XPERT MTB/RIF PERFORMANCE IN DETECTION OF *MYCOBACTERIUMTUBERCULOSIS* IN SPUTUM PELLETS USING A REDUCED SAMPLE REAGENT IN SMEAR NEGATIVE SAMPLES IN KISUMU, WESTERN KENYA

BY

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SCHOOL OF PUBLIC HEALTH AND COMMUNITY

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DECLARATION

I hereby declare that this thesis is the result of my original work and its findings have not been presented for the award of a degree certificate in any institution

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DEDICATION

To my mother Rosebella, my daughters Zilpah and Huldah, son Issachar.

ABSTRACT

Globally approximately 10 million incident cases of tuberculosis (TB) are reported annually. The emergence of multidrug-resistant tuberculosis (MDR-TB) is widely considered a major threat to global TB control. Diagnosis by solid and liquid culture has a high turnaround time that hinders timely decisions on patient management and requires laboratories with high level of biosafety and highly qualified and experienced staff. The Xpert MTB/RIF is a PCR-based diagnostic test that detects *Mycobacterium tuberculosis* and rifampicin (RIF) resistance within two hours and has beenapproved by the WHO for use in high-burden TB countries such as Kenya. However, evaluations have demonstrated low sensitivity in smear-negative samples. This study aimed to determine whether using a lower ratio of sample reagent (SR) to sputum sediments (2:1 compared with the currently recommended 3:1 ratio) would improve Xpert detection of *M. tuberculosis* in sputum pellets from smear negative patients in Kisumu western Kenya. The specific objectives of the study were to compare sensitivity and specificity of 2:1 and 3:1 sample reagent/pellet dilution, compare the positive and negative predictive value of 2:1 and 3:1 sample reagent/pellet dilution and determine influence of sputum sample quality on Xpert MTB/RIF result. This was a laboratory-based cross sectional study where 154 pellet samples collected from patients at Jaramogi Oginga Odinga Teaching and Referral Hospital, as part of tuberculosis observational study and sent to KEMRI/CDC TB laboratory were used. The samples were decontaminated using NALC sodium hydroxide,smear and culture performedand reminder pelletstored at-80^{o}C. Xpert MTB/RIF test procedure involved mixing sample pellet with SR at two ratios (2:1 and 3:1), two milliliters of the mixture was placed into the Xpert MTB/RIF cartridge, and the cartridge inserted into the Xpert MTB/RIF instrument, where fully automated PCR was completed to detect both *M. tuberculosis* and Rifampin resistance. According to this study, sensitiviy, Specificity,Negative and Positive Predictive Value of ratio 2:1 SR was 18.5% 86.0%, 66.2.% and 41.7% respectively while for ratio 3:1 was 37.5%,73.8%, 86.5% and20.9%. Results from this study showed moderate agreement between the two dilutions with Cohen kappa value of 0.476 at $p<0.001$. In terms of quality ofsputum sample appearance, tenacious samples were significantly associated with Xpert MTB/RIF performance of 2:1 sample dilution with Fishers exact test ($p = 0.053$). Tenacious or purulent samples are associated with increased probability of detection of smear negative Mycobacterium tuberculosis samples using Xpert MTB/RIF. These results have significance in TB diagnosis of smear-negative samples in that there was moderate agreement between sample reagent pellet ratio 2:1 and 3:1 so both ratios are good

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Tissue culture collection
BP	Base pair
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit
C_T	Cycle Threshold
DNA	Deoxyribonucleic acid
INH	Isoniazid
JOOTRH	Jaramogi Oginga OdingaTeaching and referral Hospital
KEMRI	Kenya Medical Research Institute
KNBS	Kenya National Bureau of statistic
MDR	Multi Drug Resistance
MGIT	Mycobacteria Growth Indicator Tube
MTB	Mycobacteria Tuberculosis
NALC	N-Acetyl-L-Cystein
NaOH	Sodium Hydroxide
PANTA	Polymixin B, Amphoterin B, Nalidixic Acid, Trimethoprim, Azilocillin
PCR	Polymerase chain reaction
RIF	Rifampin
RR	Rifampicin resistant
SR	Sample reagent
TAT	Turn Around Time
TB	Tuberculosis
TBTC	Tuberculosis Drug trial consortium
WHO	World Health Organization
WT	Wild Type
XDR	Extensively Drug Resistance

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Tuberculosis(TB) is a major problem globallyand is the leading cause of death by a single infectious agent with 1.3 million deaths, and 9.9 million incident case of TB in 2020, (WHO, 2021). The vast majority of TB cases and TB deaths are in developing countries. Risk of developing TB in people living with HIV was 20 times higher than those without HIV (WHO, 2018).Kenya is listed by the World Health Organization (WHO) among the 30 high burden TB countries. Despite the considerable investment done by the government and partners in TB care and prevention, the disease is still the 4th leading cause of death in Kenya(KNBS, 2018). Finding all people with TB disease and successfully treating them is an important priority (Masini *et al.*, 2018).

Current efforts are focused upon improving the rapidity of identification of *Mycobacterium tuberculosis* (MTB) and prompt initiation of appropriate therapy. The microbial diagnosis of TB has traditionally been carried out using two different procedures, direct smear microscopy of the sample (Ziehl-Neelsen and/or auramine-rhodamine stain), which is quick, inexpensive, and simple but has poor sensitivity requiring the presence of 5,000–10,000 bacilli per milliliter of sample to allow detection(Armand, 2011.; Desikan, 2013)and mycobacterial culture, which despite being considered the gold standard technique for TB diagnosis can take several weeks to provide a confirmation which may cause delay in diagnosis of smear-negative especially drug resistantstrains which have serious consequences for the patientas well as the community.Furthermore culture requires laboratories with high levels of biosafety and highly experienced staff (Pai *et al.*, 2003; Srisuwanvilai *et al.*, 2008).New molecular methods have thus

been developed to improve TB control, with the most sensitive ones being those based on nucleic acid amplification (Alcaide, 2011).

The XpertMTB/RIF is a PCR based diagnostic test that detects *Mycobacterium tuberculosis* and rifampicin(RIF) resistance (Helb *et al.*, 2010). It is a simple assay that can be performed with minimal training(Khalil *et al.*, 2015)and is the first rapid near-point-of-care diagnostic to be widely implemented in TB-endemic settings as the initial diagnostic test for people suspected of having MDR TB(WHO, 2014).Although the initial clinical trials of the Xpert MTB/RIF demonstrated sensitivity ranging between 98% in smear-positive TB and 72% in smear-negative cases (Helb *et al.*, 2010; Ioannidis *et al.*, 2011)subsequent evaluations have demonstrated a tremendous variability in test performance among smear negative, with sensitivity between 26 to 83% in smear-negative, culture-positive TB cases (Jafari *et al.*, 2013; Lombardi *et al.*, 2017). Xpert/MTB RIF sensitivity depends on smear status, sensitivity is high in detecting MTB in samples thatareAcid fast bacilli smear-positive but low inAcid fast bacilli smear-negative samples(Munoz *et al.*, 2013).Discovering TB in patients early and treating them in time is important as it can decrease severity of the disease, mortality and transmission to others as It is estimated that 10%-20% of TB transmission is from smear-negative cases of pulmonary TB(Campos *et al.*, 2016).

International guidelines have emphasized macroscopic sputum quality as an important determinant of performance of smear microscopy and culture (Ho *et al.*, 2015)previous studies have demonstrated substantially higher sensitivity with purulent or bloody sputum as compared with mucoid or salivary sputum on smear microscopy and culture (Yoon *et al.*, 2012).However, in Xpert MTB/RIF testing, sample quality has shown no significant difference in diagnostic yield (Meyer *et al.*, 2017).

The Xpert MTB/RIF allows rapid detection of mycobacterium in sputum, however its sensitivity is inadequate for paucibacillary specimens that are negative for acid fast bacilli (AFB) in smear microscopy. Sensitivity in smear negative samples increases with repeated testing of more than one sample(Boehme *et al.*, 2010) this may not be cost effective in resource limited settings.

Manufacturer of Xpert recommends the use of either raw, unprocessed sputa or concentrated sputum pellets (Cepheid®, 2009). To concentrate raw sputum, sample is liquefied, decontaminated either with 2% N-acetyl cysteine-sodium hydroxide (NALC-NaOH) or 4% sodium hydroxide(NaoH), centrifuged, concentrated and neutralized using phosphate buffer(Cepheid®, 2009).The increased SR/sputum ratio recommended when pellets are tested could reduce assay sensitivity by over dilution(Dharan *et al.*, 2015) in paucibacillary samples.

1.2 Statement of the Problem

Kisumu county is one of the high TB burden counties with TB incidence of 105 per 100,000, TB prevalence of 306 per 100,000 and TB /HIV coinfection rate of 59%. The current diagnostic methods lack the sensitivity to detect lower bacterial loads like in early infection, continued response to treatment or lower bacterial shedding.The Xpert MTB/RIF allows rapid detection of Mycobacterium in sputum, however its sensitivity is low for paucibacillary specimens that are negative for acid fast bacilli in smear microscopy which accounted for 60% of TB cases identified from the recent prevalence survey in Kenya.The manufacturer recomments that raw sputum be diluted with sample reagent (SR) at 2:1 and pellet/sediment at the ratio of 3:1. Increased SR/sputum ratio recommended when pellets are tested could reduce assay sensitivity by over dilution. A laboratory and clinical study reported improved performance when the volume of SR/pellet ratio was reduced (from 3:1 to 2:1) in smear negative samples. However, there is limited evidence about its performance for diagnosis of smear negative pulmonary TB in high TB burden resource-limited settings.

1.3 Objectives

1.3.1 General objective

To compare the sensitivity of Xpert MTB/RIF in detection of *M. tuberculosis* from sputum pellets using the manufacturer's recommended 3:1 SR/pellet dilution versus a reduced dilution of 2:1 in smear negative samples.

1.3.2 Specific objectives

1. To compare sensitivity and specificity of ratio 3:1 SR/pellet dilution versus a reduced dilution of 2:1 in smear negative samples in Kisumu, Western Kenya.
2. To determine the positive and negative predictive value of ratio 2:1 SR as compared to ratio 3:1 pellet dilution in smear negative samples in Kisumu, Western Kenya.
3. To determine influence of sample quality on Xpert MTB/RIF result in Kisumu, Western Kenya.

1.3.3 Null Hypothesis (H0)

1. There is no difference in sensitivity and specificity between 3:1 and 2:1 sample reagent/sputum pellet in sputum smear negative samples
2. There is no difference in positive and negative predictive value between 3:1 and 2:1 sample reagent/sputum pellet in smear negative samples
3. Sputum Sample quality does not affect MTB/RIF test result

1.4 Justification of Study

Although the initial clinical trial of the Xpert MTB/RIF demonstrated sensitivity ranging between 98% in smear-positive TB and 72% in smear-negative cases, subsequent evaluations have demonstrated a tremendous variability in test performance in smear-negative disease, with sensitivity ranging between 26% and 83%(Jafari *et al.*, 2013; Lombardi *et al.*, 2017; Moure *et*

al., 2012). People who have smear-negative disease are more likely to experience high TB-associated mortality, prolonged morbidity and may also transmit the disease to others as it is estimated that 10%-20% of TB transmission is from smear-negative cases of pulmonary TB(Campos *et al.*, 2016) and 60% of TB cases identified from a recent prevalence survey in kenya were smear negative(Masini *et al.*, 2018) highlighting the need for new tests with improved sensitivity for TB detection in smear negative.

