ANTIMYCOBACTERIAL ACTIVITY OF *PERSEA AMERICANA* AND CONVENTIONAL DRUGS SUSCEPTIBILITY AGAINST MOLECULAR ISOLATES OF MYCOBACTERIA FROM NEW CASES OF PULMONARY TUBERCULOSIS IN KISUMU COUNTY, KENYA

BY

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SCHOOL OF PUBLIC HEALTH AND COMMUNITY DEVELOPMENT

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DECLARATION

I declare that this thesis is a presentation of my original research work and has not been submitted wholly or in part for any award in any institution.

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May the Almighty God bless you abundantly.

DEDICATION

I dedicate this thesis work to my beloved Dad, Samuel Ouno Arasa and loving Mum, Monicah Kemuma, who have always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve. Special dedication to my wife Margaret Bosibori and my son Gylan Tim Nyambane for their care and love. It is also dedicated to all my siblings for their continuous encouragement during this tough period. My love for you all can never be quantified. God bless you.

ABSTRACT

Pulmonary tuberculosis (PTB) remains a major cause of morbidity and mortality worldwide. Challenges in the diagnosis and identification of the causative agent among members of mycobacteria in TB patients due to low sensitivity of smear microscopy leading to misdiagnosis and subsequently treated blindly is of great concern. Emergence and spread of multi-drug resistant *Mycobacterium tuberculosis* complex (MTBC) strains and non tuberculous mycobacteria (NTM) whose treatment is not directly analogous to that of *M. tuberculosis* poses challenges to disease control. Continued surveillance of drug susceptibility patterns help select effective or modify treatment regimen for better management of patients and timely control of the spread of MDR-TB. Tuberculosis drugs used for a long duration are toxic leading to adverse effects which has necessitated patients to look for alternative therapy which is medicinal plants such as *Persea americana*. The aim of this study was to investigate the molecular types and antimycobacterial activity of *P. americana* pod extracts and conventional first line anti-tuberculosis susceptibility pattern against mycobacteria isolates from new cases of pulmonary TB patients. Specific objectives were to determine the sensitivity of smear microscopy, geneXpert *MTB/RIF* and culture tests in sputum samples, identify the molecular types of mycobacteria isolates, determine drug susceptibility patterns of first-line anti-TB drugs against MTBC isolates and to determine the antimycobacterial activity of *P. americana* chloroform, *n*-hexane and ethanol pod extracts. A cross-sectional descriptive study was conducted between February 2016 and August 2017 that engaged 343 new cases of PTB patients at Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) and Kisumu County Hospital who gave sputum samples. Study participants were recruited consecutively from consenting individuals with smear and/or GeneXpert positive. Sputum samples were collected to saturation. Age and gender of the participants were recorded into standard data capture forms. Sensitivity of smear microscopy and GeneXpert *MTB/RIF* was done using culture as the reference standard. Mycobacteria subspecies were identified from positive MGIT tubes using GenoType® Mycobacterium common mycobacteria/ additional species and MTBC assays. Drug susceptibility test was done using BACTEC MGIT 960 SIRE (streptomycin, isoniazid, rifampicin and ethambutol) and PZA (pyrazinamide) systems. Antimycobacterial activity of *P. americana* pod extracts was performed on twenty selected MTBC (16) and NTM (4) isolates. Descriptive statistics were used to find the sensitivity of smear microscopy culture and GeneXpert test results. Fisher's exact test was used to assess the associations between patient characteristics and MTBC and NTM species identified. All statistical analyses were performed in STATA version 13.0 with significant threshold set to $P < 0.05$ at 95% confidence interval (CI). Culture results indicated that 290 (75.8%) were smear positive and 53 (24.2%) negative; while 317 (92.4%) and 26 (7.6%) were GeneXpert positive and negative, respectively, while 316/343 (92.1% were culture positive and 27/343 (7.9%) culture negative. Smear microscopy and GeneXpert had a sensitivity of 83.5% and 91.8% respectively compared to culture test. 290 (91.8%) of the 316 were MTBC and 26 (8.2%) were NTM. Subspecies among the MTBC isolates were *M. tuberculosis* 283/290 (97.6%), *M .africanum* 5/290 (1.7%) and *M. bovis* 2/290 (0.7%)*;* whereas the NTM were: *M .intracellulare* 16/26 (61.5%), *M .fortuitum* 2/26 (7.7%), *M. kansasii* 3/26 (11.5%) and *M. abscessus* 5/26 (19.2%). Out of 283 *M. tuberculosis* isolates, mono resistance to specific drugs was as follows: highest in INH and PZA 17 (6%), EMB 8 (2.8%), STR 3 (1.1%) and the least 1 (0.4%) RIF. Double resistance was: 4 (1.4%) INH + PZA and 2 (0.7%) INH + EMB. Triple resistance was: 1 (0.4%) in both STR + INH + PZA and INH + EMB + PZA. Quadrupal resistance was 1 (0.4%) STR + INH + EMB + PZA. Pentadrupal resistance was 4 (1.4%). Chloroform pod extract inhibited the growth of all isolates, while ethanol extract was active against 19 isolates with MIC range between 18.75 – 75mg/ml. *N*-hexane extract was less potent, active against 17 isolates with MIC range between 150-375mg/ml. Culture and GeneXpert tests were more sensitive in the detection of mycobacteria. About 98% of PTB due to MTBC was caused by *M. tuberculosis* and about 62% due to NTM was caused by *M. intracellulare.* The highest mono resistance was in INH and PZA and low MDR rate was revealed. Chloroform extract had the highest antimycobacterial activity, followed by ethanol, and *n*-hexane extract had the least. Accessibility to GeneXpert and culture tests in health facilities should be increased. There is need for molecular characterization of strains underlying MTBC resistance. Treatment of PTB should be guided and reviewed based on regional DST results. Chloroform should be used as an extraction solvent and further separation of the bioactive compounds in *P. americana* that can be used in the development of therapeutic agents for the treatment of pulmonary TB.

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LIST OF ABBREVIATIONS AND ACRONYMS

AFB: Acid fast bacilli

AG: Arabinogalactan ` `

AIDS: Acquired immunodeficiency syndrome

ARDS: Adult respiratory distress syndrome

AS: Additional species

ATCC: American type culture collection

ATS: American thoracic society

CDC: Center for Disease Control

CHCl: Chloroformic

CM: Common mycobacteria

Cs: Cycloserine

DALYs: Disability-adjusted life years

DLTLD: Division of Leprosy, Tuberculosis and Lung Disease

DMSO: Dimethyl sulfoxide

DOTS: Directly observed therapy short-course

DTH: Delayed type hypersensitivity

EMB: Ethambutol

EPTB: Extra pulmonary tuberculosis

ERDR: Ethambutol resistance determinant region

EtOH: Ethanol

FDA: Food and drug administration

HIV: Human immunodeficiency virus

IGRA- Interferon gamma release assay

INF-γ: Interferon gamma

INH: Isoniazid

JOORTH: Jaramogi Oginga Odinga Teaching and Referral Hospital

KEMRI: Kenya Medical Research Institute

Km: Kanamycin

KNTPR: Kenya national tuberculosis prevalence report

KNTLLDP: Kenya's National Tuberculosis, Leprosy and Lung Disease Program

LAM: Lipoarabinomannan

Lfx: Levofloxacin

LPA: Line probe assays

MAC: *Mycobacterium avium* complex

MDR TB: Multi-drug resistant tuberculosis

MGIT: Mycobacterium growth indicator tube

MIC: Minimum inhibitory concentration

MIRU: Mycobacterial interspersed repetitive unit

MOH: Ministry of Health

MTB: *Mycobacteria tuberculosis*

MTBC: *Mycobacterium tuberculosis* complex

NAAT: Nucleic acid amplification tests

NaOH-NALC: Sodium Hydroxide-N-acetyl-L-cysteine

NIAID: National Instutute of Allergy and Infectious Diseases

NTM: Non tuberculous mycobacteria

OADC: Oleic acid-albumin-dextrose-catalase

PAMPs: Pathogen associated molecular patterns

PANTA: polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin.

PAS: Para-aminosalycylic acid

PCR: Polymerase chain reactions

PLWHA: People Living with HIV/AIDS

PRRs: Pattern recognition receptors

PTB: Pulmonary tuberculosis

PTO: Prothionamide

PZA: Pyrazinamide

R: Relapse

REMA: Rasazurin microtiter assay

RFLPs: Restriction fragment length polymorphism

RGM: Rapid grower mycobacteria

RIF: Rifampicin

RRDR: Rifampicin resistance-determining region

RT-PCR: Real time polymerase chain reactions

SGM: Slow growing mycobacteria

SMP: Smear positive

STR: Streptomycin

TAZ: Thiazetazone

TB: Tuberculosis

TBM: Tuberculous menengitis

TF: Treatment failure

TLR: Toll-like receptors

TNF-α: Tumor necrosis factor-alpha

TST: Tuberculin skin test

UNAIDS: The Joint United Nations Programme on HIV and AIDS

VNTR: Variable number tandem repeat

WHO: World health organization

XDR TB: Extensively drug resistant tuberculosis

ZN: Ziehl Neelsen

DEFINITION OF TERMS

Multi-drug resistant TB (MDR-TB): Resistance to at least isoniazid and rifampicin

Extensively-drug resistant TB (XDR-TB): MDR-TB that is also resistant to any fluoroquinolone and at least one of three injectable second-line agents (amikacin, kanamycin, or capreomycin).

Incidence: The number of new and relapse cases of TB arising in a given time period, usually one year

Prevalence: The number of cases of TB at a given point in time

Mortality: The number of deaths caused by TB in a given time period, usually one year

Droplet nuclei: Tiny droplet of fluid containing tubercle bacilli

A definite case of tuberculosis: Is one in which a health worker (clinician or other medical practitioner) has diagnosed TB and has decided to treat the patient with a full course of TB treatment. Any person given treatment for TB should be recorded as a case.

New case of tuberculosis: Those patients who had no prior exposure to anti-tuberculosis drugs.

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CHAPTER ONE

INTRODUCTION

1.1. Background Information

The increasing prevalence of tuberculosis (TB) is becoming a major global health problem and has been ranked second leading cause of death after human immunodeficiency virus (HIV) (WHO, 2018). In 2017, an estimated 10 million new cases of active TB occured worldwide, equivalent to 133 cases per 100,000 population and caused an estimated 1.3 million deaths. (Global Tuberculosis Report, 2018). Women of reproductive age are the most affected by TB (WHO, 2012). Overall, a relatively small proportion (5-15%) of the estimated 1.7 billion people infected with *M. tuberculosis* will develop TB disease during their lifetime (WHO, 2017). However, the probability of developing TB disease is much higher among people living with HIV (PLWHIV), and also higher among people affected by risk factors such as under-nutrition, diabetes, smoking and alcohol consumption (WHO, 2017).

Tuberculosis is the most common presenting illness among immunocompromised people and it is the major cause of HIV-related deaths (WHO, 2013). People living with HIV are 26-31 times likely to develop TB than persons without HIV (UNAIDS, 2018). One-third of the estimated 40 million PLWHIV and AIDS are co-infected with TB, with Africa harbouring 29% of those infected (WHO, 2012). Sub-Saharan Africa bears the brunt of the dual epidemic, accounting for approximately 78% of the estimated burden in 2013 (WHO, 2018). The HIV and AIDS have profound impact on the TB epidemic in Kenya, where up to 60% of TB patients are HIV co-infected and the mortality rate attributed to TB in this group being above 130 per 100, 000 (DLTLD, 2009). Nearly 2 people die an hour from TB in Kenya despite effective treatment being available (Global Tuberculosis Report, 2018).

Kenya is ranked thirteenth among the 30 high TB burden countries globally and as the fifth highest burden in Africa. It is the 4th leading cause of death and TB epidemic affects the young (15-34yrs), who are in the economically productive age groups (WHO, 2018). In 2015, upto 81,518 TB cases were identified and treated. According to Kenya Tuberculosis Prevalence Survey 2015-2016, there is more TB in Kenya than is thought: 2015 WHO estimated 233 per 100,000 population, 558 per 100,000 population according to 2016 prevalence survey estimate which translates to approximately 138,105 TB incident cases per year. Tuberculosis prevalence in men is twice as high as women (809 vs 359 per 100,000) and are more likely to be missed. Prevalence among women aged 65 years and above was high. According to the survey, TB prevalence is highest in urban areas compared to rurals areas. The increasing TB burden in Kenya has also been attributed to other factors such as malnutrion, increasing poverty, and overcrowding (Ramachandran *et al.*, 2009). In Kisumu County the total cases notified in 2013 were 3,362 giving a case notification rate for TB at 317 per 100,000 which is above the national average of 217 per 100,000. The high burden of TB in Kisumu County has mainly been attributed to the high HIV prevalence (MOH, Annual Report 2013).

Rapid and accurate diagnosis of TB is a challenge in developing countries. Laboratory confirmed diagnosis of TB is pivotal for management of disease and reduce the transmission of infection. Improved detection of TB is considered a priority by WHO (Linda *et al.*, 2011). For the diagnosis of PTB the detection of acid fast bacilii (AFB) in expectorated sputum is still crucial, especially in developing countries of sub-Saharan Africa, where other facilities including sputum culture for *M. tuberculosis* are unavailable or are prohibitively expensive (Swai *et al*., 2011). When AFB is detected in sputum, the diagnosis of PTB is certain. However, diagnostic problem start when patients with suspected PTB have a negative sputum smear. Most laboratories use

Ziehl-Nelseen (ZN) stain to detect AFB in sputum which is a simple, rapid and cheap test but lacks sensitivity because about 5,000 bacilli per milliliter of sputum must be present for it to be positive, conversely, as few as 100 bacilli per milliliter may be required for a positive culture (Vignesh *et al*., 2007). With the sharp rise of pulmonary TB (PTB) in countries which are worst affected by the HIV epidemics, the number of patients with suspected PTB who are sputum smear negative have increased (Swai *et al*., 2011). For the diagnosis of PTB using ZN, most HIV patients have a challenge of presenting with smear negative sputum. Smear microscopy cannot differentiate *M. tuberculosis* complex (MTBC) from non-tuberculous mycobacteria (NTM) posing a challenge in diagnosis.

Kisumu County with TB prevalence rate of 31% (MOH, Annual Report, Kisumu County, 2015), AFB is the main diagnostic test used in Health facilities, arguemented with Genexpert *MTB/RIF* in some few facilities (Singh *et al*., 2013). With the invent of HIV associated TB with more frequent smear negative TB, new, rapid sensitive and specific tests such as GeneXpert *MTB/RIF* together with culture are required and should be incorporated in TB control programmes. Substantial numbers of patients are treated for TB without definitive diagnostic criteria (WHO, 2016). Regardless of great advances in molecular diagnosis, sputum cultures of mycobacteria remain the gold standard for PTB diagnosis. This formed the basis of the current study which assessed the sensitivity of culture, geneXpert *MTB/RIF* and smear microscopy diagnostic tools in sputum from new cases of PTB patients in Kisumu County.

The TB problem is compounded by the emergence of NTM as opportunistic infections in immunocompromised people (Griffith *et al*., 2007). Conventional identification of mycobacteria is achieved by standard culture and biochemical methods, all of which are time consuming. The

rise in NTM and other members of MTBC isolation demands faster methods for their identification and for selection of appropriate therapy. These NTM causes confusion in the treatment of TB in immunocompromised people as a result of misdiagnosis and over concentration in the treatment of *M. tuberculosis* TB while neglecting NTM, which acts as opportunistic infections in such patients (Varghese *et al*., 2013). The increasing cases of NTM Mycobacterioses (Mallory *et al*., 2000) could be misdiagnosed as TB and put on anti-TB chemotherapy, even though the treatment of NTM disease is generally not directly analogous to *M. tuberculosis* TB treatment (Griffith *et al*., 2007). Multi-drug regimes (second-line drugs) are used for NTM disease treatment and require prolonged durations of therapy up to two years, aimed to facilitate clearance of the mycobacteria and minimize the emergence of drug resistance (Griffith *et al*., 2007). This prolonged durations of therapy leads to toxicity and other side effects such as diarrhea, colitis and even death (Varghese *et al*., 2013).

The *Mycobacterium tuberculosis* complex (MTBC) is composed of the closely related species that causes PTB. It is important to differentiate MTBC species to distinguish between strict human and zoonotic TB and for an appropriate initiation of therapy (Djelouadji *et al.,* 2008). Therefore, clear differentiation to the species and subspecies levels should be obtained for epidemiological purposes and adequate treatment of each patient (Scorpio *et al*., 1996).

Programs such as Stop TB partnership target to reduce TB prevalence and death rates by 50% relative to 1990 and eliminating TB as a public health problem (less than 1 case per million population) by the year 2050. This requires the development of new drugs, vaccines, diagnostic approaches such as Xpert *MTB/RIF* for MDR and molecular techniques such as GenoType Mycobacterium CM/AS and MTBC assays for rapid identification of mycobacteria to species level. Because of this, new, rapid and more accessable molecular diagnostic techniques are

required for differentiation of mycobacteria species (Lee *et al.,* 2009). Therefore, the second aim of this study was identification of MTBC, NTM and their species differentiation in positive liquid cultures from new cases of PTB patients using the rapid molecular technique a commercially available GenoType CM/AS and MTBC assays.

Drug-resistant TB is a major public health problem. The emergence and spread of multi-drug resistant (MDR) MTBC strains poses significant challenges to disease control (WHO, 2010). The prevalence of MDR-TB is increasing throughout the world in both new TB cases as well as previously-treated TB cases (Prasad *et al*., 2005). Several factors cause the emergence of drug resistant TB, of which the three most important ones being previous treatment with anti-TB drugs which may be inappropriate and incomplete, high prevalence of drug resistant TB in the community and contact with a patient known to have drug resistant TB (Urassa *et al*., 2008). Treatment-naive patients are also at risk of developing MDR-TB due to either spontaneous mutations or transmission of resistant strains (Vijay *et al*., 2004). In patients with previous treatment or disease, the odds of resistant TB were 4-7 times higher than that of person with no history of past treatment (WHO, 2008). Globally, WHO estimated 630,000 cases of MDR-TB among the world's 12 million prevalent cases of TB worldwide in 2011 (WHO, 2011). Worldwide, 3.7% of new cases and 20% of previously treated cases were estimated to have MDR-TB (Global Tuberculosis report, 2013).

In Kenya, MDR-TB has been reported since the 1980s (Githui *et al.*, 1998). According to NTLD, it is estimated that there may have been as many as 1,300 (910-1,700) MDR/RR-TB among notified pulmonary TB cases in Kenya in 2016 (NTLD, 2017). Multi-drug resistant-TB patients are either not receiving treatment or have been allowed out of hospital because the government does not have money to treat them. According to the WHO, an MDR patient infects 10-15 people

every year (WHO, 2017). Treatment for MDR-TB lasts for 18 months but can extend to two years or more because it is difficult to cure and drugs used for treatment are less potent, more toxic and 50-200 times more expensive than first-line drugs (Ndung'u *et al*., 2011). If not properly treated it can result in complications that may require surgical interventions increasing period of hospitalization and raising the cost of treatment even higher (Ndung'u *et al*., 2012). The country has 146 health facilities with Xpert *MTB/RIF* used for TB and rifampicin resistance screening and 4225 treatment facilities/ sites but they do not perform drug susceptibility test (NTLD, 2017).

Drug susceptibility patterns of mycobacteria isolates is essential for the proper treatment of PTB caused by MTBC. In Kenya, drug susceptibility test for TB is not done for routine diagnosis but for research in institutions such as Kenya Medical Research Institute (KEMRI) and National Tuberculosis Reference Laboratory (NTRL). Thus little is known on anti TB drug susceptibility patterns in Kenya more especially in Kisumu County in new cases of PTB. Therefore, the current study was conducted with the specific objective to evaluate the *in vitro* drug susceptibility pattern of MTBC isolates against five conventional first line anti-TB drugs in new cases of PTB patients in Kisumu County.

Investigation of medicinal plants by ethno-botanists as an alternative to the existing synthetic medicines has been on the increase (Parekh *et al*., 2006); this is because of the fact that most synthetic/man-made medications are now progressively losing their potency to pathogens (Ncube *et al*., 2008). In addition to the multiple resistance developed by pathogens against the synthetic antibiotics, most of these drugs have a major setback due to the side effects on the patients. Medicinal plants are likely to be more tolerated by humans than the synthetic antimicrobial agents, because plants are products of nature (Krishna *et al*., 2012). Conventional anti-TB drugs

are administered for a long druration which raises challenges of adherence and the resultant possibility of developing MDR-TB strains. The emergence of drug resistance of TB drugs and the side effects caused by these drugs due to their toxicity has lead to people looking for alteranative therapy which is herbal medicine that can offer hope for the development of alternate medicines for the treatment of TB. To promote proper use of herbal medicine and determine their potential as a source of new drugs, it is essential to study medicinal plants which have folklore reputation in more intensified way (Tijjani, 2013). Plant-derived antimycobacterial compounds belong to an exceptionally wide diversity of classes, including terpenoids, alkaloids, peptides, phenolics and coumarins. Hence medicinal plants remain an important resource to find new therapeutic agents (Peng *et al*., 2015). The advantages of using antimicrobial compounds from medicinal plants include fewer side effects, better patient acceptance due to long history of use, reduced costs and cultivability rendering them renewable in nature (Hostettmann & Marston, 2000).

Persea americana is one of the 150 varieties of avocado pear (Pacific Health, 2005) with diverse application in ethnomedicine, ranging from treatment for diarrhea, dysentery, intestinal parasites and skin treatment (Gomez-Flores *et al.,* 2008). One study done by Jimenez-Arellanes *et al*., (2013) in Mexico showed that the chloroformic seed extracts of *P. americana* inhibits the growth of *M. tuberculosis.* In many parts of the world, the leaf, stem, bark and seeds of *P. americana* are used traditionally for the management, control and for treatment of an array of human disorders such as prostate cancer (Lu *et al.,* 2005), wound healing (Nayak *et al.,* 2008), parasitic skin diseases (Owolabi *et al.,* 2005). The leaf extract has also been shown to have antifungal properties (Lu *et al.,* 2005). Methanol and aqueous extracts of root and stem bark of *P. americana* has exhibited some antibacterial activity (Ching *et al*., 2013), but there is no study

that has reported the antimycobacterial activity of *P. americana* pod extract. Therefore, this formed the basis of the last specific objective of the current study which determined the antimycobacterial activity of *P. americana* chloroform, *n*-hexane and ethanol pod extracts.

1.2. Statement of the Problem

Challenges in the detection of Mycobacteria in smear negative but GeneXpert *MTB/RIF* and culture positive sputum due to its low sensitivity poses a great challenge in the treatment of TB and plays a significant role in its transmission. The emergence of NTM in people with lowered immunity whose treatment is not the same as the anti-TB drugs used for the treatment of *M. tuberculosis* TB and the emergence of MDR-TB strains is a public health concern. Also NTM pulmonary TB is misdiagnosed as *M. tuberculosis* and patients are treated blindly with first line anti-TB drugs which eventually fails because majority of the NTM are resistant to some first line anti-TB treatment. Drug susceptibility patterns of MTBC isolated from new cases of PTB patients in Kisumu County is not known and therefore these patients end up being treated with anti-TB drugs that they do not respond to because some MTBC clinical isolates from the patients have varying patterns of drug susceptibility to them. This medication with wrong and ineffective anti-TB drugs leads to the emergence of MDR-TB strains. Conventional anti-TB drugs because of its long duration of use of more than six months, raising challenges of adherence and results into toxicity with/and adverse severe side effects make patients to seek for alternative therapy which include medicinal plants. Despite the fact that *P. americana* extracts from leaves, bark, stems have demonstrated antimicrobial activity, the activity of pod extract has not been scientifically elucidated against Mycobacteria. It was therefore necessary to assess the sensitivity of the diagnostic tools used for TB screening, perform molecular identification and differentiation of mycobacteria to species level, *in vitro* determination of drug susceptibility

patterns of MTBC isolates against first-line anti-TB drugs and antimycobacterial activity of *P. americana* chloroform, ethanol and *n*-hexane pod extracts against mycobacteria isolates from new cases of human PTB in Kisumu County.

1.3. General Objective

To assess the sensitivity of diagnostic tools used in pulmonary TB screening, investigate the molecular types and antimycobacterial activity of *Persea americana* pod extracts and conventional first line anti-tuberculosis susceptibility pattern against mycobacteria isolates from new cases of human pulmonary TB patients in Kisumu County, Kenya.

1.3.1. Specific objectives

1. To assess the sensitivity of culture, GeneXpert *MTB/RIF* and smear microscopy diagnostic tools in sputum samples from new cases of pulmonary TB patients
2. To identify the molecular types of MTBC and NTM isolates from new cases of PTB patients.
3. To determine the drug susceptibility patterns of first-line conventional anti-TB drugs against MTBC isolates among new cases of PTB patients.
4. To determine the antimycobacterial activity (MIC) of *P. americana* chloroform, ethanol and *n*-hexane pod extracts on Mycobacteria isolates from new cases of PTB patients.

1.4. Research Questions

1. What is the sensitivity of culture, GeneXpert *MTB/RIF* and smear microscopy diagnostic tools in sputum samples from new cases of pulmonary TB?
2. What are the molecular types of MTBC and NTM isolates from new cases of PTB patients?
3. What is the pattern of drug susceptibility of first line conventional anti-TB drugs against patterns MTBC isolates among new cases of PTB patient from new cases of PTB patients?

4. Do *Persea americana* chloroform, *n*-hexane and ethanol pod extracts have antimycobacterial activity on clinical mycobacterial isolates from new cases of PTB patients?

1.5. Justification of the Study

The diagnosis of new cases of human PTB poses a lot of challenges more so in the case of smear negative but GeneXpert *MTB/RIF* and culture positive sputum. Non-tuberculous tuberculosis and TB causing MTBC other than *M. tuberculosis* is misdiagnosed as *M. tuberculosis* and patients are treated blindly with anti-TB drugs which may eventually fail because majority of the NTM are resistant to first line anti-TB treatment. This also leads to the emergence of MDR strains. The emergence of MDR-TB strains has lead to patients being treated with first-line anti-TB drugs which leads to treatment failure because of the little or lack of knowledge of the susceptibility patterns of MTBC isolates. Infectious diseases caused by Mycobacteria species are prevalent in Kenya especially in rural areas where a variety of plants are used by the indigenous people to treat these diseases without any scientifically determined information. Traditional medical practitioners in Nyando, Kisumu County have demonstrated the potential medicinal value of *P. americana* pod extract against Mycobacteria TB causing species MTBC and NTM. However, there lacks a scientific documentation to confirm this antimycobacterial potential. To confirm the medical potential of this underutilized product a scientific research is imperative to elucidate its medicinal value. The evaluation of *P. americana* pod extract for antimicrobial activity is necessary, and also as a possible lead for new drugs or herbal preparations. This study provides valuable information for alternative treatment for human TB. Anti-TB drugs are administered for long duration with high frequencies of adverse effects and death. This underscores the need to explore for new and effective drugs to help in the control of both MTBC and NTM strains. The

antimycobacterial activity exhibited by the pod extracts against mycobacteria clinical isolates, may provide scientific justification for the ethnomedicinal uses of the plant.

1.6. Significance of the Study

Smear microscopy arguemented with culture or/and rapid molecular diagnostic technique will reduce cases of TB misdiagnosis hence transmission and will help to differentiate NTM from MTBC TB thus aid in the selection of appropriate therapy. Knowledge of drug susceptibility pattern among members of MTBC against first-line anti-TB drugs in newly infected patients in Kisumu county will guide physicians in proper prescription of appropriate TB drugs for the proper clinical treatment of patients to avoid the emergence of MDR-TB strains. This will also prevent patients from using ineffective anti-TB drugs for a long period which are toxic with diverse effects and even death. Human PTB treatment regimens takes a long duration. The most effective and important thing is to determine an alternative medicine to solve this problem of anti-TB administration for a very long duration. Scientific clarification on the use of *P. americana* pod extract in the treatment of mycobacterial infections is very necessary. *Persea americana* pod extract was found to have antimycobacterial activity, this will form the basis for the isolation and exploitation of the active compound that will lead to drug development for the treatment of mycobacterial infections which do not respond to conventional first line and second line anti-TB drugs. Emergence of NTM MDR-TB strains calls for a speedy search of new and novel anti-mycobacteria drugs. Prolonged treatment of MDR TB by the currently used conventional second line drugs necessitates that a fast acting drug that takes a shorter period to realize positive clinical recovery is long overdue. This research will therefore be beneficial to all stakeholders with an alternative to the treatment of TB infections currently with six months treatment on the cocktail of various antibiotics. The result of the present study signifies the

potential of *P. americana* pod extract as a source of therapeutic agents, which may provide leads in the ongoing search for antimicrobial agents from plants.

1.7. Study Limitations

Decontamination with the Sodium hydroxide-N-acetyl-L-cysteine method is recommended. Digestant/decontaminant solutions may have harmful effects on mycobacteria. Low concentration of 4% Sodium hydroxide was used in reconstituting the decontaminant which is not harmful to mycobacteria. Mycobacteria growth indicator tubes which were instrument-positive may contain one or more species of mycobacteria. Faster growing mycobacteria may be detected prior to slower growing mycobacteria; therefore, it is important to sub-culture positive MGIT tubes to ensure proper identification of all mycobacteria present in the sample. While using the MGIT culture media for culturing Mycobacteria, the use of MGIT PANTA antibiotic mixture, although necessary for all non-sterile specimens, may have inhibitory effects on some mycobacteria. There was difficulty in ascertaining new cases of TB because patients were recruited based on the information they gave.

CHAPTER TWO

LITERATURE REVIEW

2.1. Epidemiology of TB

Tuberculosis is an infectious bacterial disease caused by some members of the genus Mycobacteria. The disease generally affects the lungs, but can also affect other parts of the body. Most infections do not have symptoms, in which case it is known as latent TB. About 10% of latent infections progress to active disease which, if left untreated, kills about half of those infected (Dolan *et al*., 2016). The classic symptoms of active TB are a chronic cough with blood-containing sputum, fever, night sweats, and weight loss (Getahun *et al*., 2015).

About 95% of the reported TB cases and 98% of the resulting deaths are from developing countries, of which 23% and 55% of these deaths occur in South Asia and sub-Saharan Africa respectively (WHO, 2014). The World Health Organization (WHO) confirmed the re-emergence of TB disease, and consequently declared the disease a global emergency and public health concern in 1993 as a result of the HIV and AIDS pandemic in the early 1980s (Kirimuhuzya, 2012). Women of reproductive age are the most affected by TB, with statistics of 300,000 deaths among HIV-negative and 200,000 deaths among HIV-positive individuals (WHO, 2012). Tuberculosis became a re-emerging disease to European and North-American nations due to HIV and AIDS pandemic and the influx of immigrants with no previous adequate TB prevention measures **(**Morens *et al*., 2004).

The genus Mycobacterium contains more than 100 species. They are aerobic, nonmotile, non-spore forming, Gram-positive, straight or slightly curved rods measuring 1.5 to 4 µm long and 0.3 to 0.5µm wide. Most of them are classified as tuberculous mycobacteria belonging to the *M.*

tuberculosis complex (Brosch *et al*., 2002) and non-tuberculous mycobacteria (NTM) (Tortoli, 2009).

