therefore, as observed in the study different tests are done for the diagnosis of childhood PTB along with the other clinical features.

The information about the features of the symptoms for TB, history of contact as well as all results of the routine tests done in the hospitals were taken from the patient's filed record. The routine PTB diagnostic tests done by these hospitals for children are Tuberculin Skin Test (TST), chest x-ray (CXR) and sputum ZN smear microscopy (where available) while stool Gene Xpert test was done to all by the study.

All of the children under investigation had clinical features suggestive of tuberculosis. These include 92.9% with cough for more than two weeks, 92.3% with night fever and 48.4% weight loss. History of contact was also used and was encompassing about 33% (Figure 4.1). Close contact with smear positive patients in the house hold can be probable cause of TB in young children (Marais, 2004). Moreover, symptoms and history of contact alone are not enough indications for the diagnosis of PTB. These Symptoms are not specific for PTB, but can exist in most bacterial and viral infections (Loeffler, 2003). In almost all cases children contract tuberculosis infection from adults with TB disease in their community but this may not significantly be associated with the progression of active TB disease that can be detected by the microbiological tests (Avan, 1999). This is supported by this study as it can be observed in table 4.5 and the comparisons between history of contact and

results from stool Gene Xpert assay reveal that they are not significantly associated (P value =1).

Chest radiography is used as complement for clinical evaluation in children with suspected tuberculosis and is more readily available in resource limited settings compared to the other diagnostic tests for tuberculosis (Graham, 2014). In this study Chest X- ray (CXR) was done to 75 (82.4%) children, where 68 (74.7 %) showed changes suggestive for PTB and 7 (7.7 %) did not and it can be observed in Table 4.4 and Figure 4.1 Chest X-ray is required along with clinical symptoms, but often overlaps with other respiratory diseases. Chest X-ray can be more specific when the child has been in close contact with a diagnosed case of PTB, especially smear-positive PTB (WHO, 2003), however the specificity can also be lower in high TB endemic areas where an exposure for pneumonia, severe malnutrition, HIV and other respiratory pathogenic co-infections in children are common (Graham, 2014). As it was evaluated in the previous study done (Wekesa, 2014), chest X-ray result in this study has also indicated the over estimation of the children suspected for PTB. Majority (54 out of 68) of the ones that showed CXR suggestive signs of PTB came out as negative. Only 6 by sputum ZN-smear microscopy and 14 by the stool Gene Xpert assay tested positive as indicated in Figures 4.1 and Table 4.6 respectively. Thus showing insignificant association and P-value=0.1 not significantly associated.

Tuberculin skin test has been used as the main tool for the diagnosis of latent tuberculosis for many years (David, 2013). In this study 61(67.0%) children had TST results out of these 39(42.9%) were positive while 22 (24.1%) were negative. A

positive tuberculin test does not necessarily indicate the presence or extent of tuberculosis disease; it only indicates infection and even if it is negative does not necessarily exclude TB infection (Harald, 2008). It is known that tuberculin skin test can give both false negative and false positive results (David, 2013). The tuberculin test is less likely to be positive in a child with TB if the child also has severe malnutrition, HIV infection or disseminated TB such as TB meningitis (GOK, 2013). BCG vaccination can also increase the likelihood of positive results of TST without the infection because it has low specificity for the purified protein derivative (PPD) used for the vaccination (David, 2013). Consequently the yield of the test's result can be of a wide range of result that infer the presence of the reaction in uninfected child to the absence of the reaction in a child confirmed of TB disease (Nayak and Acharjya, 2012). However, as it can be observed from the results represented in Table 4.7 of this study, though TST has some draw backs it still remains significantly associated diagnosis (P value=0.0507) with the progression of the disease which was confirmed with the smear microscopy test and stool Gene Xpert MTB/RIF assay microbiological tests.

Ziehl Neelsen (ZN) sputum smear microscopy is a microbiological diagnosis test for the detection of acid-fast bacilli (AFB). It is a rapid, inexpensive, and highly specific but with a moderate sensitivity for identifying persons with active tuberculosis (Laserson, 2005). As previously mentioned getting good quality of sample (sputum) from children is not easy, and this makes performing bacteriological test a challenge especially in hospitals like KDH that lack the needed facilities. Therefore from the 91 children suspected for pulmonary tuberculosis, 53(58.2%) did sputum ZN smear

microscopy test where available. However all of them (91) did stool Xpert test. From the tests done using sputum ZN smear microscopy, direct stool Xpert and prior extracted DNA stool Xpert, 6(11.3%), 12(13.2%) and 11(12.1%) were detected as positive respectively. In general stool Gene Xpert and sputum ZN smear microscopy tested positive 16.5% and 11.3% as shown in Figure 4.2 Stool Gene Xpert detected all the 6 positive ones from sputum ZN- smear microscopy, both tests are significantly associated (P-value =0.000) as illustrated in Table 4.9.