The current study provides data to strengthen research on the suitable concentration for smear negative patients.

1.5 Study significance

The study found no difference between the two sample reagent pellet ratios in TB detection in smear negative samples in Kisumu. There was moderate agreement between sample reagent pellet ratio 2:1 and 3:1 so both ratios are good. On effect of sputum quality on diagnostic yield,tenacious samples were associated with increased yield therefore patients should beinstructed to produce good quality sputum sample for Xpert MTB/RIF testing in pulmonary TB diagnosis.

CHAPTERTWO
LITERATURE REVIEW

2.1 Tuberculosis Pathogenisis

Tuberculosis is a communicable diseasecaused by*Mycobacterium tuberculosis*. Infection is initiated by inhalation of droplet nuclei, which are particles of 1–5 μm in diameter containing *M. tuberculosis*, expectorated by patients with active pulmonary TB when the patient coughs. The droplet nuclei, due to their small size, can remain suspended in the air for several minutes to hours. The risk of infection is dependant on several factors such as the infectiousness of the source case, the closeness of contact, the bacillary load inhaled, and the immune status of the patient(Ahmad, 2011)Mycobacteria are a distinctive rod-shaped bacteria that share a common property of a lipid-rich cell wall that avidly retains Carbol fuchsin dye even in the presence of acidic alcohol (Glickman *et al.*, 2001).*M.tuberculosis* has the ability to grow within macrophages of the potential host(Tobin *et al.*, 2008).

2.2 Tuberculosis Epidemiology

Tuberculosis (TB) is one of the most important global health problems.It is a communicable disease that cause ill health and one of the leading causes of death worldwide. Until the coronavirus (COVID-19) pandemic, TB was the leading cause of death from a single infectious agent, ranking above HIV/AID(WHO, 2021). One-third of the world's population, and 50% of adults in sub-Saharan Africa, and Asia, are infected with TB, representing an enormous pool of individuals at risk for developing active TB disease(Hood, 2013; Semba *et al.*, 2010).Tuberculosis is the leading cause of death in AIDS patients therefore TB and HIV form a deadly combination with each compounding the impact of the other. When people are infected with both TB and HIV, TB is much more likely to become active because of the person's weakened immune system (Kwan *et al.*, 2011).

Globally, 3.5% of new and 18% of previously treated TB cases were estimated to have had MDR/RR-TB in 2017. This translates into an estimated 558 000 people having developed MDR-TB and 230000 death from MDR/RR-TB in 2017 (WHO, 2018).

The resurgence of TB is fueled by the emergence of multi-drug resistance. Drug resistance in TB occurs as a result of tubercle bacillus mutations. Exposed to a single effective anti-TB medication, the predominant bacilli, sensitive to that drug are killed while the few drug resistant mutants likely to be present if the bacterial population is large multiply, another reason for resistance is overuse misuse of medications and lack of new drug development(Ventola, 2015).

Globally in 2017, 6.7 million people with tuberculosis (TB) were notified to national TB programmes, of these, just over 6.4 million had an incident episode of TB. Kenya notified 85188 cases of all forms of TB disease, of which 83599 were new and relapse cases (WHO, 2018). HIV infection is associated with fueling reactivation of TB, with an adult HIV prevalence in Kenya of 4.9% (NASCOP, 2018) and 39% of TB cases co-infected with HIV In Kenya, (WHO, 2018) Nyanza province is the leading in TB prevalence with a TB notification rate of 440/100,000. Siaya county in Nyanza has a TB notification rate of 400/100000, Kisumu County has a TB incidence of 105 per100,000 and TB prevalence of 306 per 100,000 (Burmen *et al.*, 2018).

2.3 Tuberculosis Laboratory Testing Methods

2.3.1 Microscopy

Sputum smear microscopy through Ziehl–Neelsen (ZN) staining is widely used in developing countries for the routine TB diagnosis due to cost effectiveness and high specificity, and does not require high biosafety level. However, direct smear microscopy has poor sensitivity (range, 20 to 80%), particularly in HIV-co-infected patients (Steingart, K. R. *et al.*, 2006; Steingart, Karen R. *et al.*, 2006)because it requires 5000–10,000 bacilli per mL of sputum for showing a positive

result. Almost 13% of TB transmission occurs with smear-negative, culture-positive TB patients as smear-negative TB patients often have bacterial loads substantially below 100 cfu/ml(Chakravorty *et al.*, 2005).Therefore, healthy individuals are at risk of MTB infection leading to active TB development when coming in close contact with sputum-negative TB suspects. Moreover, this test requires 3-day early morning sputum specimen collection protocol to enhance sensitivity. In addition to the lower sensitivity of sputum smear microscopy it cannot differentiate non tuberculous mycobacteria (NTM) from *Mycobacteruim tuberculosis*complex(MTBC)(Reid *et al.*, 2009).

2.3.2 Solid Culture system

Lowenstein–Jensen medium(LJ) is used for culture of *Mycobacterium* species, The medium must be incubated for a significant length of time (6 to 8 weeks) due to the slow doubling time of *M.tuberculosis*(15–20 hours) compared with other bacteria. Conventional phenotypic methods for drug susceptibility testing are based on inoculation of cultured isolates on solid media. The most widely used is the Proportion method, which is based on the exact determination in an inoculum of the proportion of organisms present that is resistant to a specific concentration of each drug, by comparing quantity of growth in a drug-containing and drug-free control media. When performed in egg-based media, the final reading is done after 42 days of incubation (Sobral *et al.*, 2011). The method does not use proprietary products or specialized equipment, is inexpensive and highly standardized for testing susceptibility to many drugs. The main disadvantage is the long period of incubationover 42 days, to report the final results(Bwanga *et al.*, 2009). When using egg-based media, a large number of tubes are inoculated for each test and the tubes incubated forover 42 days, requiring a large incubator space, it islabor intensive and

requires a careful quality control of all batches produced with drug susceptible and drug resistant strains for reliable results (Moreira *et al.*, 2015; O'Connor *et al.*, 2015).

2.3.3 Liquid culture systems

The BACTEC MGIT 960 system is a high-capacity, fully automated, continuous-monitoring instrument that can test up to nine hundred and sixty 7-ml MGIT vials for the presence of mycobacteria using nonradiometric fluorescence technology(Rodrigues *et al.*, 2009). The BACTEC MGIT 960 culture tube contains 7 ml of Middlebrook 7H9 broth base, to which an enrichment supplement containing oleic acid, albumin, dextrose, and catalase (BBL MGIT OADC), and an antibiotic mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA) are added. The enrichment supplement supplies the growing mycobacteria with nutrients while the antibiotic cocktail prevents growth of contaminants (Peres *et al.*, 2011).

The culture vials contain a fluorescent sensor that responds to the concentration of oxygen in the culture medium. The instrument's photo-detectors measure the fluorescence in each vial every 60 min. The level of fluorescence corresponds to the amount of oxygen consumed by the organisms in the inoculated specimens, and this, in turn, is proportional to the number of bacteria present. When a certain level of fluorescence is reached, the instrument indicates that the vial is positive.

With BACTEC MGIT 960 having many advantages over the other culture systems, many investigations have addressed the performance of MGIT culture systems turnaround time has improved to about 25–45 days(Diriba *et al.*, 2017; Rodrigues *et al.*, 2009; Somoskovi *et al.*, 2006).MGIT has also improved sensitivity of MTB isolation and drug susceptibility testing(Srisuwanvilai *et al.*, 2008),however due to cost, in most cases the system is not available

where the need is greatest. Even though turn around time has improved, it is still not rapid enough to allow timely decisions on patient management(Siddiqi *et al.,* 2012). Furthermore Mycobacterial culture can only be done in laboratories with high level of biosafety and highly qualified and experienced staff (O'Connor *et al.,* 2015).

2.3.4 Diagnosis of MDR-TB using Genotype MTBDR plus

The Genotype MTBDRplus (HainLifescience, Nehren, Germany) is a commercial easy-to-perform assay developed for the detection of rifampicinand isoniazid resistance in TB strains. The test is based on reverse hybridization between amplicons derived from a multiplex PCR and nitrocellulose bound wild-type and mutated probes for the mutations of interest(Miotto *et al.,* 2006). The presence of wild-type or mutated DNA sequences in the 81-bp hot spot region of rpoB and at codon 315 of katG is then shown by clear-cut hybridization signals on the membrane strips, which can easily be analyzed by using a template that is supplied with the kit(Hillemann *et al.,* 2005). Either the absence of wild-type bands or the appearance of bands targeting specific mutations indicates the presence of a resistant strain. MDR TB cases can be detected within 1 or 2 days of sputum sampling using this assay (Miotto *et al.,* 2006). LPA Has high sensitivity and can evaluate several mutations simultaneously in a single reaction(Migliori *et al.,* 2010).

Both the PCR technology and the reverse hybridization technique used for the test have been proven to be robust and reproducible, and the results are easy to interpret without the extensive expert knowledge required for the interpretation of real-time PCR data or DNA sequencing data. It can easily be implemented in routine work flows, especially when other strip assays are already established, such as in differentiation of several Mycobacterial species anddifferentiation

within the *M. tuberculosis* complex(Makinen *et al.*, 2002).However,due to high cost, it has not been widely used.

2.3.5 Xpert MTB/RIF and diagnosis of MDR-TB

Xpert MTB/RIF is a PCR based test of sputum which has been endorsed by world health organization as the initial diagnostic test of choice for people with HIV and people who have been suspected of having MDR TB. This test can identify both TB and rifampicin resistance, it does not require a high level of biosafety and highly skilled workers(Steingart *et al.*, 2014) (van Zyl-Smit *et al.*, 2011). The assay can be used to accurately measure the *M tuberculosis* load beyond the detection limit of 131 organisms per mL in an in-vitro suspension (Helb *et* al., 2010; van Zyl-Smit *et al*., 2011). The Xpert MTB/RIF assay in patients with suspected tuberculosis and newly diagnosed cases of tuberculosis has been evaluated in several studies (Boehme *et al.*, 2010; Theron *et al.*, 2011).The results of these studies suggested that the Xpert MTB/RIF assay is better than sputum-smear microscopy, its quantitative readouts correlate well with results of conventional solid and liquid cultures (Blakemore *et al.*, 2011; Boehme *et al.*, 2010). For the detection of tuberculosis and MDR-tuberculosis in HIV-positive individuals, a cost benefit was noted compared with conventional smear microscopy(Pantoja *et al.*, 2013; Weyer *et al.*, 2013). Presence of *M. tuberculosis*DNA by Xpert MTBRIF in presumptive TB patients who are culture-negative is thought to represent remnant, non-viable bacilli from previously treated culture-positive TB (Friedrich *et al.*, 2013). Xpert MTB/RIF provides semi quantitative results based on the cycle threshold (C_T) of the first positive probe that detects MTBC, which is correlated with the mycobacterial load in sputum samples as defined by smear grade and time to culture positivity(Theron *et al.*, 2011; van Zyl-Smit *et al.*, 2011).Recent studies have demonstrated that Xpert MTB/RIF-based semiquantitative measurement is efficient for both TB diagnosis and

initial evaluation of transmission risk in high and low TB burden countries (Blakemore *et al.*, 2011).