2.2. *Mycobacterium tuberculosis Complex* (MTBC)

The MTBC is composed of the closely related species. which include: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, "*M. canetti*", and *M. pinnipedi*. *Mycobacterium bovis* comprises of *M. bovis* subsp. *bovis*, *M. bovis* subsp. *caprae*, and the *M. bovis*-derived BCG vaccine strain; *Mycobacterium africanum* includes two subtypes, I (West African strain) and II (East African strain); *Mycobacterium microti*; and "*M. canetti*" may be a subspecies of *M. tuberculosis* (Brosch *et al.*, 2002; Niemann *et al.*, 2002).

Although MTBC species are closely related genetically, they differ widely in their host tropisms, geographic distribution, certain phenotypes, and pathogenicity (Tortoli, 2009). *Mycobacterium tuberculosis* is the most significant pathogen for humans in Europe and America, whereas *M. africanum* strains is widely distributed among African patients (Niemann *et al*., 2000). Both subspecies of *M. bovis* are reported to infect humans, yet they have a broad host range, including wildlife and domestic livestock like bovines and goats, *M. pinnepedii* infects seals, and *M. microti* infects rodents (Kubica *et al*., 2003; Wedlock *et al*., 2002). Smear microscopy by ZN stain cannot differentiate mycobacteria species in sputum samples from PTB patients. Yet it is important to differentiate MTBC species to distinguish between strict human and zoonotic TB and to initiate an appropriate therapy. In particular, the distinction between *M. tuberculosis*, *M. africanum* and *M. bovis* is necessary, as the latter species is naturally resistant to the first-line anti-TB drug pyrazinamide (Djelouadji *et al*., 2008). This forms the basis of the present study which involved molecular identification of mycobacteria to species level where *M. bovis* was isoalated from new cases of PTB patients.

2.3. Non-tuberculous Mycobacteria (NTM)

The NTM are ubiquitous microorganisms that are present in the body secretions due to environmental contamination, colonization or true disease. They form a group of organisms diverse in many characteristics, including pathogenicity and clinical disease (van Ingen *et al*., 2009). Historically, human infections due to Mycobacteria were due almost exclusively to *M. tuberculosis*; the extensive societal impact of this infection is legendary. More recently, other species of mycobacterium causing clinical disease have been identified and, in many geographical regions, cause greater disease burden than TB (Kankya *et al*., 2011). These organisms are referred to by a variety of collective names—anonymous or atypical mycobacteria, mycobacteria other than tuberculosis (MOTT) and NTM. They encompass all mycobacteria other than the species belonging to the MTBC and *Mycobacterium leprae.* Many NTM are saprophytic environmental species, and some may cause disease in humans (Margaret *et al.,* 2014). They are associated with a broad spectrum of clinical presentations from pulmonary to cutaneous or disseminated disease. Their pathogenic potential is based on the species and they have been isolated from TB-HIV and AIDS coinfected patients in western Kenya (Nyamogoba *et al*., 2012). There is uncertainty regarding clinical relevance of many of the species, particularly in settings where facility to isolate and identify them were recently established (van Halsema *et al*., 2015).

The *Mycobacterium avium* complex (MAC) is the most commonly encountered group of NTM where the clinically most important members are *Mycobacterium intracellulae* and *Mycobacterium avium* (Biet *et al.,* 2005). Other NTM species include *Mycobacterium chelonae, Mycobacterium kansasii, Mycobacterium marinum* and *Mycobacterium fortuitum* (van Ingen *et al*., 2009). Non tuberculous mycobacteria differ from *M. tuberculosis* in that they typically are

not transferred from patient through airborne contact (with the exception of *M. leprae*). Thus, NTM either colonizing or infecting the pulmonary tract are not considered contagious. For the majority of NTM, the most common single organ system affected is the lung. Most NTM elicit granulomatous inflammation, and many establish cavity disease that often is difficult to distinguish from TB based on clinical and radiological findings (Margaret *et al.,* 2014). In Kenya, the NTM are slowly becoming recognized as pathogens of major public health significance with the advent of HIV and AIDS (Nyamogoba *et al.,* 2012). Kisumu County is one of the counties in Kenya with the highest prevalence of HIV and AIDS at 19.9% (Kenya HIV Estimates Report, 2018).

2.4. Global Burden of Tuberculosis

An estimated 10.4 million people (90% adults; 65% male; 10% people living with HIV) fell ill with TB in 2016. Most of the estimated number of incident cases in 2016 occurred in the WHO South-East Asia Region (45%), the WHO African Region (25%) and the WHO Western Pacific Region (17%); smaller proportions of cases occurred in the WHO Eastern Mediterranean Region (7%), the WHO European Region (3%) and the WHO Region of the Americas (3%). The top five countries, with 56% of estimated cases, were (in descending order) India, Indonesia, China, the Philippines and Pakistan. Globally, the TB mortality rate is falling at about 3% per year (Global Tuberculosis Report, 2018).

Tuberculosis incidence is falling at about 2% per year; this needs to improve to 4–5% per year by 2020 to reach the first milestones of the End TB Strategy. Regionally, the fastest decline in TB incidence is in the WHO European Region (4.6% from 2015 to 2016). The decline since 2010 has exceeded 4% per year in several high TB burden countries, including Ethiopia, Kenya, Lesotho, Namibia, the Russian Federation, the United Republic of Tanzania, Zambia and

Zimbabwe (WHO, 2017). Regionally, the fastest declines in the TB mortality rate are in the WHO European Region and the WHO Western Pacific Region (6.0% and 4.6% per year, respectively, since 2010). High TB burden countries with rates of decline exceeding 6% per year since 2010 include Ethiopia, the Russian Federation, the United Republic of Tanzania, Viet Nam and Zimbabwe (WHO, 2017).

Globally, the proportion of people who develop TB and die from the disease (the case fatality ratio, or CFR) was 16% in 2016. This needs to fall to 10% by 2020 to reach the first milestones of the End TB Strategy. Between 2000 and 2016, TB treatment averted an estimated 44 million deaths among HIV-negative people. Among HIV-positive people, TB treatment supported by ART averted an additional 9 million deaths. Drug-resistant TB is a persistent threat, with 490,000 million cases of MDR-TB emerging in 2016 and an additional 110,000 cases that were susceptible to isoniazid but resistant to rifampicin (RR-TB), the most effective first-line anti-TB drug. The countries with the largest numbers of MDR/RR-TB cases (47% of the global total) were China with a prevalence rate of 5.7% new cases and 25.6% of previously treated cases (Zheyuan *et al*., 2016), India 3% (MOH, 2018) and in the Russian Federation, the prevalence of primary MDR TB varied from 5.4% to 28.3% (Raviglione & Sulis, 2016).

According to WHO (2016), South Africa is one of the countries with the highest burden of TB with an estimated incidence of 454,000 cases of active TB in 2015. About 0.8% of the population of about 54 million develop active TB disease each year. Out of the 454,000 incident cases in South Africa in 2015, it is estimated by WHO that about 57% (258,000) are HIV positive. It is also estimated that of 157,505 whose status is known, and who are known to be HIV positive, some 85% (133,116) are on antiretroviral therapy. The TB incidence rate reached its peak in 2009 at 832 per 100,000, and has since declined. The Eastern Cape, KwaZulu-Natal

and the Western Cape are the provinces which have the highest incidence rates in South Africa with reported rates of 692,685 and 681 per 100,000 respectively. The most notable decline has been in KwaZulu-Natal where the incidence has decreased from 1,185 to 685 per 100,000 over the last five years. (Global Tuberculosis Control, 2016; WHO, 2016). It is estimated that about 80% of the population of South Africa is infected with TB bacteria, the vast majority of whom have latent TB rather than active TB disease. The highest prevalence of latent TB, estimated at 88% has been found among people in the age group 30-39 years old living in townships and informal settlements (WHO, 2016).

Uganda is one of the 22 high TB burden countries in the world and in sub-Saharan Africa, which contribute 80% of the world's TB burden. It had an estimated 65,000 incident cases in 2012 (WHO, 2013). A major factor in the ongoing TB epidemic is the high burden of TB and HIV co-infected persons with about 50 per cent of the patients co-infected with HIV in sub-Saharan Africa contributing to increased progression, transmission and mortality due to TB (Kasprowicz *et al*., 2011). The true burden of TB in Uganda was not known until 2016 when it was found to be higher than previously thought (WHO, 2017). A national survey was conducted to provide a better estimate of the TB burden and assess the associated health seeking behavior among those reporting symptoms. The findings were TB prevalence was 253 per 100,000 people (previously 159 per 100,000 in 2015). Tuberculosis incidence was 234 per 100,000 people (previously 161 per 100,000) which was much higher compared to global TB incidence in 2016 at 140 cases per 100,000 population. 89,000 people get TB disease every year with 46% (40,829) undetected TB cases (missed cases) and each missed case spreads TB to 10-15 people per year. The survey also revealed that the prevalence of TB among men is 4 times higher than in women (734 per 100,000 versus 178 per 100,000). The burden of TB is highest amongst people aged 35-44 whereas the

15-24 age group has the highest missed cases. Urban areas have a higher burden (504 per 100,000) than rural areas (370 per 100,000).

In a TB screening in Uganda, the survey revealed that 16 out of 160 (10%) confirmed TB cases had a positive symptom screen alone. 63 out of 160 (39.4%) confirmed TB cases had both a positive symptom screen and an abnormal chest Xray. 81 out of 160 (50.6%) confirmed TB cases had an abnormal chest X-ray alone. Smear microscopy detected only 41.3% (66 out of 160) of the TB cases. GeneXpert detected 72 out of 73 (98.6%) culture positive TB cases. 61% (1655 out of 2714) of individuals with TB symptoms sought health care; 39% did nothing about their symptoms. 62.7% (1038 out of 1655) of individuals with TB symptoms sought care from public health facilities while 37.3% sought care from private health facilities including pharmacies and drug shops. 170 out of 1565 (10.3%) individuals with TB symptoms were investigated by sputum microscopy and 100 (6%) by chest - X-ray when they sought care (UNPBTPS, 2017).

Tuberculosis remains high in Kenya, and experts say the country lags in the fight against the disease (KNTPR, 2017). National Tuberculosis Prevalence Report (NTPR) 2017, revealed that there are more TB cases in Kenya than previously estimated/thought, with a TB prevalence of 558 per 100,000 people in 2015. In the same year (2015), the WHO had estimated that Kenya's TB prevalence rate was 233 cases per 100,000 (95% CI 188–266) which is much lower while the country was estimated to detect 80% of all TB cases (WHO, 2016; KNTPR, 2017). The prevalence of bacteriologically confirmed pulmonary TB in those above 15 years in Kenya was found to be 558 (455–662) per 100,000 population (Masini *et al*., 2018). Using the current incidence, about 169,000 (103,000–250,000) people fell ill with TB disease in 2016, but only 46% (77,376) were diagnosed and put on treatment (WHO, 2018). Kenya is thus facing a high burden of TB and 54% of the people estimated to have TB remain un-notified. Men have a

disproportionately high burden of TB- two and half times that observed in females and twice more than that reported through routine surveillance (Masini *et al*., 2018). According to Masini *et al.* (2018) there is a prevalence peak at 25–34 year age-group and the young age groups (15–44 years) that contribute to two thirds of the survey prevalent cases. This high burden of the disease in the younger age groups suggests that TB disease in Kenya is marked by active spread and transmission in the community. In addition, the prevalence to notification gap is highest in the age group 25–34 and those over 65-year-old. This indicates that there are many cases in this age groups who are not notified or not diagnosed. Majority (83%) of the prevalent TB cases were HIV-negative, suggesting that a large burden of TB also exists in the HIV un-infected population and highlighting the need to equally intensify TB case finding strategies in this population (WHO, 2016). Of the estimated 53,000 (32,000–79,000) incident TB/HIV co-infected cases in Kenya in 2016, only 43% of them were notified, implying existence of a similarly large case detection gap for TB among people living with HIV (WHO, 2017).

Tuberculosis prevalence is higher in urban areas (760 per 100,000) compared to rural areas 453 per 100,000 (MOH, 2017). These findings highlight the need to focus on urban TB care and prevention to address this skewed burden of TB among the urban population, 60% of whom live in low income informal settlements (WHO, 2018). According to Kenya's National Tuberculosis, Leprosy and Lung Disease Program (NTLLDP, 2013), the major factor responsible for the large TB disease burden is the concurrent HIV epidemic. Other contributing factors include poverty and social deprivation that have led to a mushrooming of peri-urban slums between cities and the countryside, and limited access to general health care services. The survey findings also reveal that the current practice of screening for TB cardinal symptoms (cough of more than two weeks,

fever, night sweats and weight loss) and using smear microscopy as the only test misses 40% of the TB cases.

Geographically, Nyanza region contributes up to 20% of the TB cases reported in Kenya which is partly attributed to high HIV prevalence in the region (Abongo, 2015). In Kisumu County the total cases notified in 2013 were 3,362 giving a case notification rate for TB at 317 per 100,000 which is above the national average of 217 per 100,000. According to the MOH, Kisumu County Annual Report, (2015), TB prevalence is 31% which is above the national figure. The high burden of TB in Kisumu County has mainly been attributed to the high HIV prevalence (MOH, Annual Report 2013).

2.5. Tuberculosis Mortality

Globally, substantial progress has been made in reducing mortality from TB. When an HIV-positive person dies from TB, the underlying cause is classified as HIV (WHO, 2016). Despite interaction between TB and HIV, most TB cases and deaths occur among HIV-negative people in South and Southeast Asia, where HIV prevalence is relatively low. Most of Asia, eastern Europe, and all of sub-Saharan Africa had higher TB burden than expected given their level of socio-demographic development (Nebenzahl-Guimaraes *et al*., 2017). In 2016, there were an estimated 1.3 million (range, 1.2 million to 1.4 million) TB deaths among HIV negative people (down from 1.7 million in 2000) and an additional 374,000 (range, 325,000 – 427,000) deaths from TB among HIV-positive people (WHO, 2016).

Tuberculosis is the ninth leading cause of death worldwide, and between 2012–2016 has been the leading cause of death from a single infectious agent, ranking above HIV and AIDS (WHO, 2017). Most of these deaths could be prevented with early diagnosis and appropriate treatment.

For example, among people whose TB was detected, reported and treated in 2015, the treatment success rate was 83% globally; and in high-income countries with universal health coverage the proportion of people who die from TB can be under 5%. About 82% of TB deaths occurred in the WHO African Region and the WHO South-East Asia Region in 2016; these regions accounted for 85% of the total of TB deaths. India accounted for 33% of global TB deaths among HIV-negative people, and for 26% of the combined total TB deaths in HIV-negative and HIV-positive people (WHO, 2017).

Globally, the number of TB deaths among HIV-negative people per 100,000 population was 17 in 2016, and 22 when TB deaths among HIV-positive people were included. There was considerable variation among countries, ranging from less than one TB death per 100,000 population in many high-income countries to 40 or more deaths per 100,000 population in much of the WHO African Region and in five high TB burden countries in Asia (Bangladesh, the Democratic People's Republic of Korea, Indonesia, Myanmar and Papua New Guinea) (WHO 2016). Tuberculosis incidence is either stagnant or declining more slowly than mortality in many TB-endemic countries, suggesting delays in diagnosis and treatment (Dye, 2000; Sreeramareddy *et al*., 2009; Storla *et al*., 2008; Sreeramareddy *et al*., 2014). One untreated patient with tuberculosis can infect many healthy contacts (Chun-Yu *et al*., 2010). Although only a small proportion of infected people progress to active TB, it is difficult to predict who will progress from latent infection to active disease (Blumberg & Ernst, 2016). Early diagnosis of active TB is challenging and substantial delays in diagnosis and treatment is due to; lack of awareness of symptoms, lack of access to health services, shortages of trained clinicians and laboratory personnel to make the diagnosis, and poor diagnostic tools (Storla *et al*., 2008).

Tuberculosis continues to be the leading cause of death in South Africa (Simelela & Venter, 2014). World Health Organization (2016) reported 25,000 deaths from TB in South Africa in 2015 but this excludes those people who had both TB and HIV infection when they died. These people are internationally considered to have died of HIV. Tuberculosis remained the leading cause of death in South Africa in 2014 with 8.4% of deaths nationally attributed to TB (Pretoria: StatsSA, 2015). However, the proportions of deaths due to TB has decreased significantly since 2007 when it peaked at 12.8%.

Uganda registers about 5,000 TB related deaths a year. In 2012, Uganda had a TB incidence rate of 179/100,000 and 54% of TB patients were HIV positive (WHO, 2013). The TB mortality rate was 13/100,000 (in HIV negative patients), and 25/100,000 in HIV positive patients (WHO, 2013). A number of Ugandan studies revealed that TB was the leading cause of death in HIV patients, many of whom died before TB was confirmed by laboratory methods (Amuron *et al.*, 2011; Kyeyune *et al*., 2010; Moore *et al*., 2011). Morbidity is also high: TB was ranked as the 15th and HIV the 13th highest leading age-standardized rate of disability-adjusted life years (DALYs) in Uganda relative to comparator countries in 2010 (Claudia *et al*, 2014).

Tuberculosis in Kenya is the 4th leading cause of death. Deaths among the TB patients for the year 2013 was at 9.8% and the mortality rate among the TB cases was 27 deaths per 100,000. Mortality rate among TB cases in Kisumu County in 2013 was 30 per 100,000 population which is higher than the national mortality rate (Sifuna *et al*., 2014).

2.6. Transmission of Tuberculosis

Tuberculosis most commonly affects the lungs but also can involve almost any organ of the body. The disease spreads by droplet infection when *M. tuberculosis* is carried in these airborne

particles, called droplet nuclei, of 1-5 microns in diameter (Clark-Curtiss & Haydel, 2003). Pulmonary TB is transmitted from an infected person to a susceptible person in these droplet nuclei which remain suspended in the air for up to several hours depending on the environment. Infectious droplet nuclei are generated/released into the air when persons who have active pulmonary or laryngeal TB disease exhales, coughs, sneezes, shouts, or sings (Ndungu *et al*., 2013).

A person needs to inhale only a few of these bacteria to become infected. Tuberculosis is not, however, highly contagious compared to some other infectious diseases. Factors influencing the chance of transmission include the bacillary load of the source case, as well as the proximity and duration of exposure (Escombe *et al*., 2008; 2009). Only about one in three close contacts of a TB patient, and fewer than 15% of more remote contacts, are likely to become infected. As a rule, close, frequent, or prolonged contact is needed to spread the disease. If a severely infected patient emits huge numbers of bacilli, the chance of transmitting infection is much greater. Transmission is dramatically and rapidly reduced with effective treatment (Dharmadhikari *et al*., 2014). Tuberculosis is not passed on by contact with a patient's clothing, bed linens, or dishes and cooking utensils. In general, the risk of infection among household contacts of TB patients is approximately 30 % (Singh *et al*., 2007). However, the fetus of an infected mother may contract TB by inhaling or swallowing the bacilli in the amniotic fluid. After a person inhales droplet nuclei containing *M. tuberculosis*, the droplet nuclei traverse the mouth or nasal passages, upper respiratory tract, and bronchi to reach the alveoli of the lungs where it is engulfed by the alveoli macrophages (Beers *et al*., 2004).

For reasons not clearly understood, the majority of individuals infected with *M. tuberculosis* (approximately 90 %) do not develop disease (Dharmadhikari *et al*. 2014). Following inhalation

of *M. tuberculosis* an individual may have one of the following outcomes: fail to register an infection; become infected but then clear the infection; successfully contain the infection but continue to harbour bacilli in the absence of symptomatic disease (latent TB infection); or develop progressive TB disease (Saenz *et al.,* 2013). It has been estimated that about one-third of the world population have latent TB infection, which means people have been infected by TB bacteria but are not (yet) ill with disease and cannot transmit the disease and may be at risk to develop TB disease as they age, or become immunocompromised in the future (WHO, 2014). The factors resulting in reactivation of latent TB infection in the absence of overt immune suppression are not well understood, but the huge reservoir of individuals with latent TB infection represents a major barrier to TB elimination (Dye and Williams, 2010). A definite case of TB is one in which a health worker (clinician or other medical practitioner) has diagnosed TB and has decided to treat the patient with a full course of TB treatment. Any person given treatment for TB should be recorded as a case (WHO, 2011).

2.7. Tuberculosis Disease Phenotypes

2.7.1. Primary tuberculosis

Primary (initial) infection is usually indicated by tuberculin skin test (TST) or interferon-gamma release assay (IGRA) conversion, which reflects a delayed type hypersensitivity reaction (DTH) to protein products of *M. tuberculosis* (Berry *et al*., 2010). Tuberculin skin test conversion usually occurs 3–6 weeks after exposure/infection. Primary infection remains undiagnosed in the majority of cases, as symptoms are mild, non-specific and usually self-resolving. A primary (Ghon) complex is formed, consisting of a granuloma, typically in the middle or lower zones of the lung (primary or Ghon focus) in combination with transient hilar and/or paratracheal lymphadenopathy and some overlying pleural reaction (Nayak *et al*., 2012). The primary

complex usually resolves within weeks or months, leaving signs of fibrosis and calcification detectable on chest X-ray. In general the risk of disease progression following primary infection is low, but young children and immunocompromised patients are at increased risk. A re-infection event probably triggers very similar responses to those observed with primary infection and the risk of subsequent disease progression seems to be substantially reduced. However, re-infection is likely to occur multiple times during the lifetime of an individual living in a TB endemic area, which explains its large contribution to the disease burden observed (Barry *et al*., 2009).

2.7.2. Secondary tuberculosis

Reactivation diseases or secondary or post-primary TB are often used interchangeably for TB occurrence after a period of clinical latency. However, since reactivation disease is clinically indistinguishable from progressive primary disease or re-infection disease; true reactivation disease is often preceded by an immunological impetus. Immunocompromised patients due to severe malnutrition, HIV-infection, chronic hemodialysis, immunosuppressive therapy, diabetes or silicosis are at increased risk (Grant *et al.*, 2011).

2.8. Pulmonary Tuberculosis (PTB)

Pulmonary TB affects the lungs where *M. tuberculosis* and other mycobacteria species usually attacks. Tuberculosis symptoms are usually gradual in onset and duration varying from weeks to months, although more acute onset can occur in young children or immunocompromised individuals (Davies *et al*., 2014). An infected person may at first feel vaguely unwell or develop a persistent non-remitting cough blamed on smoking or a cold. A small amount of greenish or yellow sputum is produced that is streaked with blood. Pulmonary TB patients do not run a high fever, but they often have a low-grade fever, with cold night sweat when the fever breaks. There

is loss of weight and sometimes pain in the chest (Sterling *et al*., 2010). If the infection allows air to escape from the lungs into the chest cavity (pneumothorax) or if fluid collects in the pleural space (pleural effusion), the patient may have difficulty breathing. The TB bacilli may travel from the lungs to lymph nodes in the sides and back of the neck. Infection in these areas can break through the skin and discharge pus (Fielder *et al*., 2002; CDC, 2005).

2.8.1. Parenchymal disease

Patients with cavitary lung disease typically present with chronic cough, mostly accompanied by fever and/or night sweats and weight loss. Cough may be non-productive or the patient may have sputum, that can be mucoid, mucopurulent, blood-stained or have massive haemoptysis (Knechel, 2009). Other symptoms may be chest pain, in patients with subpleural involvement. The results of the chest X-ray may be critical for treatment initiation for those patients who are sputum smear negative. Typical findings include normal chest X-ray, focal upper lobe opacities, diffuse opacities, consolidation, reticulonodular opacities, cavities, nodules, miliary pattern, intrathoracic lymphadenopathy, pleural effusion (Bianca *et al*., 2011). In HIV-infected patients, smear yield is lower and radiological abnormalities may be less typical, frustrating diagnosis. Severely immune-suppressed patients and young children are less likely to present with cavitation on chest X-ray, and more frequently have miliary (disseminated) disease (Qingliang and Jianxin, 2010).

2.8.2. Endobronchial tuberculosis

This is a specific form of PTB affecting the trachea and major bronchi. It is often misdiagnosed as bronchial asthma or bronchial malignancy. If unrecognized, the endobronchial lesions progress and cause stenosis. Symptoms are as those of pulmonary TB, however examination may

include wheezing and dyspnoea may be more prominent. There may be a female predominance, with a male: female ratio of 1:2 (Qingliang and Jianxin 2010; Xue *et al.,* 2011). Bronchoscopy and biopsy is the most useful diagnostic tool for establishing a prognosis depending on which histological subtype is found. Sputum smear and culture should be performed, but varying test sensitivities are reported. Early therapy is needed in order to prevent strictures. Treatment recommendation is standard first-line short-course regimen, but treatment prolongation may be considered on a case by case basis, for those patients with intractable disease (Xue *et al*., 2011).

2.8.3. Intra-thoracic lymphnode disease

Following first-time infection the regional lymph nodes form part of the primary (Ghon) complex. Progressive disease may occur within these affected regional lymph nodes and is typically seen in young children. Symptoms are similar to other forms of PTB, although the cough is rarely productive or the sputum blood-stained. Young children are unable to expectorate and the organism load is greatly reduced compared to adults with lung cavities, which complicates diagnosis (Perez-Velez & Marais, 2012). Enlarged peri-hilar and/or paratracheal lymph nodes may obstruct large airways with resultant collapse or hyperinflation of distal lung segments, forming cold abscesses with persistent high fever, or erode into surrounding anatomical structures such as the pericardium leading to TB pericarditis. Peri-hilar and/or paratracheal lymph node enlargement with/without airway compression is the cardinal sign of intra-thoracic lymph node disease. Lymph nodes may also erupt into the airways with aspiration of infectious caseum leading to lobar consolidation and an expansile caseating pneumonia if the airway is completely obstructed (Perez-Velez & Marais, 2012).

2.9. Extrapulmonary Tuberculosis (EPTB)

Although the lungs are the major site of damage caused by TB, EPTB affects many other organs and tissues in the body other than and outside the lung tissue. The usual progression is for the disease to spread from the lungs to locations outside the lungs (extra pulmonary sites). Body organs affected by EPTB include: lymph nodes, bones, spine, kidneys, liver, bladder, skin, eyes, and gastrointestinal system. EPTB can occur in any organ of the body except nails, hair and teeth (WHO, 2013).

2.10. Laboratory Diagnosis of Tuberculosis

The confirmation of PTB disease depends upon identification or isolation of Mycobacteria bacilli from a clinical sample which can be achieved by smear microscopy for AFB, mycobacterial culture or nucleic acid amplification tests (NAAT). The appropriate sample will depend upon the suspected site of disease. The quality of the sample may greatly affect the chances of a positive result therefore care should be taken to instruct the patient in producing a sputum sample. Children are often unable to produce sputum and in young children gastric aspirate is usually necessary (Swai *et al.*, 2011). The emergence of NTM as opportunistic infections compounds the TB problem (Griffith *et al*., 2007). Conventional identification of mycobacteria is achieved by standard culture and biochemical methods, all of which are time consuming. The rise in NTM and other members of MTBC isolation demands faster methods for their identification and for selection of appropriate therapy. Definitive diagnosis of NTM PTB is challenging due to: similarity in clinical and microbiologic characteristics to *M. tuberculosis* (TB); limited laboratory capacity to isolate and identify NTM species (Herzmann & Lange *et al*., 2010; Farhat *et al*., 2006).

2.10.1. Clinical diagnosis of tuberculosis

Chest radiography is not always helpful in smear negative patients. The radiographic distinction between active and inactive TB can be difficult and appearance may be atypical due to other infections in HIV positive patients (Soumya *et al.,* 2007). Substantial numbers of patients are treated for TB without definitive diagnostic criteria (Swai *et al*., 2011). With the advent of HIV associated TB with more frequent smear negative TB, the role of culture in TB control programs may need to be reassessed (Perkins *et al*., 2007). In countries where resources are limited, and where the use of chest X-rays may be inadequate due to the cost as well as atypical presentation found in HIV infected patients, clinical and/or laboratory characteristics which are able to identify smear negative but culture positive PTB are required (Cattamanchi *et al.*, 2009).

2.10.2. Smear microscopy by acid fast staining (AFB)

Diagnosis for the majority of patients worldwide suspected of PTB is made by sputum smear microscopy for AFB using ZN stain (Kedir *et al.,* 2018). The test was developed 100 years ago by Franz Ziehl and Frederick Neelsen. The detection of AFB in expectorated sputum is still crucial, especially in developing countries of sub-Saharan Africa such as Kenya where other facilities including sputum culture for Mycobacteria are unavailable or are prohibitively expensive. When AFB is detected in sputum, the diagnosis of PTB is certain. However diagnostic problem start when patients with suspected PTB have a negative sputum smear (Ramachandran *et al*., 2009). It has always been recognized that a proportion of patients are sputum smear negative using the ZN stain, the commonly used stain in most laboratories in the region to detect AFB in sputum. This is a simple, rapid and cheap test but lacks sensitivity of a single sputum test as it is only positive in around half of patients with active TB (Soumya *et al*., 2007). About 5000-10000 bacilli per milliliter of sputum must be present for it to be positive.

Sputum smear using ZN stain for AFB seems to be even less sensitive in children and patients with HIV associated PTB. In addition, the test is not specific for *M. tuberculosis*, but detects all AFB including NTMs and Norcadia. With the sharp rise of PTB in countries which are worst affected by the HIV epidemics, the number of patients with suspected PTB who are sputum smear negative has increased (Cattamanchi *et al.*, 2009).

The ZN smear exploits the acid-fast property of mycobacteria by staining bacilli with carbol-fuschin, using gentle heat to facilitate penetration of the dye, and then using a decolorising acid solution, which fails to penetrate the mycobacteria, leaving them stained red while other bacilli are decolorised. The slide is usually then counterstained with methylene blue to improve visualization of the mycobacteria (Habeenzu *et al*., 1998). The positive control should show good staining of AFB while the negative control should not show any AFB. Certain mycobacteria other than *M. tuberculosis*, especially the rapid growers, are easily decolorized and may give faint staining reactions. Demonstration of AFB in a smear made from a clinical specimen provides a preliminary diagnosis of mycobacterial disease, while the isolation of mycobacteria on culture provides a definite diagnosis of MTBC or disease due to NTM. Sensitivity may be increased by concentration of samples prior to microscopy, usually by centrifugation or filtration (Van Deun *et al*., 2000) but direct (unconcentrated) ZN stain is the most widely applied methodology due to resource limitations.

Traditional TB control focused on the identification and treatment of sputum smear-positive TB patients, considered to be most infectious cases, in the mistaken belief that systematic identification and treatment of smear-positive cases would be sufficient to reach eventual TB elimination. In a further policy change, WHO recommended in 2010 that two sputum samples are sufficient, rather than the standard three samples (spot-morning-spot) which had been

recommended for several decades (WHO, 2010). This is due to the low diagnostic yield of a third sputum sample and the resource limitations of TB programmes. If clinical suspicion is high repeated testing may still be warranted. A single positive smear is now also considered sufficient for a TB diagnosis (Bonnet *et al*., 2007; Mase *et al*., 2007). According to WHO, (2012) report, TB case notification for sputum positive was 48% for Kenya. The number of laboratories performing TB smear microscopy are 1, 581 and only 6 laboratories do culture with yet 1 and 3 laboratories performing drug susceptibility and Xpert *MTB/RIF* procedures, respectively (WHO, 2012).