Compared to the sensitivity evaluated for sputum smear microscopy (34 %) in the study done during TB screening for United States immigrant visa applicants (Laserson, 2005), Stool Xpert showed higher sensitivity (100%).The specificity of sputum smear microscopy was high(Laserson, 2005) while the sensitivity stool Gene Xpert MTB/RIF is 89.36%. From the WHO 2007 algorithm the pooled sensitivity and specificity for detection of smear-negative pulmonary tuberculosis for GeneXpert were 67% and 98% respectively (Walusimbi, 2013). In a study which evaluated Gene Xpert MTB/RIF assay with the microscopically negative and positive pulmonary samples the sensitivity and specificity of the Gene Xpert MTB/RIF assay was found to be 90.6% and 94.3% respectively (Ioannidis *et al.*, 2011). Therefore due to the difficulty of getting respiratory samples in children, stool Xpert MTB/RIF assay result is very encouraging can be used as the diagnosis test of PTB in children.

Compared to direct stool Xpert, prior extraction of DNA using QIAGEN stool DNA extraction the results showed no considerable difference (P-value =0.001) as can be

observed in Table 4.11. However prior extraction of DNA using QIAGEN stool DNA extraction method is a long process and may not be recommended to be used as routine diagnosis method. It can be used as a compliment for further investigation because it helps to detect cases not detected in the direct stool Xpert test.

Furthermore, stool PCR would be a rapid, non-invasive, and relatively bio-secure initial test for children with suspected pulmonary tuberculosis (Wolf, 2008). Thus using stool as a sample is a big solution to the invasive procedure for collecting respiratory samples or sputum from children. Pilot study done in South Africa indicated that stool Xpert is a promising test for diagnosis of PTB (Nicol, 2013). Gene Xpert is a portable, highly sensitive, user-friendly and rapid molecular assay with the turnaround time less than 2hours (WHO, 2012). Though the issue of affordability and the need of uninterrupted power supply can be some of the drawbacks, in settings where Xpert MTB/RIF is available, stool Xpert testing is a good solution for children of pulmonary tuberculosis with difficulty in producing respiratory samples.

5.2 Conclusions

Diagnosis of PTB in children is a challenge because all currently in use diagnosis method are not fully effective. Bacteriological tests are not easy to perform. Smear microscopy and culture look for sputum which is very difficult to obtain from children as they cannot expectorate like adults do and lacks the needed MTB load for detection. Procedures used to induce other respiratory samples are invasive and need trained personnel, as well as sophisticated facilities which are hard to get in poor

setting health cares. Sputum is swallowed MTB DNA can remain intact after intestinal transit. This study findings support that stool sample that can easily be obtained has the potential of detecting *M. tuberculosis* using Gene Xpert MTB/RIF assay.

From the previously mentioned different studies done, it is known that all methods currently in use for the diagnosis of MTB in children have deficiencies. Clinical features suggestive to PTB are not specific, history of contact is a factor for infection but not necessarily for the progression of the disease, Chest radiography can over diagnose, TST can give false positive and false negative results. As known Gene Xpert needs minimal biosafety, detects both TB and rifampicin resistance, is rapid, portable and user friendly. This study shows the detection of MTB DNA using stool sample with the high sensitivity of 100%. Therefore introduction of testing stool using Gene Xpert as the routine pulmonary tuberculosis diagnosis in children patients suspected of having PTB could be a solution to the current challenges of getting respiratory samples.

In this study stool was processed in two ways direct and with the prior extraction of DNA using QIAGEN stool extraction kit. Gene Xpert assay test results showed no significant difference. However prior extraction of DNA using QIAGEN stool DNA extraction method was a long process and took much effort compared to the direct one. Therefore prior Extraction of DNA using QIAGEN stool extraction kit may not be recommended to be used as routine diagnosis method. It can be used as a

compliment for further investigation because it can help to detect cases not detected by the direct stool Xpert test.