Previous studies, for Xpert MTB/RIF demonstrated sensitivity ranging between 98%-100% in smear positive TB(Armstrong *et al.*, 2022; WHO, 2014),While in smear negative, evaluations have demonstrated a tremendous variability in test performance, with sensitivity ranging between 26% and 68% (Jafari *et al*., 2013; Munoz *et al.*, 2013). In previously treated patients' studies have suggested lower specificities of XpertMTB/RIF(Acuna-Villaorduna *et al.*, 2017). The commonly applied protocol for detecting MTB in Xpert in most settings is the use of raw sputum to Xpert sample reagent (SR) with a ratio of 1:2(Boehme *et al.*, 2011). Manufacturer of Xpert recommends the use of either raw, unprocessed sputa or concentrated sputum sediments(Cepheid®, 2009). To concentrate raw sputum, sample is liquefied, decontaminated either with 2% N-acetyl cysteine-sodium hydroxide (NALC-NaOH) or 4% NaOH, centrifuged, concentrated and neutralized using phosphate buffer(Cepheid®, 2009).Reports have shown no difference in MTB detection in Xpert MTB/RIF using processed sputum sediments and raw sputum(Boehme *et al.*, 2011; Darwish *et al.*, 2013)when using recommended SR ratio of 1:2 and 1:3 respectively (Cepheid®, 2009).Sample centrifugation prior to using Xpert MTB/RIF increases sensitivity of the test(Boehme *et al.*, 2010), also reducing the volume of Sample Reagent/pellet ratio in processed sedimentfrom manufacturer recommended 3:1 to ratio 2:1 Xpert MTB/RIFincreases sensitivity in smear negative presumptiveTB patients(Dharan *et al.*, 2015). Freeze thaw of samples does not affect the assay sensitivity (Theron *et al.*, 2011).

2.4 Sputum Quality

Tuberculosis diagnosis is substantially increased by providing instruction on how to produce a sputum sample taken at any time of the day (Datta *et al.*, 2017). Patients are asked to take deep

breath in and hold for one second then cough deeply and vigorously when breathing out,inorder to collect thick mucoid sputum, not saliva or nasal secretion (Geldenhuys *et al.*, 2014).

Sputum qualityis affected by intrinsic and extrinsic factors, clinical and behavioral characteristics including conjunctivitis, difficulty in breathing and delay in seeking treatment (Orina *et al.*, 2019).Males produce good quality sputum than females (Acuna-Villaorduna *et al.*, 2017; Ramsay *et al.*, 2009).

International guidelines have emphasized macroscopic sputum quality as an important determinant of performance of smear microscopy and culture asprevious studies have demonstrated substantially higher sensitivity with purulent or bloody sputum as compared with mucoid or salivary sputum (Ho *et al.*, 2016). Clear instruction to patients about sputum collectionis important (Ho *et al.*, 2015).

Gross appearance has also been proposed to contribute to the increase in smear positivity (Yoon *et al.*, 2012). In addition, the number of bacilli on smear positivity can be underestimated in macroscopic poor-quality specimens (Bhat *et al.*, 2014). However, with Xpert MTB/RIF testing, salivary sputum have higher sensitivity and potentially higher yield than other sample types (Meyer *et al*., 2017). leukocytosis is a good indicator for good sputum for *M. tuberculosis* diagnosis in patents with suspected pulmonary TB(Lee *et al.*, 2015)and purulent sputum isassociated with more neutrophils and higher mycobacterial counts(Yoon *et al.*, 2012)on the contrary blood-stained sputum is less desirable for Xpert MTB/RIF testing because blood is an inhibitor of DNA amplification(Meyer *et al.*, 2017).

CHAPTERTHREE
MATERIALS AND METHODS

3.1 Study Area

Kisumu is the third largest city in Kenya, the principal city of western Kenya, headquarters of Kisumu County It is located on the shores of Lake Victoria, a fresh water lake, along the Equator within latitude 0° 02' N and 0° 10' S and longitudes 34° 20' E and 34° 65' E. Kisumu County has a population of 1,155,574(KNBS, 2019).Fishing and farming are the major economic activities among residents. There are two major health facilities in Kisumu, the Jaramogi Oginga Odinga teaching and referral hospital (JOOTRH) and the Kisumu county referral hospital (KCRH). High poverty level, lack of adequate water and sanitation facilities are the major challenges facing residents of Kisumu (Maoulidi, 2012). Other challenges are HIVand AIDS, low income and food insecurity (Bwana *et al.,* 2015). Kisumu county has the third highest adult HIV prevalence of 16.3% (NACC, 2018).HIV is a risk factor in TB infection because of the person's weakened immune system,latent TB infection is much more likely to become active.Kisumu has a TB prevalence rate of 11.2 % among people living with HIV (PLHIV) (Modi *et al.*, 2016), Siaya county which boarders Kisumu, has a TB prevalence among adolescents at 6.8/1000 population(Nduba *et al.,* 2015). Presence of slums in Kisumu such as Obunga, Nyalenda and Manyata has also contributed to the increase of TB(Sifuna, 2013).

Samples were collected from suspected TB patients who visited Jaramogi Oginga Odinga teaching and referral hospital(JOOTRH) and met the enrolment criteria of tuberculosis observational study as described under the eligibility criteria below.

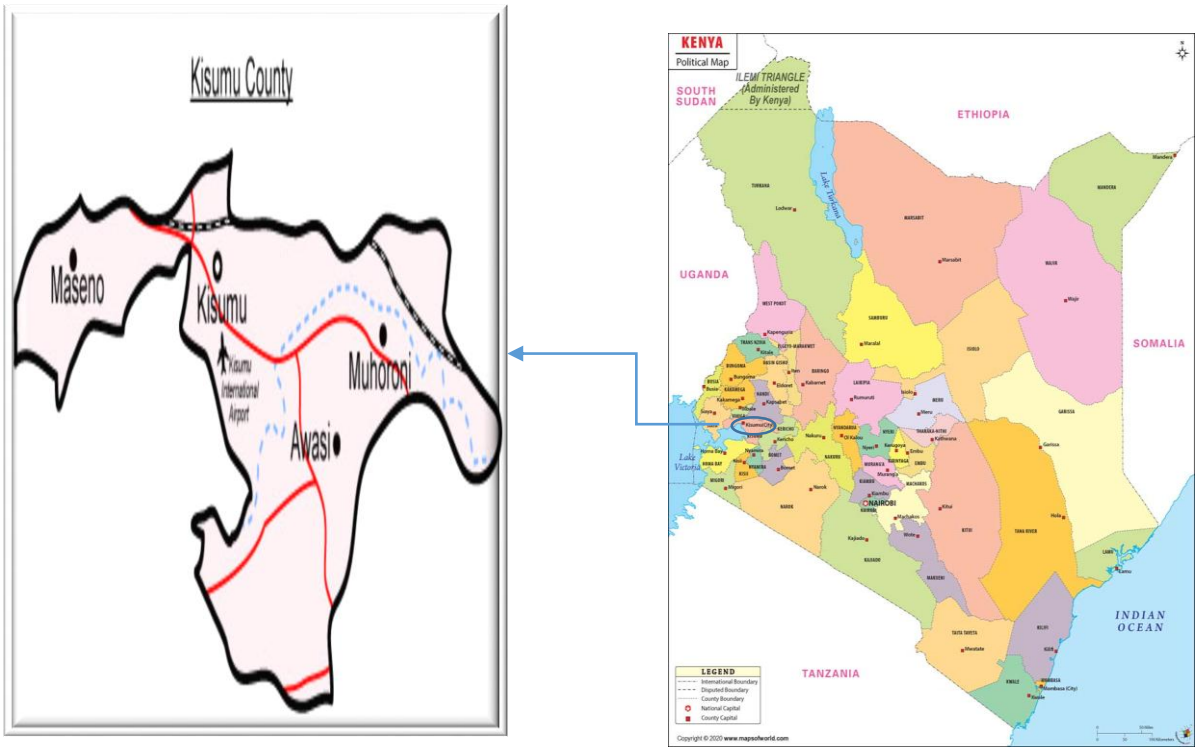

Figure 3.0: Map showing the study area of Kisumu.

3.2 Study Population

Sputum samples from eligible participants reporting to JOOTRH were collected based on the following criteria.

3.2.1 Inclusion criteria

15 years and above

Signed informed consent. For those age < 18, participant assent and informed consent signed by the legal guardian.

Sputum smear negative.

3.2.2 Exclusion Criteria

Insufficient sputum sample less than 3mls

Unwilling to sign consent form

AFB smear positive

3.3 Study Design

This was a laboratory based cross sectional study where samples collected between2017 and 2019 were used.

3.4 Sample size determination

Fisher et al (2007) formula was used for the determination of sample size which is

n= Z2pq/d2

Where:

n = desired sample size

z = standard deviation of required confident level which is given as 95% CI. Equals 1.96

p = proportion of the target population (Prevalence of TB occurrence in Kisumu county which is 11.2% (Modi *et al.*, 2016)

q = 1-p i.e. (1 – 0.112) = 0.888

d = Level of precision which is 95% with an error of 5% which equals to 0.05

n = 1.96^ (0.112*0.888)/0.05^

=3.8416*0.38207/0.025

=152.82

= 154 samples were used.

3.5 Recruitment of research participants

The study staff at the clinic were involved in identification of study participants,consenting those who met inclusion and exclusion criteria, enrollement of participants and completionof request forms. They gave sputum cups and instruction to study participants on how to collect sputum, packaged the samples for shipment to KEMRI TB laboratory.

3.6 Sputum collection

About 3mls spot sputum specimen was collected in 50ml sterile centrifuge tubes. These samples were placed in a cool box with ice packs and transported to KEMRI TB lab accompanied with their respective request forms for testing. Laboratory participates in external quality assurance (EQA) that is College of American pathologist (CAP) and smartspot

3.7 Laboratory Procedures

3.7.1 Sample reception and processing

At KEMRI/CDC TB lab samples were verified against the request form to ensure all information talliedand they (samples) were then received into an electronic database. Sputum quality was classified as salivary, mucoidand tenacious, a worksheet was developed that accompanied the sample for processing. 2% sodium hydroxide–*N*-acetyl-l-cysteine was used to decontaminate the samples in the ratio of 1:1, the samples were left to digest for 15minutes after which phosphate buffer pH 6.8 was added up to the 50ml mark and centrifuged at 3000g for 15 minutes at 4ºC. The supernatant was poured off and concentrated pellet re-suspended in 1.5 to 2.0 ml sterile phosphate-buffered saline (pH 6.8) Approximately 1.5ml of pellet was transferred into a vial and stored at -80^{o} C for future molecular work.

3.7.2 Fluorecent Microscopy smear Preparation

Smear was prepared by placing a drop of processed sample pellet on the slide and spreading, then allowed to air dry. They were heat-fixed on a slide warmer at 70°C for 2 hours, followed by staining by Auramine O for 20minutes, then 0.75% acid alcohol and counterstained by potassium

permanganate and allowed to air dry. They were examined at 20X objective of fluorescent microscope and result graded as per International Union against Tuberculosis and lung disease (IUTLD) guidelines. Positive results were confirmed by a second reader. Result were entered into an excel sheet.