In Kenya and other developing countries with a high incidence of PTB, microscopic examination of sputum smear samples is often the only available diagnostic test for PTB. As a result, patients with smear-negative TB do not receive a diagnosis in a timely manner; thus, disease may further develop, initiation of treatment may be delayed, and further TB transmission may occur (Siddiqi *et al*., 2003). Although it is not known whether HIV-TB–coinfected patients for whom the result of sputum smear is negative are as infectious as was found in one study in The Netherlands (Perkins *et al.*,2007), that patients with smear-negative TB can transmit TB has implications for countries where HIV infection is endemic. Human immunodeficiency virus infection and AIDS are accompanied by high rates of smear-negative TB. For example, 25%–61% of the HIV-infected patients with TB in sub-Saharan Africa have smear-negative PTB (Storla *et al.,* 2008). More emphasis should be placed on the development of better TB diagnosis and the improvement of culture facilities in countries with a high incidence of TB (Getahun *et al.,* 2007), including Kenya.

2.10.3. Molecular diagnosis of tuberculosis

Detection of Mycobacteria in clinical samples is generally less sensitive than nucleic acid amplification tests (NAAT) for other pathogens due to the relatively low numbers of bacilli present and the difficulty of efficiently extracting DNA from the tough mycobacteria (Cloud *et al*., 2002). New DNA sequence-based techniques for the identification of mycobacteria have been developed during the past 10 years, such as DNA sequencing (Aldous *et al*., 2005; Harmsen *et al.,* 2003), pyrosequencing and PCR-restriction fragment length polymorphism assays (Tuohy *et al*., 2005), real-time PCR assays (Shrestha *et al*., 2003), oligonucleotide arrays (Park *et al*., 2005), and commercially available tests such as the AccuProbe (Gen-Probe Inc., San Diego, Calif.). Most of these methods require either expensive equipment or extensive expert knowledge or are restricted to a limited number of species that can be identified. Further more, analysis of 16S rRNA gene sequences has limitations because of the low level of polymorphism between some closely related species. No single target gene can accurately distinguish all *Mycobacterium* species, and Devulder *et al.* (2005) have suggested that a combination of genes (such as 16S rRNA, *hsp65, rpoB* and *sod*) be used. The development of Line Probe assays (LPA) allowed the simultaneous detection of *M. tuberculosis* and determination of resistance to rifampicin and later isoniazid. However these tests are only endorsed for use on smear positive sputum and therefore do not aid greatly in the diagnosis of TB itself.

2.10.3.1 GeneXpert *MTB/RIF* test

The emergence and spread of MDR-TB strains poses significant challenges to TB disease control (WHO, 2010). In order to overcome conventional methods' low sensitivity and diagnostic delays, NAAT have been introduced. The NAATs' sensitivities are high for specimens that are AFB microscopy positive but lower for AFB-negative specimens (Rie *et al*., 2010). The

identification of mutations associated with drug resistance depends on additional NAA tests, whose application on clinical samples is indicated only for AFB-positive specimens. The rapid detection of MTBC in respiratory specimens and drug therapy based on reliable drug resistance testing results are a prerequisite for the susccessful implementation of the WHO's Stop TB Strategy (WHO, 2006). However, in many areas of the world, TB diagnosis still relies on insensitive, poorly standardized sputum microscopy methods. Ineffective TB detection and the emergence and transmission of drug-resistant MTB strains increasingly jeopardize global TB control activities (WHO, 2010). Effective diagnosis of PTB requires the availability, on a global scale, of standardized, easy- to- use, and robust diagnostic tools that would allow the direct detection of both the MTBC and resistance to key antibiotics, such as rifampicin. The rapid availability of reliable test results is likely to directly translate into sound patient management decisions that, ultimately, will cure the individual patient and break the chain of TB transmission in the community (WHO, 2010).

GeneXpert *MTB/RIF* assay is an automated, closed-cartridge system, easy to operate and user friendly molecular test for synchronized detection of TB and rifampicin resistance, recommended by the WHO. The introduction of GeneXpert *MTB/RIF* Assay test (Hillemann *et al*., 2010; Boehme *et al*., 2010, 2011) has quickly become the most widespread nucleic acids amplification test (molecular test) for diagnosing TB which detects the presence of MTBC DNA in sputum or concentrated sputum sediments and simultaneously detecting rifampicin resistance in a single reaction as indicated by mutations in the *rpoB* gene (Helb *et al*, 2010; Van Rie *et al*., 2010). Mono resistance to rifampicin is rare; however, >90% of rifampicin resistant isolates also exhibit resistance to isoniazid (Boehme *et al*., 2011). Therefore, the detection of rifampicin resistance is used as a surrogate marker for MDR *M. tuberculosis* (Watterson *et al*., 1998).

The new GeneXpert *MTB/RIF* assay targets the rifampin resistance-associated *rpoB* gene hot spot region (Boehme *et al*., 2011). It uses hemi nested real-time PCR (RT-PCR) with three specific primers to amplify this *M. tuberculosis*-specific sequence of the *rpoB* gene and combines the sensitive detection of *M. tuberculosis* DNA and determination of rifampicin resistance. To determine rifampicin resistance, the rifampicin resistance-determining region (RRDR) of the *rpoB* gene is probed with five molecular beacons spanning the *rpoB* gene 81-bp RRDR (El-Hajj *et al*., 2001). Any deviation from the wild type sequence resulting in a delay in the appearance of the signal exceeding a predetermined ΔC_T value (>3.5), between the earliest and latest cycle threshold (C_T) values, is reported as RIF resistant. The test is carried out within 2 hours in a disposable cartridge. The assay can be carried out in a nearly fully automated manner, including sample processing i.e. bacterial lysis, nucleic acid extraction and amplification, and detection of the target sequences (amplicon) using real-time PCR and reverse transcriptase PCR (Hillemann *et al*., 2010). This reduces hands-on time due to automation of bacterial lysis, DNA extraction, real-time PCR amplification, and amplicon detection in a single system. The only manual step is the mixing of a bactericidal buffer with the sample prior to addition to the cartridge. This preamplification step reduces the viability of MTBC organisms, making the assay suitable for use near patients in settings with limited biocontainment facilities (Helb *et al*., 2010). Although sensitive, specific, and easy to use, the test and equipment are expensive and the GeneXpert platform requires a stable power source limiting its value in the poor resource countries that need it the most (Boehme *et al*., 2011).

GeneXpert *MTB/RIF* has been shown to detect non-viable mycobacteria present in specimens giving both smear and culture negative as long as bacterial DNA is present (Wood *et al*., 2015). This rapid and easy to perform fully automated NAA test could prove to be an extremely helpful

diagnostic tool and detection of rifampicin resistant mycobacteria strains hence the fight against TB. The test runs on the GeneXpert platform using a disposable plastic cartridge with all required reagents (Raja *et al*., 2005). Although relatively simple to perform and rapid, the Xpert *MTB/RIF* is not a true point-of-care test and many challenges have been encountered during scale-up (Boehme *et al*., 2010). The need for a reliable electricity supply is a major barrier in some settings, problems with module calibration and maintenance, the need for the bulky cartridges to be stored below 30°C, determining optimal testing algorithms and logistics of kit supply have been some of the challenges encountered (Abdurrahman *et al.*, 2014, 2015).

Xpert *MTB/RIF* assay was endorsed by WHO in 2010 as a rapid sensitive molecular test that can diagnose *M. tuberculosis* TB and rifampicin resistant strains of TB within two hours (WHO, 2010). From the date of its endorsement in December 2010 by WHO up until June 2012 approximately 1.1 million TB tests using Xpert *MTB/RIF* were performed in resource poor developing countries including Kenya. This makes Xpert *MTB/RIF* an excellent method of choice for TB diagnosis. However, the Xpert *MTB/RIF* does not work in isolation but is used in combination with conventional laboratory diagnostic procedures for TB such as sputum smear microscopy that uses ZN staining technique in addition to culture techniques for *M. tuberculosis*. Xpert *MTB/RIF* assay was initially recommended (2010) for diagnosis of PTB in adults, but since 2013, it has also been recommended for use in children and to diagnose specific forms of EPTB. The test has much better accuracy than sputum smear microscopy (WHO, 2017).

Rapid molecular techniques have substantially changed the field of TB diagnosis and have been proven to yield rapid results while being highly sensitive (Boehme *et al*., 2010; Tadesse, 2018). The advantage of the Xpert *MTB/RIF* assay is the low probability of detection of secondary bacteria from specimens with a high contamination rate, such as stool and urine specimens. In

some cases, the Xpert *MTB/RIF* assay result was positive but the culture remained negative (Wood *et al*., 2015). The Xpert real-time technology is less prone to contamination due to the closed reaction chamber. Furthermore, the surfaces where the specimens are processed are extensively cleaned to avoid contamination with bacterial DNA. Hence Xpert *MTB/RIF* assay can be applied to pulmonary specimens with a high sensitivity and specificity, which, coupled with its speed and simplicity, make this technique a very useful tool for the diagnosis of PTB (Hillemann *et al*., 2011).

Ziehl-neelsen smear microscopy despite of being the available screening tool, it carries the risk of false negative results and incompetency to discriminate between drug susceptible and drug resistant strains of MTB. These are owing to poor sample quality coupled with a need for an experienced specialist. Meanwhile, culture being the gold standard for detecting MTB, proceeds for weeks up to months to yield results, and depends on sophisticated laboratory facilities and skilled technicians (Getahum *et al*., 2007; Hillemann *et al*., 2011). The assay can be applied to sputum samples or concentrated sputum sediments prepared from induced or expectorated sputa that are either AFB smear positive or negative (Boehme *et al*., 2010; Helb *et al*, 2010). The assay is intended for use with specimens from patients for whom there is clinical suspicion of PTB who: (1) have not received anti-TB therapy in the last 60 days, (2) had < 7 days of therapy. It should not be used for monitoring the effects of drug therapy because bacterial DNA might persist following antimicrobial therapy (Blakemore *et al.*, 2010).

Each Xpert *MTB/RIF* cartridge includes reagents for the detection of MTBC and RIF resistance as well as a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor the presence of inhibitor (s) in the PCR reaction. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and

dye stability (GeneXpert Dx System Operator Manual, 2011). The primers in the Xpert *MTB/RIF* assay amplify a portion of the *rpoB* gene containing the 81 base pair "core" region. Five differently coloured fluorogenic nucleic acid hybridization probes, called molecular beacons, interrogate the entire 81-bp core (El-Hajj *et al*., 2001). Each molecular beacon was designed to be so specific that it does not bind to its target if tne target sequence differs from the wild -type *rpoB* sequence by as little as a single nucleotide substitution. Since molecular beacons fluorescence only when they are bound to their targets, i.e. wild –type *rpoB* sequence, the absence of any one of the five colours in the assay differentiate between the conserved wild type sequence and mutations in the core region that are associated with RIF resistance (Helb *et al*, 2010). The SPC should be positive in a negative sample and can be negative or positive in a positive sample. The test results will be "Invalid" if the SPC is not detected in a negative test. Before the start of the PCR reaction, the GeneXpert Dx System measures the fluorescence signal from the probes to monitor bead rehydration, reaction –tube filling, probe integrity and dye stability. The PCC passes if the fluorescence signal from the probes meets the assigned acceptance criteria. The results are interpreted by the GeneXpert Dx System from measured fluorescence signals and embedded calculatin algorithms and are displayed in the View Results Window (GeneXpert Dx System Operator Manual, 2011), as indicated: MTB Detected: MTB target DNA is detected; both controls, SPC and PCC, meet the assigned acceptance criteria. Lower Ct values represent a higher starting concentration of DNA template; higher Ct values represent a lower concentration of DNA template. In MTB DETECTED results "RIF Resistance DETECTED", "RIF Resistance NOT DETECTED", or "RIF Resistance INDETERMINATE" will display on a separate line. MTB Not Detected: MTB target DNA is not detected; both controls, SPC and PCC, meet the assigned criteria. Invalid: Presence or absence of MTB cannot

be determined: SPC does not meet acceptance criteria, i.e. the sample was not properly processed, or PCR was inhibibited. Note: repeat test with extra specimen. Error: One or more of the PCC results failed (FAIL). Both MTB and SPC display NO RESULT. Note: repeat test with extra specimen. If the PCC passed (PASS), the error is caused by a system component failure (GeneXpert Dx System Operator Manual, 2011).

2.10.3.2. GenoType Mycobacterium CM/AS and MTBC assay

This is a new commercial kit developed to identify MTBC and NTM species from cultures. It involves DNA amplification targeting the 23S rRNA gene region, followed by reverse hybridization to specific oligonucleotide probes immobilized on membrane strips. There are two kits – the CM (Common Mycobacteria) and AS (Additional Species) kits. The CM kit identifies 15 *Mycobacterium* species, including MTBC, whilst the AS kit aims to discriminate 16 additional less common NTM pathogens. The sensitivity and specificity of the CM strip is 97 and 92.4% and of the AS strip is 99.3 and 99.4%, respectively, compared with reference methods (Lee *et al.,* 2009). Other studies have evaluated the GenoType Mycobacterium assay on NTM cultures from liquid media and showed 91–100% concordance with conventional identification methods (Padilla *et al.*, 2004). The GenoType Mycobacterium assay applied to clinical NTM isolates demonstrated rapid and accurate identification of a broad range of NTM compared with phenotypic and other molecular diagnostic techniques. This assay is relatively easy to use and does not require expensive equipment (Lee *et al.,* 2009). In study carried out by Lee (2009) and his colleagues in Australia, identification of mycobacteria species used the GenoType Mycobacterium CM/AS assay (Hain Lifescience). Rapid and reliable identification of the members of the MTBC is critical in guiding public health and primary care decisions. This is

because each organism exhibits a different epidemiology, host spectrum, geographic range, pathogenicity, and drug susceptibility pattern (Parsons *et al*., 2002).

Laboratory diagnosis and identification of Mycobacteria play a key role in the treatment of TB, but most conventional identification techniques fails to identify Mycobacteria to species level. This calls for rapid and accurate technique which is the GenoType Mycobacterium CM/AS and MTBC assay techniques which identifies Mycobacteria to species level. Thus the current study was conducted with a specific objective of characterizing and identifying the species of MTBC and NTM using the GenoType Mycobacterium CM/AS and MTBC assays.

2.10.4. Mycobacterial culture

Culture of Mycobacteria is a more sensitive technique for diagnosis but due to the slow growth of the organism with a generation time of 24–30 hours, sputum cultures take 4–8 weeks to become positive on solid media and 10–21 days in liquid media. Solid culture is usually performed on Lowenstein Jensen (LJ), Ogawa or Middlebrook 7H10/11 agar media. Liquid culture medium of Mycobacteria is more sensitive and rapid than solid culture but can be prone to contamination in some laboratories (Gomez-Flores *et al*., 2008). Certain species of mycobacteria are reported to grow in liquid medium only, thus failing to be detected on solid medium (Siddiqi *et al*., 1993).

Early commercial automated liquid culture systems for mycobacteria used radiometric assay but have now been replaced with fluorescence based quenching systems which has improved safety. The most widely used system is the BACTEC Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickinson, Sparks, Massachusetts) system which can also be used for susceptibility testing to first line drugs using a commercially available kit (Gomez-Flores *et al.*, 2008). A

culture is necessary to confirm drug susceptibility, particularly for second-line drugs in cases of MDR TB. Mycobacteria culture and phenotypic DST requires significant training, infrastructure, strict infection control and on-going quality assurance, which is only available in regional reference laboratories in most countries. It has been established that liquid medium is far superior to solid medium for recovery, time-to-detection and drug susceptibility testing. In 1993, the Centers for Disease Control and Prevention (CDC) recommended that every clinical laboratory must use a liquid medium to isolate mycobacteria in conjunction with solid medium (Tenover *et al*., 1993). Liquid medium is more prone to contamination with bacteria that are commonly present as normal flora in certain types of clinical specimens such as sputum and sometimes survive the decontamination process. Thus, addition of antimicrobials is needed to suppress contamination in liquid medium (Gomez-Flores *et al.,* 2008).

The BBL™ MGIT™ supplemented with BACTEC™ MGIT™ Growth Supplement and BBL™ MGIT™ PANTA™ antibiotic mixture is intended for the detection and recovery of mycobacteria using the BACTEC™ MGIT™ 960 System. The BBL™ MGIT™ contains 7 ml of modified Middlebrook 7H9 Broth base. The complete medium, with oleic acid-albumin-dextrose-catalase (OADC) enrichment and polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (PANTA) antibiotic mixture is added. All types of clinical specimens, pulmonary as well as extrapulmonary (except blood and urine) can be used for primary isolation in the MGIT tube using conventional methods (Siddiqi and Rusch-Gerdes, 2006). The processed specimen is inoculated into MGIT tube, placed into the BACTEC™ MGIT™ instrument for continuous monitoring until positive or the end of the testing protocol. A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round bottom tubes. This 110µl fluorescent indicator compound contains Tris 4,7-diphenyl-1, 10-

phenanthroline ruthenium chloride pentahydrate in silicone rubber base. The tubes are flushed with 10% carbon dioxide and capped with polypropylene caps. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected. Tubes entered into the BACTEC MGIT instrument are continuously incubated at 37^0C and monitored every 60 minutes for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive; i.e., the test sample contains viable mycobacteria. An instrument positive tube contains approximately 10^5 to 10^6 colony forming units per millilitre (CFU/ml). Culture vials which remain negative for a minimum of 42 days (up to 56 days) and which show no visible signs of positivity are removed from the instrument as negative and strerilized prior to discarding.

The BACTEC MGIT Growth Supplement (OADC) is added to each MGIT tube to provide substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by tubercle bacteria and plays an important role in the metabolism of mycobacteria. Albumin acts as a protective agent by binding free fatty acids which may be toxic to *Mycobacterium* species, thereby enhancing their recovery. Dextrose is an energy source. Catalase destroys toxic peroxidases that may be present in the medium. Contamination is reduced when supplementing the BBL MGIT broth base with BACTEC MGIT Growth Supplement/BBL MGIT PANTA antibiotic mixture prior to inoculation with a clinical specimen (Gomez-Flores *et al.,* 2008). After the tubes are sterilized by autoclaving, an enrichment supplement and MGIT PANTA antibiotic mixture is added according to the instructions of the manufacturer.

The identification of mycobacteria responsible for a disease and the discrimination of environmental from pathogenic species are relevant diagnostic issues that have important ramifications for the treatment of patients **(**Primm *et al.,* 2004). The American Thoracic Society and the Infectious Diseases Society of America recommend that clinically significant NTM be identified to the species level in order to determine their clinical significance and select appropriate treatments (Griffith *et al.,* 2007). The identification of mycobacterial species has traditionally been based on conventional biochemical tests and phenotypic characteristics, such as growth rate and pigmentation. Although these tests are simple to perform, they are laborious, cumbersome, error prone and require extensive incubation periods, thereby delaying prompt and accurate mycobacterial identification, which can be of serious consequence for good and timely patient care. The introduction of molecular identification techniques has revolunized bacterial taxonomy and has greatly improved the speed and accuracy of the process. However, these require a high level of expertise and a special laboratory setup (McNerney *et al*., 2012; Richardson *et al*., 2009; Slany *et al*., 2012)

2.11. Treatment of Drug-Susceptible Tuberculosis

The main aim of TB treatment is to cure the patient, to prevent the spread of TB and to prevent the development of drug resistant tuberculosis. Treatment by use of a combination of the different drugs available for treatment can cure most patients who have TB. Anti-TB drugs have various mechanisms of action which include: inhibition of protein synthesis, inhibition of cell wall synthesis, inhibition of nucleic acid synthesis, disruption of cell membrane and action as antimetabolites (Bernardes-Genisson *et al.,* 2013). Now that drugs are available, surgery is rarely used as treatment for TB. The more than twenty anti-TB drugs available are used in different combinations in different circumstances. First-line anti-TB drugs are only used for the treatment

of new cases of TB patients when there is no suggestion of any drug resistance. Second line drugs are only used for the treatment of drug resistant TB (WHO, 2010). Drug-susceptible TB can be cured in six months using a combination of first-line TB drugs (WHO, 2011). Because of the long generation time of Mycobacteria (die very slowly), the drugs have to be taken for quite a long time. Even when a patient starts to feel better they can still have bacteria alive in their body. So the patient needs to keep taking the anti-TB drugs until all the bacteria are dead. Proper medication should be adhered to for the entire period of TB treatment. Insufficient drugs will not kill the bacteria which may then lead to drug resistant strains (WHO, 2017).

Drug treatment is the only effective treatment for TB. Single drug treatment for active TB is associated with a substantial relapse rate (WHO, 2010). A patient is said to have a relapse if they improve whilst taking TB treatment but become ill again after they have finished their treatment. Single drug treatment is also associated with the development of drug resistant TB strains (WHO, 2017). Patients with active TB disease should receive at least three drugs as their initial TB drug treatment. Fewer than three drugs can result in the development of resistance.

Treating TB with streptomycin, isoniazid, or pyrazinamide monotherapy in the 1950s and 1960s led to an initial favorable response that was quickly abolished by the emergence of resistance (Selkon *et al*., 1964; Yeager *et al*., 1952). Use of combination therapy led to reduction in the emergence of drug resistance and became the standard for anti-TB therapy. Pulmonary TB has three populations of *M. tuberculosis*: bacilli in log-phase growth, slowly replicating bacilli under acidic conditions, and non-replicating bacilli under hypoxic conditions (Mitchison, 1979). Drugs such as rifampin, isoniazid, and pyrazinamide are thought to have selective action on each of these populations, making it necessary to use multiple-drug therapy to eradicate all bacilli. Isoniazid is thought to kill bacilli in log-phase growth, whereas pyrazinamide is thought to kill

slowly replicating bacilli during the first 2 months of the initial phase of therapy. Rifampicin is thought to slowly kill non-replicating persistent bacilli during the 6 months of therapy, with isoniazid added to prevent resistance during the continuation phase.

2.11.1. First-line tuberculosis treatment

Tuberculosis disease can be treated by taking several drugs for 6 to 9 months. There are 10 drugs currently approved by the U.S. Food and Drug Administration (FDA) for treating TB. Of the approved drugs, the first-line anti-TB drugs that form the core of treatment regimens are: isoniazid, rifampicin, ethambutol, streptomycin and pyrazinamide that have the greatest bactericidal activity when used for TB treatment (Coker *et al*., 2006). The amount of drug that a patient needs to take depends on the patient's weight. The treatment of new cases of TB patients with presumed drug susceptible PTB, WHO recommends that they should have six months of treatment. This consists of a two month intensive TB treatment phase followed by a four month continuation phase. For the two month intensive TB treatment phase they should receive: Isoniazid + Rifampicin + Pyrazinamide + Ethambutol followed by Isoniazid + Rifampicin for the four months continuation TB treatment phase (Zhang *et al*., 2000; WHO, 2003). It is recommended that patients take TB drugs every day for six months. Although taking the drugs three times a week is possible in some circumstances, it is essential that all the recommended TB drugs are taken. If only one or two drugs are taken, then the TB treatment probably won't work. This is because the patient has developed resistance to the TB drugs. Not only is the patient then still ill, but to be cured they then have to take drugs for the treatment of drug resistant TB. These drugs are more expensive and have more side effects (Coker *et al*., 2006).

Tuberculosis treatment fails because of the following reasons: if a patient doesn't take their TB drug treatment properly and correctly leading to the development of drug resistant TB strains; if

the patient has already drug resistant TB strain then it results in treatment failure even if the treatment is taken correctly. The 7-month continuation phase is recommended only for the following groups: Patients with cavitary PTB caused by drug-susceptible Mycobacteria and whose sputum culture obtained at the time of completion of 2 months of treatment is positive; Patients whose intensive phase of treatment did not include PZA; patients with HIV who are not receiving antiretroviral treatment (ART) during TB treatment; and patients being treated with once weekly isoniazid and rifapentine and whose sputum culture obtained at the time of completion of the intensive phase is positive (Rie *et al*., 2010). A reliable preparation containing rifampicin, isoniazid, and pyrazinamide in combination is available, as is a combination tablet of all four of these first line drugs.

2.11.2. Tuberculosis treatment failure

Patients who experience only a short improvement whilst on TB treatment, or who never respond to treatment, are said to have failed their TB treatment. Treatment failure is also sometimes defined as the continued presence of positive sputum or culture (positive result to a culture test). It can also be that positive sputum or culture appears again during the course of a patient's anti-TB drug treatment. Various factors contribute to TB treatment failure. For example, doctors as a cause of TB drug treatment failure relate to the actions of doctors in prescribing incorrect regimes, inappropriate guidelines, non compliance with guidelines and absence of guidelines (WHO, 2010). Drugs can be a cause of inadequate TB treatment if it is: poor quality of drugs, irregular supply, wrong delivery (dose/combination) and unsuitable drugs due to drug resistance. Patients as a cause of TB drug treatment failure involves lack of information, lack of money for treatment and/or transport, actual or presumed side effects, lack of commitment to a long course of drugs, malabsorption and social barriers (WHO 2010).

After three months of drug treatment for PTB caused by drug susceptible mycobacteria, 90-95% of patients will have negative sputum or culture and show clinical improvement (WHO, 2010). Normally it is considered that if a patient still has a positive culture after three months of treatment, the patient must be carefully evaluated to identify why their positive culture hasn't changed to negative. Patients whose sputum culture remain positive after four months of drug treatment should be classified as treatment failures. If drug treatment failure occurs then a sample should be sent to a reference laboratory for drug susceptibility testing for both first and second line drugs (Pozniak *et al.*, 2011).

2.11.3. Tuberculosis treatment relapse and recurrence

A patient is said to relapse if they become and remain culture negative or they become well whilst on TB treatment, but become culture positive or become ill again after finishing their TB treatment. Recurrence of active TB is usually used to refer to the situation when a person's first TB treatment appears to have been successful (Dale *et al*., 2017). There has then been a significant time interval before active TB develops again. This may either be because of reactivation of the person's previously latent TB or because they have been reinfected (Bo Young *et al*., 2016). If any of these situations occur it must be considered a real possibility that the person has drug resistant TB. Their new TB treatment programme must be decided taking this into account (WHO, 2010). This is why proper diagnosis and susceptibility testing evaluated in current study is necessary to inform decisions on the treatment programmes.

2.11.4. Tuberculosis treatment monitoring

All patients receiving TB treatment should be monitored during their treatment to assess their response to the drug treatment. Regular monitoring also helps to ensure that patients complete

their treatment. It can also help to identify and manage adverse drug reactions. Patients need to have their weight checked every month, and if the patient's weight changes the drug dosages may need to be adjusted (Maria *et al*., 2009). Patient's response to TB treatment should be monitored using sputum smear microscopy. The recommendation from the WHO is that for smear positive TB patients treated with first line drugs, the patients should have smear microscopy performed at the end of the two month intensive phase of treatment (WHO, 2008). Sputum should be collected when the patient is given the last dose of the intensive phase of treatment. If the patient has a positive sputum smear at the end of the intensive phase, then there should be a patient assessment carried out. This is because the positive smear could indicate a number of different situations. An example is that the patient might have drug resistant TB, and a change in the TB drugs they are taking might be needed. Alternatively, patient adherence might have been poor, and they might not have been taking their drugs correctly (WHO, 2004). So the assessment might result in changes being made to the patient's treatment, or to their support and supervision. Different action may need to be taken in a variety of other circumstances, such as the patient having received treatment before. New molecular tests, such as the GeneXpert *MTB/MRF*, cannot be used for TB treatment monitoring as they detect both live and dead bacteria, and cannot distinguish between them (WHO, 2010).

2.11.5. Second-line tuberculosis treatment

This refers to the treatment of drug resistant TB. For many years, the WHO defined four treatment categories. Category 1 was for new smear positive patients with pulmonary TB; Category 2 was for sputum smear positive patients who have relapsed, who have retreatment failure or who are receiving treatment after treatment interruption; Category 3 was for new smear negative pulmonary TB patients (other than those in Category 1), and patients with new less

severe forms of extra pulmonary TB; Category 4 was for chronic cases who are still sputum positive after supervised retreatment. Any patient with chronic or drug resistant-TB requiring treatment with second-line drugs falls under WHO treatment category 4 and will require specialized regimens termed Category 4 regimens in these guidelines (WHO, 2010). The choice of drugs is based on their efficacy, experience of use and toxicity. Based on this principle, the 2008 and then the 2011 WHO guidelines proposed a range of five groups, from group 1 (which included first-line drugs) to groups 2-6 which included second-line anti-TB drugs (Rendon *et al.,* 2016). Group 1 which included first-line drugs (isoniazid, rifampicin, ethambutol, pyrazinamide). Group 2 drugs included Moxifloxacin, high dose levofloxacin (fluoroquinolones); Group 3 included: Linezolid, delamanid, bedaquiline (newer drugs with increased evidence); Group 4 included: Amikacin, capreomycin, kanamycin (injectables); Group 5 included: Clofazimine, ethionamide/prothionamide, carbapenems; Group 6 included: Cycloserine, para-amino salicylic acid, amoxicillin (WHO, 2016)

Multi-drug resistant-TB treatment requires a combination of drugs from various groups. The decision on which drugs are to be used to treat the MDR-TB and on which the Kenya standardised regimen was constructed is based on: the available national anti-TB drug resistance survey data; the extent of use of anti-TB drugs in the country; prevalence of drug resistance in the different categories of TB patients; the availability of second line of anti-TB drugs, and the frequency of their use in the country. Active contact tracing should be initiated as soon as the MDR-TB diagnosis is made. In Kenya the treatment for MDR-TB is generally based on a standardized regimen. However individualized regimen is used in some special situation based on individual resistance pattern of the infecting strain. In the event this is required, the decision

should be made by a medical doctor in consultation with the TB coordinators and the PMDT team (PMDT, 2014).

Standardized regimen for TB treatment is as the follows: 8 Kanamycin (Km)-Prothionamide (Pto)- Levofloxacin (Lfx)-Cycloserine (Cs)- Pyrazinamide (PZA) / 12 Prothionamide (Pto)- Levofloxacin (Lfx)- Cycloserine (Cs)- Pyrazinamide (PZA). The number shown before each phase stands for the duration of time in months and is the minimum recommended time the phase should last. The duration of treatment is guided by smear and culture conversion. The minimum recommended duration of treatment is 20 months or 18 months after culture conversion. The treatment consists of two phases as follows:

i). Intensive Phase: This lasts for a minimum of 8 months, and should continue 4 months after sputum conversion or at least 3 consecutive negative culture results. The following drugs are recommended: (a) Injectable. Kanamycin (b) Tabs Prothionamide (c) Tabs Levofloxacin (d) Tabs Cycloserine (e) Tabs Pyrazinamide. The regimen being – 8Km-Pto-Lfx-Cs-Z

ii). Continuation Phase – This lasts for 12 months and uses the following drugs: (a) Tabs Prothionamide (b) Tabs Levofloxacin (c) Tabs Cycloserine (d) Tabs Pyrazinamide. The regimen being 12Pto-Lfx-Cs-PZA (PMDT guidelines, 2014).