5.3 Recommendations

Stool Xpert MTB/RIF assay may be introduced as one of the routine diagnosis method of PTB in children who have difficulty in producing respiratory samples. More studies in the diagnosis of TB in children in different sites with a larger number especially in more epidemic areas, for example the slums can give more reproducibility. Furthermore research to assess whether sex is a factor that influences TB infection in children like in adults should be undertaken.

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APPENDICES

APPENDIX 1 : KUERC ETHICAL APPROVAL LETTER



KENYATTA UNIVERSITY
ETHICS REVIEW COMMITTEE

Fax: 8711242/8711575
Email: kuerc.chairman@ku.ac.ke
kuerc.secretary@ku.ac.ke
Website: www.ku.ac.ke

P. O. Box 43844
Nairobi, 00100
Tel: 8710901/12

Our Ref: KU/R/COMM/51/200

Date: July 24th, 2013

Seble Haile Welday
Jomo Kenyatta University of Agriculture & Technology
JUJA.

Dear Ms. Seble,

APPLICATION NUMBER PKU/127/E16 OF 2013 – ‘GENE Xpert TESTING OF STOOL SAMPLES FOR THE DIAGNOSIS OF PULMONARY TUBERCULOSIS IN CHILDREN UNDER 15 YEARS IN GETRUDE CHILDRENS’S HOSPITAL’ – VERSION 2.

1. IDENTIFICATION OF PROTOCOL

The application before the committee is with a research topic, ‘Gene Xpert Testing of Stool Samples for the Diagnosis of Pulmonary Tuberculosis in Children Under 15 Years in Getrude Childrens’s Hospital’ version 2 dated July 23rd, 2013 received on 24th July 2013.

2. APPLICANT

Seble Haile Welday
Jomo Kenyatta University of Agriculture & Technology
JUJA.

3. SITE

Getrude’s Children’s Hospital

4. DECISION

The committee has considered the research protocol in accordance with the Kenyatta University Research Policy (section 7.2.1.3) and the Kenyatta University Ethics Review Committee Guidelines, and is of the view that against the following elements of review,

- (i) Scientific design and conduct of study,
- (ii) Recruitment of research participant,
- (iii) Care and protection of research participants,
- (iv) Protection of research participant’s confidentiality,
- (v) Informed consent process,
- (vi) Community considerations.

AND APPROVED that the research may proceed for a period of ONE year from July 24th, 2013.

5. ADVICE/CONDITIONS

- i. Progress reports are submitted to the KU-ERC every six months and a full report is submitted at the end of the study.
- ii. Serious and unexpected adverse events related to the conduct of the study are reported to this board immediately they occur.
- iii. Notify the Kenyatta University Ethics Committee of any amendments to the protocol.
- iv. Submit an electronic copy of the revised proposal to KU-ERC.


When replying, kindly quote the application number above.

If you accept the decision reached and advice and conditions given please sign in the space provided below and return to KU-ERC a copy of the letter.



PROF. NICHOLAS K. GIKONYO
CHAIRMAN: KENYATTA UNIVERSITY ETHICS REVIEW COMMITTEE

I SEBLE HAILE WELDAY.....
accept the advice given and will fulfill the conditions therein.

Signature..........Dated this day 26 of July..... 2013.

cc. Vice-Chancellor
Director: Institute for Research Science and Technology



**KENYATTA UNIVERSITY
OFFICE OF THE CHAIRMAN, ETHICS REVIEW COMMITTEE**

SEBLE HAILE WELDAY,
JOMO KENYATTA UNIVERSITY OF
AGRICULTURE AND TECHNOLOGY,
JUJA

4th NOVEMBER, 2013

Dear Ms. Welday,

RE: REQUEST FOR ADDITIONAL STUDY SITES FOR PKU/127/E 16

I acknowledge receipt of your request to have additional sites of study based on the fact that there are very few cases of MTB in the earlier mentioned study site (Gertrude's Children's Hospital).