3.7.3 Culture of Mycobacterium tuberculosis

Zero point five mls of processed sputum sediment was innoculated on to*Mycobacteria* growth indicator(MGIT) tubes containing 0.8ml Polymixin B,Amphotericin B, Nalidixic acid, Trimentoprim, Azlocillin (PANTA)(Beton, Dickinson and company)supplement, and incubated at 37^0C in the Bactec MGIT 960 instrument and monitored for up to 6 weeks. Smear microscopy using ZN, and TBc (BD) identification were performed on instrument positive MGIT tubes, to confirm the precence of mycobacterium tuberculosis.https://www.finddx.org/wp-content/uploads/2016/02/mgit_manual_nov2006.pdf

3.8 GeneXpert

Frozen samples were thawed to room temperature and vortexed for 15 seconds. Standard dilution volumes were used as recommended in the package insert. For the standard ratio 3:1 dilution, at least 0.5 ml of the total re suspended pellet and 1.5 ml of SR was added to a tube according package insertat https://www.cepheid.com/PackageInsert(Cepheid, 2019). For the experimental 2:1 dilution, 0.7 ml of re suspended sputum pellet and 1.4 ml of SR was added to a tube. The tubes were then shaken vigorously 20 times and incubated for 15 min at room temperature. After 5 to 10 min, the sample was shaken again 10 to 20 times until the samples was liquefied with no visible clumps of material. The remaining time of incubation was completed, and 2 ml of sample was then transferred into the Xpert cartridge and loaded into the automated Xpert instrument. The results was available after about two hours and were either*Mycobacterium*

tuberculosis(MTB) detected or MTB not detected. Those that were positive were quantified as very low and low by the geneXpert machine.

3.9 Data Analysis

Data was entered into an excel spreadsheet. Sensitivities and specificitywas determined using MGIT culture as the reference. MTB yield for the two sample reagent ratio was performedusingmcNemer test, while strength of agreement between the two sample reagent ratio was determined by cohen kappa.Association of sample quality and geneXpert result was done using chi square testof association (for mucoid and salivary samples)and Fisher's exact test for tenacious samples.

3.10 Ethical considerations

Approval to carry out this study was provided by the School of Graduate Studies (SGS) of Maseno University. Ethical approval was obtained from the Kenya Medical Research Institute (KEMRI) Ethical Review Committee (SSC number 2693 Appendix 4). All study participants signed consent or assent to all applicable approved informed consent forms prior to any study-related procedures. Study activities were conducted using a non-identifying study number for each participant. Participant's personal identifying information was kept in password secured database, accessible only by study personnel with training in ethical human studies research. All laboratory specimens, evaluation forms, reports, and other records that left the study site were identified by coded numbers only, to maintain confidentiality of study participants. All paper study records containing direct study participant identifiers were kept locked in an office, consistent with local clinical practice. Access to these files was limited to study personnel. Electronic study records were entered and stored on password-protected computers that were only accessed by study personnel.

CHAPTER FOUR

RESULTS

4.1 Case summary of samples used in the study

The study sampled a total of 154 pellet samples (Figure 4.1) of which 105(68.2) were mucoid, 47(30.5%) salivary and 2(3%) were tenacious. Out of geneXperttests conducted, 43 (27.9%) hadMycobacterium tuberculosis (MTB) detectedand 111(72.1) were MTB not detectedwithratio 3:1 SR/pellet dilution while 54 (35.1%) were MTB detected and 100(64.9%) were MTB not detectedwithratio 2:1 SR/pellet dilution. Of the 43 MTB detected by ratio 3:1 SR/pellet dilution, 5(3.2%) were low while 38(24.7%)were very low while of the 54 MTB detected by ratio 2:1, 11(6.5%) were low while 43(27.9%) were very low. Of the 43 MTB detected by 3:1 SR/pellet dilution, 37(24.0%) were rifampin resistance not detected and 6(3.9%) were rifampicin resistance indeterminate while of 54 positive by ratio 2:1 SR/pellet dilution, 47(30.5%) were rifampicin resistance not detected, 5(3.2%) rifampicin resistance indeterminate and 1(0.6%)was rifampicin resistant as shown in Figure 4.1 and Table 4.1.

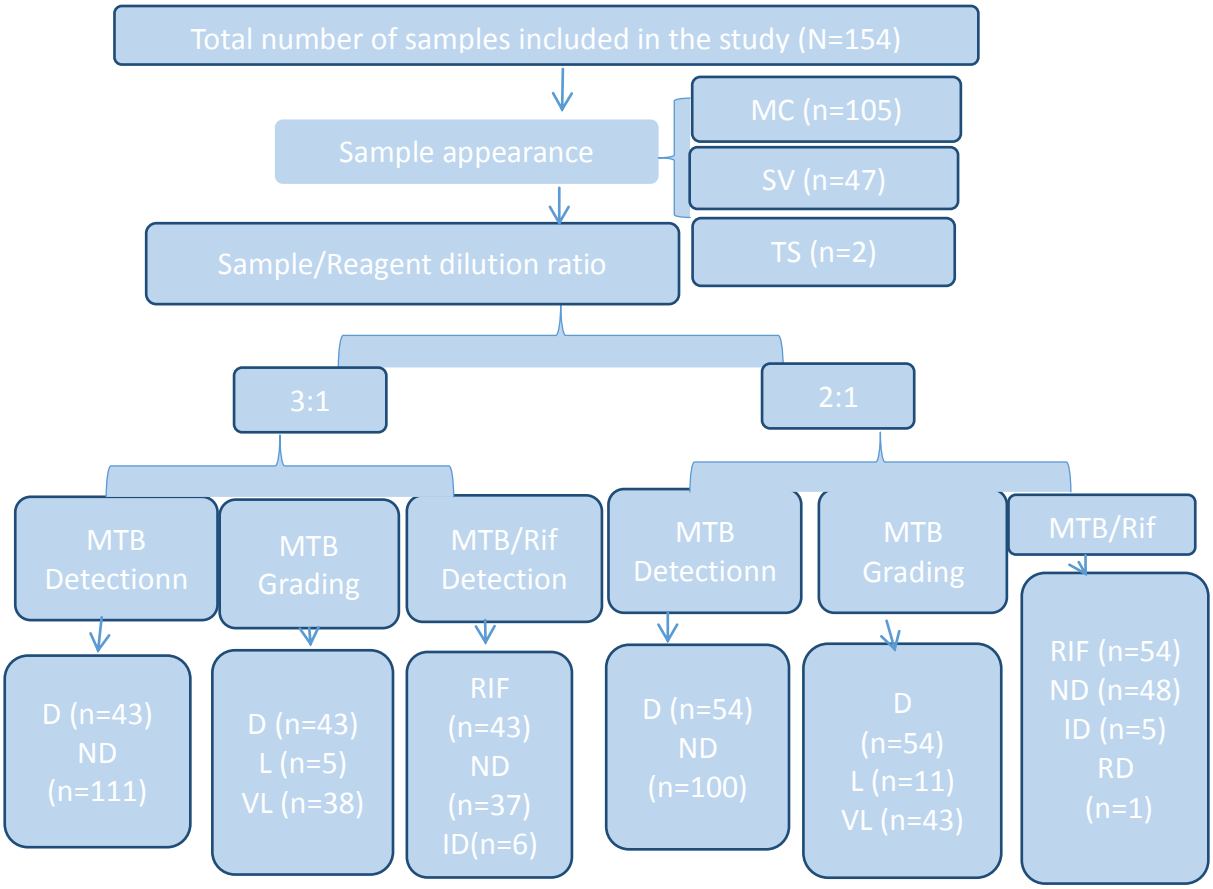

Figure 4.1: Distribution flow of samples included in the study by test procedures

MC=Mucoid**,** **SV**=Salivary**,** **TS**=Tenacious**,** **MTB**=*Mycobacteriumtuberculosis***,** **Rif**=Rifampicin**,** **D**= MTB Detected**,** **ND**=Not detected, **NA**=Not applicable**,** **L**=Low**,** **VL**=Verylow**,** **ID**=Indeterminate**,** **RD**=Rifampicin Detected

Table 4.1: Case summaries for tested samples

		Sample/Reagent dilution ratio	
Characteristic		**3:1** n (%)	**2:1** n (%)
MTB Detection	MTB Detected	43(27.9)	54(35.5)
	MTB Not Detected	111(72.1)	100(64.9)
MTB Grading	Not done	111(72.1)	101(65.6)
	Low	5(3.2)	11(6.5)
	Very low	38(24.7)	43(27.9)
Rifampicin resistance detection	Not done	111(72.1)	101(65.6)
	Not detected	37(24.0)	48(30.5)
	Indeterminate	6(3.9)	5(3.2)
	Detected	-	1(0.6)
Sputum appearance	Mucoid	105(68.2)	
	Salivary	47(30.5)	
	Tenacious	2(1.3)	

4.2 Characteristics of the sample tested.

4.2.1 Rifampicin resistance status

Table 4.2 using ratio 2:1 as the standard, percentage for rifampin resistance not applicable was 88.1%, rifampin not detected was 55.3% while rifampicin detected was 16.7%

Table 4.2: Rifampicin resistance status using ratio 2:1 as standard

Rifampicin resistance Detected 3:1 * Rifampin resistance 2:1							
			Rifampicin res 2 Code 2:1				Total
			N/A	Not Detected	Detected	Indeterminate	
Rif Detected 3:1	N/A	Count/% within Rif Detected Code 3:1	89 (80.2%	18 (16.2%	0 (0.0%)	4 (3.6%)	111 (100%
		% within Rifampin res 2 Code 2:1	88.1%	38.3%	0.0%	80.0%	72.1%
		% of Total	57.8%	11.7%	0.0%	2.6%	72.1%
	Not Detected	Count /% within Rif Detected Code 3:1	10(27)	26 (70.3)	0(0%)	1(2.7%)	37(100%)
		% within Rifampin res 2 Code 2:1	9.9%	55.3%	0.0%	20.0%	24.0%
		% of Total	6.5%	16.9%	0.0%	0.6%	24.0%
	Detected	Count	2	3	1	0	6
		% within Rif Detected Code 3:1	33.3%	50.0%	16.7%	0.0%	100.0%
		% within Rifampin res 2 Code 2:1	2.0%	6.4%	100.0%	0.0%	3.9%
		% of Total	1.3%	1.9%	0.6%	0.0%	3.9%
Total		Count% within Rif Detected Code 3:1	101 (65.6%	47 (30.5%	1 (0.6%)	5 (3.2%)	154 (100%)
		% within Rifampin res 2 Code 2:1	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	65.6%	30.5%	0.6%	3.2%	100.0%

4.2.2 MTB Grading

Table 4.3 using ratio 2:1 as the standard, MTB not detected/Not applicable was 88.1% MTB detected low was 30.0%, MTB detected very low was 51.2.