2.12. Drug Susceptibility Patterns of MTBC Isolates to First-line Anti-TB Drugs

Antimicrobial susceptibility testing is critical in prescribing an effective drug regime for a TB patient, especially in areas where drug resistance incidence is high. It is also important in the follow-up of patients who are on antimicrobial therapy but are not responding to therapy. Drug susceptibility testing (DST) is an integral part of the WHO DOTS-Plus program (MGIT MANUAL, 2006). The detection of Mycobacteria isolates in TB patients and its drug

susceptibility pattern has been increasingly recognized as an important component of global TB control (Espinal *et al*., 2000), and it is critical to test the drug susceptibility of TB bacilli in order to guide therapy (van Soolingen *et al*., 2000). Multi drug-resistant-TB is an emerging problem of great importance to public health worldwide and is defined as resistance to at least isoniazid and rifampicin. Drug-resistant TB has been reported since the early days of the introduction of antibiotics; however, the global magnititude of DR-TB had not been well studied (Espinal *et al*., 2003). The problem has also been compounded by NTM mostly in immunocompromised people which does not respond to first-line conventional anti-TB drugs (Han *et al*., 2005).

The management of drug resistance requires the surveillance of resistant cases. The amount of drug that a patient needs to take depends on the patient's weight. The WHO recommends that new cases of TB patients with presumed drug susceptible PTB, should have six months of treatment. This consists of a two month intensive TB treatment phase followed by a four month continuation phase (Coker, 2006; Enarson *et al*., 2000). Re-treatment cases of PTB due to relapse, defaulters or treatment failure, the rates of drug resistance is expected to be higher because these patients have been exposed to the anti-TB drugs leading to the emergence of resistance strains. In new cases of PTB, the resistance seen is as a result of patients acquiring drug resistant mycobacteria strains from infected MDR-TB patients. Unlike the previous earlier published studies that only focused and tested *M. tuberculosis*, the current study identified and performed DST for the three members of MTBC (*M. tuberculosis, M. africanum, M. bovis*) isolated from new cases of PTB patients.

A national TB drug-resistance survey conducted in Thailand between 2001 and 2005, found the MDR-TB rate in 2002 at 1% among 1,505 new TB patients and 20% among 172 previously treated patients. In new patients, resistance to at least isoniazid was found in 9.5% and to

rifampicin in 1.4% (Jittimanee *et al.*, 2009). In a second drug-resistance survey carried out in 2006, the rate of MDR-TB had increased to 1.7% among 1,150 new TB patients and 34.5% among 194 previously treated patients. In the new patients, resistance to at least isoniazid was found in 9.7%, and resistance to rifampicin had increased to 2.6% (WHO/SEARO, 2009).

Reechaipichitkul *et al*., (2011) conducted a similar study in a tertiary care center in northeastern Thailand to assess the local susceptibility patterns of *M. tuberculosis* and clinical outcomes of DR-TB between January 2004 and December 2008. The *M. tuberculosis* was resistant to isoniazid (2.3%), rifampicin (2.8%), ethambutol (3.8%), streptomycin (2.1%), kanamycin (0.7%) and ofloxacin (1.9%). The occurrences of MDR-TB and XDR-TB were 1.2% and 0.38%, respectively. In Bangladesh the study that investigated the drug susceptibility pattern of *M. tuberculosis* found that the drug resistance of *M. tuberculosis* to at least one drug was found in 50% of the cases. The highest resistance (26%) was found to isoniazid, which is the most popular drug in TB treatment. Resistance to streptomycin was found in 22% cases, to ethambutol was found for 20% cases, and to rifampicin was found in 12% cases (Mottalib *et al*., 2011). The study involved both new cases and treated cases where it was found that 62.2% of the new cases were sensitive to all drugs, and 37.8% were resistant to one or more drugs while drug resistant rate was higher in treated cases (84.6%) than in new cases (37.8%).

Varshney *et al*., (2014) conducted a study in India to evaluate the *in vitro* drug resistance pattern of first line anti-TB drugs and to determine the prevalence of MDR-TB in suspected cases of new and previously treated PTB patients found that out of 220 patients, 129 (58.7%) were from new cases and 91 (41.3%) were from treated cases. Totally, 44.5% were resistant to one or more than two drugs and 18.6% patients showed resistance to both isoniazid and rifampicin. The individual resistance pattern of these first line drugs were as follows: 37.7% patients were

resistant to isoniazid, 22.2% to rifampicin, 8.6% to streptomycin and 10% were resistant to ethambutol. India, designated as a high burden country for TB, has also been identified as a hot spot region for MDR-TB infection (Blower *et al*., 2004; WHO, 2005). World Health Organization estimated 8.7 million incident cases and 12 million prevalent cases worldwide in 2011. India and China accounted for almost 40% of the world's TB cases (WHO, 2012). The prevalence of MDR-TB is increasing throughout the world in both new TB cases as well as previously-treated TB cases (Prasad, 2005).

Although previous treatment for TB is the strongest risk factor for development of MDR-TB, treatment-naive patients are also at risk due to either spontaneous mutations or transmission of resistant strains (Vijay *et al.,* 2004). The risk of transmission of resistant strains from close contacts is increasing day-by-day because of overcrowding and growing burden of MDR-TB patients. Several factors have been identified in the causation of DR-TB, of which the three most important are previous treatment with anti-TB drugs which may be inappropriate and incomplete, high prevalence of DR-TB in the community and contact with a patient known to have DR-TB. In patients with previous treatment or disease, the odds of resistant TB were 4-7 times higher than that of person with no history of past treatment (WHO, 2008). Between 2000 and 2016, TB treatment averted an estimated 44 million deaths among HIV-negative people. Among HIV-positive people, TB treatment supported by ART averted an additional 9 million deaths. Drug-resistant TB is a persistent threat, with 490,000 million cases of MDR-TB emerging in 2016 and an additional 110,000 cases that were susceptible to isoniazid but resistant to rifampicin (RR-TB), the most effective first-line anti-TB drug. The countries with the largest numbers of MDR/RR-TB cases (47% of the global total) were China with a prevalence rate of 5.7% new cases and 25.6% of previously treated cases (Zheyuan *et al*., 2016), India 3% (MOH,

2018) and the Russian Federation the prevalence of primary MDR TB varied from 5.4% to 28.3% (Raviglione & Sulis, 2016).

Green *et al*. (2010) in South Africa conducted a study to assess the drug-susceptibility patterns of *M. tuberculosis* in Mpumalanga Province. In the study MTB strains from cases with PTB were tested for susceptibility against rifampicin, isoniazid, ethambutol, and streptomycin where 30.2% of the MTB strains were resistant to one or more drugs. Resistance to one drug ranged from 1.4% for ethambutol to 17.7% for rifampicin. The prevalence of MDR-TB ranged from 6.7% for three drugs to 34% for four drugs, with significant predictors being patients' age-groups of 25-54 years and >55 years. The result showed a high level (58.4%) of MDR-TB from cases in Mpumalanga province. The high number of isolated MDR-TB isolates in this study among patients is suggestive of poor management of past and existing disease (Abdool *et al*., 2009).

One study in Addis Ababa-Ethiopia to determine the prevalence and drug resistance patterns of *M. tuberculosis* among new smear positive PTB patients showed that the rate of resistance to any one drug was 23%. Any resistance to isoniazid, streptomycin, rifampicin, and ethambutol was 14%, 11.5%, 2.8%, and 0.3%, respectively. The highest proportion of mono-resistance was observed against isoniazid (9.5%) (Seyoum *et al*., 2014). Multi drug-resistant-TB was detected in 1.1% of the patients. Although the prevalence of MDR-TB was relatively low in the study area, high prevalence of isoniazid resistance is a serious concern demanding close monitoring.

In Kenya, studies reported the emergence of drug resistant TB strains from as early as 1980 (MOH, 2010b). However, information on patterns of drug resistance is very scanty (Githui *et al*., 2004). Despite the studies done in Mombasa and Nairobi, the detection rate of MDR-TB and drug resistance is very low as diagnostic methods available in the public health facilities are still

largely based on sputum slide smear microscopy (Limo *et al*., 2015). Ombura *et al*., (2016), reported some drug resistance *M. tuberculosis* strains in both new and retreated cases of TB among patients seen in Coast Provincial General Hospital, Mombasa, Kenya. The MDR-TB public health crisis continues with an estimated 580,000 cases and 250,000 related deaths in 2015. Only 125,000 were started on treatment, and just half of those people were cured (WHO, 2017). In a study conducted by Usagi *et al*. (2016) the overall resistance to the first line drugs was 6.9% while the individual TB drugs had the following resistance rates; streptomycin 6.2%, isoniazid 10%, rifampicin 9.2% and ethambutol 3.8%. The study also revealed a high MDR-TB prevalence rate of 18.46% among HIV/TB patients in Kisumu County.

Multi drug-resistant-TB is associated with higher rates of treatment failure and death; is more difficult and expensive to treat than drug-susceptible TB and has an increased risk of adverse-effects for patients (Han *et al.*, 2005). Most of the studies done to assess the drug susceptibility patterns of mycobacteria used only strains of MTBC, and NTM were not factored in showing/implying that NTM are not yet recognized as true pathogens and do not have any clinical importance (Seyoum *et al*., 2014). Also NTM are resistant to most of first-line conventional anti-TB drugs. The risk of DR-TB transmission can be reduced by efficient diagnosis and timely treatment of DR-TB patients (WHO/HTM/ TB/2006). Thus, the drug resistant status of a patient needs to be confirmed before treatment. Better access to drug resistant/susceptibility test results at the time of diagnosis of TB would facilitate appropriate selection of treatment regimens, thereby minimizing the development of drug resistant strains. Most if not all health facilities and hospitals in Kenya treat their patients only after microscopic examination which alone cannot ensure proper treatment (Mottalib *et al*., 2011).

Mono-drug resistance can be treated initially with standard first-line drugs, although the clinical course may be worse during the maintenance phase, especially if there is resistance to rifampicin. For two, three, or four drug resistance, the recommended regimen is second-line anti-TB drugs, based on the susceptibility pattern. Unfortunately, in MDR-TB, the treatment outcome is poor even when second-line anti-TB drugs are used (Orenstein *et al*, 2009). Every patient infected with MDR-TB needs strong patient support, treatment compliance, and follow-up (Ahmad and Mokaddas, 2009). Determination of the pattern of drug resistance enables health care workers to determine the type and combination of anti-TB drugs to administer to a particular patient as the treatment regimen and duration varies depending on the type of anti-TB drugs a patient is resistant to (Seyoum *et al*., 2014).

The BACTEC MGIT 960 susceptibility testing for streptomycin (S), isoniazid (I), rifampin (R) and ethambutol (E), called SIRE, and the BACTEC MGIT 960 PZA against pyrazinamide (Becton-Dickson and Company, Sparks, MD, USA) was used in the present study. This test and system works on the principle, of an increase in the fluorescence in the sensor measured automatically and designated as growth value (GV). If a drug is added to the medium which is bacteriostatic or bactericidal to the test mycobacteria, it inhibits growth and thus, there is little or no oxygen consumption, therefore little or no fluorescence of the sensor (Siddiqi & Rusch-Gerdes, 2006). Isolated cultures from TB patients were subjected to growth in the presence of a known concentration of a test drug. A control is also included with no addition of the drug. If the patient's isolate grows in the control but does not grow in presence of the drug, it is considered susceptible. If it grows in both the tubes, then it is considered to be resistant to that drug. Pyrazinamide susceptibility testing was performed on MTBC isolates using the BACTEC MGIT 960 liquid culture system in accordance with the manufacturer's recommendations.

Susceptibility testing against PZA is carried out at a lower pH (5.9) of the medium, since PZA is active only at the low pH *in vitro*. The BACTEC MGIT 960 PZA susceptibility test is a qualitative procedure to test susceptibility of MTBC against PZA. Results are obtained within 4-21 days. The MGIT 960 medium is a modified 7H9 broth with a reduced pH of 5.9. The detection of growth is achieved by the oxygen sensor at the bottom of the tube.

Based on the literature review, members of MTBC have shown to possess varying patterns of anti-TB susceptibilities. This formed the basis for this current study which was pursued to investigate/determine the current status of the susceptibility patterns of MTBC isolates against commonly used first-line conventional anti-TB drugs in newly infected PTB patients in Kisumu county. This will guide physicians in prescribing appropriate drugs for the proper treatment of TB patients to avoid the emergence of MDR -TB strains. This study supports the suggestion for accurate diagnosis through culture and identification of Mycobacteria to species level and sensitivity testing patterns of Mycobacteria isolates from new cases of PTB patients and proper prescription of anti-TB drugs for the clinical treatment of TB patients.

2.13. Antimycobacterial Activity of *Persea americana* Plant Extracts

The *P. americana* pod contain several different secondary metabolites or phytochemicals which makes the pod to poses some antimicrobial activity against microorganisms. They include: alkaloids, flavonoids, terpenoids, tannins, saponins, phenolic acids, oils and fatty acids. These phytochemicals have different mechanisms of action (Cushnie *et al*., 2014).

A study conducted in Mexico to test the effect of *P.americana* leaf extract and active fractions against *M. tuberculosis* H37Ra and H37Rv strains found that methanol extract possessed high anti-mycobacterial activity by inhibiting the growth of *M. tuberculosis* H37Ra (MIC=125µg/ml)

and H37Rv (MIC= 62.5µg/ml); furthermore, the hexane fraction inhibited the growth of both mycobacteria with MIC = 31.2µg/ml (Gomez-Flores *et al.,* 2008). It was also observed that hexane fraction of *P. americana* extract had some anti-mycobacterial activity against H37Ra and H37Rv strains. But this study did not highlight the anti-mycobacterial activity of *P.americana* extract against NTM. The study also did not highlight the anti-mycobacterial activity of *P.americana* pod extract against mycobacteria. The hexanic and methanoic extracts obtained from the stems and leaves of *P. americana* have been reported to inhibit the growth of *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra strains (Jiménez-Arellanes *et al*., 2013).

In one study done in Nigeria (Cordell, 2015) methanol and aqueous extracts of root and stem bark of *P. americana* exhibited some antibacterial activity against selected clinical isolates. In addition, their antimycobacterial activity was evaluated against four mono-resistant reference strains of *M. tuberculosis* H37Rv, two MDR *M. tuberculosis* clinical isolates and five NTM. According to the study done by Adelina Jiménez-Arellanes and his colleagues (2013) in Mexico to study the antimycobacteria activity of *P.americana* seeds showed that the chloroformic extract inhibits the growth of *M. tuberculosis* H37Rv, MDR *M. tuberculosis* SIN 4 and three out of four mono-resistant reference strains of *M. tuberculosis* H37Rv (INH-R, STR-R, and EMB-R), showing a MIC = 50 µg/ml. The extract is also active against the NTM: *Mycobacterium fortuitum, Mycobacterium avium, Mycobacterium smegmatis* and *Mycobacterium abscessus* with MIC values < 50 µg/ml. However, the ethanoic extract affect only the growth of *M. smegmatis* (MIC = 25 µg/ml) and the mono-resistant strains of *M. tuberculosis* H37Rv STR-R and EMB-R (MIC = 50 µg/ml).

The use of medicinal herbal plants in Kenya has been practiced for a long period with very little documented information. The effectiveness of most of these plants has not been scientifically

evaluated. For example, diarrhea and other infectious diseases both in children and adults, and in TB and HIV and AIDS patients are quite common in Kenya, more so in the rural areas and crowded places (Gomez-Flores *et al.,* 2008). In rural areas a number and variety of plants are used by different communities to treat these diseases without prior scientifically determined information. Studies have shown that about 30% to 40% of total plants in the plant kingdom are involved in current conventional drugs and few of them are served as nutritional supplements and more (Kirby, 1996; Hostettmann & Marston, 2002).

Different parts of *P.americana* extracts have been shown to have anti-mycobacterial activity (Tijjani, 2013). Herbalists in some parts of Nyando in Kano, Kisumu County, Western Kenya have demonstrated that aqueous pod extracts of *P. americana* may confer relief from TB related signs and symptoms such as persistent coughing, weight loss, sweating at night and blood in the sputum in HIV and AIDS persons (unpublished). However, there lacks a documented scientific evidence to elucidate the antimicrobial activity of *P.americana* pod extract against clinical isolates of mycobacteria. The current regimen of conventional anti-TB drugs are administered for a long duration of 6 to 24 months which leads to patients compliance failure, toxicity, adverse side effects and even death (Ndung'u *et al.,* 2012). Patients compliance failure leads to the emergence of drug resistance strains of mycobacteria which is very dangerous, difficult and expensive to treat (Ombura *et al*., 2016). This has lead to patients seaking for alternative drugs which are effective, non toxic, have less side effects and easily available, hence sorting to use of traditional medicines from plants (Parekh and Chanda, 2007).

These research findings provide evidence to support the antimycobacterial activity of *P.americana* plant extracts on mycobacteria and other bacteria. However, these studies did not assess the anti-mycobacterial activity of *P.americana* extracts against NTM. The studies also did

not investigate the anti-mycobacterial activity of *P.americana* pod extract against mycobacteria isolates hence the current study was set to determine the antimycobacterial activity of ethanol, *n*-hexane and chloroform *P.americana* pod extracts on mycobacteria clinical isolates from new cases of PTB patients in Kisumu County.

2.13.1. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration is defined as the lowest concentration of an antimicrobial drug that inhibits visible microorganism growth after incubation with culture media. This is a test that is used to evaluate a new chemical compound but it can be applied in evaluating the activity of crude plant extracts (Pesewu *et al*., 2008). Plant extracts such as pyrethrum plant extract are made of loop/composed of several compounds that can have inhibitory activity against various microorganism. This compounds are known as secondary metabolites. Fractionating the metabolites into individual components will alter the activity of the plant extracts (Pereira *et al*., 2016). Pyrethrum plant extract was an effective insecticide but after its "separation" into its specific constituents (pyrethrin I, pyrethrin II, cinerin I, cinerin II, jasmolin I,and jasmolin II), its activity was altered (Khazaal and Shawkat, 2011). This demonstrates that separation of a plant extract into individual metabolite alters the activity, thus the need to evaluate the activity of *P. americana* as an extract and not the specific metabolite.

2.13.2. Minimun inhibitory concentration of plant extracts against MTBC and NTM

The ethanobotanical study carried out in Mexico showed that extracts of *Citrus aurantifolia, Citrus sinensis* and *Olea europaea* were active against both drug-susceptible and drug resistant strains of virulent *M. tuberculosis* with MIC between 0.1 and 0.025mg/ml (Favela-Hernández *et al.,* 2016). According to the study done in Ethiopia the MIC of 80% methanolic root extracts of

selected plants varied as follows; *Calpurnia aurea (*25–100 μg/mL and 12.5-75 μg/mL), seeds of *Ocimum basilicum* (25–100 μg/mL and 25–50 μg/mL), leaves of *Artemisia abyssinica* (6.25-50 μg/mL and 12.5-50 μg/mL), *Croton macrostachyus* (12.5-100 μg/mL and 18.25-50 μg/mL) and *Eucalyptus camaldulensis* (6.25-50 μg/mL and 12.5-50 μg/mL) for *M. tuberculosis* and *Mycobacterium bovis* strains (Gemechu *et al.*, 2013). The investigation showed that leaves of *Eucalyptus camaldulensis* extract was the most active against both *Mycobacterium tuberculosis* and *Mycobacterium bovis* strains with an MIC of 6.25-50μg/mL.

A study done by Ogudo *et al.*,(2014) with methanol and *n*-hexane extracts of *Curcuma longa*; and dichloromethane extracts of *Zingiber officinale* Rosc. (ginger) rhizomes, determined MIC using *Mycobacterium fortuitum* ATCC 684 and *Mycobacterium abscessus.* The MIC values confirmed the existence of inhibitory effects of *Zingiber officinale* and *Curcuma longa* dried rhizome with values ranging from 6.25 mg/mL to 25 mg/mL for both extracts on selected susceptible species. Methanol and hexanic extract of *Curcuma longa* both had an MIC of 25 mg/mL against *Mycobacterium abscessus.*

Based on the literature reviewed, different plant extracts have been tested against MTBC and NTM with varying values of MIC. Methanoic, *n*-hexane and ethanoic extracts of various parts of *P. americana* plant have shown different MICs against *M. tuberculosis* and NTM but there is no literature showing the MIC of *P.americana* pod extract against *M. tuberculosis* and NTM. This calls for the investigation/determination of MIC of *P. americana* pod extract against MTBC and NTM clinical isolates from PTB patients to evaluate their potency. Thus the fourth specific objective of the current study determined the antimycobacterial activity (MIC) of chloroformic, ethanoic and *n*-hexanic *P. americana* pod extracts against MTBC and NTM clinical isolates from new cases of PTB patients in Kisumu County.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study Area

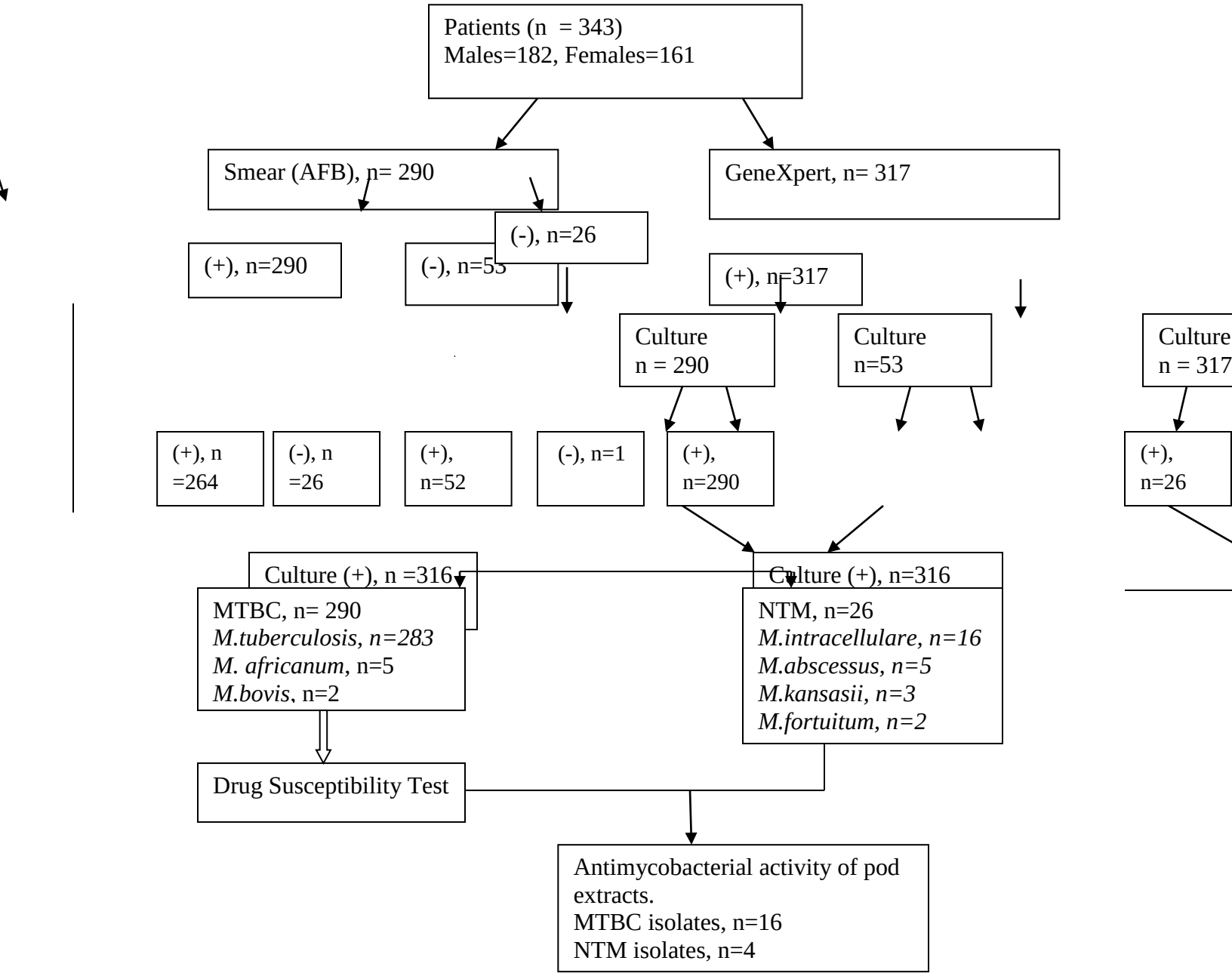
The specimens were obtained from new cases of PTB patients within Kisumu County who attended Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH) and Kisumu County Hospital. The new cases of PTB were diagnosed using smear microscopy and GeneXpert *MTB/RIF* tests in TB Laboratories. Kisumu County is one of the new devolved counties of Kenya. Jaramogi Oginga Odinga Teaching Referral Hospital is a level 5 Hospital which provides diagnostic and treatment care services which include referral services; obstetrics and gynaecology; maternal child health; medicine; surgery, including anaesthesia; accident and emergency services; non-clinical support services; contribution to region-wide information generation, collection planning, implementation and evaluation of health service programmes. Kisumu County Hospital is a level 4 Hospital which provides services such as obstetrics and gynaecology; child health; medicine; surgery, including anaesthesia; accident and emergency services; non-clinical support services; referral services; contribution to the district-wide information generation, collection planning, implementation and evaluation of health service programmes. Kisumu County is divided into administrative and political units. It is one of the 6 counties found in the former Nyanza Province with the highest TB-HIV and AIDS prevalence (MOH, 2013). Its borders follow those of the original Kisumu District, one of the former administrative districts of the former Nyanza Province in western Kenya. Kisumu City is its headquarters and is considered western Kenya's most important urban area (Maoulidi, 2011). It covers an area of 2085.9 sqkm with a population of 968,909 (according to the 2009 National

population Census). Its geographical coordinates are 0° 5' 51" South, 34° 45' 16" East. The key health indicators are high burden of HIV at 18.7% prevalence (KAIS, 2012) and TB at 31% (MOH, 2015). The main economic activities are fishing, small business and farming/agriculture. Approximately 90% of the inhabitants survive on less than a dollar per day (MOH, 2013). The total TB cases notified in the county in 2013 were 3,362 giving a case notification rate for TB at 317 per 100,000 which is above the national average of 217 per 100,000. The TB/HIV co-infection rate is 67% which is far above the national rate at 37% reflecting the prevailing high TB/HIV burden in the county (MOH, 2013). The county has an annual relief rainfall that ranges between 1200 mm and 1300 mm in different areas. The rain mainly falls in two seasons. The "Long Rains" March-April-May season and short rains in the remaining months. This seasonal rainfall highly impacts on the agricultural sector and hence food security in the country (KMD, 2019). Kisumu is warm throughout the year with a mean annual temperature of 23^0C. The temperature ranges between 20^0C and 35^0C but seldom falls below 19^0C. The humidity is relatively high throughout the year. The County neighbours Siaya County to the west, Vihiga County to the north, Nandi County to the north east, Kericho County to the east and Homa Bay County to the south west. The County has a shoreline on Lake Victoria. (Appendix 1)

3.2. Study Design

A cross-sectional descriptive study was conducted between February 2016 and August 2017 that involved three TB screening tests: GenXxpert *MTB/RIF* assay, smear microscopy and culture.

Figure 3.1: Conceptual Framework



3.3. Study Variables

3.3.1. Dependent variable

The study had the following dependent variables: Mycobacterial isolates including MTBC and NTM subspecies, antimycobacterial susceptibilities and the isolates, antimycobacterial activity of *P. americana* pod extracts.

3.3.2. Independent variable

Susceptibility profiles of first line conventional anti-TB drugs were independent variables in the study.

3.4. Study Population

The study included all patients attending chest clinic at JOOTRH and Kisumu County Hospital as they seek healthcare services for routine checkup. Individuals referred for TB screening and consented were enrolled into the study. Patients with sputum positive specimens for GeneXpert *MTB/RIF* and/or acid-fast bacilli on direct examination by ZN stain were recruited into the study.

3.5.1. Inclusion criteria

Only new cases i.e. newly diagnosed (those who had no prior exposure to anti-TB drugs) of consenting PTB patients attending the chest clinic of JOOTRH and Kisumu County Hospital and who turned out to be GeneXpert *MTB/RIF* positive and smear positive or either after spot sputum analysis were recruited. All patients were adults (18 years and above).

3.5.2. Exclusion criteria

Pulmonary TB patients who turned out to be GeneXpert *MTB/RIF* positive and smear positive or either after spot sputum analysis but on treatment with anti-TB drugs were excluded from the

study. Also those below 18 years, those who decided to consent or those with direct spot sputum analysis and GeneXpert *MTB/RIF* negative, relapse, treatment failure and on treatment were excluded from the study.

3.6. Sampling Methods

Jaramogi Oginga Odinga Teaching & Referral and Kisumu County Hospitals were conveniently chosen because the GeneXpert *MTB/RIF* testing facilities were available. All patients who fulfilled the inclusion criteria and who consented to participate were enrolled into the study and sputum samples were collected to saturation.

3.7. Sample Size Determination

Tuberculosis prevalence rate in Kisumu County is 31% (MOH, Kisumu County Annual Report, 2015). A sample size used was based on the above TB prevalence for Kisumu County. Daniel, (1999) sample size calculation formula was used in the current study.

$n = Z^2 * P(1-P)/d^2$

Where,

n = sample size

Z = statistic for level of confidence,

P = expected prevalence or proportion (in proportion of one; if 31%, P=0.31)

d = precision

(in proportion of one; if 5%, d = 0.05)

Z statistic (Z): For the level of confidence of 95%, which is conventional, Z value is 1.96. In these studies, investigators present their results with 95% confidence intervals (CI).

$$n = \frac{1.96 \times 1.96 \times 0.31 \times 0.69}{0.05^2}$$

$$= \frac{3.8416 \times 0.2139}{0.0025} = 328.69 = 329$$

Additional 5% of non-response rate was used to adjust the sample size to take care of sampling error and so the new sample size was (329 + 17) bringing the total sample size to 346 participants.

3.8. Methods of Data Collection

3.8.1. Dermographic information

Age and gender of the study participants were recorded in standard data culture form (Appendix 16).

3.8.2. Laboratory procedures

3.8.2.1. Sputum sample collection

Once a suspected PTB patient visited a chest clinic of JOOTRH and Kisumu County Hospital, sputum was collected under the supervision of a trained and competent medical staff. The patient was instructed to rinse mouth with water before sample collection to minimize contamination of specimen. Sputum specimen was collected as previously described by Nyamogoba and colleagues (2012). Briefly, at least 2 ml of three sputum specimens [(1) first spot sample in the clinic the same day, (2) early morning home-collected sample because the sample would have the highest yield at this time and (3) second spot sample in the clinic the next day] was collected. Patients were requested to cough so that expectoration would come from as deep down the chest as possible, and spit into a sterile 50 ml specimen bottles/blue falcon cap tubes (Nyamogoba *et al.*, 2012). Only expectorated sputum specimen and not saliva was collected. All the sputum

specimens were subdivided into three portions to be subjected to the following: (i) ZN staining for smear microscopy, (ii) Gene Xpert *MTB/RIF PCR* test, (iii) Culture on MGIT. Each specimen was labeled with the patient name, hospital number (patient ID), date and time of collection. The samples were refrigerated at 4^{o}C before being transported in ice cooler boxes to the Medical Microbiology Laboratory, at Moi University once weekly for analysis. The samples were processed within seven days of collection in order to minimize loss of viability of the mycobacteria. The safety for healthcare workers during collection and handling of sputum specimen was ensured by observing the WHO guidelines (Steingart *et al.,* 2007; Bock *et al*., 2007).

