Mycobacterium tuberculosis RESISTANCE TO ANTI-TUBERCULOSIS DRUGS IN COAST PROVINCIAL GENERAL HOSPITAL MOMBASA, KENYA.

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DECLARATION

I duly declare that this thesis is based on my original work and has not been presented for a degree award in any other University, or any other award.

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DEDICATION

I dedicate this work to my family and friends for their support and patience during the study period.

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LIST OF ABBREVIATIONS

AFB	Acid Fast Bacillus
AMK	Amikacin
BSL2	Bio-safety Cabinet Class 2
CAP	Capreomycin
DNA	Deoxyribonucleic Acid
DRDR	Drug resistant determining region
EMB	Ethambutol
FLD	First Line Anti Tuberculosis Drugs
FQ	Fluoroquinolone
GYR	Gyrase
HIV/AIDS	Human Immunodeficiency Virus /Acquired Immunodeficiency Syndrome
INH	Isoniazid
K	Kanamycin
KAT	Catalase-peroxidase enzyme
MDR	Multidrug-resistant
MLS	Millilitres
MTB	*Mycobacterium tuberculosis*
NAD	Nicotinamide adenine dinucleotide
NAD (H)	Nicotinamide adenine de-nucleotide hydrate

NAOH	Sodium Hydroxide
PCR	Polymerase Chain Reaction
PTB	Pulmonary tuberculosis
PZA	Pyrazinamide
RIF	Rifampicin
RPM	Rotation per Minute
SLD	Second Line Anti Tuberculosis Drugs
SM	Streptomycin
TB	Tuberculosis
tRNA	Transfer ribonucleic acid
UNAIDS	United Nations Programme on HIV and AIDS
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant Tuberculosis
TDR-TB	Total Drug Resistant Tuberculosis

OPERATIONAL DEFINITIONS

Adherence to treatment: Following the recommended course of treatment, by taking all the prescribed medications for the entire length of time necessary**.**

Adverse reaction: Negative side effect resulting from the use of a drug

Antiretroviral therapy (ARV): A lifelong combination drug treatment to improve the quality and length of life for a person living with HIV/AIDS.

Case of tuberculosis: Isa definite case of TB (defined below) or 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 anti-TB treatment.

Clinical evaluation: An evaluation done to find out whether a patient has symptoms of TB disease or is responding to treatment; also done to check for adverse reaction to TB medications.

Close contact: People who spend time with someone who has infectious TB disease.

Contact investigation: A procedure for interviewing a person who has TB disease to determine who may have been exposed to TB. People who have been exposed to TB are tested for LTBI and TB disease.

Continuation phase: TB treatment phase follows the intensive phase and commonly uses two drugs, isoniazid and rifampicin. This is the period in which sterilization of the infection site occurs with killing of the bacilli including intracellular bacilli.

Cross resistance: TB resistance to a combination of either of the injectable drugs (kanamycin, viomycin, capreomycin, and amikacin) due to a single resistance mechanism in the TB DNA that confers resistant to entire class of antibiotics.

Daily regimen: Treatment schedule in which the patient takes a dose of each prescribed medication every day.

Definite case of tuberculosis: A patient with *Mycobacterium tuberculosis* complex identified from a clinical specimen, either by microscopy, culture or by a newer method such as molecular line probe assay.

Directly observed therapy (DOT): Strategy devised to help patients adhere to treatment whereby a designated person watches the TB patient swallow each dose of the prescribed drugs.

Discordant test: This is when two test diagnostic test run parallel give conflicting results.

Epidemiology: The study of the distribution and causes of disease and other health problems in different groups of people.

Extensively drug resistant TB (XDR TB): This is a rare type of MDR-TB which is resistant to isoniazid and rifampicin, plus any fluoroquinolone and at least one of three injectable second-line drugs (i.e., amikacin, kanamycin, or capreomycin).

Follow up case: subject revisiting the hospital

Health care facilities: Institution where people receive health care, such as hospitals or clinics.

Hepatitis: Inflammation of the liver, causing symptoms such as nausea, vomiting, abdominal pain, fatigue, and dark urine; hepatitis can be caused by several drugs used to treat LTBI or TB disease.

Infection control procedures: Measures to prevent the spread of TB.

Intensive phase: Initial phase of TB treatment in which actively dividing bacilli are rapidly killed. The common regimen used is a combination of four drugs, isoniazid, rifampicin, ethambutol and pyrazinamide for eight weeks.

Intermittent therapy: Treatment schedule in which the patient takes each prescribed medication two or three times weekly at the appropriate dosage.

Latent tuberculosis infection (LTBI): Person who are infected with *Mycobacterium tuberculosis*, but do not have symptoms. However, when the immune system is weakened their status may progress to active TB.

Liver function tests: Tests done to detect injury to the liver.

Mono-resistance: Resistant to either of the first line drugs such as rifampicin, isoniazid, pyrazinamide and ethambutol.

Multi-drug resistance TB: This is dangerous form of TB that is resistance to Rifampicin and Isoniazid –the two most powerful first line anti -TB drugs. MDR can only be cured with long treatment of second line drugs.

New Cases: Patients who have never received treatment for tuberculosis or taken it for less than one month.

Poly resistance: Resistance to combination of any two first line drugs other than combination of rifampicin and isoniazid such as rifampicin / isoniazid with pyrazinamide or ethambutol respectively.

Re-Treatment Cases: These are smear-positive cases identified as “failures”, “treatment after default” and “relapses” are categorised as “retreatment” cases

Total drug resistant tuberculosis: This is the more dangerous form of TB that is resistance to all first-line drugs (FLDs) Isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin; and second-line drugs (SLDs) Ofloxacin, moxifloxacin, kanamycin, amikacin, capreomycin, para-aminosalicylic acid, ethionamide

Transmission: The spread of an organism such as M. tuberculosis from one person to another.

Tuberculosis (TB): A highly contagious chronic granulomatous inflammatory disease, caused by the bacterium called *Mycobacterium tuberculosis*.

Tuberculosis suspect: A person who has had contact with a TB patient, or presenting with symptoms or signs suggestive of Tuberculosis.

Peripheral neuropathy: Damage to the sensory nerves of the hands and feet, causing a tingling sensation or a weakened sense of touch in the hands and feet.

Primary TB: Initial diagnosed infection by *Mycobacterium tuberculosis*, typically seen in children but also occurs in adults, characterized by lungs formation of a primary complex, consisting of small peripheral pulmonary focus with spread to hilar lymph nodes.

ABSTRACT

Background: Kenya is one of the countries with a high TB infection rate and globally, it is ranked fifteenth amongst twenty-two countries with a high prevalence. The development of drug resistant *Mycobacterium tuberculosis* (MTB) strains and consequent treatment failure is a common clinical scenario in TB disease, associated with high mortality rates. However, information on drug resistance as well as both multi-drug and extensively drug resistance tuberculosis is currently very scanty in Kenya. Despite the isolated reported cases of MDR-TB in Mombasa-Kenya, detection rates are still very low, as diagnostic methods available in the public health facilities are still largely based on sputum slide smear microscopy. Therefore, the current study assessed the detection of MDR-TB and XDR-TB by molecular assay, in smear positive TB patients presenting to Coast Provincial General Hospital (CPGH), Mombasa, Kenya.

***Broad objective*:** To determine the proportion of DR-TB, MDR-TB and XDR-TB in sputa smear positive TB patients at Coast Provincial General Hospital (CPGH), Mombasa, Kenya.

Materials and methods: Two hundred and fifty-six confirmed sputa smear positive TB cases diagnosed by fluorescent microscopy technique were randomly selected for molecular Gene Lipa assay for detection of mutant genes responsible for TB drug resistance to first line and second line drugs, between January and September, 2012 at the CPGH-Mombasa, irrespective of any previous TB treatment were included in the study. But prior to start of treatment for this episode, ethical approval to conduct the study was obtained from KNH/UON and CPGH ethical research committees. Questionnaire was administered to obtain both demographic and clinical data from study participants who met the inclusion criteria. Approximately 2mls of sputum was collected in a falcon-tube, decontaminated and transported to UON, molecular laboratory for mycobacteria DNA analyses using HAINS line probe assays, to detect first line drug resistance genes. This was followed by random selection of 83 cases for second line drug resistance genes. Data was analyzed for statistical correlations using SPSS statistical package version 19.0.

***Results*:** From the 256 cases in this study, male to female ratio was 1:2. The age range was 9 to 75 years, with median age of 30. The age category of 21-30 years was found to have the

highest prevalence rate of PTB infection. Majority of the participants were new cases (98%), while the rest (2%) were retreatment cases. Seven of the new cases were PTB negative, constituting 4 (1.6%) PTB negative and 3(1.1%) NTM. For drug resistance detection, GenoType MTBDR® plus detected 91.7% new cases which showed full susceptibility to (INH) and (RIF). Of the remainder of the new cases, 8 (3.1%) and 1(0.4%) cases had mono-resistance to isoniazid (INH) and rifampicin (RIF), respectively. All the retreatment cases did not show drug resistance to first line drugs. Ethambutol mutation probes were included in the Hains Life sciences Genotype MTBDRsl Probe assay. For second line drug resistance testing using GenoType MTBDRsl Probe assay, 83(32%) specimens were analysed and one case each showed mono resistance to both ethambutol and fluoroquinolone (FQ). One case each was also detected for drug cross poly resistance to both (EMB) and (FQ) with second line injectable antibiotics. The proportion of cases that had HIV/AIDS -TB co-infection was 46 (17.8%), out of which 44 (17.1%) showed full susceptibility to TB drugs, while 2 (0.8%) were INH resistant. Equally no significance correlation was established between TB and the second line drugs (p=0.855).

Conclusion: ***Conclusion***: The findings of this study showed that proportion of various types of DR-TB was on the increase, and with the introduction of molecular tools such as MDRTB Lipa gene assay, at the patient's first point of care, TB diagnosis and management could be greatly improved.

CHAPTER ONE: INTRODUCTION

1.1. Background

The effective diagnosis and treatment of *Mycobacterium tuberculosis* (MTB) has remained a global health challenge for many years. This is further complicated by the frequent co-existence of epidemics of tuberculosis (TB) and human immunodeficiency virus (HIV), and the increasing prevalence of multidrug-resistant TB (MDRTB) (Khan, 2013). In 1993, the World Health Organization (WHO) declared TB a global emergency and public health concern as a result of the HIV/AIDS pandemics (Eduardo& Palomino, 2011). In 2011, World Health Organization (WHO) estimated the global burden of MTB infection to be 8.7 million incidents, and 14 million prevalent cases, of which 1.4 million deaths were reported. During the same period 430,000 deaths occurred among HIV positive, and one million deaths were documented among HIV negative people (World Health Organization, 2012a). The development of drug resistant TB strains and consequent treatment failure is a potential clinical scenario in TB disease and is associated with high mortality rates. This has been observed especially with various forms of Drug resistant tuberculosis (DR-TB) including Multi-drug resistant tuberculosis (MDR-TB), Extensively drug resistant tuberculosis (XDR-TB),and Total drug resistance tuberculosis (TDR-TB) (Fauci, 2015). However, in most cases MDR-TB develops during treatment of fully drug susceptible TB either due to interruption of the treatment therapy or inappropriate treatment choice (Marahatta, 2010). The resulting event is bacterial mutation and generation of genotypes that are tolerant to the toxic drug effects (Sia & Weiland, 2011).

Currently, information on drug resistance rates as well both multi-drug and extensively drug resistance tuberculosis is very scanty in the developing nations, Kenya inclusive. A few isolated cases of MDR-TB in Kenya have been reported, with low detection rate, as diagnostic methods available in the public health facilities are still largely based on sputum slide smear microscopy. This technique has low sensitivity and lacks specificity for MTB mutant strains that commonly confer resistance to the approved MTB regimens (Nyamogoba *et al*., 2012; Gunneberg et al., 2010a). Based on this background information, the present study used molecular technique of Hain Lifesciences for the

detection of MDR-TB and XDR-TB in patients who were documented to have sputum smear positive MTB at the Coast General Hospital in Mombasa Kenya, and correlated the outcome with characteristics of the patients**.** It is on this background that the current study attempted detection of MDR-TB and XDR-TB in susceptible population on pulmonary tuberculosis (PTB) to enable a stratified therapeutic approach, based on the characteristics of MTB-strains amongst patients presenting to Coast General Hospital, Mombasa, Kenya.

1.2. Problem Statement

Information on the prevalence of drug resistant tuberculosis is still very scanty and if available, is an incomplete, as screening practice at the referral health facility in Coast Provincial General Hospital lack sensitivity and specificity; and drug susceptibility testing is not performed. Physicians face a real dilemma when patients are diagnosed with active pulmonary tuberculosis (TB), and are left with no option, but start patients on first line anti-TB drug regimens, with expectations that the mycobacterium acquired are drug susceptible strains that do not develop into drug resistant mutants. If drug resistant strains develop, they are rarely noticed, resulting in transmission to other persons, poor prognosis and eventually death of the patient.

1.3. Justification

Drug resistant TB is associated with high rates of mortality due to treatment failure. Data on the prevalence of MDR-TB and XDR-TB in TB patients presenting to Coast General Hospital is lacking. This is due to lack of modern detection methods for MDR- and XDR-TB strains from sputum. Therefore, this study evaluated whether utilization of molecular techniques for detection of MTB strains in the laboratories with large patients' turnout is an effective tool for screening purposes, which can greatly improve reporting rates and turn-around-time. In addition, the findings of the study will give guidance to clinicians to stratify management of drug resistant tuberculosis, which require different treatment regimens, depending on the type of drug resistance. This could form part of Ministry of Health policy and guidelines in management of TB, MDR-TB and XDR-TB as gold standards in the diagnosis of the disease. The findings of work will also be published.

1.4. Objectives

1.4.1. Broad objective

To determine the proportion of Drug Resistance Tuberculosis in sputa smear positive TB patients presenting to, or admitted at Coast Provincial General Hospital (CPGH), Mombasa, Kenya

1.4.2. Specific objectives

1. To determine the number of DR-TB, MDR-TB and XDR-TB in sputa smear positive tuberculosis patients.
2. To determine the association between DR-TB and TB patients stratified by established patient treatment regimen classification (new and retreatment cases).
3. To determine the association between TB patients stratified by established patient treatment regimen classification (new and retreatment cases) and the various forms of DR-TB.

1.4.2. Secondary Objective

1. To determine prevalence of TB HIV/AIDS co-infection at the Coast General Hospital.

CHAPTER TWO: LITERATURE REVIEW

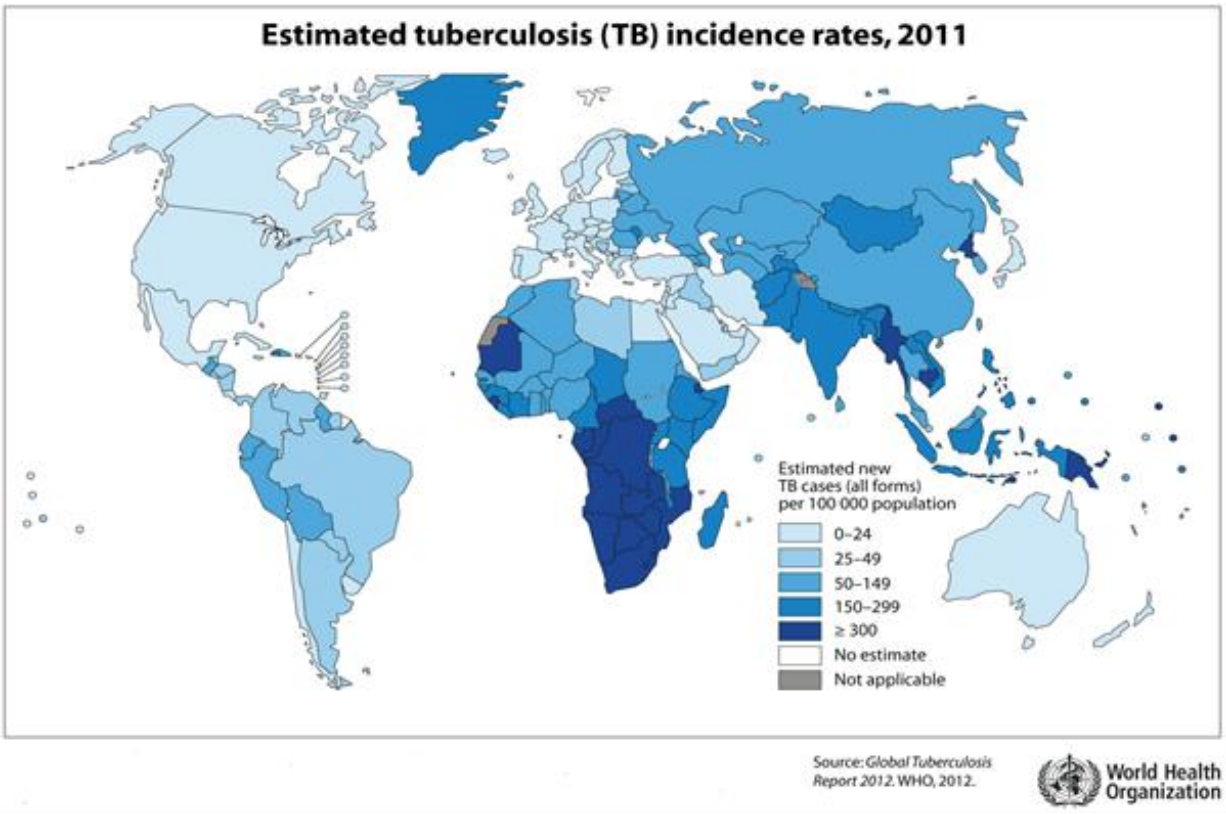
2.1. General Introduction

About one third of the human population (about 1.86 billion people) Worldwide are infected with *Mycobacterium tuberculosis* (MTB), the bacterium that causes tuberculosis; and 10% of these progress to develop active tuberculosis disease (Sia & Weiland, 2011). In addition, 95% of these reported TB cases and 98% of the resulting deaths are from developing countries, of which 23% and 55% of these death having been documented to come from south Asia and Sub-Saharan Africa respectively (USAID, 2014; World Health Organization, 2012a) (**Figure 1**).