Table 4.3: Crude (154) MTB Grading using ratio 2:1 as standard

MTBGrading ratio 3:1						
			MTB grading 2:1			Total
			N/A	LOW	VERY LOW	
MTBGrading 3:1	N/A	Count	89	2	20	111
		% within MTBGrading Code 3:1	80.2%	1.8%	18.0%	100.0%
		% within MTB grading C0de 2:1	88.1%	20.0%	46.5%	72.1%
		% of Total	57.8%	1.3%	13.0%	72.1%
	LOW	Count	1	3	1	5
		% within MTBGrading Code 3:1	20.0%	60.0%	20.0%	100.0%
		% within MTB grading C0de 2:1	1.0%	30.0%	2.3%	3.2%
		% of Total	0.6%	1.9%	0.6%	3.2%
	VERY LOW	Count	11	5	22	38
		% within MTBGrading Code 3:1	28.9%	13.2%	57.9%	100.0%
		% within MTB grading C0de 2:1	10.9%	50.0%	51.2%	24.7%
		% of Total	7.1%	3.2%	14.3%	24.7%
Total		Count	101	11	43	154
		% within MTBGrading Code 3:1	65.6%	6.5%	27.9%	100.0%
		% within MTB grading C0de 2:1	100.0 %	100.0%	100.0%	100.0%
		% of Total	65.6%	6.5%	27.9%	100.0%

Cleaned MTB grading using ratio 2:1 as standard MTB detected low was 37.5% while MTB detected very low was 95.7% table 4.4.

Table 4.4: Cleaned (31) MTB Grading using ratio 2:1 as standard

MTBGrading					
			MTB grading 2:1		
			LOW	VERY LOW	Total
MTBGrading 3:1	LOW	Count	3	1	4
		% within MTBGrading ratio 3:1	75.0%	25.0%	100.0%
		% within MTB grading ratio 2:1	37.5%	4.3%	12.9%
		% of Total	9.7%	3.2%	12.9%
	VERY LOW	Count	5	22	27
		% within MTBGrading ratio 3:1	18.5%	81.5%	100.0%
		% within MTB grading ratio 2:1	62.5%	95.7%	87.1%
		% of Total	16.1%	71.0%	87.1%
Total		Count	8	23	31
		% within MTBGrading ratio 3:1	25.8%	74.2%	100.0%
		% within MTB grading ratio 2:1	100.0%	100.0%	100.0%
		% of Total	25.8%	74.2%	100.0%

4.2.3 Rifampicin Detected

Using 2.1 as standard rifampin resistance not detected was 83.9% and rifampin resistance detected was 3.2%

Table 4.5: Cleaned (31) Rifampicin Resistance Detected using ratio 2:1 as reference

RIF Detected						
			Rifampin res 2 Code 2:1			
			Not Detected	Detected	Indeterminate	Total
Rif Detected Code 3:1	Not Detected	Count	26	0	1	27
		% within Rif Detected Code 3:1	96.3%	0.0%	3.7%	100.0%
		% within Rifampin res 2 Code 2:1	89.7%	0.0%	100.0%	87.1%
		% of Total	83.9%	0.0%	3.2%	87.1%
	Detected	Count	3	1	0	4
		% within Rif Detected Code 3:1	75.0%	25.0%	0.0%	100.0%
		% within Rifampin res 2 Code 2:1	10.3%	100.0%	0.0%	12.9%
		% of Total	9.7%	3.2%	0.0%	12.9%
Total		Count	29	1	1	31
		% within Rif Detected Code 3:1	93.5%	3.2%	3.2%	100.0%
		% within Rifampin res 2 Code 2:1	100.0%	100.0%	100.0%	100.0%
		% of Total	93.5%	3.2%	3.2%	100.0%

4.3 Specificity and Sensitivity Testing

Results by culture wasused as a standardagainst which the sensitivity of ratio 2:1 and 3:1 SR/pellet dilutionformulation for geneXpert testing was evaluated. Analysis in table 4.6shows that 86.0% turned negative by both tests, 18.5% turned positive by both tests,81.5% turned positive by Ratio 2.1 SR but negative by culture, while 14.0% turned positive by culture but negative by Ratio 2:1 SR. Therefore, the sensitivity and specificity of ratio 2:1 SR was 18.5% and 86.0% respectively while ratio 3:1 dilution had sensitivity of37.5 and Specificity 73.8% as in table 4.7 below.

Table 4.6: Evaluation of XPERT MTB/RIF performance in diagnosis of TBusing 2:1 sample reagent/pellet ratio in comparison with Culture (using Culture as a standard test)

			CULTURE TEST		Total
			Detected	Not detected	
Xpert test RATIO 2:1	Detected	Count	10	44	54
		% within RATIO 2:1	**18.5%**	81.5%	100.0%
	Not detected	Count	14	86	100
		% within RATIO 2:1	14.0%	**86.0%**	100.0%
Total		Count	24	130	154
		% within RATIO 2:1	15.6%	84.4%	100.0%

GeneXpert Sensitivity with Ratio 2:1 was 18.5% and Specificity was 86.0%

Table 4.7: Evaluation of GeneXpert MTB/RIF system performance in diagnosis of TB using ratio 3:1 diluent in comparison with Culture (using Culture as a standard test)

RATIO 3:1 * CULTURE Crosstabulation					
			CULTURE TEST		Total
			Detected	Not detected	
Xpert test RARTIO 3:1	Detected	Count	9 (37.5)	34(26.2)	43(27.9)
		% within CULTURE	**37.5%**	26.2%	27.9%
	Not detected	Count	15	96	111
		% within CULTURE	62.5%	**73.8%**	72.1%
Total		Count	24	130	154
		% within CULTURE	100.0%	100.0%	100.0%

Culture vs Xpert 3:1 ratio Specificity 73.8% and Sensitivity is 37.5%

4.4 Positive predictive value (PPV) and Negative Predictive Value (NPV)

Negative and Positive Predictive Value for ratio 2:1 was 66.2% and 41.7% while ratio 3:1 waswas 20.9% and 86.5% respectively as shown in table 4.8 and table 4.9 below

Table 4.8: PPV and NPV (Xpert Test Ratio 2:1 compared to Culture as a standard)

			CULTURE TEST		Total
			Detected	**Not detected**	
Xpert Test RATIO 2:1	Detected	Count	10	44	54
		% within RATIO 2:1	18.5%	81.5%	100.0%
		% within CULTURE	**41.7%**	33.8%	35.1%
		% of Total	6.5%	28.6%	35.1%
	Not detected	Count	14	86	100
		% within RATIO 2:1	14.0%	86.0%	100.0%
		% within CULTURE	58.3%	**66.2%**	64.9%
		% of Total	9.1%	55.8%	64.9%
Total		Count	24	130	154
		% within RATIO 2:1	15.6%	84.4%	100.0%
		% within CULTURE	100.0%	100.0%	100.0%
		% of Total	15.6%	84.4%	100.0%

Table 4.9: PPV and NPV ofXpert Test Ratio 3:1 compared to Culture as a standard

			CULTURE TEST		Total
			Detected	Not detected	
Xpert Test RATIO 3:1	Detected	Count	9	34	43
		% within RATIO 3:1 CODE	**20.9%**	79.1%	100.0%
		% within CULTURE CODE 2	37.5%	26.2%	27.9%
	Not Detected	Count	15	96	111
		% within RATIO 3:1 CODE	13.5%	**86.5%**	100.0%
		% within CULTURE CODE 2	62.5%	73.8%	72.1%
Total		Count	24	130	154
		% within RATIO 3:1 CODE	15.6%	84.4%	100.0%
		% within CULTURE CODE 2	100.0%	100.0%	100.0%

PPV was 20.9% and NPV was 86.5%

4.5 Diagnostic accuracy of the dilution tests

The strength of agreement between ratio 3:1 SR/pellet dilution and ratio 2:1 SR/pellet dilution was assessed by Cohen's Kappa statistics (ƙ) according to(McHugh, 2012): k = 0 indicating no agreement; k = 0–0.20 indicating poor agreement; k = 0.21–0.40 indicating fair agreement; k = 0.41–0.60 indicating moderate agreement; k = 0.61–0.80 indicating substantial agreement; and k = 0.81–1.0 indicating almost perfect agreement

There was a moderate agreement between the two dilutions with Cohen kappa value of 0.476 at p<0.001.However, there is no statistically significant difference in MTB detection after reducing the sample/reagent ratio used,with McNemar test, two-tailed p value was 0.090 indicating that the distribution of values 3:1 and 2:1 are equally likely.

Table 4.10: Summary of findings

	3:1	2:1
Sensitivity (%)	37.5	18.5
Specificity (%)	73.8	86.0
PPV (%)	20.9	41.7
NPV (%)	86.5	66.2
Cohen kappa	0.476 (95%CI 0.00-019, p=0.001	
McNemar test	Exact Sig (2-tailed)= 0.090	

4.6 Influence of sample Appearance on Xpert MTB/RIF result

Salivary samples were 47, mucoid 105 and 2 were tenacious. Out of 47 salivary samples 14 were MTB detected while 33 were MTB not detected. Of the 105 mucoid samples 38 were MTB detected while 67 were MTB not detected and all the 2 tenacious samples were positive for MTB. Tenacious samples were significantly associated with performance of 2:1 sample

dilutionFisher's Exact test p value of 0.053 whereas for salivary and mucoid samples, there was no statistical differencein MTB yield as inTable 4.11 below;

Table 4.11: influence of Sample Appearance on diagnostic performance of Xpert MTB/RIF

Sample quality	**2.1 TB Detected**	**2.1 TB Not Detected**	**Chi square value**	**df**	**p=value**
	Frequency (%)	Frequency (%)			
Salivary					
YES	14 (9.1)	33 (21.4)	0.828	1	0.464
NO	40 (26.0)	67 (43.5)			
Mucoid					
YES	38 (24.7)	67 (43.5)	0.184	1	0.668
NO	16 (10.4)	33 (21.4)			
Tenacious					
YES	2 (1.3)	0 (0.0)	-	-	**0.053***
NO	52 (33.8)	100 (64.9)			

*Fisher's Exact test indicating that tenacious samples were significantly associated with performance of 2:1 sample dilution.

CHAPTE FIVE
DISCUSSION

5.1 Introduction

An evaluationof GeneXpert's sensitivity in MTBdetection and rifampicin resistance in smear-negative sputum samples usingreduced sample reagent/processed pellet ratio of 2:1compared toculture as the reference.

In the current study, detection of MTB increased insignificantly at sample reagent/processed pellet ratio 2:1(35.1%) compared to 3:1(27.9%) in smear negative samples (Table 4.1). This finding was consistent with a previous study, (Dharan *et al.*, 2015)where MTB yield of XpertMTB/RIF test on concentrated sputum pellets increased by decreasing the ratio of sample reagent to sputum pellets in paucibacillary samples.Lower sensitivity of XpertMTB/RIF for detection of M. tuberculosis has been reported for sputum samples that have been induced with saline than for those that were expectorated (Theron *et al.*, 2014). The possible explanation could be over dilutionwhichmay lead to a decreased concentration of *M. tuberculosis* bacilli in the Xpert cartridge and therefore lead to lower detection by Xpert(Dharan *et al.*, 2015), lowsensitivity of Xpertfor induced specimensinsmear-negative patients, has also been reported at (40%)as compared to expectorated specimens at(79%)(Cepheid, 2019), this suggests that saline induction, cause dilution of specimens which in turn affects detection of *M. tuberculosis* by Xpert in paucibacillary specimens.