3.8.2.2. Sputum sample processing-digestion, decontamination and concentration process by NaOH-NALC method

The standard procedure recommended for MGIT to obtain optimal results was used. All clinical sputum specimens from TB patients were processed by the digestion-decontamination process as described by Steingart *et al*., (2007) using sodium hydroxide-N-acetly-L-cysteine (NaOH-NALC) prepared in the laboratory (Appendix 2, 3, 4 & 5). Sodium hydroxide-N-acetly-L-cysteine-sodium citrate solution was added in a volume equal to the quantity of specimen and the cap tightened. The mixture in a tube was slightly vortexed or hand mixed for 15–30 seconds and put on a shaker and shaken lightly during the whole time for 15-20 minutes until the specimen was completely liquefied. Phosphate buffer pH 6.8 was added up to the top ring on the centrifuge tube (falcon plastic tube has a ring for 50 ml mark), mixed well by lightly vortexing or inverting several times and centrifuged at a speed of 3000 g for 15-20 minutes. Tubes were allowed to sit for 5 minutes after centrifugation to allow aerosols to settle. The supernatant was carefully discarded/decanted into a suitable container containing a mycobactericidal disinfectant and 1–2

ml of phosphate buffer pH 6.8 was added (to neutralize NaOH) and the sediment resuspended with the help of a pipette or vortex mixer. The resuspended pellet (last suspension) was used for preparing smears for microscopic examination and for inoculation of MGIT tubes.

3.8.2.3. Smear preparation for acid-fast staining

Smears were prepared from all processed specimens before inoculation into medium as described by Siddiqi and Rusch-Gerdes, (2006). Smears were prepared on separate slides by placing a drop of the processed specimen on clean microscopic slides, spread and allowed to air dry completely then heat fixed either by passing over the flame 3 to 4 times or by heating on a slide warmer at 65-75^{0}C for 2-3 hours or overnight. The smear was not overheated or exposed to ultra violet (UV) light. All the above procedures were performed in a biological safety cabinet. The smears were handled carefully according to Mycobacteriology Laboratory Manual, (2014) since mycobacteria may still be viable (Appendix 15). Positive smears were also prepared from positive cultures of *M. tuberculosis* (H37Rv, ATCC #27294 or H37Ra ATCC 21577) and *E.coli* culture was used as negative control which was examined before examining the slides from clinical specimens.

3.8.2.4. Procedure for acid-fast staining by Ziehl-Neelsen method

This was performed at JOOTRH and Kisumu County Hospital as described by Steingart *et al*., (2007). Briefly, the prepared smears were placed on a boiling water bath then the slides were flooded with carbol fuchsin stain (Appendix 8). Slides were heated gently until steam rises (3-5 minutes). The slides were not allowed to dry out or the stain to boil, more stain was added if necessary as it dries during heating. Slides were stained for 5-10 minutes, removed from heat and allowed to cool. Then rinsed gently with distilled water for 30 seconds and drained. Smears were

decolorized by adding acid alcohol (Appendix 8) drop by drop for 2 minutes or until no more colour appears with acid alcohol/ the smear remaining only slightly pink. Slides were rinsed gently with distilled water for 5 seconds and excess water was drained. Smears were counterstained with alkaline methylene blue (Appendix 8) for 1-2 minutes, rinsed gently with distilled water for 30 seconds and excess water drained. The slides were then air-dried and observed under oil immersion microscope. Blot drying was not done as it may remove smear accidentally. Positive smears were also prepared from positive cultures of *M. tuberculosis* (H37Rv, ATCC #27294 or H37Ra ATCC 21577) and *E.coli* culture was used as negative control which were examined before the slides from clinical specimens.

3.8.2.5. Procedure for GeneXpert *MTB/RIF* PCR test

GeneXpert *MTB/RIF* Test (Cepheid, Sunnyvale, CA, USA) was performed according to manufacturer's instructions in the two health facilities. Sputum specimens were vortexed for 1 minute with glass beads and split, aliquots were frozen at -70^{0}C to be finally analyzed by the Xpert *MTB/RIF* assay, according to manufacturer's instructions. Each Xpert *MTB/RIF* cartridge was labeled with the corresponding specimen ID. Two ml of Xpert *MTB/RIF* sample treatment reagent was added to each volume of sputum in 15 ml falcon or screw-capped tube using a sterile pipette in a 2:1 ratio (2:1; v/v). The cap was replaced and the mixture in the tube was manually agitated twice. The tube was allowed to stand upright for 5 minutes at room temperature then vortexed for 30 seconds and allowed to stand upright for another 10 minutes at room temperature. The specimen was inspected to make sure it is liquefied with no visible clumps of sputum. The cartridge lid was opened and by using the sterile transfer disposable pipette provided in the Xpert *MTB/RIF* kit, 2 ml of the inactivated liquefied specimen was aspirated into the transfer pipette until the meniscus was above the minimum mark and then the sample was transferred into the

open port of the Xpert *MTB/RIF* cartridge. The catridge lid was closed. Cartridges were loaded into the GeneXpert (Cepheid, Sunnyvale, CA, USA). All subsequent steps occur automatically (sample automatically filtered and washed; ultrasonic lysis of filter-captured organisms to release DNA; DNA molecules mixed with dry PCR reagents; seminested real-time amplification and detection in integrated reaction tube). At the end of the real-time PCR run, the Xpert *MTB/RIF* assay's data analysis algorithm identified a specimen as MTB positive if at least two of the five *rpoB* probes were positive within two cycles of each other. Failure of one or more of the *rpoB*-specific molecular beacons to hybridize to the *rpoB* amplicon was interpreted as resistance. Data interpretation was received after completion of the PCR run through computed software (GeneXpert Dx System Operator Manual. Sunnyvale, CA, USA: Cepheid Inc; 2011). The results were interpreted by the GeneXpert Dx System from measured fluorescence signals and embedded calculating algorithms and were displayed in the View Results Window (GeneXpert Dx System Operator Manual, 2011), as indicated: MTB Detected: MTB target DNA is detected; both controls, SPC and PCC, meet the assigned acceptance criteria. Lower Ct values represent a higher starting concentration of DNA template; higher Ct values represent a lower concentration of DNA template. In MTB DETECTED results "RIF Resistance DETECTED", "RIF Resistance NOT DETECTED", or "RIF Resistance INDETERMINATE" will display on a separate line. MTB Not Detected: MTB target DNA is not detected; both controls, SPC and PCC, meet the assigned criteria. Invalid: Presence or absence of MTB cannot be determined: SPC does not meet acceptance criteria, i.e. the sample was not properly processed, or PCR was inhibibited. Note: repeat test with extra specimen. Error: One or more of the PCC results failed (FAIL). Both MTB and SPC display NO RESULT. Note: repeat test with extra specimen. If the PCC passed (PASS),

the error is caused by a system component failure (GeneXpert Dx System Operator Manual, Sunnyvale, CA, USA: Cepheid Inc; 2011).

3.9. Culturing of Mycobacteria in MGIT Tubes

Briefly, MGIT tubes (Appendix 7) were labelled with specimen number, then the cap was unscrewed and 0.8 ml of MGIT supplement was added. Up to 0.5 ml of well mixed processed specimen was added/inoculated into the appropriately labelled MGIT tubes of the BACTEC MGIT 960 System (Becton-Dickinson Diagnostic Instrument Systems, Towson, MD, USA) using separate pipette or pipette tip for each specimen. The tubes were immediately recapped tightly and mixed by inverting the tubes several times. The tubes and caps were wiped with a mycobactericidal disinfectant and left at room temperature for 30 minutes before incubation. The tubes were entered into the MGIT 960 instrument. Shaking of tubes during incubation was avoided as it helped to maintain the oxygen gradient in the medium. The vials were incubated at 37^0C and monitored automatically every 60 minutes for an increase in fluorescence for a maximum of six weeks until the culture flagged positive. The instrument signals a tube positive for growth, and an indicator green light shows the exact location of the positive tube in the drawer of the instrument. Any sample which was identified as positive was removed from the instrument and observed visually. Mycobacterial growth appears granular and not very turbid while contaminating bacterial growth appears very turbid. Growth of *M. tuberculosis* complex settles at the bottom of the tube. From the positive tubes, a smear was prepared for examination of acid-fast bacilli using ZN staining method as previously described to confirm the presence of mycobacteria and not contaminating bacteria. Furthermore, subculture was done by inoculation on a blood or chocolate agar, or Triple Sugar Iron Agar (TSI), to rule out the presence of

contaminating bacteria. After a maximum of six weeks, the instrument flags the tubes negative if there is no growth (Siddiqi & Rüsch-Gerdes, 2006).

3.10. Molecular Identification of MTBC and NTM

Sputa specimens from each patient were processed for isolation of mycobacteria by following standard protocols. A participant with at least one positive MGIT culture was considered as a TB case, while those with three negative culture results were regarded as not having TB. Mycobacterial isolates were identified as MTBC or NTM from the positive cultures using three commercially available DNA strip assays Hain's GenoType® Mycobacterium CM/AS and GenoType® Mycobacterium MTBC Molecular Genetic Assays, (Hain Lifescience GmbH, Nehren, Germany) which were executed consecutively.

3.10.1. GenoType® Mycobacterium CM/AS assays

3.10.1.1. DNA extraction

DNA extraction was performed by sonication in accordance with the manufacturer's instructions. In brief, 1 ml of positive bacteria culture grown in MGIT culture medium was transferred to 2 ml cryovials, pelleted by centrifugation/spinning for 15 minutes in a standard table top centrifuge with an aerosol-tight rotor in a class II biosafety cabinet at approximately 10000 x g. The supernatant (the liquid portion) was discarded and bacteria pellet resuspended in 200 µl of lysis buffer (deionized water) by vortexing followed by heating or incubation for 20 minutes at 95^0C in water bath/hot plate and further incubation for 15 minutes in an ultrasonic bath, then spun down for 5 minutes at 13,500 g/rpm (full speed) and 5µl of the supernatant was directly used for the PCR assay (Lee *et al.*, 2009). The DNA solution to be stored for an extended time period for performing other assays was transferred to a new tube.

3.10.1.2. DNA amplification

Identification of Mycobacteria species were carried out by using specific sets of primers designed to amplify a species specific 23S rRNA gene sequence of Mycobacterium species. Amplification mixture (45 µl) was prepared in DNA free room, including 5 µl extracted DNA (20-100 ng DNA) in the reaction mixture contained 35 µl primer nucleotide mix, 5 µl 10 × polymerase incubation buffer for HotStar Taq (QIAGEN, Hilden, Germany), 2 µl 25 mM $MgCl_2$ solution, 0.2 µl HotStar Taq and 3 µl biology grade water (Appendix 10 & 11). Amplification was carried out in a thermal cycler (MJ Research, PTC-100 Thermal Cycler, GMI, Inc, USA), which involved 1 cycle of denaturation solution (DEN) at 95°C for 15 min, annealing of primers at 95°C for 30 s, 2 min at 58°C for 10 cycles, then 20 cycles at 95°C for 25 s, 53°C for 40 s and 70°C for 40 s and final primer extension at 70°C, 8 min for 1 cycle. The amplified products were stored at +8 to −20°C until hybridization was done in hybridization machine (Profiblot; Tekan, Maennedorf, Switzerland).

3.10.1.3. Procedure for CM/AS assays hybridization

At least 20 µl of Denaturation Solution was dispensed in the corner of each of the wells used and then 20 µl of amplified sample was added by pipetting up and down to mix well and incubated at room temperature for 5 minutes. Upto 1 ml of pre-warmed Hybridization Buffer (HYP, green) was added carefully to each well followed by gently shaking the tray until the solution develops a homogenous color. A strip was placed in each well using tweezers in a manner to make sure complete flooding of solution over strips. The tray was then placed in shaking TwinCubator and was incubated for 30 minutes at 45^0C followed by complete aspiration of Hybridization Buffer. Washing was done by 1 ml of Stringent Wash Solution (STR, red) to each strip and incubated for 15 minutes at 45^0C in shaking TwinCubator. Hybridization was being done at room temperature

from this step forward. Stringent Wash Solution was completely removed. Again each strip was washed once with 1 ml of Rinse Solution for 1 minute on shaking TwinCubator. Then 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking TwinCubator. The solution was removed and each strip washed twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with 1 ml of distilled water on shaking TwinCubator (pour out solution each time). Approximately 1 ml of diluted substrate was added to each strip and incubated protected from light without shaking for 3-20 minutes. As soon as bands were clearly visible brief rinsing was done twice with distilled water to stop the reaction. Strips were removed from the tray using tweezers and dried between two layers of absorbent paper. Evaluation and interpretation of the results was done based on the presence and absence of different bands and compared with reference band provided in the kit (Lee *et al.*, 2009).

3.10.2. Procedure for GenoType® Mycobacterium MTBC assay hybridization

The stored extracted DNA solution that remained after pipetting out 20µl used in GenoType® Mycobacterium CM/AS assay was used in GenoType® Mycobacterium MTBC Assay for the identification of MTBC species. Hybridization and detection were carried out in a TwinCubator washing and shaking device (HAIN Lifescience, Germany). The GenoType CM/AS assay was performed as recommended by the manufacturer, and the species were identified according to the interpretation chart provided with the kit. Only those strips, that developed both control bands, UC (Universal Control) and CC (Conjugate Control), were regarded as interpretable. The GC (Genus Control) reaction zone of the membrane strip, which hybridizes with amplicons generated from all members of the Mycobacteria, should be positive for the whole set of studied strains.

Briefly, 20 µl of Denaturation Solution was dispensed in the corner of each of the wells used and then 20 µl of amplified sample was added by pipetting up and down to mix well and incubated at room temperature for 5 minutes. At least 1 ml of pre-warmed Hybridization Buffer (HYP, green) was added carefully to each well followed by gently shaking the tray until the solution develops a homogenous color. A strip was placed in each well using tweezers in a manner to make sure complete flooding of solution over strips. The tray was then placed in shaking TwinCubator and was incubated for 30 minutes at 45^0C followed by complete aspiration of Hybridization Buffer. Washing was done by 1 ml of Stringent Wash Solution (STR, red) to each strip and incubated for 15 minutes at 45^0C in shaking TwinCubator. Hybridization was being done at room temperature from this step forward. Stringent Wash Solution was completely removed. Again each strip was washed once with 1 ml of Rinse Solution for 1 minute on shaking TwinCubator. Then 1 ml of diluted Conjugate was added to each strip and incubated for 30 minutes on shaking TwinCubator. The solution was removed and each strip washed twice for 1 minute with 1 ml of Rinse Solution (RIN) and once for 1 minute with 1 ml of distilled water on shaking TwinCubator (pour out solution each time). Approximately 1 ml of diluted substrate was added to each strip and incubated protected from light without shaking for 3-20 minutes. As soon as bands were clearly visible brief rinsing was done twice with distilled water to stop the reaction. Strips were removed from the tray and dried between two layers of absorbent paper using tweezers. Evaluation and interpretation of the results was done based on the presence or absence of different bands and compared with reference band provided in the kit (Lee *et al.*, 2009).

3.11. Determination of First-Line Conventional Anti-TB Drugs Susceptibility

3.11.1. SIRE drug susceptibility testing

A total of 290 confirmed positive MTBC isolates identified using a commercially available DNA strip assay kit GenoType® Mycobacterium MTBC Molecular Genetic Assays, (Hain Lifescience GmbH, Nehren, Germany) were subjected to DST. Three MTBC species were identified *M. tuberculosis* (283/290), *M. africanum* (7/290) and *M. bovis* (2/290).

3.11.2. Inoculation and incubation

This was performed in accordance with the manufacturer's recommendations. For each test clinical isolate/test culture, 5 MGIT tubes were labeled. Four of the tubes contained the drugs (BACTEC MGIT SIRE; Becton Dickinson), and one was a drug-free growth control (GC). Aseptically 0.8 ml of BACTEC 960 SIRE Supplement was added to each of the labelled MGIT tubes and 0.1 ml (100 microliter) of reconstituted STR drug in the STR labelled tube was added. Similarly, other drugs in the other labelled tubes were added. No drug was added to the GC tube. The final critical concentrations of each drug in the test tubes was 1.0µg/ml STR, 0.1µg/ml INH, 1.0µg/ml RIF, and 5.0µg/ml EMB (Appendix 12). Each of the drug containing tubes was inoculated with 0.5ml of the inoculum (Appendix 14). No inoculum was added to the control. For the control, the test culture suspension was first diluted 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline, then 0.5ml of this diluted suspension was added into the growth control tube, the caps were tightened and the inoculated broth was mixed well by gently inverting the tube several times. Labelled tubes were placed in the correct sequence in the set carrier (GC, STR, INH, RIF, and EMB) of the MGIT machine. The susceptibility set carrier was entered into the BACTEC MGIT 960 instrument using susceptibility test set entry feature. The order of the tubes in the AST Set Carrier was ensured that it conforms to Set Carrier

definitions. For example, GC, STR, INH, RIF, EMB for the SIRE standard testing. Purity of the inoculum was checked by streaking the test culture suspension onto blood agar plate and incubate at 35^0C ± 1^0C for 48 hours and checked if there was any growth. *M. tuberculosis* H37Rv (ATCC) which is susceptible to all anti-TB drugs was used as a control organism.

3.12. Pyrazinamide susceptibility testing

Pyrazinamide susceptibility testing was performed on MTBC isolates using the BACTEC MGIT 960 liquid culture system in accordance with the manufacturer's recommendations.

3.12.1. Inoculation and incubation

This was performed as described by Siddiqi & Rusch-Gerdes, (2006) in the BACTEC MGIT 960 System Manual, 2006. Two MGIT PZA tubes were labeled, one as GC (growth control) and one as PZA (drug containing). Aseptically, 0.8 ml of PZA supplement was added to each of the two tubes followed by addition of 0.1 ml (100 µl) of the reconstituted drug into the PZA tube (Appendix 13). This gave 100 µg PZA per ml of the medium. No drug was added to the GC tube. Inoculation of 0.5 ml of the culture suspension was done to the PZA tube. For growth control inoculation, the inoculum was diluted 1:10 by adding 0.5 ml of the culture suspension (the one used for the drug tube) to 4.5 ml of sterile saline, mixed well followed by addition of 0.5 ml of the diluted suspension into the tube labelled GC and mix well. For PZA susceptibility test, the inoculum of the control is diluted 1:10 and not 1:100 as in SIRE AST. The caps were tightened and both the MGIT tubes were mixed. The tubes were then placed in a two AST Set Carriers with the sequence of first GC and the PZA and the PZA set was entered into the instrument using AST set entry feature. The GC was placed first, and PZA second, in the AST Set Carrier. Pyrazinamide was selected as the drug in the second tube AST set carrier definition

when performing the AST set entry. The purity of the inoculum was checked by streaking the test culture suspension onto blood agar plate or chocolate agar and incubated at 35^0C ± 1^0C for 48 hours.

3.13. Antimycobacterial Activity of *Persea americana* Pod Extract on Mycobacteria Isolates

3.13.1. Plant collection and preparation of extracts

Fresh mature *P. americana* fruits were picked from a tree stand in moistened plastic bags from Nyamira County with the help of a botanist and two known traditional healers. The fruits were identified, confirmed and authenticated by herbarium staff in the Department of Botany at Maseno University and a voucher specimen number was deposited in the herbarium. Fruits were transported to the Department of Chemistry at Maseno University for extraction. The inner pod was removed from the fruit by peeling the outer covering/coat with a sterile knife. The pod was cut into small pieces and 1,500 grams was weighed and dissolved in 1,000ml of the solvents. Extraction of the pod was conducted according to Mahesh & Satish, (2008). Total extraction of the pod was done using, *n*-hexane, chloroform and ethanol separately in a warring blender for 2-5 minutes. Blending involved mixing the small pieces of the pod with *n*-hexane, chloroform and 100% ethanol and then filtering the extracts. The blending and filtering processes was repeated until the extract was very fine. The pod extracts were subjected to rotary evaporator under reduced pressure (60 RPM at 80^0C) to remove the solvent during extraction process (to concentrate them) and stored at 6°C until use. The final extract obtained was in oily form that did not require preparation of stock solution. The extracts were named *n*-hexane, chloroform and ethanol *P. americana* pod extracts. The crude pod extracts obtained was used to determine its anti-mycobacterium activity where the MIC was determined.

3.13.2. Determination of antimicrobial activity of *P. americana* pod extracts

Mycobacteria isolates identified by the Hain's GenoType® Mycobacterium CM and AS assay, both MTBC and NTM were subjected to *n*-hexane, chloroform and ethanoic pod extracts for determination of antimicrobial activity as described by Jadaun *et al.,* (2007) with some modification. A total of 20 mycobacteria clinical isolates were tested against *P. americana* pod extract. Four of the isolates were NTM (*M. intracellulare, M. fortuitum, M. kansasii, M. abscessus*) and 16 were members of MTBC with varying patterns of resistance to conventional first-line anti-TB drugs. Each test clinical isolate was tested in triplicate.

3.13.3. Inoculation and incubation

This was performed as described by Siddiqi & Rusch-Gerdes, (2016) with some slight modification in the incubation process. For each the 20 test clinical isolates of Mycobacteria (Appendix 15), 12 MGIT tubes were labeled in triplicates. Ten of the tubes contained *n*-hexane pod extract with varying concentrations, the first tube was extract-free growth control (GC) and the last tube contained DMSO as a negative control. Aseptically 0.8 ml of BACTEC 960 SIRE Supplement was added to each of the labelled MGIT tubes. Approximately 200µl was added to the second tube, 175µl to the third tube, 150, 125, 100, 75, 50, 25, 12.5, and 6.25µl to the eleventh tube. In the last tube (12^{th} tube), 100µl of DMSO was added which served as a control. The same was prepared for chloroform and ethanol extracts. Each of the extract containing tubes was inoculated with 0.5ml of the test clinical isolate (well mixed culture suspension). No inoculum was added to the first tube which was growth control. The caps for all the tubes were tightened and the inoculated broth was mixed well by gently inverting the tubes several times. Labelled tubes were incubated in the correct sequence in the set carrier of the MGIT machine until they were flagged. The 1500 grams of the plant material that was dissolved in 1000 ml of

the solvents was used in the calculation of respective concentration of the extracts that was added to the tubes. The calculations were done as follows:-

1500 g = 1000ml

? = 1ml

(1500g x 1 ml)/ 1000

= 1.5 g/ml this is the concentration after adding the solvent

After evaporation, the concentration of the extract doubles, hence the original concentration becomes 3.0 g/ml.

Therefore for 200 µl

3g = 1000 µl

? = 200 µl

(3 x 200)/ 1000

= 0.6g/ml

= 600mg/ml

Table 3.1: Showing the volume with corresponding concentration of the extract used

Serial No.	Volume of extract picked (µl)	Concentration of the extract (mg/ml)
1.	200	600
2.	175	525
3.	150	450
4.	125	375
5.	100	300
6.	75	225
7.	50	150
8.	25	75
9.	12.5	37.5
10.	6.25	18.25

3.15. Data Management and Analysis

The data collected was entered in Excel, and examined to eliminate inconsistencies in the data set. The data set was then imported to STATA version 13.0 for further analysis. Simple descriptive statistics was used to assess the sensitivity of smear microscopy and GeneXpert *MTB/RIF* assay tests using culture test as the reference standards. In order to identify the molecular types of MTBC and NTM isolates from new cases of PTB patients, cross tabulation was done between molecular types and patients characteristics. A further Fisher's exact test was used to assess the associations between patient characteristics and MTBC and NTM species identified. All statistical analyses were performed in STATA version 13.0 with significant threshold set to $P < 0.05$ at 95% confidence interval (CI). Cross tabulation of the anti-TB drugs against MTBC isolates was done to determine the susceptibility patterns of MTBC isolates against the first-line anti-TB drugs.

3.16. Ethical Considerations

The proposal was initially cleared by the School of Graduate Studies (SGS), Maseno University. The study proposal was approved by Maseno University Ethical Research Committee (MUERC) [MSU/DRPI/MUERC/00280/16] and Jaramogi Oginga Odinga Teaching and Referral Hospital Ethical Research Committee (JOOTRH ERC) [ACCREDITION NO.01713]. Permission was also obtained from JOOTRH administration. Informed consent was obtained from patients before they were enrolled into the study. The purpose of the study was explained to the candidates in English or Kiswahili or a local language where necessary before written consent was sought. Code numbers were used to identify candidates in order to maintain confidentiality. The study did not expose candidates to any unusual risks as a trained hospital staff obtained sputum specimens from patients using standard procedures.

CHAPTER FOUR

RESULTS

4.1. Sensitivity of Culture, Smear Microscopy and GeneXpert *MTB/RIF* Diagnostic Tests

Out of the 346 participants, 343 (99.1%) response rate was realized. The 343 were new cases of PTB patients who gave sputa samples out of which 182 (53.1%) were males and 161 (46.9%) females. The mean age of the patients was 35.07±14.6 (standard error of mean) with a range of 18-82 years.

Of the patients who were aged less than 35 years, 93.0% (185/199) were positive for GeneXpert *MTB/RIF*, compared to 91.7% (132/144) who were above 35 years. Among the males 94% (171/182) were positive for GeneXpert *MTB/RIF* compared to 90.7% (146/161) females. 84.4% (168/199) patients aged less than 35 years were smear positive compared to 84.7% (122/144) who were above 35 years. Among the males 82.2% (146/182) were smear positive compared to 89.4% (144/161) females. Prevalence of culture positivity amongst patients less than 35 years was 95.0% (189/199) and 88.2% (127/144) for patients above 35 years. Among the patients who were culture positive 94.5% (172/182) were males compared to 89.4% (144/161) females (Table 4.1).

Table 4.1 Socio-demographic

Socio-demographic variable	**GeneXpert(n=343)** Pos n (%)	Neg n (%)	**Smear Status(n=343)** Pos n (%)	Neg n (%)	**Culture (n=343)** Pos n (%)	Neg n (%)	**Total**
Prevalence	317(92.4)	26(7.6)	290(75.8)	53(24.2)	316(92.1)	27(7.9)	**343**
Age (years)							
Category:	185 (93.0)	14 (7.0)	168 (84.4)	31 (15.6)	189 (95.0)	10 (5.0)	**199**
Less than 35	132 (91.7)	12 (8.3)	122 (84.7)	22 (15.3)	127 (88.2)	17 (11.8)	**144**
35and above							
Sex:							
Male	171 (94.0)	11 (6.0)	146 (82.2)	36 (19.8)	172 (94.5)	10 (5.5)	**182**
Female	146 (90.7)	15 (9.3)	144 (89.4)	17 (10.6)	144 (89.4)	17 (10.6)	**161**

n = frequency, % = percentage

Key: Pos = positive results; neg = negative results

Of the 343 samples evaluated, 92.4% (n=317) were GeneXpert *MTB/RIF* positive and 7.6% (n=26) negative, 75.8% (n=290) smear positive and 24.2% (n=53) negative, while 92.1% (n=316) were culture positive and 7.9% (n=27) negative. All of the 26 (100%) GeneXpert *MTB/MRF* negative were culture positive, while out of the 53 smear negative 52 (98.1%) were culture positive and 1 (1.9%) culture negative. Of the 290 smear positive, 26 (9%) were culture negative and 264 (91%) culture positive and out of the 317 GeneXpert *MTB/MRF* positive, 27 (8.5%) were culture negative, 290 (91.5%) culture positive (Tabble 4.2).

Table 4.2: Smear microscopy, GeneXpert and culture results

	Culture Results		
	Negative n (%)	**Positive** n (%)	**Total**
Smear			
Negative	1 (1.9)	52 (98.1)	**53**
Positive	26 (9.0)	264 (91.0)	**290**
Gene Xpert			
Negative	0 (0)	26 (100.0)	**26**
Positive	27 (8.5)	290 (91.5)	**317**

Values generated by cross tabulation; n = frequency, % = percentage

The results reveal that smear smear microscopy could detect only 83.5% of the TB cases detected by culture test and miss out 16.5% of the cases. (Table 4.2.1).

Table 4.2.1 Sensitivity analysis for smear microscopy vs. culture tests

	Culture Results	
Smear Results		
Positive	264 (True Positive)	26 (False Positive)
Negative	52 (False Negative)	1 (True Negative)

Sensitivity = 264/(264+52)*100

= 83.5%

The results reveal that GeneXpert *MTB/RIF* assay test was able to detect 91.8% of the TB cases detected by culture test and miss out 8.2% of the cases (Table 4.2.2).

Table 4.2.2 Sensitivity for GeneXpert *MTB/RIF* vs. culture tests

	Culture Results	
GeneXpert Results		
Negative	290 (True positive)	27 (False Positive)
Positive	26 (False Negative)	0 (True Negative)

Sensitivity = 290/(290+26)*100

= 91.8%

4.2. Molecular Characterization of MTBC and NTM Isolates

Since sputum culture is the gold standard, only culture positive samples were used to determine the molecular identity of mycobacteria isolates. Of the 316 culture positive isolates, 290 (91.8%) were identified as MTBC and 26 (8.2%) were NTM species. Of the 290 MTBC, three different species were identified, 283 (97.6%) were *M. tuberculosis*, 5 (1.7%) *M. africanum* II and 2 (0.7%) *M. bovis* as shown in Table 4.3. The analysis showed that age category of patients less than 35 years and above 35 years were statistically significant (there was a relationship between patients less than 35 years and 35 years with MTBC isolates) with MTBC species identified (p=0.020). While sex was not statistically significant with MTBC species (p=0.696) (Table 4.3). Out of the 26 (8.2%) NTM identified, four different species were identified which included 16 (61.5%) *M. intracellulare*, 5 (19.2%) *M. abscessus*, 3 (11.5%) *M. kansasii* and 2 (7.7%) *M. fortuitum*. Apart from *M. fortuitum* all the other species of NTM isolates were more common among patients aged less than 35 yrs. However, there was no significant relationship between dermographic characteristics (age and sex) and NTM species distribution (p=0.608 and 0.182 respectively) as shown in Table 4.3.

Table 4.3: The distribution of mycobacteria species (n=316) and the relationship between patient characteristics and MTBC (n=290) and NTM (n=26) species.