Tuberculosis is exacerbated by the HIV epidemic, and is currently known to be amongst one of the overarching health issues in the world (Santos, 2012). The World Health Organization (WHO) therefore, confirmed the reemergence of TB disease, and consequently declared the disease a global emergency and public health concern in 1993. This was as a result of the HIV/AIDS pandemic in the early 1980s (Kirimuhuzya, 2007). In 2009, the global burden of tuberculosis infection was estimated to be 8.7 million incidents and 14 million prevalent cases. Out of these, 1.4 million mortalities occurred as a result of tuberculosis, of which 430000 mortalities were TB co-infected HIV persons, and one million mortalities documented among HIV negative persons (World Health Organization, 2012a).

Although TB became a re-emerging disease to European and North-American nations due to HIV/AIDs pandemic and the influx of immigrants with no previous adequate tuberculosis prevention measures (Morens *et al.*, 2004). The disease is an endemic public health problem in a number of developing countries, Kenya included, with an incidence report estimated at 80% of the world TB reported cases (Borgdorff & van Soolingen, 2013; World Health Organisation, 2008b).

Figure 1: Global TB Incidence rates.(World Health Organization, 2012b)



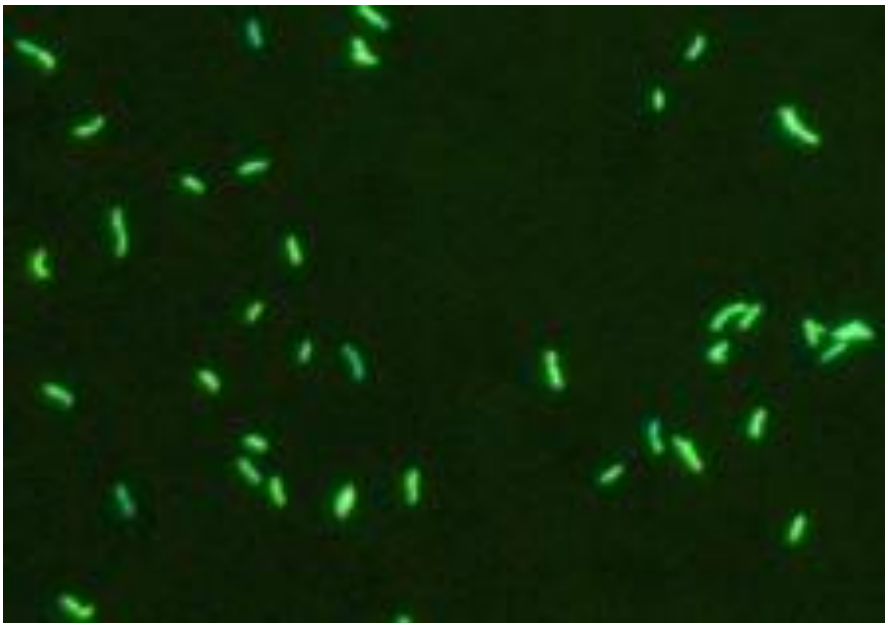
In addition, some studies reported emergence of drug resistant TB strains in Kenya from as early as 1980 (Ministry of Health, 2010b). However, information on drug resistance as well both multi-drug and extensively drug resistance tuberculosis is very scanty in Kenya (Githui *et al*., 2004). Despite the isolated reported cases of MDR-TB in Mombasa-Kenya, the detection rate is very low as diagnostic methods available in the public health facilities are still largely based on sputum slide smear microscopy detection method, which has MTB positivity reporting rate of between 40% (Iyer *et al*., 2011), and is reduced to 20% in HIV/AIDS cases (Davis *et al*., 2011)·(Sribenjalux *et al*., 2011). This technique, apart from its inherent low sensitivity, lacks specificity for drug susceptible tuberculosis strains**.**

2.2. Biology of the TB disease

Mycobacterial species that make up the MTB complex are associated with causation of TB. These includes MTB, *M. bovis*, *M. africanum*, *M. canetti and M. microti*, the latter

being commonly found in immune compromised patients (Forrellad *et al*., 2012). *Mycobacterium tuberculosis* is a small aerobic non-motile bacillus with a protective waxy mycolic cell wall that protects it from adverse environmental condition (Veyron-Churlet*et al*., 2010). When stained with auramine rhodamine (fluorochrome stains), and observed under microscopy, the mycolic acid accounts for its fluorescence characteristics, appearing yellow against a black background (**Figure 2, Figure 3**), which scientist have used as a diagnostic tool in the identification of mycobacterium in clinical specimens**.**

Figure 2: Fluorescence microscopy of *Mycobacterium tuberculosis* adapted from slide share Acid fast staining in tuberculosis: principles practices and application Dr.T.V Rao MD)



The mode of transmission for majority of MTB complex species is usually by inhalation of infectious airborne material of persons with active tuberculosis when they cough, sneeze, talk, sing or spit freely in the environment (Ndungu *et al*., 2013). However, the above mentioned chain of transmission can be disrupted by isolating infectious patients and initiation of an effective anti-tuberculosis therapy (Herchline & Thomas, 2014). During the initial stage of infection with the TB bacilli, an immune response will control the spread of TB infection, resulting in a localized and non-symptomatic infection, which is clinically referred to latent TB. Latent TB is also known to remain dormant in the body

for many years, and the majorities of such patients have positive immunologic test results forMTB**,** and present a diagnosis of latent TB infection (LTBI) **Appendix1**. A battery of clinical examinations, microbiology/bacteriology, radiography and histopathology tests are used to diagnose active TB disease (CDC, 2011) **Appendix2**.

The symptoms for both drug-susceptible TB and MDR/XDR-TB are similar; patients may present prolonged cough, cloudy sputum that is sometimes blood stained, shortness of breath with chest pain, fever, chills, night sweats, fatigue and muscle weakness as well as weight loss.(Sterling *et al*., 2010). Generally, susceptible strains respond to therapy with anti TB drugs within two weeks and become non contagious (Ahmad & Morgan, 2000). Conversely, TB patients whose symptoms do not improve upon completion of TB treatment course, which take 6 months, could be candidates of drug resistance or multidrug resistant TB (Davies, 2001).

2.3. Clinical significance of MTB infection

Mycobacterium tuberculosis is an obligatory aerobic and intracellular pathogen. The tubercle bacilli enter the lung via the host's airway before colonising the alveolar, at the apex of the host lung, as these tissues are rich in oxygen supply. The bacilli then spread from the site of primary infection in the alveolar, through the lymphatic or blood vessel to other parts of the body, with the apex of the lung and the regional lymph node being the most favoured sites.On this ground, some studies have documented the existence of miliary form of TB, usually as a result of latent tubercle in the lung apex that burst and get disseminated through the lymphatic and cardiovascular system, leading to infection of other body organs (Sharma & Mohan, 2004), resulting in extra- pulmonary TB of the pleura, lymphatics, bone, genito-urinary system, meninges, digestive system, liver, spinal, heart, spinal, or skin, that occurs in about ten percent of TB patients (Serafino, 2013).

During the immune response to infection by MTB, the alveolar macrophages are the first phagocytic cell released to destroy the bacilli. This host-pathogen association is important, as it also define the disease outcome. Within two to six weeks of infection, cell-mediated immunity (CMI) response triggers the influx of activated macrophages and inflammatory cells, into the lung lesion (**Figure 4)**, resulting in the formation of

granuloma (Cooper *et al.*, 2011). The exponential growth of the bacilli is inhibited in the granuloma, as the bacilli remain confined in the middle area of the granuloma. Fibrosis, which is the last defence mechanism of the host to finally wall off the infection, may be formed around the necrotised caseous lung tissue, made up of dead macrophages and inflammatory cells, (Kapoor *et al*., 2013).The bacilli may remain inactive, or be reactivated later in some individuals with challenged immune system. On reactivation, the bacilli increase in number in the macrophages and are discharged from the bronchi cavitation into the airways, resulting in the local progression or disseminated TB (Salgame, 2011; Huynh *et al*., 2011).

In addition, many anti-mycobacterial effector functions of activated macrophages, such as production and release of reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), have also been known to be involved in bacterial destruction (Nathan & Ding, 2010;Voskuil *et al*., 2011). Overall, macrophage-Mycobacterium interactions and the role of macrophage in host response can be summarized under the following sub-headings as shown in **Table 1**(Herbst*et al.*, 2011). Equally, other important group of effector cells recruited as a result of innate immune response are natural killer cells (NK). Theses cell play an import role in the controlling bacilli spread by releasing lethal chemical substance to surrounding infected monocytes, or directly target the invading bacilli.

For *Mycobacterium tuberculosis* to successfully cause disease, it must have a strategy on how to reside and multiply in the host, without recognition by host immune system in order to avoid elimination. Therefore, to survive, the *Mycobacterium tuberculosis* has armed itself with numerous immune evasion strategies. One such mechanism is the modulation of antigen presentation cells (APC), and preventing its interaction with the T helper cell (Korbel *et al*., 2008). Despite its ability to determine virulence, the mycolic acid is part of lipid Mycobacterial cell wall alongside cord factor and wax-D (Favrot & Ronning, 2012; Matsunaga & Moody, 2009). Together, they confer different survival mechanisms and enable the bacteria to cause disease. The cell wall also has hydrophilic property that resists antibiotics, alkaline and acidic compounds known to be injurious to the cell (Barkan*et al*., 2009).

Figure 3: Pathogenesis of Pulmonary tuberculosis (Nature Reviews Microbiology; and modified with reference notes)

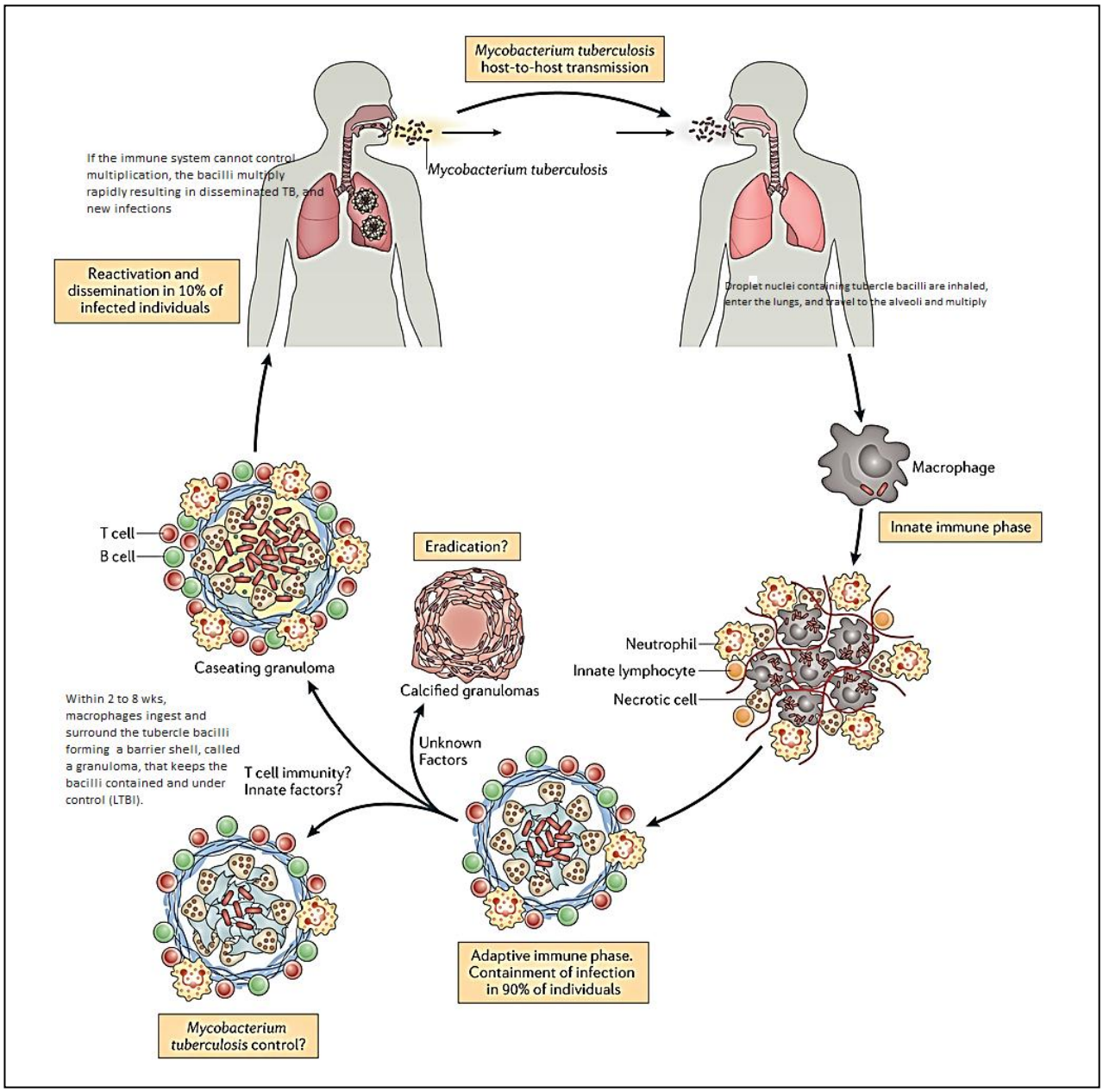


Table 1: Macrophage Mycobacterium interactions in the host response against tuberculosis (adapted from Herbst *et al.*, 2011)

Phases	Phagocytosis process in host immune cell
Phase 1.	Surface binding of MTB to the macrophage Complement receptors CR1, R3, CR4 Mannose receptors Surfactant protein receptors CD14 Scavenger receptors
Phase 2.	Phagosome-lysosome fusion
Phase 3.	Mycobacterial growth inhibition and/or killing Production of reactive nitrogen species Production of reactive oxygen species Apoptosis
Phase 4.	Recruitment of accessory immune cells and development of a local inflammatory response Elaboration of cytokines TNF-α Elaboration of chemokine (IL-8) Antigen presentation

Other proteins secreted by MTB such as superoxide dismutase and catalases have been reported to be antagonistic to reactive oxygen intermediaries (ROI). In order to defend itself from the oxidative stress radical from the phagocytic respiratory burst, MTB also releases antioxidants protein alkylhydroperoxidase reductase encoded by the *AhpC* gene. Alkylhydroperoxidase reducatse reduces organic peroxides, therefore, protecting mycobacteria from reactive nitrogen intermediates (resistance to RNI) produced during its phagocytosis and provide anti-tubercular immunity (Nathan & Shiloh, 2000). MTB may also produce ammonia as a metabolite, neutralizing the acidification in the intra-

lysosomal compartment. This will in turn deactivate the killing effect of lysosomal enzymes (Gordon *et al*., 1980).