You can now carry out your study in Gertrude's Children's Hospital and in the other requested hospitals as follows;

1. Kiambu District Hospital
2. Mbagathi District Hospital
3. St. Mary's Mission Hospital

Thank you



**PROF. NICHOLAS K. GIKONYO
CHAIRMAN ETHICS REVIEW COMMITTEE**

APPENDIX 2: GCH ETHICAL APPROVAL LETTER



September 10, 2013

REF: GCH/ERB/VOLXIII/13

Seble Haile Welday
Jomo Kenyatta University of
Agriculture and Technology
JUJA

Dear Ms Welday,

RE: GENE Expert Testing of Stool Samples for the Diagnosis of Pulmonary Tuberculosis in Children under 15years in Gertrude's Children's Hospital (RESUBMISSION)

Date of first review – 11th August 2013

We are in receipt of your revised proposal for the above study.

The Hospital's Ethical Review Board has reviewed the revised proposal and notes that you have addressed all the issues raised during the previous review.

The board has hereby **approved** your request to conduct the study.

Please note that this approval is only to conduct the study and is not an approval for publication or presentation of findings. A separate approval will be required for this purpose.

The Hospital will require the write up of your study findings upon completion as this will form part of our database for future references. You are also required to report back to the board on your progress after 6months. **Meeting these requirements will be a condition for granting approvals for publications or presentations of research findings in the future.**

On behalf of the Hospital I wish you a fruitful research.

Regards

GERTRUDE'S CHILDREN'S HOSPITAL
P.O. Box 42385 - 00100, NAIROBI
Tel: 7206000 / 601 - 7

Dr. Thomas Ngwiri
SECRETARY
GERTRUDE'S CHILDREN'S HOSPITAL
ETHICAL REVIEW BOARD

APPENDIX 3: INFORMED CONSENT FORM

This Informed Consent Form has two parts:

- Information Sheet (to share information about the study with you)
- Certificate of Consent (for signatures if you agree that your child may participate)

PART I: Information Sheet

Introduction

I am from a research team established by Department of Medical Microbiology, JKUAT, Department of Pathology-The Aga Khan University Hospital, Nairobi, and Gertrude's Children's Hospital and its satellite clinics. I am here to conduct a study on Tuberculosis infection among children less than 15 years of age admitted to Gertrude's Children's Hospital. The study is trying to find out the factors associated with tuberculosis infection among children and to assess the effectiveness of stool as diagnosis sample for pulmonary tuberculosis infection that we could apply in routine diagnosis. Since your child is having these symptoms he/she is identified as potential participant. Therefore we are asking if you would allow your child to participate. Since the child is too young to decide on his/her own. Your decision to have your child participate in this study is entirely voluntary. You can withdraw from the study at any stage without any consequence for treatment if you do not wish to continue. If you agree to be recruited as a participant into study, I would like to interview you, and ask you for your permission to collect stool sample from your child. Because we do not know whether tuberculosis can be detected using stool sample better than the currently

available diagnosis method, the hospital will need to take sputum while the stool as samples is for the study to make comparisons. Children taking part in this research will then give both samples to help us diagnose the disease well. The hospital staff members may need to take sputum in the early morning hours but the stool at the time the child defecates. There will be no side effects, risk or any experience of discomfort from giving us the stool as a sample.

All information you provide will be handled as confidential and your individual answers will not be known, except by the interviewer and the coordinator of this study. Your contact details are held on an electronic database for study management purposes only, you could inspect this information. The results will be used only to improve strategies for diagnosis and prevention of tuberculosis, one of the most burden diseases leading to mortality and mobility in Kenya. We will need at least 30 minutes to discuss and record the information. You will be given a copy of the full Informed Consent Form. Will you participate in this study?

Yes

No

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask the doctors or the staff members of this hospital.

Do you have any question?

Thank you.

Date:

Interviewee

signature.....

Interviewer

signature.....

Name of Principal Investigator:

Name of Organization: JKUAT

Name of Sponsor: ADB and NBH

Who to Contact

If you have any questions you may ask to the staff members of this hospital now or later, even after the study has started. If you wish to ask questions later, you may contact any physician who is treating your child and the following: Seble Haile Welday, (0718092420), Dr.K.A.Nyerere, (0722689073), Prof. G Revathi (0722829517), Dr. Joseph Kariuki Mbuthia (0720963887) and the Kenyatta University Ethics Review Committee, e-maile kuerc.chairman@ku.ac.ke, or kuerc.secretary@ku.ac.ke

PART II: Certificate of Consent

A. Parent or Guardian Informed consent form

I have been invited to have my child participate in research of TB diagnosis using stool sample. I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily for my child to participate as a participant in this study.