	MTBC n = 290 (91.8%)				**NTM n = 26 (8.2%)**				
Demographics	*M.africanum II* n=5 (1.7%)	*M.bovis* n=2 (0.7%)	*M.tuberculosis* n=283 (97.6%)	*p*-value	*M.abscessus* n=5 (19.2%)	*M.fortuitum* n=2 (7.7%)	*M.intracellulare* n=16 (61.5%)	*M.kansasii* n=3 (11.5%)	*p*-value
Age									
< 35	1 (20.0)	0 (0)	174 (61.5)	**0.020 ***	3 (60.0)	0 (0)	9 (56.3)	2 (66.7)	0.608
≥35	4 (80.0)	2 (100.0)	109 (38.5)		2 (40.0)	2 (100.0)	7 (43.7)	1 (33.3)	
Sex:									
Male	2 (40.0)	0 (0)	127 (44.9)	0.696	3 (60.0)	1 (50.0)	11 (68.8)	0 (0)	0.182
Female	3 (60.0)	2 (100.0)	156 (55.1)		2(40.0)	1 (50.0)	5 (31.2)	3 (100.0)	

Key: MTBC = *Mycobacterium tuberculosis complex*, NTM = non tuberculous mycobacteria, < = less than, ≥ = equal to or more than/above.
* *p*-value significant at p<0.05 using Fisher's Exact test
n = frequency, % = percentage

4.3. Drug Susceptibility Patterns of MTBC Isolates against the First-Line Conventional Anti-TB Drugs

The present study included a total of 290 confirmed MTBC isolates from new cases of infected PTB patients. The MTBC species were: *M. tuberculosis* 283/290 (97.6%), *M. africanum* II 5/290 (1.7%) and *M. bovis* 2/290 (0.7%). A total of five different first line conventional anti-TB drugs were used in the current study: streptomycin (STR), isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). Comparing drug type to MTBC species, *M. tuberculosis* was highly sensitive to all the anti-TB drugs as follows; streptomycin 274 (96.8%), isoniazid 254 (89.8%), rifampin 278 (98.2%), ethambutol 267 (94.4%), pyrazinamide 254 (89.8%). *M. bovis* was 100% sensitive to all drugs except pyrazinamide where there was 100% resistance. *M. africanum* II varied in its sensitivity to anti-TB drugs; streptomycin 4 (80%), isoniazid 3 (60%), pyrazinamide 4 (80%). Resistance was streptomycin 1 (20%), isoniazid 2 (40%), and pyrazinamide 1 (20%). *M. africanum* II was neither resistant to rifampicin nor ethambutol (Table 4.4).

Table 4.4: The MTBC species and any resistance to one anti-TB drugs

Drugs	***M.africanum II***		***M.bovis***		***M.tuberculosis***	
Any resistance to one drug	Sensitive n (%)	Resistance n (%)	Sensitive n (%)	Resistance n (%)	Sensitive n (%)	Resistance n (%)
Streptomycin(STR)	4 (80.0)	1 (20.0)	2 (100.0)	0	274 (96.8)	9 (3.2)
Isoniazid (INH)	3 (60.0)	2 (40.0)	2 (100.0)	0	254 (89.8)	29 (10.2)
Rifampin (RIF)	5 (100.0)	0	2 (100.0)	0	278 (98.2)	5 (1.8)
Ethambutol (EMB)	5 (100.0)	0	2 (100.0)	0	267 (94.4)	16 (5.6)
Pyrazinamide(PZA)	4 (80.0)	1 (20.0)	0	2 (100.0)	254 (89.8)	29 (10.2)

n = frequency, % = percentage, *Mycobacterium africanum II* = east African strain of *Mycobacterium africanum*

A total of 59/283 (20.8%) of *M. tuberculosis* strains showed resistance to at least any one drug tested, while 224/283 (79.2%) were sensitive. 46/283 (16.3%) were resistant to one drug (mono resistance), 6/283 (2.1%) to two drugs (double resistance), 2/283 (0.7%) to three drugs (triple resistance), 1/283 (0.4%) to four drugs (quadruples) and 4/283 (1.4%) to five drugs (pentadruple-resistance). Two isolates of *M. bovis* were resistant to one drug. Two isolates of *M. africanum* II were resistance, one case to one drug and another one case to three drugs. At least four strains of *M. tuberculosis* were confirmed to be multi-drug resistant (MDR) (resistant to isoniazid and rifampicin). Interestingly all the MDR strains were resistant to all the five drugs (Table 4.6).

Table 4.5: The MTBC species and the anti-TB drug resistance levels

Drugs resistance levels	***M.africanum*** n (%)	***M.bovis*** n (%)	***M.tuberculosis*** n (%)
None	3 (60.0	0	224 (79.2)
Mono	1 (20.0)	2 (100)	46 (16.3)
Double	0	0	6 (2.1)
Triple	1 (20.0)	0	2 (0.7)
Quadrupal	0	0	1 (0.3)
Pentadrupal	0	0	4(1.4)
Total	**5 (100.0)**	**2 (100.0)**	**283 (100.0)**

n = frequency, % = percentage

Resistance to only one specific drug by *M.tuberculosis* was as follows: streptomycin 3/283 (1.1%), isoniazid 6% (17/283), rifampicin 1/283 (0.4%), ethambutol 8/283 (2.8%), pyrazinamide 17/283 (6%), with *M. bovis* showing 2/2 (100%) resistance to pyrazinamide and *M. africanum* II showing 1/5 (20%) resistance to isoniazid. Resistance to two specific drugs was as follows: isoniazid and pyrazinamide 4/283 (1.4%); isoniazid and ethambutol 2/283 (0.7%), but *M. bovis* and *M. africanum* II did not show any resiatnce to two drugs. Resistance to three specific drugs in *M. tuberculosis* was as follows: streptomycin, isoniazid and pyrazinamide 1/283 (0.4%);

isoniazid, ethambutol and pyrazinamide 1/283 (0.4%). In *M.africanum II* resistance to three drugs was 1/5 (20%) which was not seen in *M.bovis.* Resistance to four drugs (streptomycin + isoniazid + ethambutol + pyrazinamide) was only seen in *M. tuberculosis* with a prevalence rate of 1/283 (0.4%). Resistance to all the five drugs was seen in *M. tuberculosis* with a prevalence rate of 4/283 (1.4%) and they were considered to be multi-drug resistant strains (MDR-TB) as they were resistant to at least isoniazid and rifampicin. *M. bovis* and *M. africanum* II did not show any MDR (Table 4.6).

Table 4.6: Pattern of Drug Susceptibility in MTBC Isolates to Different Combination of First-line Drugs

Drugs levels	***M.tuberculosis***	***M.bovis***	***M.africanum II***
	n (%)	n (%)	n (%)
None	224 (79.2)		3 (60.0)
Resistant to only one specific drug			
STR only	3 (1.1)		
INH only	17 (6.0)		1(20.0)
RIF only	1 (0.4)		
EMB only	8 (2.8)		
PZA only	17 (6.0)	2 (100.0)	
Resistant to 2 drugs			
INH + PZA	4 (1.4)		
INH + EMB	2 (0.7)		
Resistant to 3 drugs			
STR + INH + PZA	1 (0.4)		1(20.0)
INH + EMB + PZA	1 (0.4)		
Resistant to 4 drugs			
STR + INH + EMB + PZA	1 (0.4)		
Resistant to 5 drugs			
STR + INH + RIF + EMB + PZA	4 (1.4)		
Total	**283 (100.0)**	**2 (100.0)**	**5 (100.0)**

Key: STR = streptomycin, INH = isoniazid, RIF = rifampicin, EMB = ethambutol, PZA = pyrazinamide; n = frequency, % = percentage

4.4 Antimycobacterial Activity of *P. americana* Pod Extract Against Mycobacteria Isolates

Upto 20 selected Mycobacteria clinical isolates were subjected to *P. americana* chloroform, *n*-hexane and ethanol pod extracts. Four different NTM test isolates were: *M. intracellulare, M. abscessus, M. kansasii* and *M. fortuitum*. The remaining 16 isolates were three species of MTBC namely 12 *M. tuberculosis*, *3 M. africanum II* and 1 *M. bovis* with varying patterns of drug susceptibility to first-line conventional anti-TB drugs. Among the twelve *M. tuberculosis* isolates 1 was sensitive to all the five anti-TB drugs, 5 were mono resistant to a specific drug, 2 were double resistant (isoniazid + pyrazinamide; isoniazid + ethambutol), 2 were triple resistant (streptomycin + isoniazid + pyrazinamide; isoniazid + ethambutol + pyrazinamide), 1 quadrapal resistant (streptomycin + isoniazid + ethambutol + pyrazinamide) and 1 resistant to all the drugs were selected. The three selected *M. africanum* II were: 1 was sensitive to all the drugs, 1 was isoniazid mono resistant and 1 was triple resistant (streptomycin + isoniazid + pyrazinamide) and *M. bovis* with mono resistance to pyrazinamide. Hexanic, chloroformic and ethanoic extracts obtained from *P. americana* pod demonstrated antimycobacterial activity against the tested clinical isolates.

Chloroformic extract exhibited appreciable antimycobacterial activity against all the tested clinical isolates with MIC range (18.75mg/ml – 75mg/ml) with the highest potency against *M. africanum II* (MIC = 18.75mg/ml) which was sensitive to all five anti-TB drugs.

Ethanoic extract also exhibited an appreciable antimycobacterial activity against 19 isolates with MIC range 18.75mg/ml – 75mg/ml, but 1/19 (5.3%) isolate of *M. tuberculosis* resistant to all five drugs (pentadrupal-resistance) showed complete resistance against ethanol extract.

N-hexane extract, however exhibited the least antimycobacterial activity against 17 clinical test

isolates with high concentrations (MIC range = 150mg/ml – 375mg/ml), but 3 isolates showed complete resistance with *M. tuberculosis* resistant to a combination of four drugs i.e. isoniazid + streptomycin + ethambutol + pyrazinamide, pentadrupal resistance i.e. isoniazid + rifampicin + streptomycin + ethambutol + pyrazinamide and *M. abscessus* strains showing complete resistance. Ethanoic extract was more potent against pyrazinamide mono resistance *M. bovis* with MIC of 18.75mg/ml while it had slightly less potency against NTM isolates with MIC = 75mg/ml. These results of MIC showed that ethanol and chloroform extracts had the lowest MIC values (18.75mg/ml) against Mycobacterial clinical test isolates, thus indicating their high potency.

The extracts demonstrated antimycobacterial activity against the NTM isolates (MIC range = 37.5-225mg/ml), but *M. abscessus* showed complete resistance to *n*-hexane extract. Chloroform and ethanol extracts demonstrated high potency/ good antimycobacterial activity compared to *n*-hexane. *N*-hexane demonstrated some antimycobacterial activity with low potency (high concentration 225mg/ml) against Isoniazid and Rifampicin mono resistance. Double and triple resistance strains of *M. tuberculosis* (isoniazid + pyrazinamide, isoniazid + ethambutol) were also sensitive to *n*-hexane extract with MIC range 300mg/ml and 225mg/ml respectively. However, quadrupal resistance (streptomycin + isoniazid + rifampicin + ethambutol + pyrazinamide) showed complete resistance to *n*-hexane extract.

Chloroform and ethanol extracts demonstrated good antimycobacterial activity against all the NTM test isolates with MIC range of 37.5-75mg/ml and 75mg/ml respectively. This was also the case in *M. tuberculosis* where all the test isolates with different patterns of drug resistance were sensitive to chloroform and ethanol extracts with MIC range 37.5-75mg/ml, except for *M. tuberculosis* pentadrupal resistance which was resistant to ethanol extract.

Mycobacterium africanum II and *M. bovis* test isolates demonstrated sensitivity to the extracts with the same trend as in *M. tuberculosis* test isolates. *N*-hexane demonstrated low potency with MIC range 150-300mg/ml, however, chloroform and ethanol extracts demonstrated high potency, MIC range 18.75-75mg/ml but the majority MIC being 37.5mg/ml against the test isolates. DMSO used as a control did not inhibit the growth of the clinical test isolates.

Table 4.7: Minimum inhibitory concentration (MIC) of *Persea americana* Chloroform, Ethanol, and n-hexane pod extracts against NTM and selected Patterns of Anti-TB Susceptibility MTBC Clinical Isolates

Test organisms	**Pattern of anti-TB drug susceptibility**	***n*-hexane MIC (mg/ml)**	**Chloroform MIC (mg/ml)**	**Ethanol MIC (mg/ml)**
NTM				
M. intraellulare		150	75	75
M. abscessus		**R**	75	75
M. kansasii		150	37.5	75
M. fortuitum II		225	37.5	75
MTBC				
M. tuberculosis	Sensitive	150	37.5	37.5
	Mono-resistance			
	STR	150	37.5	37.5
	INH	**225**	**37.5**	**75**
	RIF	**225**	**75**	**75**
	EMB	**150**	**75**	**75**
	PZA	**150**	**37.5**	**37.5**
	Double resistance			
	INH + PZA	**300**	37.5	37.5
	INH + EMB	**225**	**75**	**75**
	Triple resistance			
	STR + INH + PZA	**300**	75	37.5
	INH + EMB + PZA	**375**	**75**	**75**
	Quarter resistance			
	STR + INH + EMB + PZA	**R**	75	75
	Pentagon resistance			
	STR + INH + RIF + EMB + PZA	**R**	75	**R**
M. africanum	Sensitive	150	18.75	37.5
	Mono resistance			
	INH	225	37.5	37.5
	Triple resistance			
	STR + INH + PZA	300	37.5	75
	Mono-resistance			
M. bovis	PZA	150	37.5	18.75

Key: MIC = Minimum inhibitory concentration, NTM = Non tuberculous mycobacteria, MTBC = *Mycobacterium tuberculosis* complex, R = resistance, STR = streptomycin, INH = isoniazid, RIF = rifampicin, EMB = ethambutol, PZA = pyrazinamide; mg/ml = concentration

CHAPTER FIVE

DISCUSSION

5.1. Sensitivity of Culture, Smear Microscopy and GeneXpert *MTB/RIF* Screening

In the current study, the results revealed that overall sensitivity of smear microscopy and GeneXpert MTB/RIF assay tests was 83.5% and 91.8% respectively. Smear microscopy test could detect only 83.5% of the TB cases detected by culture test and miss out 16.5% of the cases and GeneXpert MTB/RIF assay test could detect only 91.8% of the cases detected by culture test. The assay could miss out 8.2% of the cases detected by culture test. These results show that smear microscopy was less sensitive than Gene Xpert *MTB/MRF* and culture tests. Low sensitivity of smear microscopy is due to the fact that 10^4/ml of bacilli is required for AFB to be seen using smear microscopy. Patients with smear-negative but culture and GeneXpert-positive PTB are capable of transmitting *M. tuberculosis* (Hernandez-Garduno *et al.,* 2004) to susceptible people. A study done in the Netherlands showed that patients with smear negative results but culture positive were able to transmit PTB (Tostmann *et al*., 2008). A study conducted in Dar es laam, Tanzania by Swai *et al*. (2011) showed that among suspected PTB patients found to have a negative result for AFB, a significant proportion (27.2%) had sputum culture positive for *M. tuberculosis*. These results are in agreement with the current study which revealed a lower rate of 16.5% smear negative but culture positive PTB. Therefore in the current study and other studies, the data indicates that smear microscopy is not highly sensitive to detect a good number of PTB patients with low mycobacterial levels as compared to culture test.

Using sputum culture as the gold standard for the diagnosis of PTB disease (Getahun *et al*., 2007) the results shows that sputum smear is not a very sensitive tool in the diagnosis of PTB. This has been shown by other studies where sensitivity has been described to be between 51% to

53.3% (Ramachandran *et al.*, 2007; Cattamanchi *et al.*, 2009). Although the gold standard for the diagnosis of TB involves the isolation and identification of *M. tuberculosis* using cultures (WHO, 1997), the cost and facilities of doing cultures are prohibitive in most developing countries. Due to the slow growth of *M. tuberculosis* and need for sophisticated laboratory facility, culture is available only in reference laboratories. The Xpert *MTB/RIF* assay has been introduced with the aim to increase the detection of TB especially in smear negative, but the assay does not detect NTM species (WHO, 2015). Sputum smear microscopy remains the main diagnostic tool for PTB that allows initiation of treatment and monitoring of patient progress (Cattamanchi *et al.*, 2009). As sputum smear microscopy is not a very sensitive tool in the diagnosis of PTB, presumptive diagnosis is usually made based on an algorithm of clinical and radiological criteria. In some cases when sputum smears are negative but the patient has clinical features highly suggestive of PTB, broad-spectrum antibiotics are recommended for at least 10-14 days and sputum smears repeated. If the patient's condition does not improve while sputum smear remains negative, a chest radiograph is done and if found to be abnormal, a presumptive diagnosis of PTB is made and the patient is started on anti-TB treatment as AFB negative PTB (Swai *et al.*, 2011). Most rural health facilities in Kenya do not have machines for a chest radiograph and therefore PTB patients who are smear negative are set free to go home without any treatment as the AFB turns to be negative.

Instituting a more sensitive diagnostic tool will prevent the unnecessary cost of treating individuals who do not have TB and at the same time it will prevent further spread of TB. This emphasizes the need of culture and the use of rapid molecular identification techniques such as Genotype Mycobacterium CM/AS and MTBC Assay kits and GeneXpert for the identification and diagnosis of AFB negative PTB. More studies should be conducted to investigate clinical

presentation of patients associated with smear negative PTB which can be used to predict PTB in such patients (Swai *et al.*, 2011).

In Kenya and other developing countries in the sub-Saharan Africa with a high incidence of PTB, microscopic examination of sputum smear samples by ZN method is often the only available diagnostic test for PTB. As a result, patients with smear-negative TB do not receive diagnosis in a timely manner; thus, disease may further develop, initiation of treatment may be delayed, and further TB transmission may occur to susceptible people without prior knowledge (Siddiqi *et al.*, 2003). One study has shown that HIV-TB–coinfected patients for whom the result of sputum smear is negative are as infectious as smear positive PTB (Perkins & Cunningham, 2007). Patients with smear-negative TB and with the potential to transmit TB has implications for countries such as Kenya where HIV infection is endemic more especially in counties with high prevalence of TB-HIV and AIDS coinfection such as Kisumu and Homabay. Human immunodefiency virus infection and AIDS are accompanied by high rates of smear-negative TB. For example, 25%–61% of the HIV-infected patients with TB in sub- Saharan Africa have smear-negative PTB (Storla *et al.*, 2008). The rates of smear negative culture positive in the current study is not surprising, Kisumu County being one of the counties in Kenya with the highest prevalence of 19.9% HIV and AIDS infection (Kenya HIV Estimates Report, 2018). Globally, use of rapid molecular tests such as GeneXpert is increasing, and many countries are phasing out the use of smear microscopy for diagnostic purposes although microscopy and culture remain necessary for treatment monitoring. Despite advances in diagnostics, a considerable proportion of the TB cases reported to WHO are still clinically diagnosed rather than bacteriologically confirmed. In 2016, for example, only 57% of the pulmonary cases reported to WHO were bacteriologically confirmed (WHO, 2017).

By facilitating early detection of TB, prior to smear positivity, the application of Xpert *MTB/RIF* should have a significant impact on transmission chains and push back the epidemic. However, many of the patients diagnosed by Xpert *MTB/RIF* would have been initiated on treatment due to chest X-ray findings or clinical findings consistent with TB and the extent to which the use of Xpert *MTB/RIF* will increase case finding is not yet clear. The application of the test will improve targeting of treatment, with less patients who do not have TB being incorrectly started on treatment and a greater number of smear negative 'true TB' cases being detected.

In this study, 8.5% of the specimen were smear negative, culture negative but GeneXpert *MTB/RIF* positive. This could be due to the fact that GeneXpert *MTB/RIF* detects both viable and non-viable mycobacteria. This is a case of non-viable mycobacteria where only the DNA was present to be detected. This could be cases where patients were using streptomycin for the treatment of other bacterial infections. Streptomycin being a broad-spectrum antibiotic, bacteriostatic, interfered with protein synthesis inhibiting mycobacterial growth, exposing the DNA. It could even suggest false positive GeneXpert results or false negative or missed diagnosis by smear and culture tests.

All of the 26 GeneXpert negative were culture positive. They were confirmed by GenoType CM/AS assay to be NTM species which are not detected by GeneXpert test assay. GeneXpert is so specific to the species of interest and that is why it missed to detect NTM species in the 26 isolates which all posted negative results. The assay only detects members of MTBC and rifampicin resistance but does not detect non tuberculous mycobacteria in TB patients.

5.2. Molecular Characterization of MTBC and NTM Isolates

Both MTBC and NTM were identified from the cultures. The most prevalent MBTC was *M. tuberculosis* and for NTM was *M. intracellulare*. Treatment and management of patients with MTBC and NTM are entirely different, therefore prompt isolation, detection and differentiation is necessary for effective treatment and suitable management of the disease and for epidemiological purposes (Hasegawa *et al*., 2002; Park *et al*., 2010). *Mycobacterium tuberculosis complex* and NTM may or may not have the same clinical presentations, but the treatment regimens are always different (Singh *et al*., 2013; Marzouk *et al.*, 2011). Laboratory differentiation between MTBC and NTM by routine methods are time consuming and cumbersome to perform (Singh *et al*., 2013). For example conventional biochemical tests used to identify different mycobacterial species are complex and time consuming (Marzouk *et al*., 2011). Hence, there is need for rapid differentiation and identification of different Mycobacterium species for effective treatment and management of the disease.

In the current study 91.8% cases were confirmed to be MTBC and 8.2% NTM using GenoType Mycobacterium CM/AS assay. The results in the current study were similar to the findings of a study conducted in Serbia where 88.8% were identified as MTBC and 11.2% NTM, however, *M. tuberculosis* was the only MTBC species recognized (Živanović *et al*., 2014). This implies that in Serbia *M. tuberculosis* is the only member of MTBC causing pulmonary TB in the study region. In other parts of the world the prevalence of NTM is: Belgium 1.4% (De Keukeleire *et al*., 2017), India 4.23% (Narang *et al*., 2005), and Ghana 8% (Bjerrum *et al*., 2016) which is similar with the current study. Higher rates of NTM have also been isolated from TB patients in India 29% (Umrao *et al*., 2016). Most of the NTM species reported in these studies was *Mycobacterium avium* complex (MAC). This also implies that members of *Mycobacterium*

avium complex are predominant non tuberculous mycobacteria causing TB where *Mycobacterium intracellulare* is the most common among MAC. The reported prevalence rate of NTM in the current study was much lower than previous studies reported in Kenya. Limo *et al.* (2015) reported 42.4% prevalence of NTM and 57.6% MTBC in tuberculosis retreatment cases in National Tuberculosis Reference Laboratory (NTRL), Nairobi**.** A possible explanation of the high prevalence of NTM in Limo's study could be attributed to the fact that the target population at the reference laboratory were patients whose first-line anti-TB treatment had failed and now they were seeking diagnosis to know the cause of their treatment failure. Non-tuberculous mycobacteria do not respond to first line anti-TB drugs (ATS, 2007) and therefore this could be the contributing factor in treatment failure. This implies that there is a high proportion of TB patients who fail to seek retreatment in NTRL or any other health facility after treatment failure. Furthermore, after treatment failure in TB patients, they are regarded as MDR-TB patients. Lower NTM prevalence rates have also been reported in western Kenya where a study was done to investigate TB-HIV co-infection found 4.2% of NTM (Nyamogoba *et al*., 2012).

5.2.1. Prevalence of *Mycobacterium tuberculosis* complex (MTBC)

Three different MTBC species were identified. The most predominant species was *M. tuberculosis* (97.6%), being by far the most frequently causative agent of PTB worldwide. *M. africanum* subtype II accounted for 1.7% and *M. bovis* (0.7%). A study conducted in Serbia to isolate mycobacterial species from respiratory specimens found only *M. tuberculosis* strain among MTBC in all samples (Živanović *et al*., 2014). *Mycobacterium tuberculosis* has also been identified as the major cause of PTB in reports from various previous studies in Uganda, Tanzania and Kenya (Asiimwe *et al*., 2008; Mbugi *et al*., 2015; Katale *et al*., 2017; Usagi *et al*., 2016). This suggests its wide distribution in the regions. As it is, most people know that *M.*

tuberculosis is the only etiological agent of human PTB. *Mycobacterium tuberculosis* prevalence at 61.5% was significantly higher in patients less than 35 years. This is likely linked to the high HIV prevalence in the study area- Kisumu County.

Mycobacterium africanum subtype II was the second predominant MTBC species isolated accounting for 1.7% of the isolates causes up to half of human PTB in West Africa. In Kenya and other sub-Saharan countries, *M. africanum* II has been isolated from TB patients. Recent surveys showed highly variable prevalence of *M. africanum* in different regions of Africa as follows: Ivory Coast 5%; Cameroon 10% (Niobe-Eyangoh *et al.*, 2003); Gambia 39% (de Jong *et al.*, 2010), with alarming prevalence being in Guinea-Bissau 60% (Bonard *et al*., 2000). These results shows a higher prevalence of human PTB caused by *M. africanum* compared to Kenya. A previous study in Kenya reported a prevalence of *M. africanum* II at 8.9% and *M. bovis* at 6.7% (Limo *et al.*, 2015). The commercial GenoType MTBC DNA strip assay (Hain Lifescience GmbH) used in this study does not differentiate *M. canettii* from *M. tuberculosis* and *M. africanum* type I from *M. pinnipedii*, but *M. africanum* type I being a West African strain has minimal chances of being isolated from PTB patients in Kenya. It is an infrequently isolated MTBC species, mostly from patients from, or with connection to Africa (Brosch *et al*., 2002). *Mycobacterium africanum* has infrequently been isolated in some European countries, such as Germany, England, France and Spain (Kamel *et al*., 2014). The presence of *M. africanum* in areas outside the West African region such as Gambia which causes 39% of TB was mostly detected in specimens from immigrants from this region, is common among older patients and people with severe malnutrition (de Jong *et al*., 2010). *Mycobacterium africanum* grows more slowly than *M. tuberculosis*, with cultures occasionally yielding growth only after 10 weeks, compared to 3–4 weeks in *M. tuberculosis* (Kamel *et al.*, 2014).

Mycobacterium bovis which is the causative agent of bovine tuberculosis (bTB) a bacterial zoonosis was the least dominant MTBC species isolated, accounting for 0.7%. This results show a very low contribution of *M. bovis* to human PTB in this region. The low prevalence of *M. bovis* could be attributed to people no longer practicing old cultures such as consuming raw animal products. It has a broad host range including wildlife, domestic livestock such as cattle, farmed buffalo, goats and farmed dear. While goats are considered reservoir hosts of *M. bovis*, other animal species such as pigs, cats, dogs, horses and sheep are considered spillover hosts (Kamel *et al*., 2014).

It has been estimated that about 3.1% of human TB cases worldwide are caused by *M. bovis* which is higher than in the current study. While <1% of human infections are caused by *M. bovis* in Spain, the prevalence in Africa ranges from 3.9% in Nigeria, up to 7% in Uganda, and even 16% in Tanzania (Romero *et al.*, 2006; Malama *et al*., 2014). It was reported that in some developing countries, *M. bovis* is responsible for 5–10% of all human TB cases and 30% of all TB cases in children (Wedlock *et al*., 2002). *Mycobacterium bovis* was isolated from approximately 3.9–5% of human TB patients in Nigeria (Cadmus *et al*., 2006; Mawak *et al*., 2006) and 0.6–1.85% of patients with PTB in Burkina Faso (Sanou *et al*., 2014). The high prevalence rates of *M. bovis* in these studies could be due to frequent contacts between livestock, wildlife and humans which favours disease transmission. It can also be due to people/pastrolist practicing the old tradition customs of consuming raw animal products such as milk and meat. The higher rates also suggests potential of drug resistance to conventional first line anti-TB drugs and pathogenicity that promote its persistence in the regions. *Mycobacterium bovis* is treated similarly to *M. tuberculosis* but it is usually naturally resistant to pyrazinamide. In fact, healthcare providers might not know that a person has *M. bovis* instead of *M. tuberculosis.* High

direct or indirect contacts between livestock and wildlife favours disease transmission between species. Moreover, other factors include the husbandry practices, proximity to wildlife and tradition customs of consuming raw meat or milk by the pastoralist communities (Katale *et al.,* 2017). Drinking contaminated milk, feeding on infected carcasses or even coming into contact with pus secreted from draining fistulae of lymph nodes can be the means of disease transmission of *M. bovis* (Michel *et al.,* 2006, 2007). The higher prevalence of *M. bovis* in those aged over 35 years may be due to their interaction with domestic animals such as cattle in rural communities and practicing some tradition old customs of consuming raw animal products such as meat, milk and blood.

However, the isolation of *M. bovis* from human sputum in some reports may indicate its potential for human-to-human infection, especially in closed populations such as prisons (Gumi *et al*., 2012). In most African countries and more especially East Africa, infections caused by *M. bovis* is underdiagnosed as it is assumed that it is less virulent and has less transmission potential among human populations than other MTBC species specifically *M. tuberculosis*. Nevertheless, *M. bovis* remains a serious opportunistic infection in HIV-infected persons. Therefore, it is important for public health policy makers to be able to differentiate human infections caused by *M. bovis* from those due to *M. tuberculosis* (Wedlock *et al*., 2002). Interaction between wildlife which act as reservoirs that spread the infection to domestic animals especially cattle make eradication more complicated and extremely difficult. This necessitates special programs to control the disease (More *et al*., 2015). *M. bovis* direct transmission from animals to humans through the air is rare, but it can be spread directly from person to person when pulmonary tuberculosis patients cough or sneeze. Symptoms of TB disease caused by *M. bovis* are similar to the symptoms of TB caused by *M. tuberculosis*; this can include fever, night sweats, and weight

loss. Other symptoms might occur depending on the part of the body affected by the disease (Gonzalo Asensio *et al*., 2014).

5.2.2 Identification of non-tuberculous mycobacteria (NTM)

Four different species of NTM were identified in the current study: *M. intracellulare* (61.5%), *M. abscessus* (19.2%), *M. kansasii* (11.5%) and *M. fortuitum* (7.7%). *Mycobacterium intracellulare* has been reported to be predominant in Australia and South Africa (Hoefsloot *et al.*, 2013). In India, the predominated species was *M. abscessus* (31.3%), *M. fortuitum* (22%) and *M. intracellulare* (13.6%) (Umrao *et al.*, 2016) which is in contrary to the current study. The distribution of NTM species in the current study differs from that found in previous studies in Kenya. In 2015, Limo *et al.* (2015) reported seven different NTM species: *M. intracellulare*, followed by *M. absessus, M. fortuitum, M. sacrofuloceum, M. kansasii, M. interjectum* and *M. xenopi*. However, in both studies *M. intracellulare* was the most frequently isolated NTM species. Nyamogoba *et al.,* (2012) isolated three NTM species with notable absence of *M. kansasii* and *M. absessus, M. peregrinum* being among the isolates.

The rate of 61.5% isolation of *M. intracellulare* which is a member of MAC was the most predominant NTM species in the current study. This prevalence rate was much higher compared to the 20.8% in Turkey (Martin-Casabona *et al.,* 2004), New Delhi 2.6% (Singh *et al*., 2007), Southern Asia 17.4%. In Brazil 15% NTM isolated from AIDS patients, 57.8% cases was found to be *M. avium* (Lima *et al*., 2013). Non-tuberculous mycobacteria isolation has also been reported in other studies; in the Netherlands (van Ingen *et a*l., 2012), Poland (Safianowska *et al*., 2010) and Great Britain (Moore *et al*., 2010). Based on the two studies conducted in Kenya both of which reported high prevalence rates of *M. intracellulare* isolated from PTB patients suggests potential of drug resistance to conventional first line anti-TB drugs and pathogenicity that

promote its persistence in the regions. The high isolation rate of *M. intracellulare* noted among the NTM strains tested in the present study is not surprising, since this species is typically the most common causative agent of severe PTB in immunocompromised people especially in the later stages of AIDS. Kisumu County where the study was conducted is one of the counties in Kenya with the highest HIV/AIDS prevalence (15.3%). *M. intracellulare* is primarily a pulmonary pathogen that affects individuals who are immune compromised (e.g. AIDS, hairy cell leukemia, and immunosuppressive chemotherapy). It is also responsible for 40% of lung diseases in immunocompetent patients (Griffith *et al*., 2007).