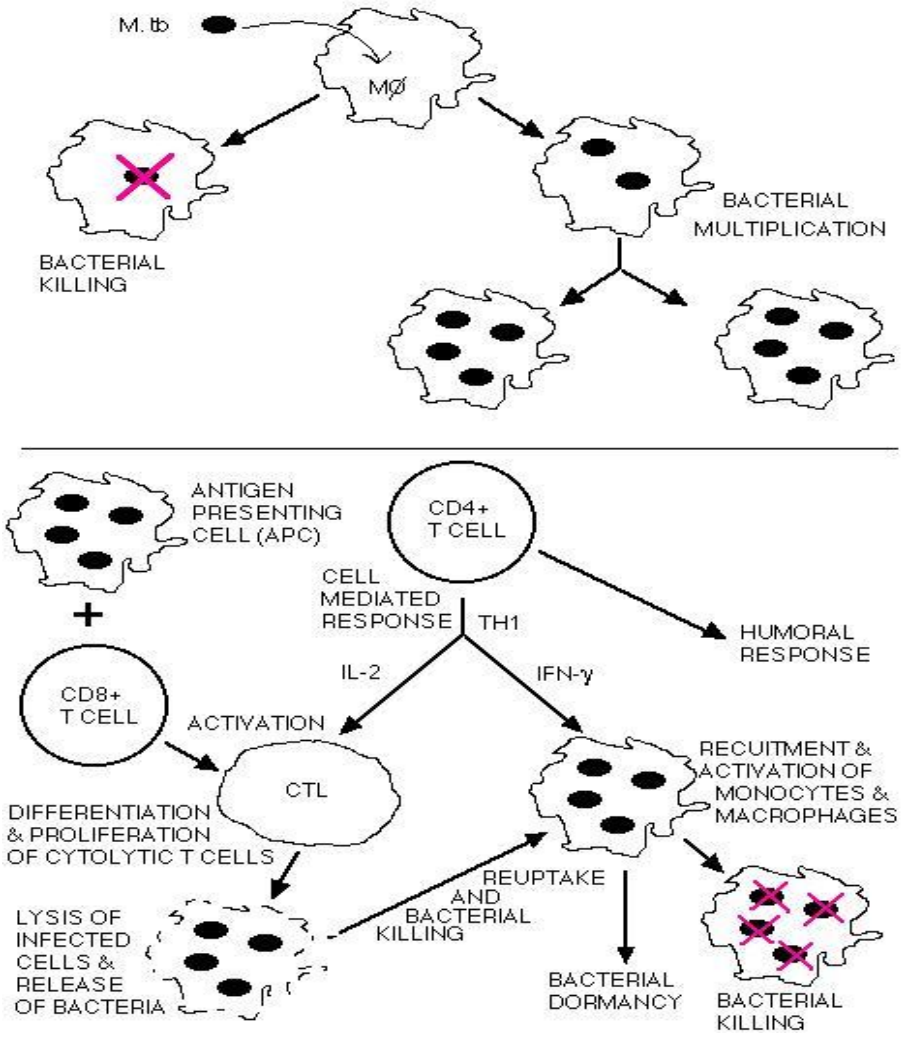
Mycobacterium components such as sulphides, lipoarabinomannan (LAM) and phenolic-glycolipid I (PGL-I) are also reported to be potent oxygen radical scavengers, causing MTB-infected macrophages to be incapacitated during the oxidative burst, and inability to present antigens to CD4+T cells resulting to persistent infection (Raja, 2004). In addition, mycobacteria can block the proton pump (ATPase) responsible for transportation of the acidic degradation compound from the lysosome to the phagosome containing the bacilli (Meena & Rajni, 2010). Apart from prevention of phagolysosomal fusion, it has also been reported that virulent mycobacteria are able to escape from fused phagosome and multiply in the macrophage cytoplasm (Scherr *et al*., 2009). This can be achieved by the production of phagosomal membrane surface protein, which inhibits phagosome maturation. An example of such a protein is the tryptophan-aspartate containing coat protein (TACO), which blocks cytoskeleton vesicular movement of phagocyte (Anand, 2005). Therefore, it is on this basis, that vitamin D3 and retinoic acid are often recommended as supplements, as they suppress TACO gene transcription that is responsible for inhibiting MTB entry into the target cell (Gesensway, 2000; Chunaa, 2011).

A different mechanism by which antigen presenting cells (APCs) contribute to the dysfunctional T cell activation and proliferation is by the production of cytokines, such as Tumour Growth Factor (TGF-β), Interleukin 10(IL-10) or Interleukin 2(IL-2). (Redford *et al*., 2011; Bonecini-Almeida *et al*., 2004). Therefore, a small number of Mycobacterium may multiply intracellularly and be released when the macrophages die. If alive, these bacilli may spread by way of lymphatic channels or through the bloodstream to more distant tissues and organs. This process primes the immune system for a systemic response (Schluger & Rom, 1998)**.**

Identification of diagnostically relevant antigens from Mycobacterial antigens using murine monoclonal antibodies points to humoral immune response. However, *M. tuberculosis* is a classic example of a pathogen for which the protective response relies on

CMI, and an increase in CD4+ and CD8+ T cells in the lung draining lymph nodes has been demonstrated in mouse models four to eight weeks post infection by some authors (Feng*et al*., 1999). The activated T cells also migrate to the site of infection and interact with APCs as shown in **Figure 5**.

Figure 3: Immune response to tuberculosis infection Nature Reviews Microbiology; and modified with reference notes



The tuberculoma contain macrophages, and activated inflammatory cells such as CD4(+) and CD8(+) cells that contains the infection within the granuloma and prevent reactivation (Pawlowski *et al*., 2012). It is in this context that a reduction in NK activity

has been reported to be associated with multidrug-resistant TB (MDR-TB) (Kee *et al*., 2012).The cell mediated response to Mycobacteria in the macrophages results in hypersensitivity reaction at the localized site, due to excessive lymphokine-mediated macrophage activation. The cytokines released in the process in turn become destructive to the surrounding tissues, leading to necrosis. In the bronchus, in particular, the dead material may break away and get discharged into the airway, causing capitations in the lungs **Figure 5.**Infection of other areas of the lung or pleural cavity may lead to the bacilli being disseminated to other parts of the body as disseminated miliary TB, or be coughed out and transmitted to other susceptible persons **Figure 5** (Caccamo *et al*., 2009)

2.4. Treatment for TB infection

Early diagnosis and prompt treatment of TB infection is important as it ensures that TB infection does not progress to debilitating TB disease. Therefore to successfully mitigate TB infection, both health workers and patients are required to adhere to the CDC latent TB treatment guidelines (www.cdc.gov/tb/). Treatment for LTBI is recommended for persons deemed to be at relatively high risk of developing active TB, and should be initiated only after active TB has been excluded by clinical and radiographic evaluations as shown in **Appendix 3**.

Failure to rule out TB on time, may result in inadequate treatment and development of drug resistance (Magdalena *et al*. 2012), that is currently at a crisis level, especially for those with multidrug resistant form of tuberculosis. It is reported that only 3% of MDR patients have access to effective treatments, though when used, these drugs have side effects. However, the success rate when compliance is met is only 50% (Kliiman & Altraja, 2009). Since MDR-TB exhausts all available treatment, it is very hard to treat, and treatment may extend to two years, with patient drug intake totaling to thousands of pills, including hundreds of injections administered, as compared to the treatment of drug susceptible TB that takes only nine months (Kant *et al*., 2010).

TB drug regimen implementation and categorization **(Appendix 4)** is guided by drug potency and how they inhibit mutation of drug susceptible genes. Rifampicin, the first-line of anti TB drugs of choice, has mutational frequency of one drug resistant mutant in

every in 108 cell of division (bacilli), compared to intermediate drugs such as Isoniazid (INH); 1 in 105-106 bacilli, streptomycin (SM) 1 in 105-6 bacilli, ethambutol (EMB) 1 in 105-6 bacilli, kanamycin (KAN) and pyrazinamide (PZA) whose mutational frequencies are 10-3. Other less effective drugs categorized as second-line anti-TB drugs such as thiacetazone, ethionamide, capreomycin, cycloserine and viomycin have resistance frequency of 10-3 mycobacterial replications(Canetti *et al*., 1969).

When determining TB treatment regimen, it is important to first identify the contact person, and align the treatment regimen by considering whether the contact is a diagnosed latent TB infection, or undiagnosed case (Schluger &Burzynski, 2010). As a rule any person who has had close contact with an active progressive TB infectious person should immediately be started on TB treatment. This, however, is based on the drug susceptibility test result of the infectious patient. The contact person would also be required to undertake sputum smear test for confirmation of TB, and drug susceptibility test report if available. In addition, it is also necessary for a clinician to verify if it is an active progressive TB infection, and consider factors such as coexisting medical conditions of contact person, potential drug interaction and complication (Leung *et al*., 2012). Equally, worth consideration are the physiological state of the patient such as age (infant, child and adolescent), weight, nursing mother, pregnant mothers and any use of contraceptives that may result in drug interactions **Appendix 5** (Cohn Richard, 2000).

The commonly used treatment regimen in latent drug susceptible TB infection, is isoniazid (INH), which should be administered daily for nine months, or twice a week for the same period under direct observation treatment (Holland *et al*., 2009). An alternative is the use of INH daily for six months, considering case adherence. However, the nine months' period is recommended because of its high efficacy, compared to the six months' course, with estimated 70% efficacy. The nine months daily dosage of INH is also preferred for drug susceptible TB infection in children aged 2-11years, and HIV/AID persons on anti-retroviral (ARV) drugs who do not develop adverse side effect of the drug (Cohn *et al*., 2000). Another treatment regimen for latent TB is drug combination of isoniazid and rifapentine also recommended for children aged two years and above, or recent converters whose previous results of TB skin test and interferon gamma release

assay are negative. However, this treatment regimen is not recommended for mothers on contraceptives, pregnant women, persons with HIV/AIDS prescribed on HAART (protease inhibitors) drugs, children aged below two years, and those with TB drug resistant strains of INH and rifampicin (CDC, 2011; David and CohnRichard, 2000).

The third treatment regimen for latent TB is the use of rifampicin, which is recommended for daily usage for either four or six months. In addition, rifampicin may be used when contact person cannot tolerant INH, as well as in the treatment of INH mono-drug resistant cases. However, the six months' daily treatment course is preferred for infants, children and adolescents who have mono-resistance and intolerance to INH. Nevertheless this regimen is not recommended for HIV/AID patients on ARV-drugs, as drugs counteraction may result in hepatotoxicity and hypersensitivity reactions (Pozniak *et al*., 2005).

During the treatment for latent TB, contacts should be re-tested at least 8-10 weeks after exposure. Children below five years who are at higher risk of progressing to TB and immunosuppressed persons should be put on treatment immediately, even if their chest X-ray report is negative (Graham, 2006). In the event that repeat laboratory test and chest X-ray report remains negative for this category, it is advised that the treatment be stopped. However, for persons with HIV/AIDS, TB treatment may be continued even if the second test report is negative (Iyer *et al*., 2011).

Contacts with HIV/AIDS are recommended to use INH for nine months and avoid rifampicin, due to its adverse drug reaction (Zilly *et al*., 2003). But for a normal healthy working person with HIV/AID who is not on Anti-retroviral drugs, the twelve months' dose of INH is sufficient. If the contact person is expectant, it is also important to immediately start the patient on TB treatment, even as the laboratory TB and HIV investigations are being done (Centers for Disease Control and Prevention (CDC)).In the event that the laboratory test results remain negative, and there is still no other risk factors of sero-conversion, then 2-3 months after postpartum, the clinician should still consider the twelve months' dosage of INH daily, or twice weekly by directly observed therapy (Cooke, 1985). During treatment in pregnancy and post-partum period, vitamin B6

supplement should also be prescribed and liver function tests done to monitor hepatotoxicity levels. However, the use of INH is not contraindicated in nursing mothers as very little INH passes through breast milk and, in fact, INH may also be prescribed for infants suspected of LTBI (Hall*et al*., 2009). In case of a progressive active pulmonary TB, treatment of the patient is done with the objective to cure the patient, prevent patient disability, death from its late effects, prevent relapse as well as development of acquired drug resistant strains, and reduce the transmission.

WHO recommends that a combination of four drugs is used for the complete cure of TB infection and control (Prasad & Srivastava, 2013). In principal, the guiding features in this regimen is that, by taking the drug combination of these four most effective first line anti TB drugs for drug susceptible TB (isoniazid, pyrazinamide, ethambutol and rifampicin) **Table 2**, the organism is eliminated quickly (Peloquin, 2002). Furthermore, the prolonged therapy administered over many months reduces chances of mutation, and consequently the emergence of TB drug resistance strain (Connolly*et al*., 2007). The initial, intensive phase of treatment lasts two months, and ensures that the four drug combination of bactericidal effects quickly kills the MTB. This initial phase is followed with withdrawal of pyrazinamide and ethambutol, with the continued usage of isoniazid and rifampicin for four months, otherwise termed the consolidated phase (Hall *et al*., 2009).

Table 2: Drugs used in treatment of pulmonary tuberculosis(Hall, Richard D, & Gumbo, 2009).

INTENSIVE PHASE TREATMENT	**CONTINUATION PHASE**
2 months of HRZE	**4 months of HR**
(H) Isoniazid	(H) Isoniazid
(R) Rifampicin	(R) Rifampicin
(P) Pyrazinamide	
(E) Ethambutol	
Note. Only in cases of TB meningitis , shall ethambutol be replaced with streptomycin	

Following confirmation of drug resistance by drug-susceptibility tests which involve molecular assays, cultures or clinical examination, individual patient's therapy is adjusted immediately, based on the advice of TB expert, from the empirical treatment regimen and according to confirmed drug susceptibility test results (Albert *et al*., 2010) **Table 3**. In the event that the organism shows resistance to INH or rifampicin (mono-resistant), then the subsequent drug is withheld and replaced with streptomycin, or preferable a secondary drug such as fluoroquinolone, with the treatment period extended beyond six months (Devasia *et al*., 2009).On the other hand, if MTB is resistant to both INH and RIF ((MDR-TB), then the drugs used in standard therapy are withheld, while second line drugs such as fluoroquinolone (Ofloxacin Levofloxacin Moxifloxacin and Gatifloxacin), aminoglycosides (kanamycin and amikacin), polypeptides (capreomycin, viomycin), D-cycloserine, ethionamide and terizadone recommended (World Health Organisation, 2008). In case MTB organism is found to be resistant to the second line drugs, the medication for XDR-TB is advised (Cox *et al*., 2007). These categories of patients, although rare, have unfavorable outcomes in terms of mortality, and the treatment is difficult, as drugs are still on clinical trials. In 2011, WHO reported that so far only 48% of the documented 3.5 % of MDR-TB cases had complete treatment success (WHO, 2014).

In special instances patients may be required to have an extended treatment period due to severity of the disease. Such cases occur in complicated disseminated extra pulmonary (miliary) TB, typical of HIVAIDS and TB co-infection of pleural effusion, lymphadenitis, peritonitis, testicular, pericarditis, meningitis, and spinal TB of the bones(Bass *et al*., 1995). During the treatment, adherence to drug is necessary, yet the challenges the patient face are also considered. These include intake of combination of drugs and longevity of period for use. However some of these factors can be solved through direct observation treatment, that ensures high cure rates, and reduced chances of development of drug resistant strains (Centers for Disease Control, 2003). In addition, constant monitoring of infection outcome in patients through regular physical examination, chest X-rays and sputum smears where culture and sensitivity tests cannot be performed, is necessary (Surendra*et al*., 2012; Quyen Ton, 2008).

Table 3: Standard treatment regimen for pulmonary tuberculosis (Curry international tuberculosis centre, drug resistant tuberculosis: a survival guide for clinicians)

RESISTANCE PATTERN	TREATMENT REGIMEN	DURATION OF TREATMENT (MONTHS)
INH,RIF	PZA,EMB, FQ,+ Inj (6M)+SLD agents if disease is extensive	18-24M
INH,RIF,+(EMB /PZA)	(PZA /EMB) +FQ, 2 SLD agents l +Inj SLD(6 months)	18-24 M(consider high dose of INH,)
INH,RIF,	FQ, INJ SLD+ STR (6 month)	24 M (consider high dose of INH,)
INH,RIF, PZA,EMB	FQ, 3 SLD agents + Inj SLD (6-12 month)	24M
INH,RIF, PZA,EMB,FQ	STR +3 SLD agents + TLD	>24M(consider high dose of INH, and surgery)
INH,RIF, PZA,EMB,INJ	FQ, +available SLD agents/Inj ;+any TLD	>24 M (consider surgery)
Key : Inj = injectable , M= months, SLD= second line drugs, TLD=third line drugs		

Drug monitoring and evaluation is also an important aspect, as some patients may develop adverse drug side effects, which require withdrawal of drug and consequent substitution (**Appendix 4)**. For example, patients using rifampicin may experience hepatitis, jaundice, thrombocytopenia, hypotension, and pruritis. Other than being a potent enzyme inducer, rifampicin and rifapentine used in the treatment of latent TB, may also counteract drug activity and neutralize the effectiveness of warfarin, methiodine, and hormonal contraceptives (Baciewicz*et al*., 2013). Use of rifapentine may also result in hypersensitivity reactions, with clinical manifestations of fever, headache, dizziness, body aches, petechial spots, and pruritis in special cases. Similarly, side effects of isoniazid may result in hepatoxicity and peripheral neuropathy as commonly seen in diabetics, alcoholics, persons with poor malnutrition and those with renal failure (Quyen Ton, 2008). The use of pyrazinamide may cause elevated serum uric acid, resulting in

arthralgia and ultimately gouts, while ethambutol side effects may include optic neuritis (Gadkowski, 2014).