Print Name of Participant_____

Print Name of Parent or Guardian_____

Signature of Parent or Guardian _____Date _____

If illiterate

I have witnessed the accurate reading of the consent form to the parent of the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness _____ AND Thumb print
of parent

Signature of witness _____



Date _____

Day/month/year

B. Informed Assent Form: (for children under 15 years of age who attend in the study site hospitals and clinics in and around Nairobi).

I,.....understand
that my parents (mom and dad)/guardian have/has given permission (said it's okay)
for me to take part in a project about diagnosis of PTB using stool sample. I am
taking part because I want to. I have been told that I can stop at any time I want to
and nothing will happen to me if I want to stop.

.....

Signature

I have accurately read out the information sheet to the parent of the potential participant, and to the best of my ability to make sure that the person understands it well. A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent _____

Signature of Researcher /person taking the consent _____

Date _____

Day/month/year

APPENDIX 4: PATIENT DEMOGRAPHIC DATA

TB Screening Questionnaire for children

Information on Children:

Clinic Patient No. K/L _____ Study Patient No. _____ Hospital/Clinic _____

Name: _____ IP No: _____

D.O.B: _____ Date: _____

The child's sex (put \surd when applicable)

Male Female

Child's weight?.....

Child's height?.....

1. Does your child have clinical evidence of TB?

Yes No

2. Have you or your child had any tuberculosis treatment before?

Yes No

If yes, When?

Where?

For how long..... What was the result?.....

3. How long days (weeks) did your child has symptoms of cough before being diagnosed with TB?

From 1 to 4 weeks

Over 4 weeks

4. Does your child have night fever?

Yes

No

5. Does your child have abnormalities on chest x-ray suggestive to TB? (from filed record).

Yes

No

6. Mantoux /TST examination result? (From filed record).

Positive

Negative

7. Does your child have BCG scar?

Yes

No

8. Does your child spit sputum? Yes No If yes for how long?

From 1 to 4 weeks from 5 to 10 weeks more than 10 weeks

9. Sputum examination? (from filed record)

- Microscopy results categorized according National Tuberculosis program:

Scanty + ++ +++

- Cultured result:

Negative Positive

- Gene Xpert

Negative Positive

10. Does your child have a family member or been in contact with a person with a history of confirmed or suspected TB?

Yes

No

• Relationships:

Mother

Father

Grandmother

Grandfather

Other

11. Does your child live in out-of-home placements?

Yes

No

12. Does your child lives among, or is frequently exposed to, individuals who are homeless, migrant farm workers, users of street drugs or residents in nursing homes?

Yes

No

13. How many people live in your house?

1 to 4

More than 4

14. How many rooms are there in your house?

One

More than one

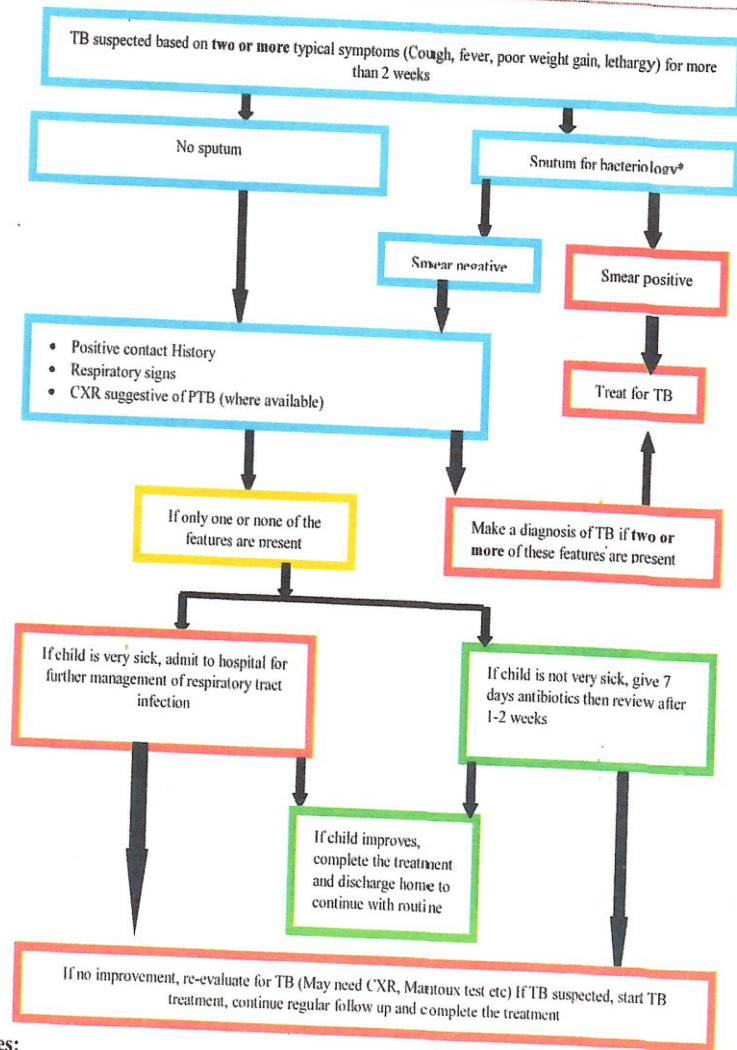
15. Room of index case ventilation.