Mycobacterium abscessus was the second most common NTM species isolated which accounted for 19.2%. A previous study in Kenya reported a similar trend, but lower prevalence of 13.4% (Limo *et al*., 2015). A study in India reported higher prevalence rates of *M. abscessus* 31.3%. The higher prevalence reported in current study may be because *M. abscessus* is particularly difficult to treat medically, because the disease often progresses slowly over years and because older adults are typically affected (Umrao *et al.*, 2016).

Mycobacterium kansasii was the third most common NTM species isolated. Comparing with other studies, the isolation rates of *M. kansasii* was higher at 11.5% in the current study. Low *M. kansasii* prevalence rates have been reported: in Europe 5% (Sester *et al*., 2014), Poland 35% (Wassilew *et al*., 2016), India 1.9% (Umrao *et al.*, 2016) and Israel 33.9%, (Shitrit *et al*., 2007). Elsewhere in Kenya, *M. kansasii* was also reported; Limo *et al*. (2015) found 3.4% among tuberculosis retreatment cases in Kenya, but a study done in Western Kenya to isolate NTM species of Mycobacteria did not isolate any *M. kansasii* (Nyamogoba *et al*., 2012). Clinically, *M. kansasii* is a slow-growing acid fast bacilli that causes a chronic, upper-lobe cavitary disease, resembling that from *M. tuberculosis*. The infections are also prevalent in areas where HIV

infection is common such as Kisumu County due to the susceptibility of the hosts. *Mycobacterium kansasii* is the second most common slow growing NTM causing pulmonary infection and is treated differently from *M. tuberculosis* infection, and it does not require contact tracing because it is not transmitted by person-to-person contact. The recommended regimen for treating pulmonary *M. kansasii* disease includes daily rifampin (600 mg/day), isoniazid (300 mg/day), and ethambutol (15 mg/kg/day) for a duration that includes 12 months of negative sputum cultures. Amikacin, streptomycin and clarithromycin can also be included. Pyrazinamide should not be used to treat *M kansasii* infection since it is resistant to it (Broaddus *et al*., 2015). Thus, the clinical or molecular identification of *M. kansasii* would be helpful, given the delay before bacteriological confirmation is available.

Mycobacterium fortuitum was the least predominant NTM species reported in the current study with an isolation rate of 7.7%. This is slightly higher than 5.7% that was reported in New Delhi-India (Singh *et al.*, 2007), but lower than a second study in Lucknow-India which reported 22% (Umrao *et al*., 2016). In other parts of sub-Saharan Africa, *M. fortuitum* has been isolated from TB patients as follows: South Africa 41.5% (Van Halsema *et al*., 2015); Nigeria 15% (Pokam & Asuquo, 2012); Tanzania 2.9% (Katale *et al.*, 2014). In the previous two studies in western Kenya, 6.7% *M. fortuitum* prevalence was obtained (Nyamogoba *et al.*, 2012; Limo *et al*., 2015). The increasing importance of NTM in the clinical laboratory is now generally recognized. Among the many factors that may contribute to such an increase and difference in the prevalence of NTM are the HIV and AIDS pandemics and other immunocompromising diseases, the technical improvements in NTM recovery and identification, the increased interest in NTM identification and the improvement of public health services for tuberculosis.

In sub-Saharan Africa, especially in East Africa, most of the Mycobacterial species reported in the current study have also been isolated from both new and retreated cases of PTB. Kenya, Uganda and Tanzania have experienced a gradual rapid increase in the prevalence of PTB and increase in NTM infections, but it is still unclear if this trend is real or is the result of improvement in the use of new rapid molecular diagnostic techniques. Previous studies conducted in the three countries concluded that mycobacterial infections caused by NTM were rare in Africa, but recent studies in African countries with the major objective of isolating mycobacteria species responsible for TB using rapid molecular diagnostic methods have reported a high prevalence of NTM and other MTBC species other than *M. tuberculosis* in immunocompromised patients such as HIV and AIDS (WHO, 2009). This shows that TB caused by NTM in HIV and AIDS patients is as common as TB caused by *M. tuberculosis*. Correct and sufficient data of human NTM infections in Kenya is yet to be unveiled, but the already conducted studies in specific regions in the country indicates that NTM problem is bigger than previously documented.

5.3. First-Line Anti-Tuberculosis Drug Susceptibility Patterns of MTBC Isolates

The current study was conducted to also determine the patterns of drug resistance of first-line conventional anti-TB drugs against MTBC isolates from new cases of PTB patients in Kisumu County. All anti-TB drug resistance detected in this study was primary resistance. This is resistance among newly diagnosed PTB cases with no previous history of treatment or retreatment with anti-TB drugs or treatment for less than one month. The true assessment of drug resistance in Kenya particularly in Kisumu County is limited by inadequate culture and drug sensitivity facilities.

In the current study, any resistance to isoniazid and pyrazinamide was the most prevalent both accounting for 10.2%. The overall resistance of *M. tuberculosis* to at least one drug tested was 20.8% which was lower than Bangladesh 37.8% (Mottalib *et al*., 2011), India 44.5% (Varshney *et al.,* 2014), Abu Dhabi, UAE 23% (Alfaresi & Hag-Ali, 2010), South Africa 30.2% (Green *et al*., 2010), Ethiopia 23% (Seyoum *et al*., 2014), and Kenya 30.6% (Ndung'u *et al*., 2011). However, it was relatively higher than one study in Kenya where resistance to at least one drug was 18.3% (Githui *et al*., 2000). The low resistance could basically be due to new cases of TB and low access and exposure to these anti-TB drugs in this population in cases of other microbial infections. This is an indication that *M. tuberculosis* mono resistance in Kenya has risen slightly over the past two decades. In Indonesia 18.9% of *M. tuberculosis* isolates were resistant to at least one first-line TB drug (Wiwing *et al*., 2015). Lower resistance rates were also revealed in other previous studies compared with the current study. In Tanzania 5.83% of the isolates were resistant to at least one drug (Urassa *et al*., 2008). Based on the data from different studies, it shows that the rates of drug resistance is higher in Asian countries compared to African countries. All the data in these studies were obtained from studies conducted from new cases of PTB patients. The WHO has reported that the proportion of resistance to at least one anti-TB drug ranges from 0% to 56.3% in new cases and from 0% to 85.9% in previously treated cases. 10.9% and 20.1% of new and previously treated cases, respectively, are resistant to streptomycin around the world (WHO, 2008).

Any resistance to streptomycin in the current study was 3.2% which was much lower than Bangladesh where resistance was 22% (Mottalib *et al*., 2011), Abu Dhabi, UAE 25.6% (Alfaresi & Hag-Ali, 2010), Iran 23.1% (Shamaei *et al*., 2009), India 8.6% (Varshney *et al*., 2014) and Ethiopia 11.5% (Seyoum *et al.,* 2014). A previous study conducted in Nairobi- Kenya reported a

resistance rate of 5.2% (Ndung'u *et al*., 2011). However, some studies reported low rates of resistance compared to the current study. In North Eastern Thailand the prevalence rate was 2.1% (Reechaipichitkul *et al*., 2011). One study in Turkey reported almost similar rates of 3.4% in new cases but higher rates of 24.5% in re-treatment cases (Komurcuoglu *et al*., 2013). Streptomycin has been in use since the beginning of TB chemotherapy in 1943 (Amukoye, 2008). Furthermore, streptomycin being a broad spectrum antibiotic it is widely and commonly used in the treatment of other bacterial infections apart from TB. Inadequent treatment due to poor compliance by patients has led to bacteria developing resistance by spontaneous or induced mutations altering their drug-target sites (ribosomes) targeted by streptomycin. Its use is not restricted hence, high rate of resistance to streptomycin is expected (Gillespie, 2002). Currently streptomycin is administered as the first substitute in first line anti-TB treatment, in combination with isoniazid, rifampicin, and ethambutol. It is bacteriostatic when given parentally and also known to reduce bacterial load (Jindani *et al*., 2003). Low any resistance to streptomycin in the current study indicates that the drug is not misused in the treatment of other bacterial infections in the this population.

Any resistance to isoniazid in the current study was 10.2%. This was unexpected results because all TB patients were new cases who had not been exposed to isoniazid. Isoniazid is thought to kill bacilli in log-phase growth i.e. only active against metabolically-active replicating bacilli (Pradhan *et al*., 2014). Isoniazid is not a common drug used in the treatment of other bacterial infections and therefore its use is restricted to mycobacterial infections hence low TB resistance. This finding is consistent with previous studies which was slightly lower than earlier studies in Kenya where any resistance to isoniazid was reported to be 12.9% (Ndung'u *et al*., 2011). However it was higher than North Eastern Thailand where *M. tuberculosis* resistance to isoniazid

was 2.3% (Reechaipichitkul *et al*., 2011), and Turkey 4.3% (Komurcuoglo *et al.*, 2013). The highest resistance to isoniazid was observed in India at 37.7% (Varshney *et al.*, 2014) followed by Abu Dhabi, UAE 34.8% (Alfaresi & Hag-Ali, 2010), Bangladesh 26% (Mottalib *et al*., 2011), Bangladesh 76.03% (Rahman *et al*., 2009), Ethiopia 14% (Seyoum *et al*., 2014) and Iran 11.6% (Shamaei *et al*., 2009), which is the most popular anti-TB drug used in the treatment of TB. Lower rates of any resistance to isoniazid than in the current study were also observed in new cases of TB patients such as in Nepal 7.1% (Thapa *et al*., 2016) and 1.4% (Pradhan *et al.*, 2014).

WHO 2008 reported a 5.9% resistance rate worldwide (WHO/IUATLD, 2008). According to WHO isoniazid resistance rate higher than 10% can predict the development of MDR-TB (Githui *et al*., 1998). In the current study, isoniazid resistance rates being slightly above 10% is an indication of slow development of MDR-TB strain in Kisumu County, Western Kenya. This high resistance may be due to the fact that isoniazid is used widely in the treatment of TB as a first-line drug both in intensive and continuous phase and poor compliance by patients can select for drug resistant mutant strains. Since all the patients were new cases of PTB meaning that they had not been exposed to any anti-TB drug, then patients most likely acquired resistant strains from other TB patients. Resistance to isoniazid in this study might be due to the isoniazid resistance prior to the current treatment (Nasiri *et al*., 2014) but not because of poor management as all patients in this study were new cases of TB. The higher prevalence of isoniazid resistance has important implications as it is the cornerstone drug used throughout the course of non-MDR-TB treatment. It is also the drug of choice for chemoprophylaxis of TB in developing countries for treating latent TB infection. Isoniazid is considered crucial in the consolidated phase of treatment, and has the ability to synergize with other first-line drugs as recommended in the intensive phase of treatment (Hall *et al*., 2009). Loss of the effectiveness of this drug

compromises both the preventive therapy and treatment of TB disease. Moreover, it is predictor for MDR-TB in the future since MDR-TB often develops from initial isoniazid mono-resistant strains. Isoniazid together with rifampicin remains the basis for the treatment of TB (Jenkins *et al*., 2009). Resistance to isoniazid can lead to MDR-TB unless control programs are carried out carefully (Ndung'u *et al.,* 2012).

Any resistance to rifampin was 1.8% which is similar one study conducted in Nairobi, Kenya that reported 1.3% resistance (Ndung'u *et al*., 2011). Higher resistance rates have been reported in various countries Abu Dhabi, UAE 32.5% (Alfaresi & Hag-Ali, 2010) reporting the highest resistance, in South Africa 17.7% (Green *et al*., 2010), India 22.2% (Varshney *et al*., 2014), Bangladesh 12% (Mottalib *et al*., 2011), Nepal 9.5% and 4.2% (Thapa *et al*., 2016; Pradhan *et al.,* 2014), Turkey 4.2% (Komurcuoglu *et al*., 2013), Thailand 2.6-2.8% (Jittimanee *et al*., 2009); WHO/SEARO, 2009; Reechaipichitkul *et al*., 2011), Iran 3.9% (Shamaei *et al*., 2009), and Ethiopia 2.8% (Seyoum *et al*., 2014). The low resistance to rifampicin in the current study compared to other countries could be the design and policy differences in the drug use. In Kenya, rifampicin is restricted to mycobacterial infections only, thus infrequently used, whereas in other countries, the drug could be used in the treatment of other bacterial infections frequently, leading to mutations.

Rifampicin is one of the most effective anti-TB antibiotics and together with isoniazid constitutes the basis of the multidrug treatment regimen for TB. Rifampicin is active against both actively growing and non-growing slow metabolizing bacilli. It is thought to slowly kill non-replicating persistent bacilli during the 6 months of therapy, with isoniazid added to prevent resistance during the continuation phase (Mitchison & Coates, 2004). It is therefore, a drug of choice both in the consolidated phase of progressive TB treatment, and in short-course therapy.

However, its widespread application as a broad spectrum drug, and administration as a single drug in the short-course chemotherapy has contributed to an increase in drug-resistance (Van Ingen *et al*., 2011) which could explain the high resistance observed in other countries.

Any resistance to ethambutol in this study was 5.6% which was almost similar with one study conducted in Kenya by Ndung'u *et al,* 2011 that reported a rate of 4.5%. Outside Kenya, resistance has been observed at a lower level such as in North Eastern Thailand 3.8% (Reechaipichitkul *et al*., 2011), Iran 3% (Shamaei *et al*., 2009), Turkey 2.6% (Komurcuoglu *et al*., 2013), South Africa ranged from 1.4% (Green *et al*., 2010) and Addis Ababa-Ethiopia the rates were much lower 0.3% (Seyoum *et al*., 2014). It was however much lower than studies carried out in Bangladesh where 20% resistance was reported (Mottalib *et al*., 2011) and India 10% (Varshney *et al*., 2014). Abu Dhabi, UAE 20.9% (Alfaresi & Hag-Ali, 2010) reported the highest resistance. The low and higher levels of resistance to ethambutol could be policy differences in various countries and the frequency of use of this drug. High ethambutol resistance will exclude it from second line drug to treat MDR-TB. Ethambutol is the first-line drug included in the regimen of second-line drugs to treat MDR-TB cases. It is used alongside other first-line anti-TB drug regimens with good synergism in TB treatment. Together with isoniazid, rifampicin and pyrazinamide, ethambutol has been described as "fourth drug" for empirical treatment in *M. tuberculosis* and *M. avium* with disseminated infections and HIV co-infection (Jain *et al*., 2008). The high rate of ethambutol resistance would challenge its inclusion in MDR-TB therapy as this may lead to unintentional wrong therapy (Hoek *et al*., 2009).

Any resistance to pyrazinamide in the current study was also high 10.2%, however various studies conducted to determine the patterns of drug susceptibility of first line anti-TB drugs failed to include pyrazinamide in the test. From the Netherlands, 0.8% (van Klingeren *et al*.,

2007) resistant rates was observed and the results were not in agreement with the findings in the current study. A recent multicenter study has shown a rate of 5.1% PZA resistance among isolates from patients with PTB in Bangladesh (Zignol *et al*., 2016). Alfaresi and Hag-Ali, (2010) in Abu Dhabi reported a much higher resistance rate of 34.8% to pyrazinamide. High rates of pyrazinamide resistance in some countries might be due to the earlier introduction of the drug in the treatment regimen of TB patients in the national TB control programme making it to be administered for longer duration. The high proportion of PZA resistance may also be contributed to the high rate of re-treated TB patients because pyrazinamide resistant rate increases in prevalence as risk of resistance to other drugs increases. Loss of the effectiveness of pyrazinamide compromises both drug sensitive and MDR-TB treatment. Pyrazinamide is a frontline anti-TB drug used in both first- and second-line treatment regimens. Though the prevalence of PZA resistance is higher in MDR-TB cases, due to technical difficulties in the laboratory and the possibility of false susceptibility results, most TB laboratories do not perform or rarely perform PZA susceptibility test (Zhang *et al.*, 2012; Aono *et al*., 2002). As a result, most patients infected with PZA resistant strains fail to get appropriate treatment (Zimic *et al*., 2012). The introduction of pyrazinamide reduced the length of treatment to six months (Sia and Wieland, 2011.

Mono-resistance to the five anti-TB drugs was also observed in the current study. Resistance to only one specific drug by *M. tuberculosis* was as follows: mono resistance to only one specific drug was highest in isoniazid and pyrazinamide (6%) followed by ethambutol (2.8%), streptomycin (1.1%) and rifampicin being the lowest (0.4%). Mono-resistance associated with isoniazid, streptomycin, and rifampicin in the current study is lower than the 9.5%, 7% and 1.7% reported in Ethiopia respectively, however, lower ethambutol resistance (0.3%) was observed

(Seyoum *et al*., 2014). The high proportion of isoniazid mono resistance in this study might be due to the common use of the drug in the national TB control programme for longer time because of its accessibility. This implies that the precursors of isoniazid resistance are accumulating in the study setting which can increase the likelihood of MDR-TB if rifampicin resistance rises. Therefore, mono resistance to isoniazid should be properly monitored in order to minimize the spread of MDR-TB strains in Kisumu County.

Mono-resistance of *M. tuberculosis* to isoniazid (6%) and ethambutol (2.8%) in the current study is slightly higher than a study by Agarwal *et al.* (2015) in India where isoniazid and ethambutol resistance were 3.6% and 1.1% respectively. However, streptomycin resistance in both studies is similar (1.1%). Mono-resistance to isoniazid is higher than that reported in another study in India (1.7%), where ethambutol and streptomycin mono-resistance was not observed (Sharma *et al*., 2011). 0.4% rifampicin resistance in the current study is not consistence with the earlier mentioned studies in India where mono resistance was never observed (Agarwal *et al*., 2015; Sharma *et al*., 2011; Pereiira *et al*., 2005). Usagi *et al*., (2016) in Kenya reported high resistance rate to isoniazid (10%), rifampicin (9.2%), streptomycin (6.2%) and ethambutol (3.8%) among HIV positive TB patients in Kisumu County. This results suggests that mono-resistance to rifampicin is not common compared to other first-line anti-TB drugs, and almost all rifampicin-resistant strains are also resistant to other drugs, especially to isoniazid, forming the basis of screening rifampicin's resistance for MDR-TB (Traore *et al.*, 2000). The 1.8 % rifampicin resistance rate of non-MDR rifampicin resistance in this study supports the use of rifampicin drug and is considered as a surrogate marker for MDR-TB. It is also in line with WHO recommendation of non MDR-TB rifampicin resistance less than 3% as good quality performance indicator (Traore *et al*., 2000, 2006). The high rate of rifampicin resistance might be

due to its adverse effects such as nausea, vomiting, rashes, hepatitis, gastrointestinal tract (GIT) upset, flu-like symptoms, fever and jaundice, which could result in patient non-adherance and hence may lead to the selection of resistant strains. Rifampicin mono-resistance is uncommon but increasing in some areas of the world, more especially in sub-Saharan Africa, Kenya being among them because of its widespread application as a broad spectrum drug, and administration as a single drug in the short-course chemotherapy (Van Ingen *et al*., 2011). The countries with the largest numbers of MDR/RR-TB cases (47% of the global total) were China, India and the Russian Federation (WHO, 2015).

Mycobacterium bovis showed 100% resistance to pyrazinamide. This was expected results because *M. bovis* is naturally resistant to pyrazinamide as it does not produce an enzyme that activates pyrazinamide. Rijal *et al*., (2005) in Nepal reported a mono-resistance rate of 1%, 2% and 6% to ethambutol, isoniazid and streptomycin respectively, however rifampicin did not display any mono resistance.

Resistance pattern in a combination of two specific drugs in the current study revealed a resistance of 0.7% for the combination of isoniazid + ethambutol which was lower than a study conducted in India where resistance was 2.6% (Agarwal *et al*., 2015) and Nepal 2.63% (Rijal *et al*., 2007); isoniazid + pyrazinamide revealed a resistance of 1.4%. These resistance patterns involving isoniazid are dangerous because second-line regimen is used for treatment, which is very expensive, used for a long duration and very toxic. Therefore, resistance pattern in combination of two drugs involving isoniazid should be properly monitored to minimize the spread of MDR-TB in the study area.

Mycobacterium bovis and *M. africanum* did not show resistance to a combination of any two specific drugs. Resistance to a combination of two anti-TB drugs isoniazid + rifampicin which essentially termed as MDR-TB was not observed in the current study. Isoniazid and rifampicin are the two most powerful first-line anti-TB drugs.

Resistance to a combination of three specific drugs in *M. tuberculosis* was also revealed where streptomycin + isoniazid + pyrazinamide was 0.4%; isoniazid + ethambutol + pyrazinamide 0.4%. In *M. africanum* resistance to streptomycin + isoniazid + pyrazinamide was 20% but was not seen in *M. bovis.* The higher percentage (20%) could be as a result of the low number of *M. africanum* subjected to the test. Preliminary results from the current study shows that resistance to a combination of three drugs, pyrazinamide resistance appeared in both combinations. This implies that pyrazinamide resistance is spreading among MTBC strains (PMDT guidelines, 2014).

Resistance to a combination of four specific drugs (streptomycin + isoniazid + ethambutol + pyrazinamide) was only seen in *M. tuberculosis* with a prevalence of 0.4%. There is limited literature/information on resistance to a combination of anti-TB drugs including pyrazinamide because most studies do not include pyrazinamide in their studies.

Resistance to all the five drugs was seen in *M. tuberculosis* with a prevalence rate of 1.4% and they were considered to be MDR-TB as they were resistant to at least isoniazid and rifampicin. Such patients with drug resistance TB to all the five first-line anti-TB drugs poses a challenge to health care workers. The use of new drugs such as Bedaquiline should only be used after the national MDR TB clinical team has agreed on it and minutes of the same availed (PMDT guidelines 2014). *M. bovis* and *M. africanum* did not show any multi-drug resistance. For the

treatment of a combination pattern of two, three, four and five drug resistance, the recommended regimen is second-line anti-TB drugs based on the susceptibility pattern.

The prevalence rate of MDR-TB in the current study was 1.4% observed only in *M. tuberculosis* but not seen in other MTBC subspecies tested. The low resistance rate could be attributed to patients receiving regular monitoring to ensure that they complete their treatment which makes patient adherence to drugs good in Kisumu county. This is slightly below the 3.7% world MDR-TB prevalence (Usagi *et al*., 2016). This was similar to studies in Jimma, Sourth western Ethiopia where the rate of MDR-TB among newly diagnosed TB patients was 1.5% (Abebe *et al*., 2012), in Thailand 1.2% (Reechaipichitkul *et al*., 2011) and 1.1% (Seyoum *et al*., 2014). This prevalence was much lower than previous studies in Addis Ababa, Ethiopia where the prevalence of MDR-TB was 31.4% (Abdella *et al*., 2015) which can be explained by the study target population in Ethiopia employed in the study which were referral cases of drug susceptibility test (DST) and culture due to treatment failure. Multi-drug resistance-TB strains acquired resistance during the intensive or/and continuation phase of treatment (Abdella *et al*., 2015). Studies conducted in various states of India reported higher MDR-TB as follows: in Gujarat, there were 2.4% in the newly diagnosed cases and 17.4% in previously treated cases (Ramachandran *et al*., 2009); Kerala 2% in newly diagnosed cases (Joseph *et al*., 2007). Kashmir reported an initial drug resistance of 5.4% and a secondary drug resistance of 36.5% among the study population (Dutta *et al*., 2010). In Turkey, MDR-TB prevalence in new cases was 2.16% slightly higher than the current study (Komurcuoglu *et al*., 2013).

Higher MDR-TB prevalence has also been reported: in Bangladesh 2.7% (Mottalib *et al*., 2011), in Turkey 4.8% (Ozturk *et al*., 2005) and in Kenya 18.46% among TB-HIV co-infected patients in Kisumu County (Usagi *et al*., (2016). This high prevalence rate of MDR-TB compared with

the current study both of which were conducted in Kisumu county could be due to the difference in the target population employed. There is a possibility that some patients were referred cases whose initial treatment had failed and they were seeking retreatment in the facility. Lower MDR-TB has also been reported in North Eastern Thailand 1.2% (Reechaipichitkul *et al*., 2011), which was a reduction from the previous study conducted at the same site that reported 2.4% MDR prevalence (Reechaipichitkul *et al*., 2011). The decrease may be due to the adherence to appropriate strategies for the treatment of TB in Thailand. Compared to national data, the prevalence of MDR-TB in the current study is lower.

The problem of drug resistance can be overcomed by DOTS strategy where anti-TB medications are swallowed by patients under the supervision of a health worker thereby ensuring that proper medication are given at proper intervals and at the right doses. It is said that effective TB control program leads to decrease in resistance pattern. Studies have shown that there is a direct association between previous TB treatment and an increased prevalence of drug resistance (Irfan *et al*., 2006). To decrease resistance, adequate and complete therapy for patients diagnosed with TB should be ensured. Based on the studies, it shows that previous treatment in TB is the strongest risk factor which includes failed previous TB treatment, relapsed after treatment or default during previous treatment, for the development of MDR-TB. MDR-TB cases are higher in retreatment patients than in newly diagnosed patients. Isoniazid and rifamicin are the two most powerful first-line anti-TB drugs. Fluoroquinolones (FQs) and pyrazinamide are key drugs being tested as part of new TB and MDR-TB regimens. Understanding the background prevalence of resistance to these drugs is essential. However, *M. bovis* will pose a challenge in the treatment in case a patient is suffering from PTB caused by *M. bovis* (WHO, 2014). Globally, nearly half a million newly infected patients harbour MDR-TB strains (WHO, 2016).

Extensively drug resistant TB (XDR-TB) strains were not detected in the current study because DST was not performed for second line anti-TB drugs. Mono resistance to *M, africanum* was observed in streptomycin (20%), isoniazid (40%), and pyrazinamide (20%). All the five species of *M. africanum* were neither resistant to rifampicin nor ethambutol. There was limited data available on the previous studies involving drug susceptibility pattern of *M. africanum* to first line anti-TB drugs. Most studies did a general study on MTBC strains without taking into consideration the different species of MTBC.

In the current study, *M. bovis* was 100% sensitive to all the four anti-TB drugs except pyrazinamide. However, resistance to just pyrazinamide does not usually cause problems with treatment, because TB disease is treated with a combination of several antibiotics. Latent infection without disease is not treated with pyrazinamide. *Mycobacterium bovis* is usually susceptible to most antibiotics used to treat human PTB, caused either by infection with *M. tuberculosis* or *M. africanum.* The susceptibility of *M. bovis* reported here is consistence with the reports from various previous studies: In Brazil none of the isolates of *M. bovis* was resistant to any of the five anti-TB drugs used in the determination of DST, excluding pyrazinamide (Parreiras *et al*., 2004). In Michigan, the results were also identical to findings in the current study, where all the isolates were susceptible to isoniazid, streptomycin, rifampin, and ethambutol but resistant to pyrazinamide (Fitzgerald *et al.*, 2011). Nevertheless, it should be recognized that TB cases caused by MDR *M. bovis* may result in disease that is harder to treat on a second- line drug regimen. This highlights the need for performing species-level identification and drug susceptibility testing whenever *M. bovis* is suspected.

Identification of mycobacteria to species level is not mandatory in Kenya and therefore PTB patients infected with NTM fail to respond to conventional first line anti-TB drugs. These

patients end up being classified as MDR-TB without knowing the root cause of failure to respond to anti-TB drugs. The resistance of *M. tuberculosis* to rifampicin and isoniazid as seen in the current study is an alarming marker of MDR-TB. First-line drug susceptibility pattern differences of MTBC in various regions in Kenya and other countries, may be due to the difference in effectiveness of TB control program in different countries.

Mycobacterial species in the MTBC may undergo low-frequency spontaneous and induced chromosomal mutations which result in genetic resistance to anti-TB drugs (Kurbatova *et al*., 2013). For example, MTBC species undergo a mutation in the β-subunit of their RNA polymerase which is the target site of rifampicin. However, it is the application of anti-TB drugs which creates pressure for selection of these strains with mutations. This is generally due to improper therapeutic applications such as insufficient length of drug treatment, poor patient adherence to dosing schedules, using a single anti-TB drug which does not clear the bacilli instead of the recommended multiple drug therapy, and failure to recognize pre-existing resistance in a TB case (Jain & Dixit, 2008). These problems then lead to the emergence of MDR strains of mycobacteria.

5.4. Antimycobacterial Activity of *P. americana* pod extract

In the current study the antimycobacterial potentials of *P. americana* chloroform, ethanol and *n*-hexane, pod extracts were investigated against 20 selected clinical isolates of mycobacteria. The extracts demonstrated antimycobacterial activity against the tested clinical isolates. Chloroformic extract exhibited appreciable antimycobacterial activity against all the tested isolates with MIC range (18.75mg/ml – 75mg/ml) with the highest potency against *M. africanum* (MIC = 18.75mg/ml) which was sensitive to all five anti-TB drugs. This implies that chloroform used as

a solvent for extraction is able to extract ideal phytochemicals from the pod that are capable of interfering with a number of different crucial metabolic processes even in resistant strains of mycobacteria, that leads to their death. Isolation of individual phytochemicals could lead to the discovery of compounds for development of drugs effective against both drug sensitive and drug resistant TB strains. Ethanoic extract also exhibited appreciable antimycobacterial activity against 19 isolates with MIC ranging from 18.75mg/ml – 75mg/ml. However, one isolate which was resistant to all the five drugs (pentadrupal-resistance) *M. tuberculosis* showed complete resistance. This implies that ethanol was not able to extract the phytochemical that was able to kill or inhibit the growth of pentadrupal resistant strains of mycobacteria.

N-hexane pod extract, however demonstrated the least antimycobacterial activity against 17 test isolates (MIC = 150mg/ml – 375mg/ml) with *M. tuberculosis* resistant to a combination of four drugs (quadrupal resistance), five drugs (pentadrupal resistance) and *M. abscessus* strains showed complete resistance. The less activity of the *n*-hexane fraction relative to the chloroform and ethanol extracts may be supported by the fact that most phenolic compounds dissolve readily in polar solvents than non-polar solvents. And thus, the polarity of solvents is known to affect the composition of the secondary metabolites of an extract (Parekh *et al.,* 2006).

The antimycobacterial activity of the extracts was observed to be dependent on the type of solvent used for extraction which affect the composition of the crude extract. Various studies have shown that methanoic and ethanoic plant extracts have more antimicrobial activity compared to other solvents (such as *n*-hexane, water, dichloromethane, etc.) because they extract both polar and non-polar phytochemical compounds (Ncube *et al*., 2008). The current results shows great antimycobacterial activity in ethanol and chloroform extracts. This indicates that the solvents were able to extract bioactive constiuents with antimycobacterial activity thus, higher

potency compared to *n*-hexane. This is in agreement with the work of Krishna *et al.*, (2012), where chloroformic extract of *Callistemon citrinus* was more potent in inhibiting the growth of both Gram positive and negative bacteria (Krishna *et al*., 2012). It can be seen from the results in this study that the *n*-hexane extracted low concetrations of the bioactive constituents, hence high quantity of the extract is needed to demonstrate the antimycobacterial activity of the extract (MIC being 150 – 375mg/ml). Based on the results, it can be concluded that the therapeutic activity of *P. americana* pod can be harnessed by using chloroform and ethanol as solvents for extraction.