2.5. Treatment targets and development of drug resistance

2.5.1 Current concepts of TB drug resistance

Effective medical therapy for tuberculosis (TB) has existed for more than half a century, yet TB remains among the most pressing public health issues today. Challenges to effective solutions include lack of access to diagnosis and treatment, the frequent coexistence of epidemics of TB and human immunodeficiency virus (HIV), and the increasing prevalence of multidrug-resistant TB (MDRTB) (Dye *et al*., 2005).

The relationship between drug resistance in *M. tuberculosis* strains and their virulence and transmissibility has not been fully investigated. However, drug resistance in *Mycobacterium tuberculosis* arises from spontaneous chromosomal mutations at low frequencies required for antibiotic action (Somoskovi *et al*., 2001). For the bacteria to survive the micro-environmental stress, they acquire new genes, or delete certain genes (mutate) that expose them to harmful effects of the drugs, and thereafter produce off-springs that can proliferate in such adverse conditions set by drug stress (Zhang & Yew, 2009). Clinical drug-resistant TB largely occurs as a result of improper decision during disease treatment in these genetic alterations through inconsistent drug supply, sub-optimal prescription and poor patient adherence. The resultant event is a compounded problem, especially with the increasing emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB with human immunodeficiency virus (HIV) infection (Jason *et al*., 2008).

Molecular mechanisms of drug resistance have been elucidated for the major first- and second-line drugs, including rifampicin, isoniazid, pyrazinamide, ethambutol, the injectable fluoroquinolone (Ofloxacin Gatifloxacin levofloxacin), capreomycin, viomycin, and amino glycoside kanamycin amikacin (Velayati *et al*., 2009). Anti TB drug isoniazid, for instance, is an important drug used in TB treatment, and together with rifampicin can serve as prophylactic agents in latent TB cases (Surendra *et al*., 2014). It

also constitutes the backbone of first line anti TB treatment, due to its selective intracellular killing activity, and sterilizing effect on mycobacteria 'persisters' (Bernardes-Génisson et al., 2013; Ying Zhang *et al*., 2012). INH 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). Contrarily, INH resistance may arise through different genetic mutations commonly found in the *katG*, *inhA* and *ahpC* gene loci, the drug target sites. To be effective against the bacilli, INH requires activation by catalase peroxidase coded by the *katG* gene, mutation which has been reported to confer resistance to the drug (Isakova, 2008). Other studies have also reported resistance to INH due to mutation in bacterial *inhA* gene (Tseng*et al*., 2013).

Unexpectedly, it has been postulated that 80% of mutation associated with INH resistance occur in *katG*, at codon S315T. This is the major region determining drug resistance, as a result of gene deletion or missense reading, leading to abnormal amino acid insertion (de Siqueira *et al*., 2009). Moreover, it has been reported that most of these mutations are associated with MDR-TB rather than mono-resistant strains, and mutation in *katG* gene in particular, is responsible for high resistance while *inhA* exhibit a minimum inhibition concentration level of resistance (Unissa et al., 2015)

Lack of *katG* gene consequently represses gene code for catalase and peroxidase release, and this result in inactivation of the pro-drug, with limited toxic effect. Adjacent to the *katG* gene locus of the mycobacterial cell is *ahpC* gene locus that is responsible for production of alkylhydroperoxidase. This enzyme is necessary for protecting the cell wall against the oxidative stress exerted by catalase, and as well as other environmental stress produced by the host immune defense, particularly phagocytic cells (macrophages) (Flynn, 2013). Missense reading or deletion of *katG* gene further leads to over expression of alkylhydroperoxidase, which protects organisms from destructive effects of the drug on the cell wall fatty acid (**Table 4**) (Catherine*etal*., 2007).

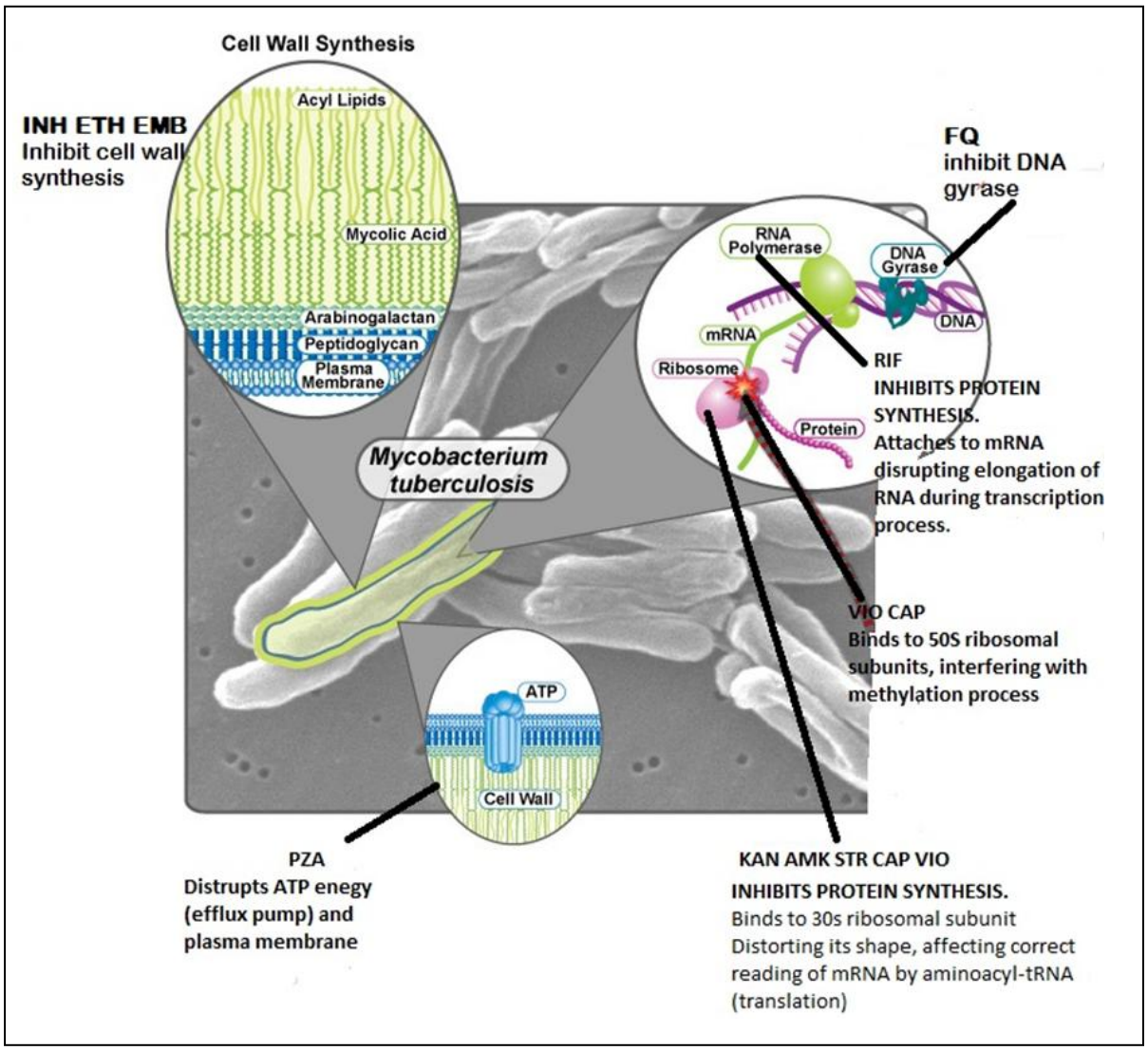
Although a second line drug, ethionamide has structural similarities to isoniazid. As a pro-drug, it requires activation via *ethA* gene encoded mono-oxygenase to exert its activity. Ethionamide adduct competes with NADH-enoyl-ACP reducatse for NADH

binding site on cell membrane, needed in transportation of fatty acid biosynthesis of mycolic acid. Consequently, mycolic acid which is responsible for protecting bacteria cell wall against catalase released during the oxidative burst (Heath & Rock, 2004; Campbell & Cronan, 2001). By inhibiting this enzymatic process, a porous and permeable cell wall is formed, which in turn increases the ethambutol uptake, resulting to bacilli death. Therefore, mutation in *ethA* and *inhA* genes alter drug attachment side of the bacteria and confers resistance to ethionamide, prohibiting drug entry by action of the efflux (Roberto *et al*., 2005; Hazbon *et al*., 2006).

Rifampicin, also first line drug, was introduced in 1972, and found to have efficient antimicrobial action, with specific intracellular killing ability against *M. tuberculosis* in the macrophages. Rifampicin (RIF) is also a sterilizing drug, that is also effective against both actively growing and slowly metabolizing (non-growing) bacilli (Mitchison & Coates, 2004). It is therefore, a drug of choice both in the consolidated phase of progressive TB treatment, and in short-course chemotherapy. However its widespread application as a broad spectrum antibiotic, and administration as a single drug in short-course chemotherapy, has contributed to increased reported cases of drug resistances (Van Ingen *et al*., 2011). The target for RIF in the mycobacterium is the beta sub-unit of RNA polymerase in nucleic acid, to which it binds and prevents the RNA polymerase from synthesizing and/or elongation of the mRNA. Consequently this disrupts transcription process, and protein synthesis **Figure 6 and Table 4**, ultimately leading to cell death (Pang *et al*., 2013; De La Iglesia & Morbidoni, 2006). In order to circumvent the effect of the drug, mycobacteria interfere with *rpoB* gene expression that is used as an attachment site for the drug (Wehrli, 2013).

To determine gene mutation causing resistance to RIF in MTB isolates, attention is usually focused in the "hot-spot' region of 81 base pairs (bp) of *rpoB* gene, between codon 507- 533. This is known as rifampicin resistant determinant region (RRDR) and accounts for 92% of RIF's resistance. It is estimated that 86% of RIF's resistance mutation are as a consequence of changes in codon 526 and codon531.A study by Traore and others reported the resistance to rifampicin can be associated with resistance to other drugs, and especially isoniazid, forming the basis of screening RIF's resistance for MDR.

Figure4: Drug targets for *Mycobacterium tuberculosis* cell wall and metabolism (First line treatment of drug sensitivity www.naid.nih.gov.)



Ethambutol (EMB) is a broad spectrum bacteriostatic and antimycobacterial drug used alongside other first line drug regimens with good synergism, in the treatment of tuberculosis (Jadaun *et al*., 2007). Together with RIF, INH, and PZA, EMB has been described as "fourth drug" for empirical treatment in *M. tuberculosis* and *M. avium*, with particular preference to those with disseminated infection and HIV co-infection (Jain *et al*., 2008). The drug has the ability to penetrate the cerebral spinal fluid, interfering with biosynthesis of mycobacterial cell wall arabinogalactan, through inhibition of arabinosyltransferase enzyme encoded in *embCAB* gene (Goude *et al*., 2009). The

enzyme arabinosyltransferase is vital in transfer of arabinogalactan (AG) and lipoarabinomannan (LAM) that forms part of the mucolyl-AG-peptidoglycan layer that surrounds the outer cell wall, conferring its integrity **Figure 6 and Table 4**. By holding down this enzymatic process, a porous and permeable cell wall is formed, which in turn increases the ethambutol uptake, resulting to bacilli death (Korycka-Machała*et al*., 2005).

Interestingly, almost 4% of all M. tuberculosis clinical isolates have been shown to display resistance to EMB (Telenti *et al*., 1997). Studies conducted by Ramaswamy *et al*., using panel isolates of ethambutol resistant strains reported that 68% of EMB drug resistance isolates were associated with mutational changes in codon 306 in *embB* gene, making it the 'ethambutol resistance determinant region' (ERDR) (Ramaswamy *et al*., 2000). It has been reported that when the amino acid methionate M306 (required for methylation) present in wild type *embB* gene of arabinosyl transferase is replaced with leucine or valine in variant strains. This enzyme subsequently transfers arabinogalactan to the mycolic acid complex in the cell wall, and is crucial for the cell wall integrity. Some studies have also related resistance in both high (>40μg/ml) and minimal (<20 μg/ml) inhibition concentration level to mutation in codon 306 (Plinke *et al*., 2011)**.**

Used as a substitute for the first line drugs, streptomycin (STR) is an amino glycoside that was first used in the treatment of TB in 1945. However, rapid resistance emerged due to its use as a mono-therapy (Crofton & Mitchison, 1948). Currently it is administered as the first substitute in first line anti TB treatment, in combination with INH, RIF, and EMB. It is bacteriostatic when given parentally and also known to reduce bacterial load (Jindani *et al*., 2003). Like amikacin and kanamycin, streptomycin binds to both 16S ribosomal nucleic acid (rRNA) and the 12S ribosomal protein in the 30S ribosomal subunit **Figure 6.** This disrupts the decoding enzymatic activity of aminoacyl-tRNA on messenger RNA, resulting in deficient translation and inhibition of protein synthesis **Table 4** (Demirci *et al*., 2013).

Another FLD effective against TB, used to shorten therapy from 9 to 6 months period is pyrazinamide (Sia & Wieland, 2011) whose key characteristic is its ability to inhibit replication of semi-dormant bacilli in the acidic environment (Heifets, 1992). Just Like

isoniazid, pyrazinamide is a pro-drug that requires activation by the enzyme pyrazinamidase/nicotinamidase (Pzase) present on mycobacterium cell to its active form, pyrazionic acid (Hu *et al*., 2006) Figure 6.Pyrazionic acid targets two bacterial cell metabolic pathways. First, its pH creates an acidic surrounding that disrupts the bacterial membrane, interfering with fatty acid synthesis required in the all replicating cell Table 4. Secondly it targets and weakens the efflux pump resulting in tolerance and drug resistance (Machado *et al*., 2012). The resistance to PZA, mainly due to mutation in codon 561*PncA* gene results in inability of the organism to convert pro drug pyrazinamide to pyrazionic acid. Normally, this gene is responsible for catalysing the enzyme pyrazinamidase-nicotinamidase (Pzase) needed for drug conversion. Mutation in 561 nucleotide *pncA* gene detected in PZA –resistant strain results in poor catalytic activity, and metal binding ion capacity of Pzase (Zhang & Mitchison, 2003).

One class of the currently used second-line drugs (SLD) in treatment of DR-TB and MDR-TB is fluoroquinolone, two major examples which are Ofloxacin, Levofloxacin Moxifloxacin and gatifloxacin (Keller *et al*., 2000). Both drugs are synthetic derivatives of nalidixic acid, and also by-products of the purification of chloroquine, the anti-malarial drug (Bisacchi, 2015). Fluoroquinolones were first used in 1984 after the emergence of MDR-TB reported cases, and currently recommended as a substitute drug when INH or RIF is withdrawn (Ginsburg*et al*., 2003). In such instances, moxifloxacin and gatifloxacin, administered as second line TB drugs can as well serve as alternative FLD. This is also applicable to TB patients who suffer adverse side effects to INH and RIF, and require a shorten treatment period (Ziganshina*et al*., 2013).

Fluoroquinolones are effective in blocking translation of mycobacterium chromosomal DNA gyrase by the enzyme gyrase topoisomerase, expressed in loci *gyrA* and *gyrB* of the bacteria sub-unit A and B, **Figure 6**. The topoisomerase is responsible for super-coiling of the DNA and, its inhibition disrupts subsequent addition of nucleotides at the replication folk for the translation purpose, causing cell death. Therefore, mutation on the gyrase gene itself will alter drug attachment sites, resulting in drug resistant strains (Aubry*et al*., 2004) **Table 4**. This was confirmed by studies performed by Sun *et al.*, and Zhang *et al.*, who observed that mutation at the drug binding site by quinolone resistant

strains occurred mostly at DNA *gyrase A* at the conserved regions on 320 base pairs (bp), and 375 base pairs (bp) also known as quinolone resistant determinant regions (QRDR REGION) (Santos, 2012; Sun *et al*., 2008).

Apart from fluoroquinolones, the second group of SLD for TB treatment is the aminoglycosides class, kanamycin and its derivative amikacin. They have similar structure and have antibacterial activities similar to streptomycin, although classified in different families. These drugs bind to 16S rRNA and exert their antimicrobial activity at the protein translation level, disrupting the protein synthesis at the reticulum **Figure 6** and, when administered parentally, have bacteriostatic activity against actively dividing mycobacteria in the juvenile stage of infection (Cole & Telenti, 1995). Aminoglycosides generally bind to ribosome's at the 16s rRNA and disrupt elongation of peptide chain in the mycobacteria leading to misreading of the mRNA by the tRNA. This causes incorrect addition of amino acid to the peptide bond and results into cell protein starvation and subsequent cell death (Caminero *et al*., 2010). Mutation in the *rrs* gene encoding for 16s rRNA are therefore associated with resistance to KAN and AMK. Nucleotide changes at positions 1400, 1401 and 1483 of the *rrs* gene have also been found to be associated with KAN resistance (Suzuki *et al*., 1998). Other studies have also reported that a change from arginine to guanine at the codon 1400 in the *rrs* gene was associated with resistance to KAN of MIC more than 200µg/ml (Suzuki *et al*., 1998).