Good (more than one opened window)

Bad (no window)

APPENDIX 5: TB DIAGNOSTIC APPROACH IN CHILDREN (GOK, 2013)

APPROACH TO DIAGNOSIS OF PULMONARY TB DIAGNOSIS IN CHILDREN



Notes:

1. All children should be tested for HIV
2. Mantoux test should be interpreted as follows
 - >5mm diameter of induration in high risk children (includes HIV-infected children and severely malnourished children)
 - >10 mm diameter of induration in other children (whether they have received vaccination or not)
3. Please note that a mantoux may be negative despite the child having TB especially in severe disseminated TB, malnutrition and HIV disease.

APPENDIX 6: GENE XPERT

The image displays the GeneXpert diagnostic system. On the left, a GeneXpert cartridge is shown in a blue holder, labeled "Xpert MTB/RIF". A handheld device is shown in the center, connected to the cartridge. On the right, a large laboratory machine is shown, labeled "GeneXpert". The machine has a screen displaying "GeneXpert InVivo-48" and a grid of sample slots. Below the machine, a bar chart shows the number of samples per shift for different models: 5, 20, 80, and 500-1000.

GeneXpert

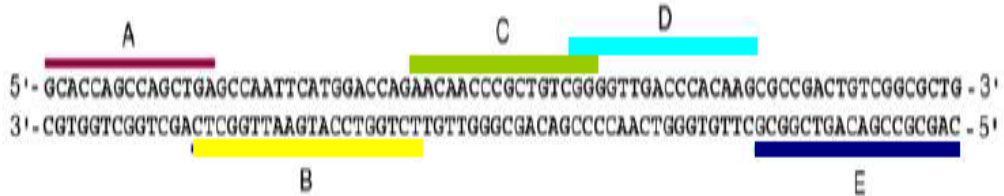
Xpert MTB/RIF

GeneXpert InVivo-48

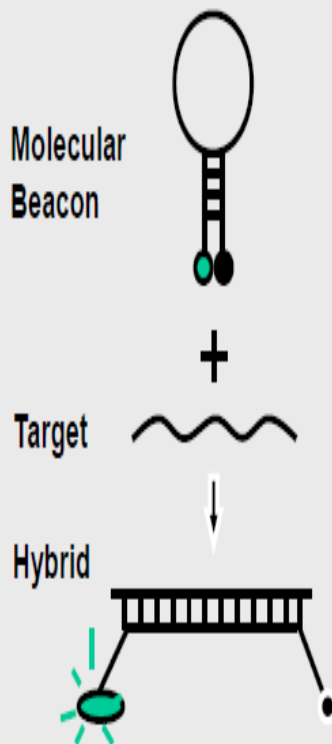
Model	Samples per shift
GeneXpert	5
GeneXpert InVivo-12	20
GeneXpert InVivo-24	80
GeneXpert InVivo-48	500-1000

FIND
foundation
for innovative new diagnostics

Xpert MTB/Rif Molecular Beacon Assay



The PCR target is the 81 bp region of the rpoB gene: 5 probes bind to wildtype, but not mutant target



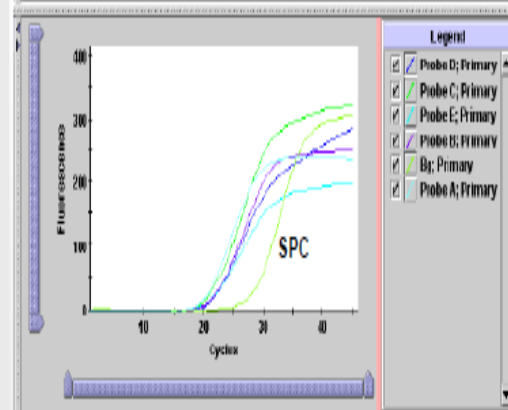
Each probe is labeled with a different fluorescent dye, permitting simultaneous detection