5.2 Sensitivityand Specificity

The present study had a sensitivityof 18.5%(Table 4.6) for ratio 2:1and 37.5% (Table 4.7) by ratio 3:1 in smear negative using culture as the standard.This islowerthan reported sensitivity byratio 2:1 of 67% (Dharan *et al.*, 2015) in detection of mycobacterium tuberculosis compared to the conventional ratio.This couldbe due to presence of residual mycobacterial DNA in the respiratory tract or the detection of non-viablebacilli(Costantini *et al.*, 2020) leading to false positive results by Xpert MTB/RIF,as some samples included in the current study were from patients previously on anti TB treatment. Another possibility for culture negative could be loss of viable bacteria during NALC-NaOH processing(Ullah *et al.*, 2017).Studies done in Ethiopia and South Africareported slightly higher sensitivities of 48.6 % and 43% in smear negative culture positive samples(Geleta *et al.*, 2015; Lawn *et al.*, 2011).Other studies that have used the conventional ratio have reported higher sensitivities in smear negative samples,a previousstudy reported sensitivity of 62.6%(Dorman *et al.*, 2012), whileother studies have reported sensitivities of 60% and 63.2%in smear negative culture positivecases(Liu *et al.*, 2021; Tadesse *et al.*, 2016).Another study from China reported a higher sensitivityof 88.8%(Ou *et al.*, 2015), difference in sensitivities may be due to differences of study populations, genetic differences involved and prevalence of tuberculosis among study populations(Rasheed *et al.*, 2019).Another possible explanation could be differences in study designs and the presence of PCR inhibitors or insufficient nucleic acid material in the specimens(Geleta *et al.*, 2015).The quality of the sputum collected by the patient,and medical staff's instructions to the patient is another reason as it may differ, and thereforeaffect sensitivity(Liu *et al.*, 2021)

Similar to other tests, a negative result cannot exclude the diagnosis of TB therefore Clinicians should be aware of Xpert MTB/RIF limitations so that patients with a high clinical probability of TB despite a negative Xpert are started on anti-TB treatment(Tadesse *et al.*, 2016).

Specificity, in the present study was 86.0 % (Table 4.6), for ratio 2:1 and 73.8% (Table 4.7) for ratio 3:1.This is lower than what has been reported previously as 93%,(Agrawal *et al.*, 2016; Reechaipichitkul *et al.*, 2016).However other studies have also reported lower specificities among smear-negative pulmonary tuberculosis cases,(Ullah *et al.*, 2017) another study reported specificity of 71.6%(Liu *et al.*, 2021), this could be attributed to difference in the population studied and sputum sample volume used(Scott *et al.*, 2011).Another possible explanation could be the laboratory level of MTB/RIF testing, according to report by Cochrane review, Xpert MTB/RIF specificity was lower at point of care and in peripheral laboratories compared to intermediate and central laboratories(Horne *et al.*, 2019).Lower specificity of Xpert MTB/RIF has also been reported in people with a prior history of tuberculosis(Theron *et al.*, 2018). This observation could be dueto false positive results by Patients previously on anti TB treatment who may have DNA in sputum that can be extracellular.

5.3 Positive and Negative Predictive values

Positive predictive value (PPV) and negative predictive value (NPV) of Xpert MTB/RIF ratio 2:1 for the diagnosis of pulmonary tuberculosis in smear negativein the current study was41.7% and 66.2% respectively (Table 4.8), while ratio 3:1 had PPV 20.9 and NPV of 86.5. this is relatively low in comparison with other studies that have reported high positive and negative predictive value for tuberculosis diagnosis in smear negative using conventional ratio (Chew *et al.*, 2016; Geleta *et al.*, 2015; Rasheed *et al.*, 2019; Ullah *et al.*, 2017).However,similar to the current study, low positive predictive value of 44.44% has been reported in China when Xpert

MTBRIF was compared to smear microscopy(Shao *et al.*, 2017).Still in chinaanother study reported much lower PPV of 19.4% in smear negative pulmonary TBusing culture as the gold standard(Liu *et al.*, 2021).

Wide variations may be due to differences in samplecollection, transport, and testing times(Tang *et al.*, 2017). Another possible explanation could be false positive results by Patients previously on anti TB treatment who may have DNA in sputum that can be extracellular or associated with non-intact cells, whichdo not grow in culture, since Xpert MTB/RIF test amplifies any DNA, of live or dead bacilli(Omar *et al.*, 2019). HIV infectedindividuals have compromised pulmonary immune clearance and could have DNA for long(Theron *et al.*, 2018) therefore clear history of treatment with anti TB is required to avoid false positive results.

Quality of sputum sample may influence the diagnostic performance of the Gene Xpert MTB/RIF assay, the presence of heme compounds and other endogenous PCR inhibitors could inhibit DNA amplification resulting in false negatives results which has been reported to be in range of 3% - 15%(Souza D *et al.*, 2020).Cross-contamination is known to cause false positive resultshowever, PCR in Xpert MTB/RIF is less prone to contamination due to the closed reaction chamber.

A moderate agreement between the two dilutions was observed, Cohen kappa value 0.476 at p-value<0.001. The reason why MTB in some specimens was not detected by ratio 3:1 sample reagent/pellet,but detected by ratio 2:1 SR/pellet could be that there were low numbers of bacilli which were under the lower limit of detection by Xpert MTB TB/RIF assay at ratio 3:1 dilution but when the sample reagent was reduced, the bacilli were detected.

5.4 RIF Resistance Detection

In the presentstudy,XpertMTB/RIF using SR/pellet ratio 2:1 detected one RIF resistant which was not detected on SR/pellet ratio 3:1this could be attributed to higher DNA concentration as a result of lower sample dilution.This is in line withLawn and Nickol's proposition that the detection of RIF resistance by the GeneXpert(MTB/RIF) requires a minimum threshold of DNA concentration of 65%and 100% (Lawn *et al.,* 2011).The current study was performed in smear-negative pulmonary tuberculosis that excluded many of the MDR-TB patients, this may be the reason for the low frequency of drug-resistant TB.There were indeterminate rifampicin resistance result in both dilutions this could be due to low DNA quantity in the sample (Mwanza *et al.*, 2018) as all that were RIF indeterminate and MTB detection was very low. Our results therefore suggest that the performance of the MTB/RIF test for rifampin resistance was influenced by the amount of bacteria in the sputum samples.

5.5 Influence of sample Quality on Xpert performance

Most of the samples used 68.2% were mucoid, 30.5% salivary and 3% were tenacious, this is similar to a previous study that reported high number of mucoid samples, followed by salivary then purulent/tenacious samples(Meyer *et al.,* 2017). In the current studythere was no significant difference in MTB yield of Xpert testing in salivary and mucoid specimens in smear negative sputum samples and this is consistent with previous studies which found no difference between mucoid, salivary and purulent samplesin Xpert MTB/RIF testing(Meyer *et al.*, 2017; Zimba *et al.,* 2019). This similarity could be due sputum timing as in both these studies spot sputum was used.

However, inthe current study there was a significant differencein MTB detection using Xpert MTB/RIF in tenacious samples (p= 0.053).Our findings in terms of sample quality and MTB

yield are consistent with a recent study which demonstrated higher yield in purulent and muco purulent samples in patients presumed to have tuberculosis(Kiptoo *et al.*, 2017). Contrast to our findings, the study that was done in Ugandareported higher diagnostic sensitivityon salivary samples than mucoid and mucopurulent samples and this seems contrary to biological plausibility(Zimba *et al.*, 2019) showing higher diagnostic yield fromsalivary samples which are regarded as poor quality samples. However, this was consistent when compared to mycobacterial culture where they reported higher culture positive among salivary than non-salivary(Meyer *et al.*, 2017).

Findings from the present study show that purulent or tenacious samples are associated with increased probability of detection of mycobacterium tuberculosis in XpertMTB/RIF. In previous reports, improving sputum quality increased TB diagnostic yield (Bhat *et al.*, 2014; Ho *et al.*, 2016),weare therefore in agreement with Ho *et al* and Zimba *et al*that TB programs should continue to train providers on high quality sputum collection techniques(Ho *et al.*, 2016; Zimba *et al.*, 2019). patients need to be informed that a good quality sputum specimen consists of material brought up from the lungs after a productive cough. leukocytosis has been found as a good indicator for good sputum for *M. tuberculosis* diagnosis in patents with suspected pulmonary TB(Lee *et al.*, 2015).purulent sputum is associated with more neutrophils, a higher degree of inflammation, and greater mycobacterial counts(Yoon *et al.*, 2012). Any deficiency in the key steps, in sample collection including the medical staff's instructions to the patient may affect the quality of the sputum collected by the patient (Liu *et al.*, 2021). There has been a lot of investments by countries in rolling out Xpert MTB/RIF since its endorsement by WHO Such investments may be compromised if TB diagnostic algorithms do not encompass collection of good quality sputum(Kiptoo *et al.*, 2017).

5.6 Study Limitations

The present study had limitation in that most of the samples used were mucoid and salivary.Purulent samples used were very few and there was no blood stained specimen. To overcome this limitation of small samples size analysis was done using Fishers exact test. However future studies should examine performance of tenacious sample in Xpert MTB/RIF using large sample size. Some of the samples used were from patients who were on anti TB treatment.

CHAPTER SIX

SUMMARY, RECOMMENDATION AND CONCLUSION

6.1 Summary of Findings

Sensitivity, specificity, positive and negative predictive value for ratio 2:1 was18.5, 86.0, 41.7 and 66.2 respectively, while for ratio 3:1 was 37.5,73.8,20.9 and 86.5 there was moderate agreement between the two dilutions Cohen kappa value of 0.476 at $p<0.001$.There was no statistically significant difference in MTB detection with McNemar test, two-tailed p value of 0.090 .

The present study reported increased probability of detection of *Mycobacterium tuberculosis* in purulent or tenacious samples.

6.2 Conclusion

i. Ratio 2:1SR pellet dilution in smear negative has a high specificity but low sensitivity compared to ratio 3:1SR pellet dilution.

ii. Positive predictive value of ratio 2:1 SR pellet dilution in smear negative sampleswas higher compared ratio 3:1 while negative predictive value was lower than that of ratio 3:1

iii. Tenacious or purulent samples are associated with increased probability of detection of smear negative Mycobacterium tuberculosis samples using Xpert MTB/RIF in pulmonary tuberculosis.

6.3 Recommendationfrom this study

i. There was moderate agreement between sample reagent pellet ratio 2:1 and 3:1 so both ratios are good.

ii. Ratio 3:1 sample reagent /pellet dilution has relatevely better negative predictive value compared to 2:1

iii. patients should be instructed to produce good quality sputumsample for gene Xpert testing since purulent samples are associated with increased probability of detection of pulmonary TB.

6.4 Recommendation for future Studies

i. There is need to evaluate sputum sample quality using largesample size of tenacious samples byfuture studies in smear negative TB.

ii. There is need to evaluate role of reduced sample reagent in smear negative using large sample size and in other sample types like gastric aspirate induced sputum and extra pulmonary samplesin smear negative.