Viomycin (VIO) and capreomycin (CAP) are basic peptide antibiotics whose potent activities against persistent forms of MTB contribute to their importance, as SLD and are used alongside fluoroquinolones. They specifically bind to 30S or 50S ribosomal subunits of prokaryotic mycobacterium, inhibiting protein synthesis at translation stage (Chaoui *et al*., 2012). Other studies conducted by Plikaytis BB, *et al.*, reported that mutations in the *rrs* gene that is responsible for activating rRNA methyltransferase for methylation process is often disrupted due to alternating drug target sites (Johansen et al, 2006). In addition, over expression of the gene *rrs* may result in abnormal enzyme activity, resulting in diverse drug resistant region codons (Javid *et al*., 2014). Increased resistance to VIO and CAP, specifically occur in *rrs* gene, when guanine is substituted with

arginine, or thiamine in codon 1401 and 1484 (Brossier*et al*., 2010). However, cross-resistance has also been reported between KM, AMK, CAP and VM (Maus*et al*., 2005).

Table 4: Genes involved in the conferring drug resistance to standard tuberculosis drugs (Yew & Leung, 2008).

INTENSIVE PHASE TREATMENT	**CONTINUATION PHASE**
2 months of HRZE	**4 months of HR**
(H) Isoniazid	(H) Isoniazid
(R) Rifampicin	(R) Rifampicin
(P) Pyrazinamide	
(E) Ethambutol	
Note. Only in cases of TB meningitis , shall ethambutol be replaced with streptomycin	

CHAPTER THREE: MATERIALS AND METHODOLOGY

3.1. Study design

This was a descriptive cross sectional study carried out at Coast General Referral Hospital, Mombasa between January and September, 2012. Following ethical approval and signed informed consent from the patients, demographic data was obtained using administered questionnaires during the routine clinical check-up, and clinical history extracted from the files (**Appendices 6 and 7)**. Sputa smear positive samples for TB were then subjected to molecular analyses for drug resistance strains at the medical microbiology department of the University of Nairobi.

3.2. Study population

The study population included all sputa smear (SM) TB positive patients identified using direct fluorescent staining and microscopy, using auramine and rhodamine stains, at the microbiology section of the CPGH.TB Patients who met the inclusion criteria, and voluntarily gave informed consent to participate in the study were recruited sequentially**.** Inclusion criteria required that participants were sputa smear positive, gave adequate sputum sample (2mls), were 18 years old and above and gave written informed consent to participate in the study. Patients below 18 years of age, the parent/ guardian give written informed consent to participate in the study.

A total of 256study participants were recruited and their clinical samples processed using Genotype MTBDR®*plus* assay (Hain Life-science, Nehren, Germany) for identification of MTB strains and drug resistant mutant gene patterns responsible for resistant to first line anti-tuberculosis agents, isoniazid and rifampicin. Subsequently 83samples were selected for SLD resistance test using GenoTypeMTBDRsl® (Hains Lifesciences, Germany). In order to minimize bias, samples from FLD tests were organized into four-sample series, from which the fourth was selected for the SLD test. In addition, samples which were positive for FLD resistance as well as those referred for gene Xpert were included to obtain the required number for SLD test.

3.3. Ethical approval

The study approval was obtained from the Kenyatta National Hospital/University of Nairobi Ethical and Research Committee (KNH/UON ERC) and Coast General Hospital administration prior to commencement of the study

3.4. Laboratory Techniques

3.4.1. Demographic and clinical data collection

Eligible patients were interviewed using structured questionnaire to collect data on their demographic characteristics and medical history **(Appendix 7)**. In addition, data generated from the patients laboratory request forms were recorded into the data collection forms. The data was later cleaned and entered into database.

3.4.2. Specimen collection and laboratory analyses

Sputum samples that were positive by fluorescent microscopy were stored at 4°C for subsequent molecular analyses. Each sample was labeled with a unique study number corresponding with the questionnaire. Approximately 2mls of the sputum was aliquoted into a clean falcon test-tube and decontaminated/digested using 4% sodium hydroxide (NaOH) according to modified Petroff's procedure (**Appendix 8**). The aliquots were stored at -20^0C in the microbiology laboratory, Coast General Referral Hospital, before being transported to the University of Nairobi, molecular laboratory in accordance with the International Air transport Association (IATA) protocol for clinical infectious specimens for TB strains DNA analyses (**Appendix 9**). In the molecular laboratory, DNA was extracted, amplified and hybridized to probes on an immobilized phase using GenoType MTBDR®*plus* assay and subsequently MTBDR®*sl plus* assay (Hain Life-science, Nehren, Germany), **Appendix 9**. PCR was repeated for sputa specimens that showed inconsistent development of bands on the MTBDR®*plu*s strip, and/or no MTB control band.

3.4.3. Quality assurance for sputum sample collection and processing

Strict adherence to standard operating procedures (SOPs) was observed during sample collection, decontamination, and during transportation. In addition, the manufacturer instructions during processing were maintained throughout the study duration. Aseptic technique was strictly adhered to, and all reagents were prepared in accordance with standard operating procedures (SOPs).

3.4.4. Bio-safety measures

Sample processing was carried out under specified control areas as indicated in the Hain Lifescience manual. In addition, laboratory work was carried out in Bio-safety Level Cabinets (BSL2), and dust free PCR work stations. Clean gowns and face mask were also worn in every room. Sterile material and 70% alcohol was used to decontaminate equipment before and after use. Sodium hypochlorite solution was used to sterilize infectious liquid waste before pouring the content carefully down the sink and solid infectious laboratory wastes was disposed according to the institution's guidelines.

3.4.5. Genotyping for MTB drug resistance

Analyses with GenoType test kits were conducted to screen for mutant MTB strains and predict mutational gene patterns associated with drug resistance. The test kit GenoType MTBDR®*plus* assay (Hain Life-science, Nehren, Germany) is a commercially available multiplex PCR DNA strip assay, designed to simultaneously detect TB, MTBC (Mycobacterium TB Complex), Non-Tuberculosis Mycobacteria (NTM), and commonly known gene mutations conferring resistance to antitubercular antibiotics administered during TB therapy. It is based on the principle of a multiplex PCR in combination with reverse hybridisation to identify amplicons complimenting biotinylated probes on a nitrocellulose membrane, for detecting resistance gene patterns (MTBDR Geno Type Version 2.0).

The MTBDR®plus assay was used to analyze genes associated with drug resistance to Isoniazid (INH) and Rifampicin (RIF), the backbone drugs for first line therapy, and MTBDR®*sl plus* assay for ethambutol and second line drug resistance gene patterns, as

previously described (Lacoma *et al*., 2012; Hauck, et al., 2009). In brief, to run MTBDR®plus assay, 500µl of sputa positive clinical samples was decontaminated, and block heated at 95^0C for 20 minutes, followed by sonification for 15minutes and centrifugation at 13,000 revolutions per minute for 5 minutes to extract the mycobacteria cell DNA. This was then followed with 5µl addition of supernatant to 45µl of PCR master mix. The mixture was then loaded into a programmed real time thermocycler for amplification of the drug resistance-determining region (DRDR) of the gene, with addition of biotinylated primers (3' TGA CCTGAAAAGAC 5') shown in **Appendix 11**. The thermocycling conditions involved first step at 95°C for 15 minutes, followed with twenty cycles at 95°C for 30 seconds, 65°C for 2 minutes; thirty cycles at 95°C for 25 seconds, 50°C for 40 seconds, 70°C for 40 seconds and elongation step of one cycle at 70°C for 8 minutes. The amplicons were then stored at 4°C in the refrigerator.

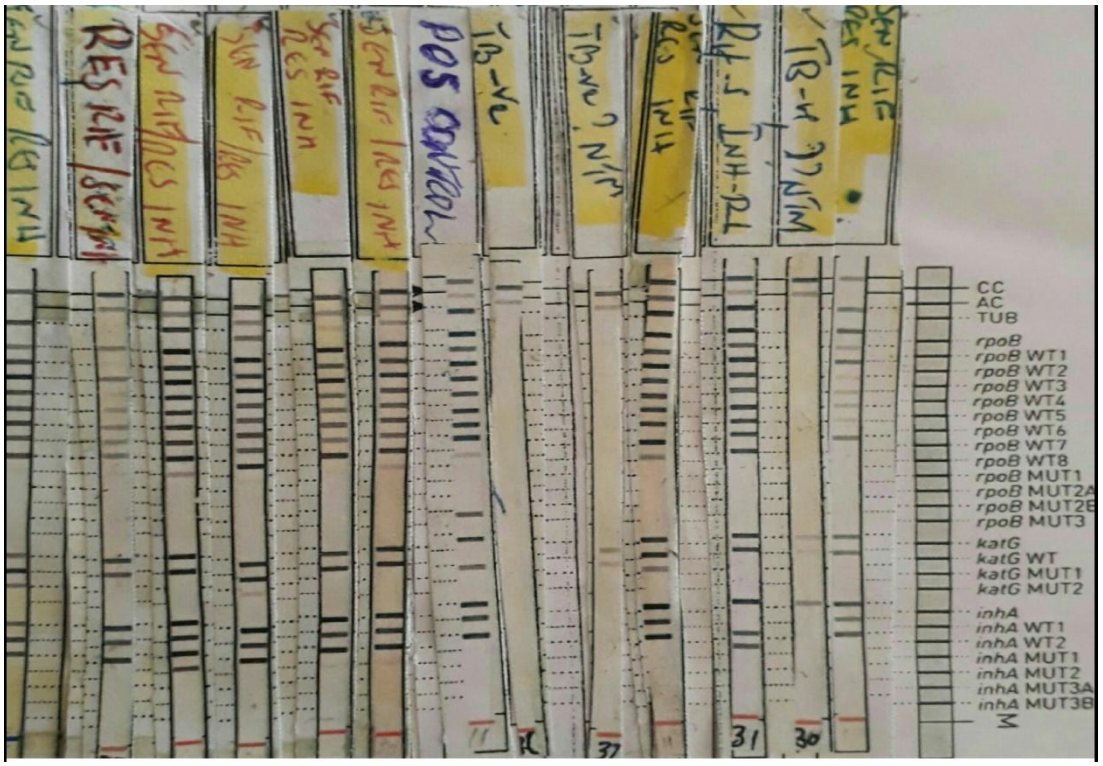
For MTBDRsl assay, 500µl of the decontaminated sputum of positive clinical samples were block heated at 95^0C for 20 minutes, followed by sonification for 15minutes and centrifugation at 13,000 revolutions per minute for 5 minutes, to extract the mycobacteria cell DNA. This was followed with addition of 5µl of the supernatant to 45µl of PCR master mix. The mixture was then loaded into a programmed real time thermocycler for amplification of the drug resistance-determining region of the gene, with addition of biotinylated primers. The thermocycling conditions involved first step of heating 95°C for 15 minutes (1 cycle), followed with ten cycles at 95°C for 30 seconds, 58°C for 2 minutes; thirty cycles at 95°C for 25 seconds, 53°C for 40 seconds, 70°C for 40 seconds and elongation step of one cycle at 70°C for 8 minutes. The amplicons were then stored at 4°C in the refrigerator.

Post-PCR analyses of the drug resistance genes were done using hybridization processes. The first step was addition of 20µl of denaturation solution to 20µl of DNA PCR products, and mixed by pipetting the mixture up and down, before incubating at room temperature for 5 minutes. After incubation, 1ml of hybridization solution was added to each tray well containing the denatured DNA, and mixed by tilting the mixture in the tray up and down, using a twin incubator for 30 minutes at 45^0C. The test kits have probes embedded in the strips that result in attachment of similar complementary DNA

sequences. Primers used in the amplification process are biotinylated, and when corresponding amplicons are correctly subjected to prescribed protocol conditions, the complementary sequences are visible as bands on the strips. These bands were further interpreted to define positive diagnosis; or absence of susceptible TB, MTBC (Mycobacterium TB Complex), NTM (Non-Tuberculosis Mycobacteria), any mono-drug resistant TB, MDR-TB, or XDR-TB as the case may be (WHO, 2013).

The test strips were labeled with the study identifiable number using a DNA strip marker. The labeled strips were placed in corresponding trays, containing samples with denatured DNA, and incubated for 30 minutes at 45^0C in the Twincubator. This was followed by complete aspiration of the mixture, and 1 ml of STR (fixing and washing solution) added to each tray, and incubated again for 15 minutes in the Twincubator. The STR solution was aspirated, and 1 ml of RIN (rinsing solution) added to each tray and further incubated for 1 minute at room temperature in the twin incubator, before aspirating the whole quantity of RIN from the well. This was followed by the removal of the DNA strips from each tray, and air dried on absorbent paper. Using a transparent cellophane paper, the dried strips were attached onto a result sheet labeled with each sample's identifiable number and interpreted using the respective interpretation charts provided with the test kit. MTBDR®plus result chart was used to interpret first-line and MTBDRsl for second-line drug resistance mutant strains respectively (**Figure 7 and Figure 8**). For instance, where mutation was present on the target regions (wild type probe band), and the amplicon did not hybridize with the relevant probes on the nitrocellulose strip, this mutation was observed as lack of binding gene to wild-type probes. Equally, if hybridization occurred on any of the designed mutated probes specifically bound to mutation probes in MTB strains.

Figure 5: MTBDR®plus Genotype test strip and interpretation of results



MTBDR®*plus* gene probe test strip that was used to identify INH and RIF mutant /drug resistant strains contained 27 reaction zones **(**bands on strips on **Figure 7**). This included six control signal probe bands such as conjugate control (CC), amplification control AC, *M. tuberculosis* complex (MTB) control (TUB), *rpoB* gene amplification control (rpoB), *katG* gene amplification control (katG) and *inhA* gene (inhA*)* amplification control. Also included are 21 signal probe bands with both wild and mutant probes to for the drug susceptibility testing, as observed in RIF and INH drug target gene interpretation chart. For a test to be reported valid the six control bands were observed at their designated sites, and drug resistant strain was identified by missed probe in the wild type probe, or addition of mutant probe. For example, INH mutant strains resistance was interpreted as presence of INH gene locus, missing wild type probe band or having mutant probe band.

Figure 6: Genotype MTBDRsl for Second line drugs and interpretation of results

MTBDR*sl* gene probe test strip was used to identify EMB, FQ, KAN, AMK, CAP and VIO drug resistant mutant strains, contained in 22 reaction zones (**Figure 8)**. This included six control signal probe bands such as, conjugate control (CC), amplification control (AC), *Tuberculosis* complex control, *gyrA* gene locus control, *rrs* gene locus control and *embB* gene locus control. Also included are 16 signal probe bands with both wild and mutant probes for drug susceptibility testing, as observed in drug target gene interpretation chart. For a test to be reported valid, six control bands were observed at their designated sites of CC, AC, MTB complex, *gyr A*, *rrs*, and *embB* locus). Drug resistant strain was identified by missed probe in the wild type probe, or addition of mutant probe. For example, FQ mutant strains resistance was interpreted as presence of gene locus, missing wild type probe band but having mutant probe band. Whereas EMB and aminoglycoside resistant (cross polyresistant) was interpreted as presence of gene locus for both drugs, with missing wild type probe band and presence of mutant probe band for both drugs.

3.4.6. Statistical analysis

Data was imported to SPSS version19 from excel spread sheet and analyzed using SPSS v.19. Demographic characteristics such as age and sex were summarized into means and percentages. The Pearson Chi-Square analysis was performed to test significant association between age, sex, patient category and drug resistance. *P* values of < 0.05 were considered statistically significant.

CHAPTER FOUR: RESULTS

The findings of the study were organized in various sections to outline the numerical values, percentages and correlations between the demographic and clinical data with the resistance patterns of *Mycobacterium tuberculosis* (MTB), for both first and second line drugs anti-tuberculosis drugs.

4.1. Demographic details and clinical data

The demographic and clinical data were obtained during the routine clinical check-up, after obtaining informed consent from the study participants. Cases which were reported as sputum smear positive for TB by direct fluorescent microscopy, were then subjected to molecular analyses for first line resistance drug (FLD) and Second Line Drug (SLD) drug resistance strains at the medical microbiology department of the University of Nairobi. A total of 256 study participants were included in this study of which 172 (67.2 %) were males and 84(32.81%) females. The age of the patients studied ranged from 9 to 75 years, with a median of 30 years. The clinical data are as shown in table. Of the 5 out of 256 clinical samples previously reported as MTB positive by fluorescent microscopy technique during patient clinic check-up. Genotype MTB technique (MTBDR®plus assay for MDRTB) confirmed 3 of these study participants as Mycobacterium Tuberculosis (MTB) negative, due to lack of identifiable bands on the test strip, and 2 Non Tuberculosis Mycobacterium (NTM).