Test and Analyte Result Detail Errors History

Assay Name: MTB Beta Version: 3

Test Result: **MTB POSITIVE BELEUM**
Rif Resistance - NOT DETECTED

Analyte Name	Ct	EndPt	Analyte Result	Probe Check Result
Probe D	21.9	267.0	POS	PASS
Probe C	20.7	324.0	POS	PASS
Probe E	21.6	198.0	POS	PASS
Probe B	21.7	249.0	POS	PASS
Dx	27.7	268.0	NA	PASS
Probe A	20.4	295.0	POS	PASS



Example of Rif-Sensitive Profile - 5 probes & SPC show fluorescence

APPENDIX 7 : STOOL GENE XPERT TEST RESULT SHEET

GeneXpert PC

27/01/14 12:42:06

Test Report

Sample ID: Q31-114
Test Type: Specimen
Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-RIF Assay G4	5	In Vitro Diagnostic

Test Result: **MTB DETECTED VERY LOW;**
Rif Resistance INDETERMINATE

Test and Analyte Result

Analyte Name	Analyte Result	Probe Check Result
Probe D	NEG	PASS
Probe C	POS	PASS
Probe E	NEG	PASS
Probe B	POS	PASS
SPC	NA	PASS
Probe A	POS	PASS
QC-1	NEG	PASS
QC-2	NEG	PASS

User: Margaret.W. Wanyeki
Status: Done
Expiration Date*: 02/02/14
S/W Version: 4.4a
Cartridge S/N*: 108724622
Reagent Lot ID*: 05012
Start Time: 27/01/14 10:23:04
End Time: 27/01/14 12:07:37
Instrument S/N: 801585
Module S/N: 621246
Module Name: B4

Notes:

Errors
<None>

For In Vitro Diagnostic Use Only.

Test Report

Sample ID: S31-214
 Test Type: Specimen
 Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-RIF Assay G4	5	In Vitro Diagnostic

Test Result: **MTB NOT DETECTED**

Test and Analyte Result

Analyte Name	Analyte Result	Probe Check Result
Probe D	NEG	PASS
Probe C	NEG	PASS
Probe E	NEG	PASS
Probe B	NEG	PASS
SPC	PASS	PASS
Probe A	NEG	PASS
QC-1	NEG	PASS
QC-2	NEG	PASS

User: Margaret.W. Wanyeki
 Status: Done
 Expiration Date*: 27/07/14
 S/W Version: 4.4a
 Cartridge S/N*: 214185895
 Reagent Lot ID*: 11713
 Start Time: 24/02/14 13:35:00
 End Time: 24/02/14 15:19:03
 Instrument S/N: 801585
 Module S/N: 612550
 Module Name: B1
 Notes:

Errors
 <None>

For In Vitro Diagnostic Use Only.

Test Report

Sample ID: Q22-114
 Test Type: Specimen
 Sample Type:

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-RIF Assay G4	5	In Vitro Diagnostic

Test Result: **ERROR**

Test and Analyte Result

Analyte Name	Analyte Result	Probe Check Result
Probe D	NO RESULT	PASS
Probe C	NO RESULT	PASS
Probe E	NO RESULT	PASS
Probe B	NO RESULT	PASS
SPC	NO RESULT	PASS
Probe A	NO RESULT	PASS
QC-1	NO RESULT	PASS
QC-2	NO RESULT	PASS

User: Margaret.W. Wanyeki
 Status: Aborted
 Expiration Date*: 02/02/14
 S/W Version: 4.4a
 Cartridge S/N*: 108724651
 Reagent Lot ID*: 05012
 Start Time: 27/01/14 13:25:30
 End Time: 27/01/14 14:35:57
 Instrument S/N: 801585
 Module S/N: 621247
 Module Name: B3
 Notes:

ERROR

#	Description	Detail	Time
1	Post-run analysis error	Error 5011: Signal loss detected in the amplification curve for analyte [Probe B]. 19.8 decrease in signal with 20.7% decrease at cycle 16.	27/01/14 14:35:11

For In Vitro Diagnostic Use Only.