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APPENDIXES

Appendix 1 Ethical Approval

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KEMRI/RES/7/3/1 **January 07, 2020**

TO: **DR. ELISHA OKEYO**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CGHR**
KISUMU

Dear Sir,

RE: **SSC PROTOCOL NO. 2693 *(REQUEST FOR ANNUAL RENEWAL)*: PLATFORM FOR ASSESSMENT OF TUBERCULOSIS TREATMENT OUTCOMES: AN OBSERVATIONAL STUDY OF INDIVIDUALS TREATED FOR PULMONARY TUBERCULOSIS (TB)**

Thank you for the continuing review report for period **January 21, 2019** to **November 29, 2019.**

This is to inform you that the Expedited Review Team of the KEMRI Scientific and Ethics Review Unit (SERU) was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **January 21, 2020** for a period of **one (1) year.** Please note that authorization to conduct this study will automatically expire on **January 20, 2021.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval by **December 09, 2020.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI
THE ACTING HEAD
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 2 CONSENT AND ASSENT FORM

Protocol Title:

TBTC Study 36

Platform for Assessment of TB Treatment OutcomesAn Observational Study of Individuals Treated for Pulmonary Tuberculosis (TB)

Tuberculosis Trials Consortium (TBTC)

Funded by the Centers for Disease Control and Prevention (CDC),

Atlanta, Georgia, USA

Short Title of the Study: "TBTC Study 36" or "Platform Study"

CONSENT FOR PARTICIPATING IN A CDC-FUNDED RESEARCH TRIAL

SITE LEADER: Kevin Cain **PHONE: +254 057-2022902**

PRINCIPAL INVESTIGATOR: ELISHA OKEYO **PHONE: +254721410654**

INTRODUCTION

The Kenya Medical Research Institute (KEMRI) and Centers for Disease Control and Prevention (CDC) are working together on this study to improve treatment of tuberculosis (TB). You are being asked to take part in this study because your doctors believe that you have TB of the lung.

We want to better understand how to test for TB and how to predict and prevent TB treatment failure and relapse. Taking part in this study is your decision. This document includes information on the purpose of the study, how it may help others, any risks to you, and what is expected of you if you decide to take part in the study.

YOUR PARTICIPATION IS VOLUNTARY

The information in this consent form will be discussed with you. Once you understand the study, and if you agree to take part, we will ask you to sign this form. You will be given a copy of the signed consent form to keep.

Before you learn about the study, it is important that you know the following:

Your participation is your choice (voluntary). You may decide not to take part in the study;

You may decide to withdraw from the study at any time without losing the benefits of your routine medical care.

WHY IS THIS STUDY BEING DONE?

There are two main purposes of this study. The first purpose is to collect sputum from persons with TB. The sputum will be tested at a local TB laboratory to improve our ability to detect TB before and during treatment. This research will help us know how best to use new laboratory methods to test for TB in your sputum.

The second purpose is to help better predict and prevent TB treatment failure and relapse. We will follow you closely while you take your TB medications.

HOW MANY PEOPLE WILL TAKE PART IN THIS STUDY?

Enrollment in this study is open-ended. We may enroll more than 1,000 participants.

HOW LONG WILL YOU BE IN THE STUDY?

You will be in the study for the length of your TB treatment plus about 12 additional months, which is about 18 months in total.

HOW WILL THE STUDY WORK?

We will collect medical information that you provide before enrollment. This information will include your medical history, smoking history, use of alcohol and recreational drugs, symptoms from TB, and current medications. You will also have height, weight, and temperature taken and undergo a physical exam. We will ask you to have a chest X-ray, if you have not already had one.

To qualify for the study, we will ask you to provide at least one sample of sputum before enrollment. We will also ask you to provide up to 15 mL (3 teaspoons) of blood to check for anemia, liver disease, and kidney disease.

We will also ask you to have an HIV test if:

You have never been tested, or

Your previous HIV tests were negative but you have not been tested in the past 3 months, or

You are HIV positive, but you do not have documentation of test results.

You may be asked to provide up to 5 mL (1 teaspoon) of blood for the HIV test. If the first test **result is** positive, we will repeat blood sample testing to confirm that your test is positive.

If **the result of** your first HIV test is positive, we will ask you to provide an additional 10 mL (2 teaspoons) of blood. This blood will be used to check the number and percent of your CD4 blood cells (lymphocytes) and the HIV viral load.

If you are a woman who can get pregnant, we will ask you to provide 10 mL (2 teaspoons) of urine or 5 mL (1 teaspoon) of blood to check for pregnancy.

For you to qualify for the study:

Your sputum must show the presence of TB, and

Your sputum culture must be positive for TB, and

Your sputum culture must show no resistance to one of the most important TB drugs currently available (rifampin/rifampicin).

If you do not qualify for the study, your doctor will make sure you receive treatment for your TB outside the study.

If you qualify for this study, you will receive the usual care for TB as determined by your doctor. This study does not affect your doctor's treatment decisions.

After enrollment, we will ask you to come to the clinic for a total of 12 study visits:

Weeks 2, 4, 6, 8, 13, 17, 22, 26

Months 9, 12, 15, and 18.

If your TB medicines are prescribed beyond Week 26, you will have monthly study visits until your TB treatment is completed and an additional study visit 12 months after completing treatment.

At these study visits, we will ask you about symptoms, review your TB medications (when you are on treatment), check your temperature and weight. Your doctor will conduct a physical exam. At Weeks 8 and 26 and at Months 12 and 18, we will ask you about recent smoking.

We will ask you to provide at least one sputum sample at Weeks 2, 4, 6, 8, 13, and 17. If you are not able to cough up anything, we will have you breathe in a mist of moist air with sterile salt water to help loosen any phlegm in your lungs so you can cough it up. This is **generally** not painful.

We will also ask you to provide up to 15 mL (3 teaspoons) of blood at Weeks 4 and 8 to check for anemia and liver disease.

If you have HIV infection, we will also ask you to provide 10 mL (2 teaspoons) of blood to check the number and percent of your CD4 blood cells (lymphocytes) and the HIV viral load at Week 26, Month 12, and Month 18.

If you have HIV infection, your doctor will make sure that you get the care you need for your HIV. You may continue to participate in this study.

If your TB gets worse during treatment, or if it comes back after going away (called a relapse), we will ask you to provide at least three more sputum samples. You may refuse to provide more sputum samples. We will also ask you to get a new chest X-ray. If you are a woman who can get pregnant, we will ask you to provide 10 mL (2 teaspoons) of urine or 5 ml (1 teaspoon) of blood to check for pregnancy. If your HIV tests were negative at enrollment, we will ask you to provide up to 5 mL (1 teaspoon) of blood or for a new HIV test. If the first test is positive, we will repeat the blood sample testing to confirm that your test is positive. If your second HIV test is positive, we will ask you to provide another 10 mL (2 teaspoons) of blood. This blood will be used to check the number and percent of your CD4 blood cells (lymphocytes) and the HIV viral load.

If your TB medicines stop working or if your TB returns, your doctor will make sure that you get the care you need for your TB.

In summary, before enrollment, 20 to 40 mL (4 to 8 teaspoons) of blood will be collected. At Weeks 4 and 8, 15 mL (3 teaspoons) of blood will be collected. For those with HIV infection, 40 mL (8 teaspoons) more of blood will be collected during of the study. Up to 10 mL (2 teaspoons) of blood may be collected to test women for pregnancy. This makes for a total of 50 mL to 120 mL (3 to 8 tablespoons) of blood that may be collected over the entire study. Up to 20 mL (4 teaspoons) of urine may be collected to test women for pregnancy in the study.

WILL ANY OF YOUR SAMPLES BE USED FOR TESTS OF YOUR GENES?

None of your samples collected in this study will be used for testing of your genes (DNA).

HOW WILL YOU GET TB MEDICATIONS FOR THE STUDY?

Your TB medicines will be given to you by your doctor or from other local sources.

WHAT WILL HAPPEN TO THE RESULTS OF THIS STUDY?

Your study doctor can provide you with the results of all blood and urine samples. Your study doctor can also provide you with the results of all standard tests for TB in your sputum samples.

You will not receive results for any research tests for TB in your sputum, since these results will not affect your clinical care.

Results of this study may be published in international medical journals so that other doctors and health workers might learn from this work. Please let KEMRI/CDC study staffs know if you would like to know the results of this study

WHAT ARE THE RISKS AND DISCOMFORTS OF THIS STUDY?

We will attempt to collect at least 3 mL (less than 1 teaspoon) of sputum at baseline, and at Weeks 2, 4, 6, 8, 13, and 17 of TB treatment (as applicable). If you are not able to cough up anything, we will have you breathe in a mist of moist air with sterile salt water in it. It takes about 15 minutes. This may help loosen any phlegm in your lungs so you can cough it up. This is generallynot painful, but coughing is sometimes uncomfortable. We understand that you may not always be able to provide this amount of sputum. There are no expected risks associated with providing a sputum sample.

The risks associated with taking blood could include bleeding, pain, infection, bruising, and inflammation of your vein.

There are no risks associated with providing a urine sample.

WHAT ARE THE BENEFITS OF THIS STUDY?

The results of research conducted on your sputum samples are not expected to directly benefit you. They will not change the way that your TB is being treated. However, these results may help with the future treatment of other people who have TB.

WHAT ABOUT PREGNANCY AND BREASTFEEDING?

If you are a woman who is able to become pregnant, we will ask you to take a pregnancy test before you join this study. Women who are pregnant or who are breastfeeding may join the study.

If you become pregnant during the study, tell study staff right away**. If you wish to remain in the study, we will continue to follow you in the study during your treatment.** You may go to another doctor to help manage your pregnancy if you choose to do so.

ARE THERE ANY COSTS OR PAYMENTS FOR BEING IN THIS STUDY?

There is no cost to you for being in this study. You will not have to pay for any medicines or tests or supplies provided to you as part of this study.

You will receive a payment of up to 500Kshs for participation in the study to cover travel expenses.

WHAT IF YOU DON'T WANT TO BE IN THE STUDY ANY LONGER?

Taking part in this study is your choice. You do not have to be a part of this study if you do not want to. If you choose to be a part of this study, you may change your mind and choose to stop taking part in this study at any time. Withdrawing from this study will not affect the benefits of your regular medical care. If you leave the study early, we may use your health information that we already have if it is needed for this study. Any samples already collected will remain part of the study.

WHO IS SPONSORING AND FUNDING THIS STUDY?

This study is sponsored and funded by the CDC through the Tuberculosis Trials Consortium (TBTC).

WHO HAS REVIEWED THIS STUDY?

This study has been reviewed and approved by the KEMRI Ethical Review Committee. The study has also been reviewed and approved by the CDC IRB and by the IRBs or Ethics Committees (ECs) at other participating sites.

CAN YOUR STUDY PARTICIPATION BE STOPPED WITHOUT YOUR CONSENT?

You may be taken off the entire study without your consent if:

Your study doctor decides that continuing in the study would harm you;

The study is cancelled by the sponsor (the CDC), or KEMRI ERC

You are in jail or prison.

HOW IS YOUR PRIVACY PROTECTED?

We will takeevery reasonable step to protect the privacy of your health information and to prevent misuse of this information. We will not use your name in any speech or paper about the study. You will be identified only by a code. Personal information from your records will not be released without your written permission.

Efforts will be made to keep your personal information private, but we cannot guarantee complete confidentiality. Your personal information may be released if required by law. We will not use your name in any speech or paper about the study.