Table 5: Drug resistant testing for first line drugs (rifampicin and isoniazid) stratified by Patients' gender, age, TB patient classification and HIV status.

Characteristic	Category	MTB negative	NTM	Fully susceptible	INH resistant	RIF resistant	Total
		[n (%)]	[n (%)]	[n (%)]	[n (%)]	[n (%)]	N=256
Gender	Male	2 (0.8)	1 (0.4)	163 (65.6)	5 (2.0)	1 (0.4)	172 (69.2)
	Female	1 (0.4)	1 (0.4)	79 (30.9)	3 (1.2)	0 (0.0)	84 (32.8)
Age in years	>10	0	0	2 (0.8)	0	0	2 (0.8)
	11-19	1(0.4)	0	21 (8.2)	0	0	22 (8.6)
	20-45	2(0.8)	2 (0.8)	190 (74.2)	7 (2.7)	1 (0.4)	202 (78.9)
	>45	0	0	29 (11.3)	1 (0.4)	0	30 (11.7)
TB Patient classification	New cases	3 (1.2)	2 (0.8)	237 (92.5)	8 (3.1)	1 (0.4)	251 (98.0)
	Follow-up	0	0	1 (0.4)	0	0	1 (0.4)
	Relapse	0	0	3 (1.2)	0	0	3 (1.2)
	Defaulters	0	0	1 (0.4)	0	0	1 (0.4)
HIV/AIDS status	Negative	2 (0.8)	0	89 (34.8)	5 (1.9)	1 (0.4)	97 (37.9)
	Positive	1 (0.4)	1 (0.4)	44 (17.2)	2 (0.8)	0	48 (18.8)
	Unknown	0	1 (0.4)	109 (42.5)	1 (0.4)	0	111 (43.3)

4.1.1. MTB confirmation and drugs resistant testing for first line drugs

With the MTBDR®plus assay for MDRTB, all the 256 cases were subjected to first line drug drugs resistanttesting for the drugs isoniazid (INH) and rifampicin (RIF). Out of these, 251 were confirmed positive cases for MTB with MTBDR®plus assay, 3 had MTB negative results, and two had non tuberculous mycobacterium (NTM), despite initially being reported as Acid Fast Bacillus positive by the fluorescent microscopy technique. In terms of susceptibility, a total of 242 cases had no drug resistant to the first line drugs (RIF and INH), while 8(3.2%)cases were found to be INH monoresistance. Monoresistance to RIF was also detected in 1(0.4) case. No drug resistance to both RIF and INH (MDR-TB) was identified from study sputa samples. Pearson Chi-Square analysis indicated no Statistical significant correlation between gender and MTB drug resistance, $p = 0.320$

4.1.2. Age categorization and drug resistance test for first line drugs

The study cases were further categorized according to age groups and first line drug resistant testing. In this context, two cases (0.8%) below 10(0.8) years, showed no drug resistant to the first line drugs (INH and RIF). In the age group of 11- 19 years, considered as adolescents of in this study, 22(8.6%), and 21(8.2%)cases were showed no drug resistant to the first line drugs, and one (0.4%) was found to be MTB negative. The age group 20-45 years, also considered as reproductive age, had 202 (78.9%) cases, of which seven cases (2.7%) and one (0.4%) cases had mono resistance to INH and RIF respectively. Nonetheless, there was no age group with drug resistance to both INH and RIF (MDR-TB). See table 6 for more details.

4.1.3. TB Patients' classification by resistance test for first line drugs

The 256 study participants were classified as either new TB patients with no previous anti-TB treatment, or retreatment TB patients that included follow-up, relapse or defaulter cases. Of the 251(98%) new TB patients, 8 (3.1%) cases showed resistance to isoniazid, 1case (0.4%) was resistant to rifampicin, while 237 (92.5%) were fully susceptible to INH and RIF. The remaining five TB patients classified as follow-up (1), relapse (3), and defaulter (1), all of which showed no drug resistance to first line drugs INH and RIF. However, no significant statistical correlation between the categorization of cases and first line drug resistance testing was established, $p = 1.0$

4.1.4. HIV/AIDS status and resistance to first line drugs INH and RIF

Out of the 256 recruited MTB positive cases, 111 (43.3%) cases did not know their HIV/AIDS status, and of these 109(42.5%) showed no drug resistant to INH and RIF. However, one (0.4%) INH resistant, and one (0.4%) NTM case was detected. No RIF was detected.

Amongst 48(18.8%) TB cases co-infection with HIV/AIDS, 44(17.2 %) cases showed no drug resistant to INH and RIF, 2(0.8%) cases had INH resistant, and No RIF resistant was detected. However, 1(0.4%) case each for MTB negative and NTM was detected.

Amongst the 97(37.9%) HIV/AIDS negative cases, 89(34.8%) showed no drug resistant to to INH and RIF, 5(1.9%) had INH resistant, and 1(0.4%) showed RIF resistant. However, 1(0.4%) case was MTB negative.

Also found in the HIV/AIDS unknown category, was 1 (1.2%) case of EMB resistance, from the 83 selected samples for the GenoType MTBDR*sl* VER 1.0, second line drugs kit and EMB resistance. Pearson Chi-Square analysis, with two sided asymptomatic significance showed no significant statistical correlation association between HIV/AIDS status and TB Co-Infection; and HIV/AIDS with drug resistance to first line TB drugs (p value = 0.968).

4.2. Drug resistant identification test for second line drugs

From the 256 study cases, majority were patients attending the hospital as new cases (251of 256). Eighty-two (82) cases were conveniently selected by randomly selecting every fourth case whose DNA specimen was stored after performing FLD drug resistant test, all cases request for geneXpert DNA specimen, and those with drug resistant mutant genes identified by the MTBDR®plus as positive study cases. Selected cases for MTBDR*sl* gene probe testing for identification of SLD were all new cases, as they were the majority of study sample (251of 256).

For second line drug (SLD) resistant testing, it is important to point that that although ethambutol (EMB) is a FLD, EMB resistant gene probe is included in the MTBDR*sl* version 1.0gene probe test kit, used in second line drug (SLD) drug resistant testing identification. Of the 82 conveniently selected DNA specimen tested, 73(89.0%) cases were fully susceptible to both SLD and EMB. However, a total of 3 (3.6%) cases had drug resistance to SLD. of these three DNA specimen with SLD drug resistant 1 one (1.2 %) case had mono resistant to fluoroquinolones, 1 (1.2 %) case had cross resistant to fluoroquinolones and SLD injectable, 1(1.2%) showed FQ mono resistant, 1(1.2%) case had cross resistant to EMB and SLD injectable, and 1(1.2%) case showed mono resistant to EMB; A total of five 5(6%) had invalid test results with unreadable gene probe bands on the test strip (Table 6).

Table 6: Second line drug resistant testing stratified by Age, Patient classification and HIV status

Characteristic	Category	Invalid Test results n=5	Fully susceptible [n (%)]	EMB Res [n (%)]	FQ Res [n (%)]	FQ & inj Res [n (%)]	EMB& inj Res [n (%)]	Total N=82
Age	<10	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0 (0.0)	0 (0.0)
	11 – 19	0 (0.0)	12 (14.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (14.6)
	20 – 45	3 (3.7)	50 (61.0)	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.2)	57 (69.6)
	>45	2 (2.4)	11 (13.4)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	13 (15.8)
TB Patient classification	New cases	5 (6.1)	73 (89.0)	1 (1.2)	1 (1.2)	1 (1.2)	1 (1.2)	82 (100.0)
HIV status	Negative	2 (2.4)	31 (37.8)	0 (0.0)	1 (1.2)	0 (0.0)	1 (1.2)	35 (42.7)
	Positive	0 (0.0)	16 (19.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	16 (19.5)
	Unknown	3 (3.7)	26 (31.7)	1 (1.2)	0 (0.0)	1 (1.2)	0 (0.0)	31 (37.8)

***Note: Invalid tests were those that had technical problems during analysis with unreadable gene probe bands.**
Injectable drugs; KAN, AMK, VIO, CAP

4.2.1. Age categorization and resistant identification for second line drugs

Below 10 years, no case was tested for Second line drugs (SLD) susceptibility pattern (KAN, AMK, VIO, CAP and FQ). In the age group of 11-19 years, also considered as adolescents, there were 12 (14.6%) cases, and none showed drug resistant mutant gene probes for SLD. In the reproductive age group of 20-45 years, 3(3.7%) samples had invalid test results. Of the 54 tested, 50 (61.0%) cases were fully susceptible to SLD, and three had drug resistant to SLD; of which one case (1.2%) was detected to be FQ mono resistant, cross resistant was detected for FQ and SLD injectable in one case (1.2%) including one case of resistance to EMB and SLD injectable(1.2%). Above the age category of 45years, no SLD resistance was found among the 11(13.4%) samples tested. However, 2 of the 13 samples tested, revealed that their results were invalid and therefore the results could not be interpreted (See table 7).

4.2.2. Patients' classification and drug resistant identification to second line drugs.

All conveniently selected 82 cases from 256 preserved DNA isolates, previously tested for FLD resistant were tested for SLD and ethambutol (EMB) were all from new TB patients. Of which 73 cases showed no drug resistant to SLD. Of the four (4.8%) drug

resistant cases, one (1.2%) case each showed mono resistant to fluoroquinolones FQ and EMB, In addition, one case was resistant to combination of fluoroquinolones and injectable antibiotics (CAP, VIO KAN, AMK), while one case was resistant to combination of ethambutol and injectable antibiotics (CAP, VIO KAN, AMK). Five clinical samples had invalid findings during the analysis (**Table 6**). Since the selected cases were not classified as MDR-TB, there was no case of extensively drug resistance (XDRTB) identified. Pearson chi square test was not run, since all samples collected were classified as new TB patients.

4.2.3. HIV/AIDS status and drug resistant identification to second line drugs

The study also revealed that 16(19.5%) TB co-infected HIV/AIDS cases tested for Second line drugs (SLD) showed no drug resistant. Of the 35(42.7%) HIV/AIDS negative cases 31(37.8%) showed no drug resistant of which two drug resistant cases; one (1.2%) had FQ mono-resistance, while one case was resistant to combination of ethambutol and injectable antibiotics (CAP, VIO KAN, AMK) also known as cross resistance. Thirty-one (37.8%) cases did not know or were unclear about their HIV/AIDS status and of these 26(31.7) % were fully susceptible to SLD, one (1.2%) case had cross resistance to FQ+INJ. However, of the 5 invalid test results 3 were from this category. See Table 7.

The combined analysis of both first and second line drug resistance, showed no significant statistical correlation between drug resistance and HIV/AIDS status (p value =0.907).

CHAPTER FIVE: DISCUSSION

Pulmonary tuberculosis (TB) is a communicable disease caused by *Mycobacterium tuberculosis* (MTB). Although it affects any part of the body, only active pulmonary type of the disease can be transmitted from one person to the other through droplets carrying the TB bacillus (Zachary, 2015). Kenya is one of the high burden TB countries ranking tenth among the 22 high burden countries in the world and 5th in Africa (Sitienei *et al*., 2013). In 2012, the WHO reported 8.7 million new cases of TB globally, of which 0.5 million were in children aged less than 15 years, and 26% of those cases occurred in the Africa region (World Health Organization, 2012a). In South Africa, for instance, TB notifications have increased six-fold over the last two decades, largely as a result of increasing human immunodeficiency virus (HIV) prevalence (Wood *et al*., 2010).

Unexpectedly, data on age and sex dependent prevalence of TB infections as well as drug resistance have not been well documented. Worldwide differences in sex-specific tuberculosis case rates remain fundamentally un-explained. However, the notification rates for pulmonary *Mycobacterium tuberculosis* infection for both sexes combined, in many developing countries are usually similar to those of industrialized countries, with men's disease rates exceeding women's after the age of 15 years (Martien*et al.*, 2000; Cassels *et al*., 1982). These findings raise the possibility that cases of tuberculosis among women are being under-reported in developing regions, and are supported by study results that compare active and passive case-findings, in which women with tuberculosis are under-notified to public health authorities due to various challenges (Yassin *et al*., 2013; Borgdorff *et al*., 2000).

This study showed that females constituted 32.8%, while males were 69.2%, an approximate ratio of 1:2. Similarly, some studies have also reported that the prevalence of infection with *Mycobacterium tuberculosis* remains constant among different genders until adolescence, after which males reporting rates become higher than those of females (Hamid *et al*., 2004). In other studies though, it has documented that in industrialized countries, females aged 15 to 34 years showed higher tuberculosis notification rates than males of the same age. However, with decreased notification rates in females with time, it

became apparent that the rates in males became higher than those of females for all ages above 15 years (Ukwaja*et al*., 2011). Nevertheless, further research comparing sex differences in tuberculosis rates would be necessary to determine whether young women are under notified in developing countries.

In this study, although the age of the patients ranged from 9 to 75 years, a higher number of patients were in the 31-40 years category. This is in line with other studies which reported that for many years, the prevention, diagnosis and treatment of TB among children have been relatively neglected, with great attention given to the detection and treatment of infectious cases, mostly occurring in adults (World Health Organization, 2012a). According to WHO report on global TB care, the reasons why TB disease burden remains difficult to estimate in children is due to the inefficient notification methods available in developing countries, which are biased towards detecting active TB in adults, and do not favour detection of TB in children who mostly have latent TB, that progresses to active TB in later years (World Health Organization, 2012a).

Currently, HIV/AIDS infection is the greatest risk factor for new TB infection, and a potential risk for recurrence of TB (Getahun *et al*., 2010b; Datiko*et al*., 2008; Mallory*et al*., 2000). Tuberculosis is a serious opportunistic infection among people living with HIV/AIDS (PLWHA) worldwide, whereas HIV co-infection modifies the natural history, clinical presentation, and adversely affects the outcome of TB (Schutz *et al*., 2010). In turn, TB is the leading cause of respiratory morbidity (Desalu *et al*., 2009) and mortality in HIV/AIDS infected persons globally, and accounts for 44% of all HIV/AIDS-related deaths annually in Kenya (Ministry of Health, 2010a).

In Kenya, HIV/AIDS has profound impact on the TB epidemic, where up to 60% of TB patients are feared to be HIV/AIDS co-infected, with the mortality rate attributed to TB in this group being above 130 per 100, 000 (Nyamogoba *et al*., 2012). On the other hand, the increasing TB burden in Kenya has been attributed to the concurrent HIV/AIDS epidemic, with the 2007, 2008 and 2009 Division of Leprosy, Tuberculosis and Lung Disease (DLTLD) annual reports indicating persistently high rates (44–60 %) of TB-HIV/AIDS co-infection (Ministry of Health, 2010a).

In this study, although a large proportion of the patients were of unknown HIV/AIDS status, 111 (43.3%) cases, 48 (18.8%) cases were positive for HIV/AIDS. However, of the three MTB negatives one case (0.4%) was found in HIV/AIDS positive cases. The annual report of 2009 by the Division of Leprosy, Tuberculosis and Lung Disease (DLTLD) indicated high rates (44–48%) of TB-HIV/AIDS co-infection than this study, findings which could be attributed to the large study population and difference in methodology. In addition, their study obtained data from a rural set up, which has been reported to have less crowding and low mycobacteria infection transmission. However, our findings are closely in agreement with UNAIDS world HIV/AIDS day report in 2012 on global TB-HIV co-infection rates, of 14.8% new TB treatment cases. This report showed there was a decline in TB/ co HIV/AIDS infection as a result of the scale up in HIV/AIDS prevention and treatment programmes (Clavagnier, 2012; World Health Organization, 2013).

The TB-HIV/AIDS burden is further compounded by the emergence of non-tuberculous mycobacterium (NTM) as opportunistic infections in the HIV/AIDS patients, and their treatment is not directly analogous to that of TB (Claire *et al*., 2007), (Saritsiri *et al*.,, 2006). Infections due to non-tuberculosis mycobacteria (NTM) are increasing worldwide, detrimentally affecting both HIV/AIDS sero-negative and immune compromised individuals. Cases of non-tuberculous mycobacteria are reported mainly from European countries and America, where tuberculosis is not endemic. In TB endemic regions such as Southeast Asia and sub-Saharan Africa, the occurrence of NTM is under-reported (Gopinath & Singh, 2010). However, not all acid fast bacilli represent mycobacteria, let alone *M. tuberculosis* complex. Non tuberculous mycobacteria (NTM) and some other bacterial species including *Nocardia* species may as well give false positive results in fluorescent microscopy (Snelling *et al*., 2013).

Recently, several molecular techniques have gained a wide application in detection of PTB and extra pulmonary tuberculosis. GeneXpert MTB/RIF was introduced by WHO in many high burden countries, including Kenya. Majority of the higher referral level health facilities use it in to detection of TB and identification of rifampicin resistance based on DNA sequences.