The antimycobacterial activity of chloroform and ethanol extracts in the current study is impressive as it was not expected more especially in NTM and resistant strains of MTBC clinical isolates. This is because NTM are considered to be naturally resistance to most of first-line anti-TB drugs, and in this case expected to be resistant to *P. americana* pod extracts used. The low MIC demonstrated by chloroform and ethanol extracts in this study (18.75mg/ml) implies that the phytochemicals present in *P. americana* extracted by the two solvents possess therapeutic potential. Further studies are required to define the active compound(s) responsible for the antimycobacterial activity of chloroform, ethanol and *n*-hexane extracts. In the current study, the pod extract was not screened to isolate individual compounds. Besides the solvent used for extraction, other factors that have been shown to affect antimicrobial efficacy of the plant extract include: (i) the part of the plant used because different parts of plants have different concentrations of the secondary metabolites, (ii) age of the plant part during harvest, and (iii) environmental conditions (Arukwe *et al*., 2012). The pod part of *P. americana* contains phytochemicals such as alkaloids, saponins, tannins, flavonoids, phenols, essential oils and steroids. Phytochemicals are important chemicals found virtually in plants and their different

parts and at different concentrations (Arukwe *et al*., 2012). The antimycobacterial activity exhibited by the *P. americana* pod extract in the current study may be due to the presence of the above mentioned secondary metabolites which are known to be pharmacologically active and contribute to the antimycobacterial activity of medicinal plants (Aherne *et al*., 2009). Alkaloids are important plant secondary metabolites and various studies have shown that alkaloids contain anti-bacterial activity (Erdemoglu *et al*., 2007). It is believed that single compounds may not be responsible for the bioactivity, but rather a combination of compounds interacting in an additive or synergistic manner (Javid *et al.*, 2015; Javed *et al.*, 2012).

Though the mechanisms of antimicrobial actions of the plants secondary metabolites are not fully understood, some investigators have reported secondary metabolites from plants have varied mechanism of action such as cell membrane disruption, inhibition of cell wall synthesis causing cell lysis, inhibition of nucleic acid synthesis, and inhibition of protein synthesis (Javed *et al.*, 2012).

The *n*-hexane pod extract did not show any antimycobacterial activity against *M. abscessus* in the current study. This could be attributed to the failure of the solvent to extract the active component/ or in sufficient concentrations to bring effect on the pathogen. However, the susceptibility of the other rapidly growing Mycobacteria such as *M. fortuitum* in the current study offers the chance for possible chemotherapeutic alternatives, since some of these rapidly growing mycobacteria, are known to show resistance to some anti-TB drugs, such as Telithromycin, meropenem and fluoroquinolones (Yang *et al.*, 2003).

Sensitive mycobacteria species showed an MIC of between 37.5 – 75mg/ml in chloroform and ethanol, 150 - 375mg/ml in *n*-hexane, suggesting that the crude extracts contain compounds that could be used to develop a better antimycobacterial drug. This preliminary investigation agrees

with the reports of Adeleye *et al*., (2008) that the ethanoic and aqueous extracts of *A. ascalonicum* inhibited the growth of *M. tuberculosis.* Also, Mansour *et al.,* (2009) reported that partially purified extract of *Allium ascalonicum* showed significant antimycobacteria activity against *M. tuberculosis*. With this MIC, this suggest that the extracts contain compounds that could be developed to elicit better antimycobacteria activity.

Most of the medicines currently in the market are derived from plants, which are modified in the laboratory to make them less sensitive to inactivation by enzymes produced by microorganisms that inactivates drugs by hydrolyzing the pharmacologically active part with pharmacological activity (Nessar *et al*., 2012). From the time immemorial before the invention of synthetic medicine, people relied on herbal medicine as the only remedy of managing and treating various illness by herbalist or traditional healers. Herbal medicine has been and still will be the main provider of medicines, as it has been since human existence.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATIONS

6.1. SUMMARY OF THE MAIN FINDINGS

From the findings of this study, 92.4% of the samples from PTB patients were positive for GeneXpert *MTB/RIF* assay and 7.6% were negative, 75.8% were positive in smear microscopy and 24.2% were negative, while 92.1% were culture positive and 7.9% culture negative. Sensitivity of smear microscopy and GeneXpert *MTB/RIF* assay tests was 83.5% and 91.8% respectively compared to the culture test. New cases of pulmonary TB in Kisumu County is caused by members of MTBC and NTM with the overall prevalence of 91.8% and 8.2% respectively. Three members of MTBC that causes PTB are: *M. tuberculosis* the most predominant species accounting for 97.6%, followed by *M. africanum* 1.7% and *M. bovis* 0.7% was the least. Four species of NTM isolated where *M. intracellulare* which was the most predominat accounting for 61.5%, followed by *M. abscessus* 19.2%, *M. kansasii* 11.5% and *M. fortuitum* 7.7% was the least. *Mycobacterium tuberculosis* and any resistance to one anti-TB drugs was highest in both isoniazid and pyrazinamide (10.2%), followed by ethambutol (5.6%), streptomycin (3.2%) and rifampicin had the least (1.8%). *Mycobacterium africanum* was resistant to streptomycin, isoniazid and pyrazinamide at the rates of 20%, 40% and 20% respectively. *Mycobacterium bovis* was sensitive to all the five anti-TB drugs tested except pyrazinamide. Drug susceptibility patterns in *M. tuberculosis* revealed the highest resistance to one specific drug in isoniazid and pyrazinamide at 6% followed by ethambutol 2.8%, streptomycin 1.1% and rifampicin had the least 0.4%. Resistance to a combination of drugs was also observed. The prevalence rate of MDR-TB was 1.4%. Chloroform pod extract of *P. americana* had the highest antimycobacterial activity against all the mycobacteria isolates tested

(MIC range 18.75mg/ml – 75mg/ml) followed by ethanoic extract which had antimycobacterial activity against 19 isolates (MIC range 18.75 – 75mg/ml), and *n*-hexane had the least antimycobacterial activity with MIC range 150 – 375mg/ml against 17 isolates.

6.2. CONCLUSIONS

1. Culture and GeneXpert *MTB/RIF* tests were more sensitive than smear microscopy in the detection of mycobacteria in new cases of pulmonary TB patients.
2. New molecular tools are able to characterize new PTB cases from MTBC and NTM which are normally difficult to identify using conventional techniques.
3. *Mycobacterium tuberculosis* treatment should encampus the use of multiple anti-TB drugs to avoid the observed resistance to single drug regiments.
4. Chloroformic, ethanoic and *n*-hexanic pod extracts of *P. americana* demonstrated antimycobacterial activity against MTBC and NTM clinical isolates some of which were resistant to conventional anti-TB drugs demonstrating potential of *P. americana* pod extracts as a source of therapeutic anti-TB agents.

6.3. RECOMMENDATIONS

1. To increase accessibility to genexpert and culture tests in health facilities.
2. GeneXpert cannot differentiate mycobacteria to species level, therefore GenoType Mycobacterium CM/AS and MTBC Assay tests should be introduced into routinely employed laboratory diagnostics in the speciation of mycobacteria infections as a useful tool for diagnostic and effective management of infections in Kenya.
3. Appropriate multi-drug use for specific mycobacteria species based on drug susceptibility patterns to avoid drug resistance.

4. Chloroform should be used as a solvent for extraction of the *P. americana* pod extract and further separation and identification of the bioactive compounds that can be used in the development of therapeutic agents for the treatment of pulmonary TB.

6.4. SUGGESTION FOR FURTHER STUDIES

The current study suggests that chloroform, ethanol and *n*-haxane pod crude extracts of *P. americana* have some antimycobacterial activity hence further studies are required for separation and purification to define the individual bioactive compounds present in these extracts responsible for the antimycobacterial activity.

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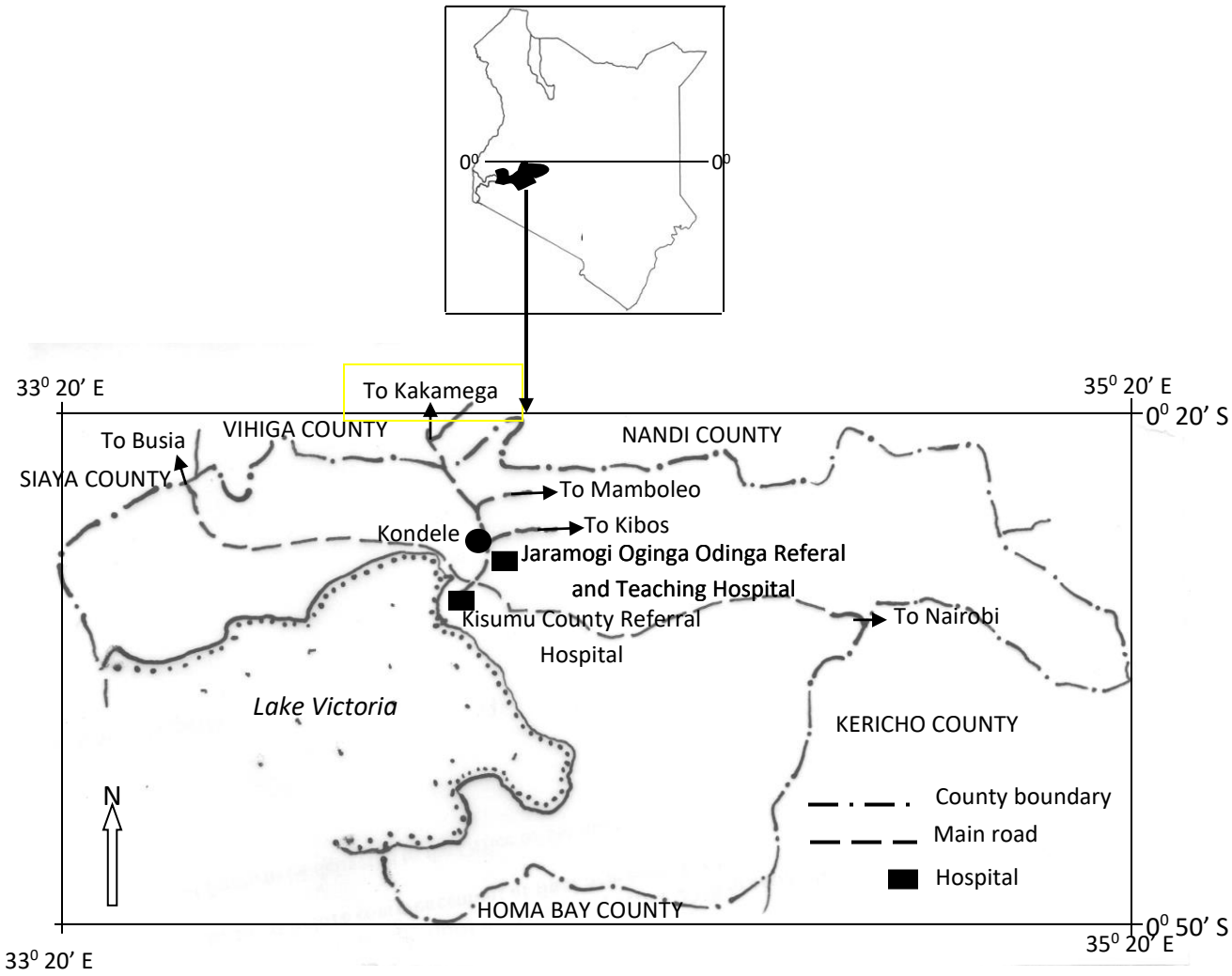
APPENDICES

Appendix 1: Map of Study Area- Kisumu County



Procedures for Preparation of Laboratory Reagents

Appendix 2: Preparation of 4% NaOH Solution

1. Dissolve 4g NaOH pellets into 100 ml of distilled/deionized water
2. Sterilize by autoclaving
3. Concentration of NaOH may be varied (3-6%) NaOH solution at the beginning).

Appendix 3: Preparation of 2.9% Sodium Citrate Solution

1. Dissolve 2.9g sodium citrate in 100 ml distilled/deionized water
2. Sterilize by autoclaving.

Appendix 4: Preparation of Sodium hydroxide-N-acetly-L-cysteine (NaOH-NALC)

1. Mix equal quantities/volumes of sterile NaOH and 2.9% sterile sodium citrate solution
2. Immediately prior to use, add N-acetyl-l-cysteine (NALC) powder to achieve/yield a final concentration of 0.5% (for 100 ml NaOH-Na citrate solution, add 0.5 g NALC powder)
3. Mix well and use the same day (NALC activity is lost if left standing for more than 24 hours).

Appendix 5: Table showing the amount of NALC powder added in different volumes of NaOH-Na citrate.

Volume of digestant needed	**Mix indicated amounts (ml) of**		**Add NALC (g)**
	4% NaOH	**2.9% Na citrate $2H_2O$**	
50	25	25	0.25
100	50	50	0.50
200	100	100	1.00
500	250	250	2.50
1000	500	500	5.00

Appendix 6: Preparation of Phosphate Buffer (pH 6.8)

1. Dissolve 9.47g of anhydrous disodium phosphate (Na_2HPO_4) in 1000 ml (1 liter) distilled/deionized water, using a volumetric flask (Solution A)
2. Dissolve 9.07 g of mono-potassium phosphate (KH_2PO_4) in 1000 ml (1 liter) distilled/deionized water, using a volumetric flask (Solution B)
3. Mix equal quantities of the two solutions
4. Check the pH by adding more solution A to raise the pH; more solution B lowering the pH
5. The final pH is 6.8 followed by sterilization by autoclaving.

Appendix 7: Reconstituting MGIT PANTA

1. Reconstitute MGIT PANTA with 15 ml MGIT growth supplement, mix well until completely dissolved
2. Add 0.8 ml of this enrichment to MGIT tube
3. Add the enrichment with reconstituted PANTA to the MGIT medium prior to inoculation of specimen in MGIT tube
4. Do not add PANTA/enrichment after the inoculation of specimen.
5. Do not store MGIT tube after the addition of enrichment/PANTA.

Appendix 8: Preparation of Ziehl-Neelsen (ZN) Stain

1. Prepare primary stain (basic fuchsin) by completely dissolving 1gm of basic fuchsin in 10 ml of 95% ethyl alcohol
2. Filter the final staining solution in case of any particles or precipitate
3. Prepare decolourizer (acid alcohol) by dissolving 5 gm phenol in 95 ml of 95% ethyl alcohol and mix gently

4. Prepare counter stain (methylene blue) using two solutions (solution A and B)
5. Prepare solution A by dissolving 0.3gm methylene blue chloride in 30ml of 95% ethyl alcohol
6. Prepare solution B by completely dissolving 0.01gm potassium hydroxide in 100ml distilled/deionized water
7. Gently mix solutions A and B to make counter stain and filter to remove the particles.

Appendix 9: Preparation of Hybridization Reagents

1. Pre-warm shaking TwinCubator to 45^0C
2. Pre-warm HYB and STR solutions to 37-45^0C before use, mix if necessary {The reagents must be free from precipitates (note, however, that solution CON-D is opaque)}
3. Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature
4. Using a suitable tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed
5. Mix well and bring to room temperature
6. For each strip, add 10µl concentrate to 1 ml of the respective buffer
7. Dilute CON-C before each use
8. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

Appendix 10: Preparation of Master Mix (Amplification Mixture)--25 samples

1. Transfer 875µl of primer nucleotide mix (PNM) to a clean crayovial

2. Add 50µl of $MgCl_2$ followed by addition of 125µl buffer
3. Add approximately 75µl of molecular grade water to the mixture
4. Add 5µl of Taq DNA.

Appendix 11: Table showing the number of samples in the preparation of PCR master mix

Sr.NO	**No.of samples**	**1**	**3**	**5**	**6**	**7**	**12**	**13**	**14**	**24**	**25**
1	PNM(µl)	35	105	175	210	245	420	455	490	840	875
2	$MgCl_2$(µl)	2	6	10	12	14	24	26	28	48	50
3	Buffer(µl)	5	15	25	30	35	60	65	70	120	125
4	Mol.grade H_2O (µl)	3	9	15	18	21	36	39	42	72	75
5	Taq DNA (µl)	0.2	0.6	1.0	1.2	1.4	2.4	2.6	2.8	4.8	5.0

Appendix 12: Reconstitution of SIRE lyophilized drugs

1. Reconstitute each critical concentration drug vial with 4 ml of sterile distilled water
2. Mix thoroughly to dissolve completely
3. Add 0.1 ml (100 µl) of reconstituted drug solution into each of the labelled BACTEC MGIT 960 tubes which result in the following critical concentration of drugs in the medium: streptomycin (S) 1.0 µg/ml of medium; isoniazid (I) 0.1 µg/ml; rifampin (R) 1.0 µg/ml; ethambutol (E) 5.0 µg/ml.

Appendix 13: Reconstitution of lyophilized PZA drug

1. Reconstitute each of the PZA drug vials with 2.5 ml of the sterile distilled/deionized water and mix well
2. The reconstituted drug solution will contain 8000 µg/ml of PZA.

Appendix 14: Inoculum preparation for the (DST) assays

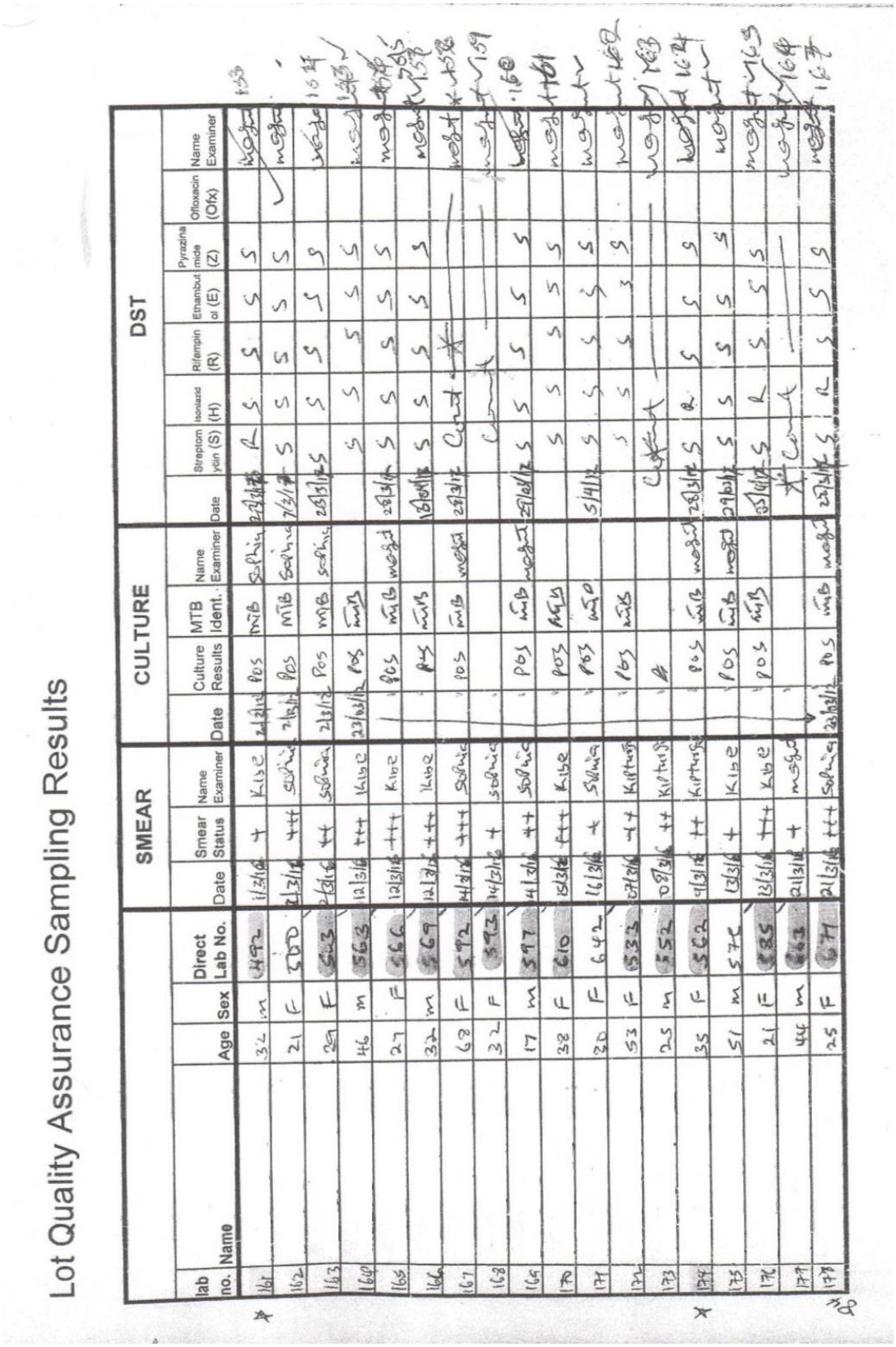
1. The day a MGIT tube is positive by the instrument is considered DAY 0.
2. Keep the tube incubated for at least one more day (DAY 1) before being used for the susceptibility testing (may be incubated in a separate incubator at 37^0C $\pm 1^0$C)
3. A positive tube may be used for DST up to and including the fifth day (DAY 5) after it becomes instrument positive.
4. A tube that has been positive for more than five days should be subcultured in a fresh MGIT tube supplemented with MGIT 960 Growth Supplement and should be tested in a MGIT 960 instrument until it is positive.
5. Use this tube from one to five days of instrument positivity
6. If growth in a tube is on DAY 1 or DAY 2, mix well (vortex) to break up clumps. Leave the tube undisturbed for about 5-10 minutes to let big clumps settle at the bottom
7. Use the supernatant undiluted for inoculation of the drug set
8. If growth is on DAY 3,4, or 5, mix the tubes well to break up the clumps, let the large clumps settle for 5-10 minutes and then dilute 1.0 ml of the positive broth with 4.0 ml of sterile saline. This will be a 1:5 dilution, then use this well mixed diluted culture for inoculation.

Appendix 15: WHO guidelines on sputum handling to minimize the risk of contracting TB in the laboratory

1. Collect sputum in a falcon tube (screw capped tube)
2. Wearing of protective gown by staff working in TB laboratory (gloves must be worn and must be long enough to overlap the sleeves of the gown, use hair covers (caps) and shoe covers, wearing a nose mask)

3. Class II biosafety cabinets (BSC) must be used for all manipulations of clinical MTB specimen (sputum).
4. Equip clinical centrifuges with biosafety canisters (buckets with aerosol containment lids)
5. Aerosol-generating procedures such as centrifugation, vortexing, mixing, pipetting, pouring, and inoculation of media be done only inside the BSC, delay opening caps until aerosols have settled, eject micropipette tips down inside discard bucket.
6. Heat-fix slides on a warmer in the BSC
7. Keep arms parallel inside the BSC to the work surface, work in the center, and minimize arm movements; once beginning work, do not move hands out of the hood until work is completed. These precautions will minimize interruption of airflow inside the BSC.
8. Keep the amount of equipment inside the BSC to a minimum so as to not interfere with the airflow pattern.
9. Disinfect the BSC and all work surfaces with a tuberculocidal disinfectant, 15 minutes before and after every procedure.
10. Place all wastes containing MTB in a leak-proof container or autoclavable plastic bag that contains disinfectant solution which can be sealed before being removed from the BSC and autoclaved
11. Remove all outer protective clothing when leaving the containment laboratory.

Appendix 16: Standard data culture form for demographic data collection



Appendix 17: Consent Form

I am a PhD student in Maseno University, Department of Biomedical Sciences & Technology and conducting a research on "Molecular Characterization and Antimycobacterial Activity of *Persea americana* and Conventional Drugs Susceptibility Against Mycobacteria Isolates from New Cases of Pulmonary Tuberculosis in Kisumu County, Kenya", as a fulfillment of the requirements for the award of the degree in Doctor of Philosophy in Medical Microbiology. I would like to recruit you to partcipate in my study as a respondent. As a respondent, your participation in the study is voluntary, and access to health care will not be dependent on participation, Moreover, you are free to withdraw at any time without having to incur any penalty. Confidentiality and privacy will be strictly safeguarded and observed. Furthemore, this research is for the purpose of my PhD study work and will not be used for any other purpose whatsoever. If you agree to partcipate in the study then append your signature.

Respondents signature/finger print ..... Date .....

Thank you in advance

Yours sincerely

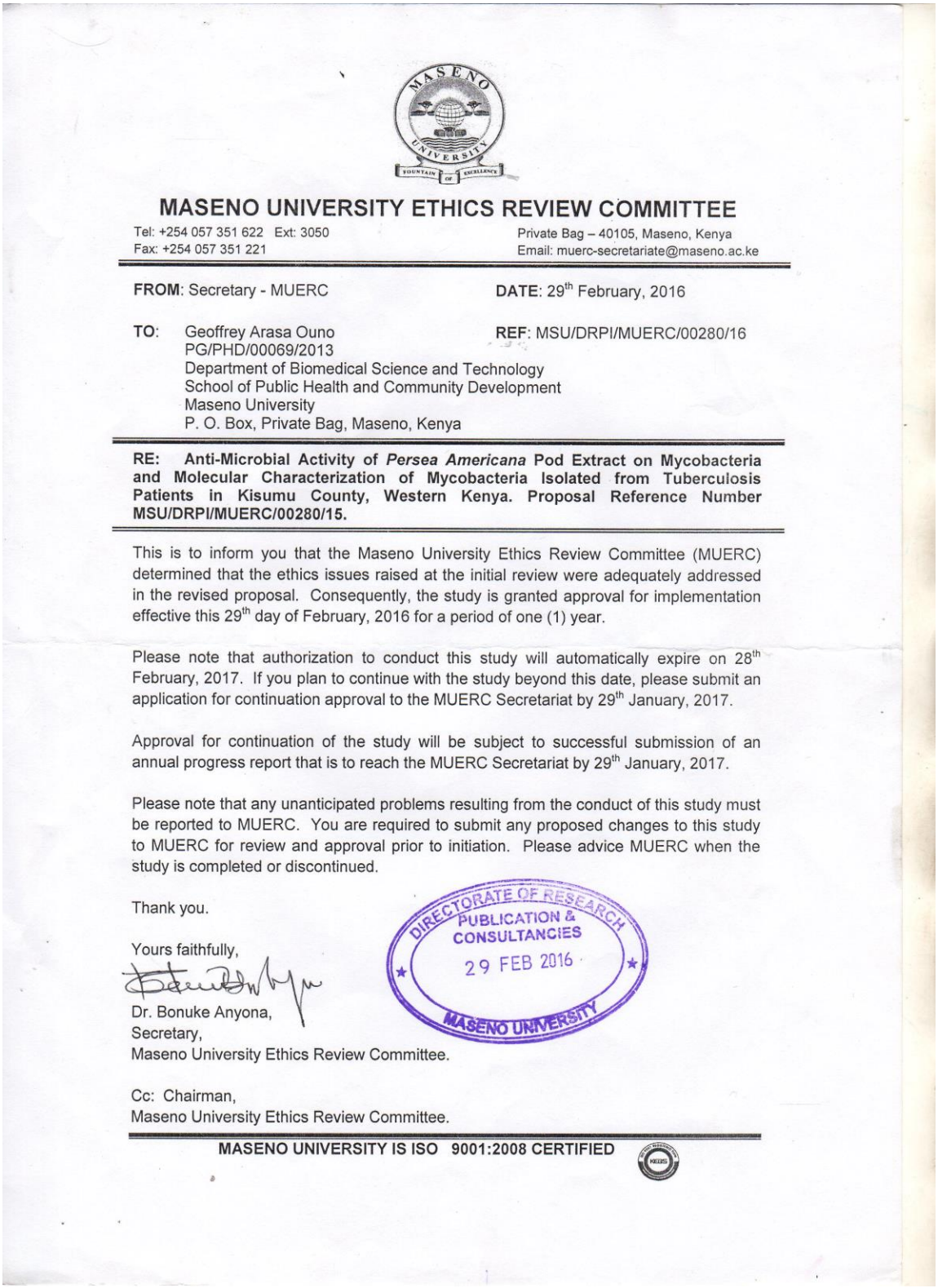
Geoffrey Arasa Ouno

NOTE: Below are the key contacts

Principle investigator: Mr. Geoffrey Arasa Ouno (+254 722718915)

Maseno University Ethics Review Committee (MUERC): +254 57 351 622- Ext.3050

Appendix 18: Ethical Approval by Maseno University



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 29th February, 2016

TO: Geoffrey Arasa Ouno
PG/PHD/00069/2013
Department of Biomedical Science and Technology
School of Public Health and Community Development
Maseno University
P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/00280/16

RE: Anti-Microbial Activity of *Persea Americana* Pod Extract on Mycobacteria and Molecular Characterization of Mycobacteria Isolated from Tuberculosis Patients in Kisumu County, Western Kenya. Proposal Reference Number MSU/DRPI/MUERC/00280/15.

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 29th day of February, 2016 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 28th February, 2017. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 29th January, 2017.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 29th January, 2017.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES 29 FEB 2016 MASENO UNIVERSITY

Yours faithfully,

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED

Appendix 19: Ethical Approval by JOOTRH

MINISTRY OF HEALTH

Telegrams: "MEDICAL", Kisumu
Telephone: 057-2020801/2020803/2020831
Fax: 057-2024337
E-mail: ercjootrh@gmail.com
When replying please quote

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849
KISUMU

Ref: P/1/VOL.XIV /250

Date March 9th, 2016

PI..... Geoffrey Arasa Ouno,

PG/PHD/000/2013.
MASENO UNIVERSITY.

Dear Geoffrey,

RE: FORMAL APPROVAL TO CONDUCT A STUDY ENTITLED: *"anti- microbial activity of Persea Pod extract on Mycobacteria and Molecular Characterization of Mycobacteria isolated from Tuberculosis patients in Kisumu county, Western Kenya. Proposal Ref: no.MSU/DRPI/MUERC/00280/15"*

The JOOTRH ERC (ACCREDITATION NO. 01713) has reviewed your article on above subject and granted permission for the study to be conducted Kisumu County-Western Kenya,. You are therefore, permitted to commence your study immediately. Note that permission is granted for a period of 1 year wef. March 9th, 2016 to March 9th 2017.

Also note that you will be required to notify the committee of any amendment(s), serious or unexpected outcomes related to the conduct of the study or termination for any reason.

Finally, note that you will also be required to share the findings of the study with us in both hard and soft copies upon completion.

The JOOTRH ERC takes this opportunity to thank you for choosing the institution for review of your protocol and wishes you the best in your endeavours.

Yours sincerely,

MAKUNDA W. NANCY,
For: SECRETARY - ERC,
JOOTRH – KISUMU.

JOOTRH ETHICS & REVIEW COMMITTEE
P. O. Box 849 - 40100
KISUMU