Your medical and research records may be reviewed by the KEMRI EC/IRB, the CDC, the U.S. Office for Human Research Protections (OHRP), research staff, study monitors, and their designees. Also, the research staff at KEMRI/CDC is required to make sure that people not involved with this study do not have access to your research and medical records while

collecting personal information about you. They will keep your files in a locked cabinet in a safe place and will handle your personal information very carefully. This will also help to protect your privacy.

WHAT IF YOU ARE INJURED?

If you are injured because of being in this study, JOOTRH will give you immediate necessary treatment for your injuries. The cost for this treatment will be charged to you or your insurance company. The study cannot pay you for any care for study related injuries or for ill effects of TB.

WHAT IF YOU HAVE PROBLEMS OR QUESTIONS?

Questions about this research study, contact (Dr Elisha Okeyo KEMRI/CDC) by calling *(+254721410654*) or send an email to *(Eokeyo@kemricdc.org)*

Questions about your rights as a research subject, contact (the secretary of KEMRI Nairobi Ethical Review Committee) by calling ((020) 2722541 or 0722205901 fax (254) (020) 2720030) or send mail to (P.O. Box 54840- 00200 Nairobi Kenya) on advice on how to proceed.

If you think you are having a problem with the medicines used in this study or have been harmed by this study, contact (Dr Elisha Okeyo KEMRI/CDC) by calling ((+254721410654) or send an email to ((Eokeyo@kemricdc.*org*)

SIGNATURE PAGE FOR CONSENT TO PARTICIPATE IN STUDY 36: PLATFORM FOR ASSESSMENT OF TB OUTCOMES STUDY

If you have read the consent or if you have had it explained to you and understand the information, and you voluntarily agree to join this study, please sign your name below. You have been given a chance to ask questions and feel that they have been answered. You agree for your sputum sample and isolates to be stored or shipped for tests at the CDC laboratories at CDC, Atlanta. You know that after choosing to be in the study, I may withdraw at any time. I may also withdraw my stored samples at any time. You will receive a signed copy of this consent.

_____ _____
Name of Subject (please print)
_____ _____ _____

KEMRI/CDC ASSENT FORM: **Protocol Title:**

TBTC Study 36

Platform for Assessment of TB Treatment Outcomes

An Observational Study of Individuals Treated

for Pulmonary Tuberculosis (TB)

Tuberculosis Trials Consortium (TBTC)

Funded by the Centers for Disease Control and Prevention (CDC),

Atlanta, Georgia, USA

Short Title of the Study: "TBTC Study 36" or "Platform Study"

ASSENT FOR PARTICIPATING IN A CDC-FUNDED RESEARCH TRIAL

Flesch-Kincaid Grade Level 6.1

SITE LEADER: Kevin Cain **PHONE: +254 057-2022902**

PRINCIPAL INVESTIGATOR: Elisha Okeyo **PHONE: +254721410654**

INTRODUCTION

You are requested to participate in this study because your doctors believe you have TB of the lung. We would like to understand how to test for TB and how to predict if someone who is being treated for TB is not responding to medications or has become sick with TB shortly after completing treatment. Your participation in the study is voluntary and we will also request your parent or guardian to allow you to participate in the study. If you decide not to participate in the study or leave the study, you will still receive treatment for your TB disease.

How will the study work?

Before you join the study, we will collect information about your medical history. We will want to know how long you have been sick. We also want to know if you may have any other diseases and if you are taking any other medications. We will also measure your weight, height, temperature and check your body.

For you to be able to join the study, you will be requested to provide at least one sample of sputum before you start treatment. You will also be requested to provide up to 3 teaspoons of blood to check the state of your blood and body organs.

We will also ask you to get a chest X-ray. Women who can get pregnant will be asked to be tested to see if they could be pregnant**.** Those who are pregnant can continue to be in the study if they wish to be. We will refer to another doctor if they wish who will take care of their pregnancy**.**

You may also be requested to have HIV testing done. If the tests show you are HIV positive, we will also ask you to provide another 2 teaspoons of blood.

For you to be part of the study, your sputum must show that you have TB.

If you do not join the study, your doctor will make sure that you continue to receive treatment for your TB outside the study.

If you join this study, you will receive the usual care for TB as determined by your doctor.

After you join the study, we will ask you to come to the clinic for a total of 12 or 13 visits. These visits will be at weeks 2, 4, 6, 8, 13, 17, 22, 26, and, then at Months 9, 12, 15, and 18. If you must take TB medications after Week 26, you will have monthly study visits until your TB treatment is completed.

You will also have an additional study visit 12 months after completing treatment. At these visits, study staff will ask how you have been feeling and will also check your body.

We will ask you to provide sputum at Weeks 2, 4, 6, 8, 13, and 17. If you are not able to cough up anything, we will ask you to breathe in a mist of moist air with sterile salt water to help loosen any phlegm in your lungs so you can cough it up. This is generallynot painful.

At Weeks 4 and 8, we will also ask you to provide up to 15 mL (3 teaspoons) of blood to check your blood cells and the state of your body organs.

If you are HIV positive, we will ask you to provide 2 teaspoons of blood at Week 26, and at Months 12 and 18.

If you are HIV positive, your doctor will ensure that you get care for your HIV. You can still be in this study

If during treatment, your TB gets worse or if your TB comes back after going away we will ask you to provide three or more additional sputum samples. We will also ask you to get a new chest X-ray. Women who can get pregnant will be asked to be tested to see if they could be pregnant.

If your TB medications are no longer working or if your TB comes back again after it had gone,

your doctor will ensure that you get care for your TB.

WHAT ARE THE BAD THINGS ABOUT THIS STUDY?

We don't expect any bad thing to happen. There are no bad things with providing a sputum sample. If you are not able to cough up anything, we will ask you to breathe in a mist of moist air with sterile salt water to help loosen any phlegm in your lungs so you can cough it up. This is **generally** not painful.

In blood collection, there may be pain, or swelling of the place that was used to collect blood. The study staff will ensure that if anything bad happens, it is handled as required.

WHAT IF YOU DON'T WANT TO BE IN THE STUDY ANY LONGER?

Taking part in this study is your choice. You do not have to be a part of this study if you do not want to. If you choose to be a part of this study, you or your parent or guardian may change your mind and choose to stop taking part in this study at any time. Moving out from this study will not affect the medical care.

PERSONS TO CONTACT

If you have any questions about the study, feel free to ask them or you can request your parent or guardian to ask them on your behalf.

If you have more questions later, contact Dr Elisha Okeyo at +254721410654 or email Eokeyo@kemricdc.org. If you have questions about research participants' rights, you can call the secretary of the KEMRI Nairobi Ethical Review committee by calling (020) 2722541 or 0722205901 or send a mail to P.O. Box 54840-00200 Nairobi Kenya

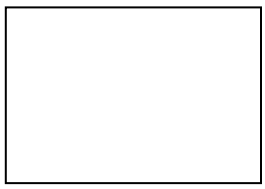
We will give you a signed copy of this form to take home.

AGREEMENT AND ASSENT TO PARTICIPATE IN <u>STUDY 36: PLATFORM FOR ASSESSMENT OF TB OUTCOMES STUDY</u>

Your signature below indicates that you had a chance to discuss this study and ask questions and that they were well answered and you agree to participate in this study. You also understand that you will only participate in the study if your parent/guardian also agrees. You agree for your sputum sample isolates to be shipped for tests at the CDC laboratories at CDC, Atlanta. You know that after choosing to be in the study, you may withdraw at any time. I may also withdraw my stored samples at any time. You will receive a signed copy of this consent.

Name of Subject (please print)

Signature of Subject **Date / (Time)**



Thumb-print of Subject **Date / (Time)**

Name of Witness (please print)

Signature of Witness ***Date / (Time)***

Signature of Person Obtaining Consent **Date / (Time)**

Name of Person Obtaining Consent

Appendix 3 laboratory standard operating procedures

6. Procedures

6.1. Preparation of decontamination reagent

6.1.1. Sodium hydroxide (NaOH), 4%

6.1.1.1. Sodium hydroxide pellets (analytical grade) 4 g Distilled water 100 ml

6.1.1.2. Dissolve NaOH in the distilled water. Sterilize by autoclaving at 121 ºC for 20 minutes.

6.1.2. Tri Sodium citrate, 2.9%

6.1.2.1. Weigh Tri Sodium citrate analytical grade 2.9 g 29g

6.1.2.2. Dissolve in the distilled water. Sterilize by autoclaving at 121 ºC for 20 minutes.

6.1.3. NALC–NaOH solution freshly prepared

6.1.3.1. Mix equal volumes of solutions 1 and 2. Add 0.25 g *N*-acetyl *L*-cysteine (NALC) to 50ml falcon tube just before use.

6.2. PreparationPhosphate buffer PH 6.8

6.2.1. Formula for phosphate buffer per 500ml purified water.

6.2.1.1. Dissolve 9.47g of Na_2HPO_4 into 1000mls of distilled water.

6.2.1.2. Dissolve 9.04g of KH_2PO_4 into 1000mls of distilled water

6.2.1.3. Mix the two solutions

6.2.1.4. Autoclave buffer at 121 ºC for 20 minutes

Check PH with meter.PH should be 6.8-7

6.2.2. Phosphate buffer tablets

6.2.2.1. Dissolve one tablet in 100ml of distilled water

6.2.2.2. Confirm PH for every new lot

6.2.2.3. Autoclave and aliquot for use

6.3. Preparation of auramine staining solution

Auramine – phenol stain Auramine is a potential cancer causing agent – always wear gloves and clean any spills immediately. Phenol crystals and vapor are corrosive, toxic, and may cause burns; avoid contact with skin and mucous membranes, prepare in a well ventilated area

6.3.1. Add 100ml of ethanol (or methanol) to a one-litre glass flask

6.3.2. Add 1.0g of auramine powder, mix until dissolved completely

6.3.3. Do not use heat since this can inactivate the auramine

6.3.4. Label “1.0% auramine in alcohol”, date and initial

6.3.5. Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

6.3.6. Solution B

6.3.7. Dissolve 30g of phenol crystals in 900ml distilled water, mix

2. Label the bottle “3% phenolic solution for auramine”, date and initial

Store in a dark bottle in a cupboard at room temperature (expiry 12 months)

6.3.8. Decolorizing solution: Acid Alcohol 0.75%

6.3.8.1.	Sodium Chloride	0.5 g.	10 g.
6.3.8.2.	Distilled water	20 ml.	200 ml.
6.3.8.3.	Hydrochloric acid (conc.)	0.75 ml.	15 ml.
6.3.8.4.	Ethyl alcohol (absolute)/methanol	100 ml. To	2000 ml.

6.3.8.5. Dissolve Sodium chloride in distilled water.
6.3.8.6. Add gradually the acid to NaCl solution
6.3.8.7. Top up to the required volume with absolute ethyl alcohol.
6.3.8.8. Label and store at RT (stable for three months)

6.3.9. Counter stain: Potassium Permanganate

6.3.9.1.	Potassium permanganate	0.1 g.	1 g.
6.3.9.2.	Distilled water	100 ml.	1,000 ml.

6.3.9.3. Dissolve potassium permanganate crystals in distilled water.
6.3.9.4. Filter using whatman filter paper.
6.3.9.5. Label the stain with the name and date of preparation and expiry.
Store in an amber bottle at room temperature. (Stable for three months