Despite its high cost, molecular techniques using the line probe assay (MTBDR®plus and MTBDRsl) continues to gain popularity among health providers because of its wide selection of drug resistance information. This is crucial information for TB treatment regime, based the antimicrobial susceptibility pattern of the pathogen. Of importance, the reporting time using the conventional method has been reduced to 24 hours from 8 to 16 weeks.

With some of the reported smear positive cases turning out as MTBDR®plus genotype negative, suggests that the existence of NTM or other acid-fast bacterial diseases was previously underscored , and many of these cases previously misdiagnosed as TB in the past (Shah *et al*., 2009). In this study, we found 2 NTM positive (0.8%), and 3 negative (1.2%) giving a total of 5(2.0%) cases using MTBDR®plus genotyping method. These were cases initially reported as smear positive, implying that such cases are frequently misdiagnosed as PTB (Gopinath & Singh, 2010). In addition, such patients do not get appropriate treatment, considering the facts that smear microscopy is used in most of our health care facilities. The overlapping clinical manifestations of the diseases caused by *M. tuberculosis* make the specific diagnosis of NTM difficult, considering the increasing cases of HIV/AIDS associated NTM mycobacterioses which could be misdiagnosed as TB and mistakenly managed on anti-TB chemotherapy (Griffith *et al*., 2007).

Treatment for pulmonary tuberculosis has existed for decades, yet TB remains among the most pressing public health issues today. Some of the challenges in TB treatment have been lack of access to rapid diagnosis, emergence of drug resistance (DR), multidrug-resistance (MDR) strains, extensively drug resistance (XDR). The frequent co-existence of epidemics of TB and human immunodeficiency virus (HIV), has also contributed to the increasing prevalence of multidrug-resistant TB (MDR-TB) (Havlir, 2008). However, the main target of TB treatment is to prevent development of active TB disease and mitigate disease transmission to healthy person, as well as reduce morbidity and mortality resulting from drug resistance (Rosales-Klintz *et al*., 2012; Almeida Da Silva & Palomino, 2011). Latent TB infections are treated with the regimens suited to meet their needs, which include isoniazid (INH), rifampicin (RIF), and rifapentine (RPT) for various durations. On the other hand, treatment for active tuberculosis involves four main front-

line drugs, which are isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA). It is imperative that patients complete their drug regimens, failure which potentiates re-infection or drug resistance (Centers for Disease Control, 2003).

Drug resistance of Mycobacterium tuberculosis is usually thought to arise during multidrug chemotherapy and, over the past decades, the tuberculosis bacilli has become resistant to various anti-TB drugs, making infection control increasingly difficult (Smith and Kerstin, 2013). The multidrug resistant TB (MDR-TB), defined as resistance to Isoniazid (INH) and Rifampicin (RIF), while extensively drug resistant TB (XDR-TB) implies resistance to INH, RIF, plus any fluoroquinolone and at least one of the three injectable second–line drugs (amikacin, kanamycin and capreomycin) (Banerjee *et al*., 2008). In fact, according to a weekly report published in 2006 by Centers of Disease Control (CDC), it was found that during 2000-2004, 20% of the TB strains collected worldwide were MDR and one tenth of those strains were XDRTB strains (Rabia, 2007). During the study recruitment, 251(98%) of the study population were new cases, of these 237(92.5%) showed full susceptibility to both INH and RIF , a total of 9 mono drug resistant cases was found in both INH 8 (3.1%) and RIF 1(0.4%). Similarly one case of EMB drug resistant was found in the 83conviniently selected samples for SLD resistance gene pattern.

When TB patients were classified, no drug resistant was found in follow up, relapse and defaulter cases, respectively. However, all drug resistance cases were in new cases, suggesting that exogenous infection for in both FLD and SLD. In addition, this study found that out the 48 reported HIV/AIDS infected cases 44 (17.2%) were fully susceptible to the FLD, and 2 (0.8%) were resistant to INH. Additional 2 (0.8%) HIV/AIDS-positive cases showed no reaction for TB using the molecular technique. Our findings revealed that 2 (0.8%) of the 5 erroneous test previously reported as PTB by fluorescent microscopy, were from HIV/AIDS positive patients. This gives the impression that they were victims of wrong drug therapy for a disease they did not have, at the expense of antiviral therapy and drug counteraction, as one (0.4%) each for NTM and MTB negative. For the second line drugs, 73(89.0%)out 82 cases analyzed found not to have drug resistant, of which 16(19.5%) were HIV/AIDS positive, while 31(37.8%)

and 35(42.7%) cases were of unknown and negative HIV/AIDS status, respectively (Berhan *et al*., 2013 and Suchindran*et al*., 2009).

Spontaneous gene mutations in *M. tuberculosis* underlie the bacteria's resistance to the most commonly used anti-TB drugs. Among the reasons for this, the non-compliance with the treatment regimens is signalled as the first cause (Koch *et al*., 2014). Notwithstanding the fact that mutations in a number of genes are clearly associated with drug resistance in *M. tuberculosis*, there are still many cases where resistant strains do not harbour any known mutation (Rosales-Klintz *et al*., 2012; Böttger*et al*., 2005). For example, a recent study using whole-genome sequencing identified new genes and intergenic regions associated with drug resistance and its evolution, showed that TB drug resistance is a phenomenon more complex than previously assumed (Warner & Mizrahi, 2013). Therefore, the development of new molecular techniques targeting specific molecular mutations associated with drug resistance has created a valuable adjunct to conventional drug susceptibility testing (DST) for M. tuberculosis (Hazbon *et al*., 2006). These techniques can be performed directly on clinical samples without a culturing step and therefore, allowing a reliable diagnosis of drug-resistant TB to be achieved within a 24-hour period (Sandgren *et al*., 2009).

Drug resistance was frequent with isoniazid, with the 8 (3.2%) reported cases all which were new TB treatment cases showing resistance. Two (0.8%) of these cases were HIV/AIDS positive, and 1 (0.4%) with unknown HIV/AIDS status. Isoniazid resistance in TB and HIV/AIDS co-infection was therefore 20%-30%, but rifampicin resistance which is known to be rare, as well as ethambutol resistance were not identified in the category of TB and HIV/AIDS co-infection cases. However, in the HIV-negative category, drug resistance was found out to be 10% each for rifampicin and ethambutol (Tessema*et al*., 2012). In addition, none of the re-treatment (follow up, relapse, defaulters) cases was identified as drug resistant TB. Therefore, this study did not find any causal association of TB and HIV co-infection to drug resistance development (Suchindran *et al*., 2009) Besides, from the molecular analyses, five out of the eight isoniazid resistant cases had mutation in the katG MUT1 codon 351, in which the amino acid serine was replaced with thiamine Ser315Thr. This usually result in inactive drug due to lack of catalase peroxidise

from the mycobacteria, necessary to convert pro-drug to its active form. The remaining three cases of INH resistance had mutation in inhA MUT1, modifying the drug target attachment site (van Soolingen *et al*., 2000). This observation is consistent with the hypothesis that isoniazid resistant is most common ancestral condition in *M. tuberculosis* (Rabia, 2007).

Limitation of Hain Life science technique requires the need for modern laboratory infrastructure, trained skilled laboratory personnel to correctly analyse and interpret TB results. In addition, the kits are slightly expensive compared to fluorescent stain and GeneXpert assay. For this reason, it has not been widely adopted for routine testing of TB resistance as compared with the Gene Expert assay in most diagnostic laboratories.

CONCLUSION

Previously, no work has been done at the Coast Provincial General Hospital in Mombasa, Kenya to assess the existence of drug resistance in TB patients attending the TB clinic. Although no multi-drug and extensively drug resistance was established in this study, 13 drug resistance mutant genes were detected. Equally important was that two isolates had cross resistance genes for anti-tubercular injectable plus FQ/EMB, 11 had mono drug resistance, and 3 NTM isolates that missed detection previously, was identified during the study, hence making the technique an important tool that can guide clinicians manage TB patients.

Consequently, because the molecular techniques enabled the detection of mono and poly resistance to anti TB drug regimen among the study cases, we also established that there was no association between TB/HIV co-infection with development of drug resistance resistant strains as elucidated in previous studies.

Despite the high cost running molecular tests when compared to sputa smear microscopy, this study showed that molecular tests can be adopted in management of TB patients, owing to its rapid results turnaround time and accuracy, in the detection of presenting drug resistant genes.

RECOMMENDATIONS

Molecular techniques can be routinely used in management of tuberculosis, especially when drug resistance is suspected. This is because other techniques used for drug resistance testing are time consuming, over burdening techniques with high risk of exposure, and also take long durations before laboratory results can be evaluated. Hence, TB testing facilities that receive high sputum volume for analysis more likely to have results compromised and inaccurately reported due to technician's fatigue. Of important, is that this technique has lesser risk of exposure to laboratory workers, on condition that sample processing guidelines are followed, and handled under controlled environments.

In the diagnosis of TB, the existence of NTM needs to be considered, in light of the fact that it is commonly associated with extra and pulmonary fibrosis which gives poor prognosis in HIV/AIDS patients. In addition, there is need to improve accessibility to HIV/AIDS testing services, in order for patients to learn their HIV/AIDS status in a timely manner, improved disease progress management and viral load suppression. The option of having comprehensive and integrated health service will also strengthen the coordination of HIV and TB co-infection, data reporting, that can subsequently be used to make policies and decision on programme, both at national government and with programme specific implementing partners.

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APPENDIXES

Appendix 1: Charateristics of latent and active TB diseases(CDC, 2011)

Latent TB Infection (LTBI)	**TB Disease (in the lungs)**
Inactive tubercle bacilli in the body	Active tubercle bacilli in the body
Tuberculin skin test or QuantiFERON®-TB Gold test results usually positive	Tuberculin skin test or QuantiFERON®-TB Gold test results usually positive
Chest x-ray usually normal	Chest x-ray usually abnormal
Sputum smears and cultures negative	Sputum smears and cultures may be positive
No symptoms	Symptoms such as cough, fever, weight loss
Not infectious	Often infectious before treatment

Appendix 2. Auramine staining technique for detection of acid-fast bacilli using LED microscopy.

The property of acid-fastness is based on the presence of mycolic acids in the mycobacterial cell wall. Primary stain (auramine) binds cell-wall mycolic acids. Mycobacteria retain the fluorescent bright yellow colour of auramine even after intense decolourization with strong acids, and alcohol, as they do not release the primary stain from the cell wall. Potassium permanganate is then used to quench fluorescence in the background providing contrast for focusing under the LED microscope.

Rejection criteria for sputa specimens collected.

1. Any incoming specimen that was not clearly labelled or not labelled was rejected.
2. Sputa from new cases suspect was rejected if found to be salivary, clear and with no mucous particles material, unless collected from follow-up patients.

Equipment and materials

1. Alcohol sand jar (only if a loop is used, not needed with disposable sticks).
2. Bunsen burner
3. Diamond pencil
4. Forceps
5. Lens paper or soft tissue paper
6. Plastic bag for waste disposal
7. clean applicator sticks
8. Fluorescence microscope
9. Slide staining rack
10. Slide boxes
11. New, clean slides (rinse in alcohol and dry if necessary)
12. Timer
13. Staining reagents
14. Staining bottles, 250 ml, with spout
15. Beaker for rinsing water

16. Sink and clean water supply
17. Freshly prepared 0.5% Sodium hypochlorite solution

Reagents and solutions

1. Auramine staining solution, 0.1%
2. Acid-alcohol decolourizing solution, 0.5%
3. Counterstaining solution
4. Potassium permanganate, 0.5%,

PROCEDURE

1. The working area in the BSL2 cabinet was disinfected using freshly diluted 0.5% Sodium hypochlorite solution.
2. Slides were labelled using the marker pen according to the laboratory register serial number marked on the sputum container.
3. Using a clean applicator stick, a smear was made, after selecting a small portion of purulent or mucopurulent material and spread uniformly, by the action of making circular movements repeatedly, over an area of to about 2–3 cm x 1–2 cm.
4. The smeared slides were left to stand and dry on a slide drying rack at room temperature.
5. After preparing the slide smears the BSL2 cabinet was disinfected using freshly diluted 0.5% Sodium hypochlorite solution.
6. The slides were arranged on a staining rack and fixed by passing a touch of flame three times slowly under the slides smear.
7. The smeared slides and control slides (positive and negative control) were placed on the staining rack over a sink, and flooded with filtered 0.1% Auramine staining solution, and left to stand for 15 minutes, before rinsing off the stain with clean tap water from a beaker.
8. Each smeared slides was decolourised by flooding with Acid-alcohol 0.5% solution for 3 minutes before rinsing using clean tap water from a beaker.
9. Potassium permanganate (counter stain) was flooded on each smear for 1 minute, before rinsing with clean tap water from a beaker.

10. The stained slides were left to stand on a drying rack, in a dark room to air dry.

Reading, recording and reporting

The dried slides were loaded on the LED microscope stage, and examined using the power 100 x objectives and 40 x objectives for confirmation of AFB.

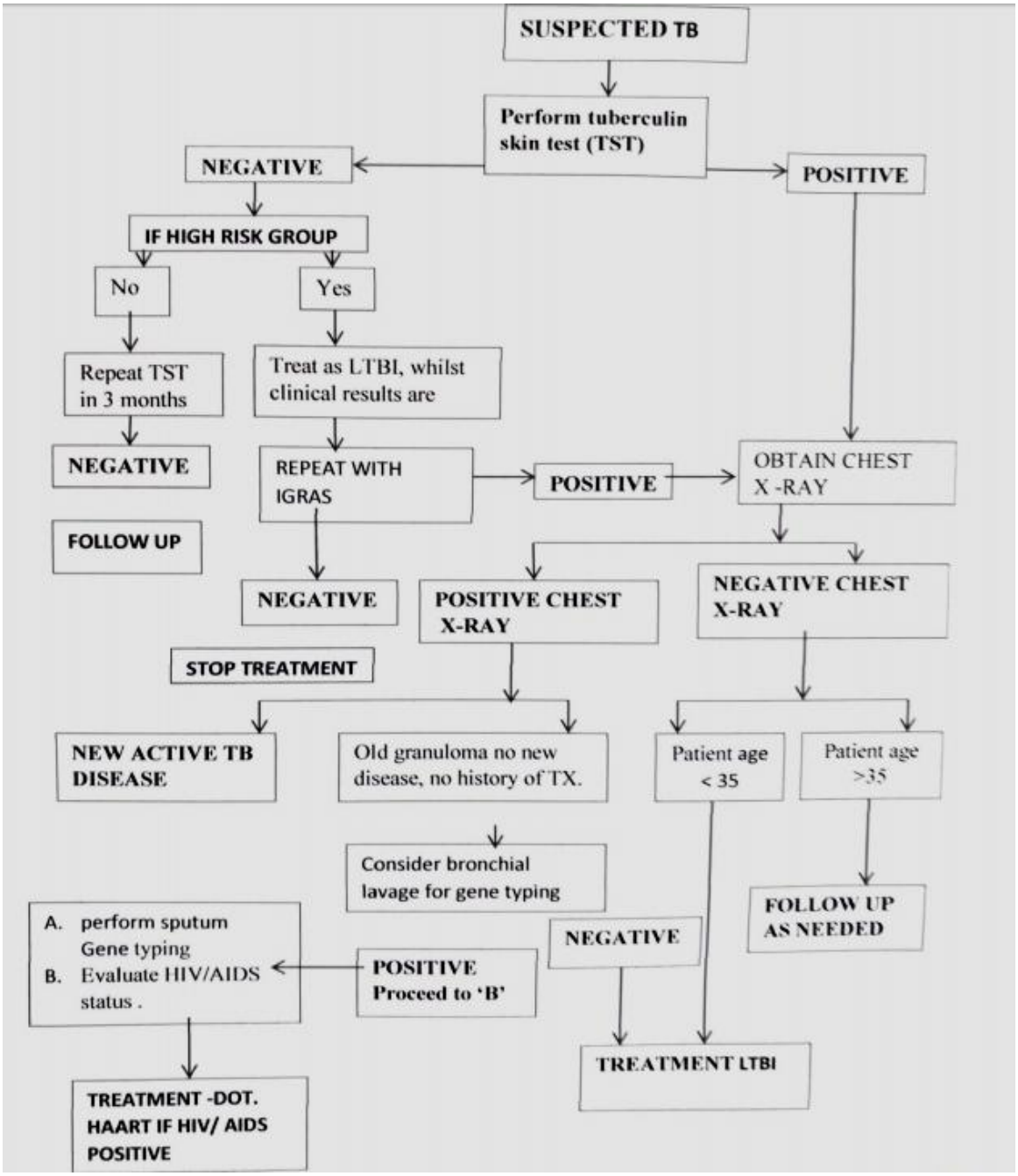
1. Negative result was reported if acid-fast bacilli not seen.
2. Positive results were reported if acid-fast bacilli appeared as bright yellow/greenish against the dark background with long, slender or short fragmented slightly curved rods in small groups, or in large clumps.
3. The LED microscope power supply was turned off and examined slides stored in a slide box in-order of the numbers of the laboratory register for further references.
4. Using power 200X, magnification, AFB seen is recorded is divided by 10, and quantified according to the IUATLD/WHO reporting scale.(http://www.google.com/iuatld/who scale)

Quality control

The following practices would result into false negative reporting.

1. Poor specimen collection, and sample picking during smear preparation.
2. Overheating during fixation
3. Excessive decolourization
4. Poorly prepared staining solution;
5. Inadequate staining time with auramine;
6. Over-staining with permanganate;
7. Exposure of slide to daylight for too long;
8. Reading less than one length;
9. Prolonged duration between staining and reading, particularly if slides were poorly stained or not kept in the dark.

Appendix 3: Screening and Treatment for pulmonary TB



Appendix 4: Drugs recommended for management of pulmonary tuberculosis (from Tuberculosis chemotherapy, JoshiJyotsna and modified with reference notes).

Categorization of tuberculosis agents		
Grouping	Drugs	
1. Firstline line drugs	Isoniazid (H) (Oral) Rifampicin(R) (Oral) Ethambutol (E) (Oral)	Pyrazinamide (Z) (Oral) Streptomycin (S) (Oral
2. Secondline drugs	Ofloxacin (IM) Levofloxacin(IM) Moxifloxacin (IM) Gatifloxacin (IM) Kanamycin (IM) Amikacin (IM)	Capromycin (IM) Viomycin (IM) Ethionamide(Oral) Cycloserine (Oral) Para Amino Salicyclic Acid - PAS(Oral)
3. Agents with unclear efficacy considered, but not recommended by WHO for routine use in XDR-TB, patients	Clofazimine Linezolid Amoxicillin/Clavulanate	Clarithromycin imipenem/cilastatin

Appendix 5: Adverse side effects to first line anti tubercular drugs

SIDE EFFECTS AND TOXICITIES OF ANTI-TB MEDICATIONS		
Isoniazid Rash Hepatic enzyme elevation Hepatitis Peripheral neuropathy Mild CNS effects	**Streptomycin** Ototoxicity (hearing loss or vestibular function) Congenital deafness Kidney problems Hypokalemia Hypomagnesemia	**Kanamycin/Amikacin** Auditory and renal toxicity Hypokalemia Hypomagnesemia Vestibular toxicity (rare)
Rifampin/Rifabutin Rash Hepatitis Fever Thrombocytopenia Flu-like symptoms Orange discolorization of :- urine,tears, sweat Photo sensitivity	**Ethionamide** GI upset Bloating Hepatotoxicity Metallic taste Hypothyroidism (esp. with PAS)	**Capreomycin** Auditory, vestibular, and renal toxicity Eosinophilia Hypokalemia Hypomagnesemia
Pyrazinamide Gastrointestinal upset Hepatitis Rash Arthralgias Hyperuricemia Gout (rare	**Cycloserine** Psychosis Seizures Headache Depression Other CNS effects Rash	**Para-aminosalicylic acid** GI disturbance Hypersensitivity Hepatotoxicity Hypothyroidism
Ethambutol Optic neuritis (decreased red-green color discrimination) Decreased visual acuity Rash	**Levofloxacin** Abdominal cramps GI upset Insomnia HeadachePhotosensitivity	

Appendix 6: Participants Consent Forms

TITLE OF STUDY: PULMONARY DRUG RESISTANT TUBERCULOSIS IN GENERAL HOSPITAL; MOMBASA.

Informed consent explanation for patients aged 18 years and above or guardians to those give assent.

My name is Idah Pam Ombura, and I am undertaking a post graduate study at the department of Medical Microbiology at the University of Nairobi. I would like to request you to participate in a medical research study.

The objective of this study is to determine the level of drug resistant tuberculosis. The information obtained from your participation in my study will assist obtaining my study objectives.

You will be requested to collect only one sputum sample of approximately 2ml in the provided specimen container for drug resistant analysis.

Any positive findings from the study will be brought to the attention of your physician for purposes of better client management strategies. These results will also benefit the policy makers in their provision and distribution of resources to affected areas; and guidelines on management of multi-drug resistant tuberculosis.

1. You will not be given any monetary benefits; neither will you incur any costs.
2. Please understand that the following principles apply to all participants of the study:
3. Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you
4. Your confidentiality will be safeguarded, and no names of the participants will appear in any final report or publication resulting from the study

5. No risk will be incurred in participating in the study
6. This study will only require one sputum sample

Contact information

If you have concerns now or in the future regarding your rights or this study; or research related injury, you may contact me or my supervisor through this number 0722276588, or the Secretary to the Kenyatta National Hospital Research and Ethics committee, P.O Box 20273, Nairobi. Telephone number-020726300 ext. 9

SECTION TO BE SIGNED BY THE STUDY PARTICIPANT

The above details about the study and the basis of participation have been explained to me in English/ Kiswahili and I agree to take part in the study. I understand that I am free to choose to be part of the study or not. I give my consent to be screened for Multi drug resistance tuberculosis.

Patient signature/ Thumb mark: …….

Witness' signature: …….....

Person obtaining consent signature : ……

Date : …….

Appendix 7: Participants Questionnaire Form.

PULMONARY DRUG RESISTANCE TUBERCULOSIS IN GENERAL HOSPITAL, MOMBASA.

DEMOGRAPHIC DATA:

Study No: ☐☐☐

Patient initial: ☐☐☐☐☐☐

Clinic No: ☐☐☐☐☐☐

Date of interview: DD MM YY ☐☐ ☐☐ ☐☐

DOB: DD MM YY ☐☐ ☐☐ ☐☐

Religion: Christian ☐ **Muslim** ☐ **IF Other Specify**_____

Nationality: _____

Marital Status: Single ☐ Married ☐ Divorced ☐

Separated ☐ Widowed ☐

Educational Level: None ☐ Primary ☐ Secondary ☐

College ☐ University ☐

Occupation: Professional…… Housewife ☐ Unemployed ☐

CATERGORY OF STUDY PARTICIPANT:

New case (no previous treatment) ☐

Follow ups (those currently on treatment) ☐

Previous case (treatment) treatment failure ☐

Defaulter ☐

Others specify _____

DATA FROM LABORATORY ANALYSIS

	POSITIVE	NEGATIVE
Sputum microscopy (FM stain)….	☐	☐

	POSITIVE	NEGATIVE
HIV status……	☐	☐

TB Genotypes (Hains test):

Mono-resistant ☐ Poly-resistant ☐ MDR ☐ XDR ☐

Signature of the technologist: _____ Date: _____

Appendix 8: Procedure for sputum decontamination by Modified Petroff's technique

1. Approximately 1ml of sputum was added to 3ml of 4% of sodium hydroxide in a labeled falcon tube and the lids fastened.
2. Each tube was vortex to homogenize the sample, then left to stand in the BSL class 2 cabinets for 15 minutes.
3. Tubes were centrifuge at 3000 rpm (rounds per minute) for 15 minutes.
4. The supernatant was discarded into the 10% (jik) sodium hypochloride solution liquid disposal container.
5. 20ml of sterile distilled water was added to re-suspend the sediment and the content vortexed, followed by Centrifugation at 3000rpm for 15 minutes.
6. The supernatant was decanted leaving approximately 1 ml of the deposit.
7. The deposit was aliquoted into two labeled cryovial and frozen at -70^{o}C.

Appendix 9: Hains life-sciences Genotype protocol for performing LIPA assay PCR

STEP 1.DNA EXTRACTION

Materials and Methods

Consumables

1. 1.5 ml screw capped tubes
2. Pipettes and corresponding tips
3. Gloves
4. Disinfectant (10% Dettol solution and Distilled water)
5. Lab coat

Mechanical extraction

1. Bio safety cabinet class II
2. Heat block
3. Centrifuge
4. Vortex
5. Ultra sonic bath
6. Timer

A). DNA EXTRACTION BY MECHANICAL METHOD

Sputum

1. 500 µl of decontaminated sputum sample was transferred into 1.5 ml screw capped tube and centrifuged at 10,000 RPM for 15 minutes.
2. The supernatant was discarded and deposit (pellet) re-suspended in 100 µl of sterile distilled water.
3. The sample was vortexed to homogenize.
4. The sample was transferred into the heat block at 95^0C for 20 minutes, and incubated in the ultrasonic bath for 15 minutes at room temperature.
5. The tubes were centrifuge at 13,000 revolutions per minute (RPM) for 5 minutes.

6. 10µl of supernatant (the DNA extract) was aliquoted into new labeled cryovial and at -20^{0}C.

B). PRE-PCR STATION

This step deals with preparation of Master Mix to be used for DNA amplification.

1. New dust-free gloves and laboratory gown is worn.
2. The work area BSC2 or UV hood in the Pre- PCR room is decontaminated with freshly diluted 0.5% Sodium hypochlorite solution.

Material and Methods

1. BSC, class II or UV hood
2. Micropipettes (0- 10 µl ; 2-20 µl, 20-200 µl, 100-1000 µl)
3. Pipettes tips
4. Waste biohazard container
5. Gloves powder free
6. 1.5 ml Micro tubes
7. PCR tubes

Reagents

1. PNM buffer
2. PCR buffer
3. 25mM $MgCl_2$ (magnesium chloride)
4. molecular biology grade water
5. Taq polymerase

Procedure

1. The number of samples was determined in order to calculate the master mix working volumes.
2. Reaction tubes (cryovial) were labeled with respective identification numbers
3. Using master mix per reaction tube, the amplification mix was calculated for the total working reactive solution.

Calculation of total reactions

The total reactions is given as the total number of specimens to be processed, plus one positive control, one negative control, and one extra correction volume.

Example: Suppose 6 samples are to be processed the total (master mix aliquoted into PCR reaction tubes**)** reactions will be for 9 reactions and calculation of the volume to be used for each reagent is calculated as below.

Preparation of master mix

Reagent	**For 1 reaction (µl)**	**For 9 reactions (µl)**
PNM	35	35x9 = 315
10 x PCR Buffer	5	5x9 = 45
25m M$MgCl_2$	2	2x9 = 18
H_2O (molecular biology grade water)	3	3 X 9 = 27
Taq polymerase (= 1 unit)	0.2	0.2X 9 = 1.8

1. 45 µl aliquot mixture of the master mix was dispensed into each labeled PCR tube.

Precaution

1. All the materials should be Cleaned and decontaminated before and after the use.
2. All used material should be Cleaned and Removed from BSC cabinet, and the BSC cabinet cleaned before putting off the lights of the BSC.
3. Hands should be washed and used laboratory coats left behind.
4. Doors should always remain closed.

C). AMPLIFICATION (PCR-AREA)

At the end of this step, the amplicons obtained will be used for hybridization and detection.

Materials and Methods

1. Thermo-cycler
2. Micropipettes (20-200 µl, 100-1000 µl)
3. Pipette tips (20-200 µl, 100-1000 µl)
4. Gloves powder free
5. DNA

Precautions

Decontaminate all instruments, racks, bench space with freshly prepared 0.5% of hypochlorite solution before and after loading the thermo-cycler.

Transfer 5 µl of respective DNA into 45 µl of master mix and close the caps tightly

Place or load the reaction tubes on to Thermo- cycler and closer the lid

On the thermo-cycler, select appropriate program for the amplification procedure and start. For more refer the manual (SOP).

D). HYBRIDIZATION AND VISUALIZATION

In the hybridization steps, probes are embedded in the strips which will complement the correct DNA sequence, if present in the amplicons. The primers used in the amplification process are biotinylated, when these amplicons are subjected to all conditions as prescribed in the hybridization procedure; these complementary sequences are visible as bands on the strips. These bands are further interpreted to define positive diagnosis or absence of susceptible TB, MTBC (Mycobacterium TB Complex), NTM (Non-Tuberculosis Mycobacteria), any mono-drug resistant TB, MDR-TB, or XDR-TB as the case might be.

Methods and Material

1. Tray for hybridization
2. Twincubator
3. Water bath
4. Timer

5. Micropipettes (20 µl, 200 µl, 1000 µl)
6. Pipettes tips
7. Waste biohazard bag
8. Waste container containing 10% bleach
9. White paper
10. Permanent tube Fine Markers
11. DNA strip markers
12. Set of Tweezers
13. Gloves

Reagents

1. DNA strips
2. Amplicons or DNA amplification product
3. DNA Denaturation reagent (DEN)
4. Hybridization reagent (HYB)
5. STR Solution
6. RIN solution
7. Conjugate concentrate (CON C)
8. Conjugate Diluent
9. Concentrated Substrate concentrate (SUB C)
10. Substrate Diluent
11. Distilled water
12. Bleach solution

Precaution

Wear new gloves and a lab coat (disposable), and decontaminate working area with fleshly prepared 5% Sodium hypochlorite.

1. Pre-warm hybridization buffer (HYB) and Stringent wash (STR) at 45^0C to dissolve all crystals.
2. Bring to room temperature Rinse (RIN) solution and distilled water.
3. Freshly dilute CON-C and SUB-C; 1:100 in the respective diluents provided in the kit and protect them from light.

Denaturation step

Dispense 20 µl of denaturation solution (DEN) in each well of the tray; add 20 µl of DNA PCR products in each tray and mix by pipetting up/ down. Incubate at room temperature for 5 min. (at the bench).

Hybridization step

1. Add 1ml of hybridization solution (HYB) to each well containing DEN + amplified DNA.
2. Mix the solution by tilting the mixture up and down.
3. Label the strips according to the samples labeling, using DNA strip marker
4. Place the labeled strips to each tray corresponding to each sample (the well contains already the mixture DEN, DNA, HYB) and incubate for 30 min. at 45^0C in the Twincubator.
5. Aspirate the whole quantity of HYB completely
6. Add 1 ml of STR (fixing and washing solution) to each tray and incubate for 15 min. in the Twincubator
7. Remove the whole quantity of STR completely, then add 1 ml of RIN (rinsing solution) to each tray and incubate for 1 min. at room temperature in the Twincubator.
8. Remove the whole quantity of RIN.

E). Detection/Visualization

1. Add 1 ml of the diluted conjugate. For dilution: (10µl Con-c + 1ml Con-D) and incubate for 30 minutes at room temperature in the Twincubator.
2. Aspirate the conjugate completely.
3. Add 1ml of RIN (rinsing solution) to each tray and incubate for 1 min. at room temperature in the Twincubator.
4. Aspirate the whole quantity of RIN completely, and then rinse once with water for 1 min.
5. Add 1 ml of substrate. Dilution: (10µl SUB-c + 990 µl SUB-D) to each tray and incubate for 2-10 minutes at RT in the Twincubator.
6. Aspirate the substrate completely.
7. Stop the reaction by rinsing twice with water for 1 minute in each rinse.
8. Remove DNA strips from the tray and air Dry DNA strips on absorbent paper.
9. Stick dried strips to result sheet and interpret.

NB: Interpretation should be done according to respective manual in the insert.

Appendix 10: Primers applied in Hains Life-sciences GenoType MTBDR plus and MTBDRsl Assay.(Farooqi et al. 2012 and Hillemann et al. 2009)

Primer target	Primer sequence (5′-3′)	Mution region
katG (F) **katG (R**	CCATGGCCGCGGCGGTCGACATT GTCAGTGGCCAGCATCGTCGGGGA	MUT1 (S315T1) MUT2 (315T2)
InhA (F) **InhA (R)**	CCTCGCTGCCCAGAAAGGGA ATCCCCCGGTTTCCTCCGGT	inhA MUT1 (C15T) inhA MUT2 (A16G) inhA MUT3A (T8C) inhA MUT13B(T8A)
RpoB (F) **rpoB (R)**	GACGACATCGACCACTTC GGTCAGGTACACGATCTC	rpoB MUTI (D516V) rpoB MUT2A (H526Y) rpoB MUT2B (H526D) rpoB MUT3 (S531L)
GyrA (F) **GyrA(R)**	GATGACAGACACGAC GTTGC GGGCTTCGGTGTACCTCAT	gyrA MUT1 (A90V) gyrA MUT2 (S91P) gyrA MUT3A (D94A) gyrAMUT3B(D94N/D94Y) gyrA MUT3C (D94G)
rrs (F) **rrs(R)**	AAACCTCTTTCACCATCGAC GTATCCATTGATGCTCGCAA	rrs MUT1(A1401G) cap, amk, kan (C1402T)res cap, vio, kan rrs MUT2 (A1401G)cap vio, amk, kan
Emb(B)	CGTTCCGGCCTGCAT CACCTCACGCGAC AGCA	MUT1A (M306I-ATGATA) M306V M306I (ATG – ATC/ATT)
Legend ;F=forward primer, Reverse primer		