DETECTION OF MUTATIONS ASSOCIATED WITH ANTIMICROBIAL RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS* IN SELECTED HEALTH FACILITIES IN KENYA

JOSHUA KIBET ROTICH

(H56/70624/2011)

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT FOR THE AWARD OF MASTERS OF SCIENCE IN MEDICAL MICROBIOLOGY FROM THE UNIVERSITY OF NAIROBI, SCHOOL OF MEDICINE

University of Nairobi

2019

DEDICATION

This work is dedicated to my family, wife Evelyn and sons Nathan and Noah for their consistent encouragement and prayers that made this study possible.

DECLARATION

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

Signature…….. Date……

Name: **Joshua Kibet Rotich**

Reg. No.: Registration No. H56/70624/2011

This thesis has been submitted for review with our approval as the University supervisors.

Signature…….. Date……..……

Prof. Omu Anzala

Director KAVI/ICR,

University of Nairobi

Signature…….. Date……

Ms. Winnie C. Mutai

Department of Medical Microbiology, College of Health Sciences,

University of Nairobi

ACKNOWLEDGEMENT

First and foremost, I thank God for the numerous blessings He has bestowed upon me throughout my dissertation journey. I would first like to thank my thesis advisor Prof. Omu Anzala of KAVI/ICR the School of Medicine at the University of Nairobi. The door to Prof. Anzala's office was always open whenever I ran into any challenge or had a question about my research writing. He consistently allowed this paper to be my own work, but steered me in the right direction whenever he thought I needed it. Special thanks goes to my supervisor Ms. Winnie Mutai for the step by step detailed guidance on the development of this work and laboratory work and writing of this dissertation.

I take this opportunity to say special thanks to the Department of Medical Microbiology Chair Prof. Walter Jaoko and his great staff who were always equipped with kind words of encouragement throughout the project period.

I give special thanks to the county laboratory teams and to the Qiagen-KAVI/ICR specialized molecular laboratory for the tireless efforts to ensure the laboratory work was done seamlessly: Monica Pettersson applications specialists from Qiagen, Martin Koome, Abigael Njoki, Isabel Muthoni you were all great.

I finally salute contributions of the bioinformatics specialist Joyce Njuguna of ILRI for interpreting DNA sequences and Richard Kiplimo the statistician at DLTLD for data analysis without who's work this dissertation would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

AFB	Acid Fast Bacilli
AMR	Antimicrobial Resistance
AIDS	Acquired Immunodeficiency Syndrome
AMK	Amikacin
DLTLD	Division of Leprosy, Tuberculosis and Lung Disease
DNA	Deoxy-Ribonucleic Acid
DST	Drug Susceptibility Testing
EAPHLN	East Africa Public Health Laboratory Networking Project
EMB	Ethambutol
HCWs	Health Care Workers
HIV	Human Immunodeficiency Virus
INH	Isoniazid
MDR-TB	Multi-Drug Resistant TB
MoH	Ministry of Health
NAAT	Nucleic acid amplification test
PCR	Polymerase Chain Reaction
PSQ	Pyro-sequencing
PZN	Pyrazinamide
OFL	Ofloxacin
RIF	Rifampicin
RMS	Resistance Mutation Study
RRDR	Rifampicin Resistance Determining Region

SM	Streptomycin
TB	Tuberculosis
TST	Tuberculin Skin Testing
WHO	World Health Organization
XDR	Extensively Drug Resistant
ZN	Ziehl – Neelsen
rpoB	Gene encoding β subunit RNA polymerase
glpK	Glycerol kinase gene
pyKA	Pyruvate kinase gene
pncA	*M. avium* coded pyrazinamide resistance gene
KatG	Gene encoding the enzyme catalase-peroxidase
rrs	Ribosomal RNA gene
SNPS	Single nucleotide polymorphism
WRD	WHO-recommended rapid diagnostic
Xpert MTB/RIF	GeneXpert MTB/RIF test
USD	United States Dollars

ABSTRACT

Background: Drug resistant tuberculosis is a huge medical issue in several countries of the world and has continuously become a challenge to its prevention and control. Kenya is ranked as the fifteenth in position of the twenty-two countries with the highest prevalence of tuberculosis with about two thousand three hundred patients suffering from multidrug resistant tuberculosis in 2016. Status of the patterns of mutations associated with drug resistance of *Mycobacterium tuberculosis* in the country is limited although it has been a threat to national tuberculosis control program and a major public health problem in Kenya.

Objective: The main objective of this study was to determine mutations in genes coding for drug resistance in *M. tuberculosis* from sputum samples.

Methodology: This was a cross sectional study carried out in five public health hospital laboratories. A total of 288 routinely analyzed TB smear positive sputum samples were collected and analysed by pyrosequencing.

Results: Mutations were detected in 9% (27/288) samples tested. Mutations were detected in genes associated with five of the antibiotics RIF, INH, SM, EMB and AMK and there were no mutations in genes associated with OFL. Mutations were detected on all the four first line antibiotics tested. 85% (23/27) of the samples with mutations were on the *rpoB* gene (associated with resistance to Rifampicin) from four of the counties. One sample (1/27) had a mutation on *katG* gene (associated with Isoniazid), eight samples (8/27) had mutations on *rpsl* gene (associated with Streptomycin), two samples (2/27) had mutations on *embB* gene (associated with Ethambutol), one sample (1/27) had mutation on *rrs* (associated with Amikacin) and there were no mutations detected on the *gyrA* gene (associated with Ofloxacin). Mutations on more

than one gene associated with two or more drugs were detected in seven samples (7/27) and multiple multi-locus mutations were detected on *rpoB* and *rpsl*. 98% of the codon changes reported were on the genes associated with 1st line antibiotics and only one on the genes associated with the 2nd line antibiotics.

Conclusion: Mutations associated resistance to all the four 1st line TB antibiotics were detected on sputum samples that could hamper treatment of patients with the antibiotics. Mutations on the genes associated with resistance to second line antibiotics were low with 1/288 (0.3%) mutation.

Recommendations: Pyrosequencing test for drug resistance genes is an invaluable technology that should be included in the testing algorithm for susceptibility testing in TB reference and research laboratories in Kenya.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the study

Infection with multidrug-resistant *Mycobacterium tuberculosis* (MTB) has become a worldwide health pandemic according to World Health Organization (WHO, 2012). The continent of Africa, Eastern Europe and Asia is home to the highest number of tuberculosis (TB) disease cases in the world in which both China and India carry almost half of those cases. The WHO zones of South East Asia and the African region recorded the highest occurrence of new cases in 2016 of forty five per cent and twenty five per cent respectively (WHO, 2016).

Current data shows that in the last decade, there has been an annual increase in TB case notification of 16% since 1987 that is attributed to Human Immunodeficiency Virus (HIV) in Sub-Saharan Africa. Since tuberculosis usually establishes in people with HIV/AIDS, the infection increases the chances of rapid disease development in newly acquired infections. Recurrence of TB after successful treatment due to immunosuppression leads to considerable morbidity and mortality (World Health Organization, 2012). Out of the six hundred thousand rifampicin resistant cases picked by GeneXpert/RIF test (Xpert MTB/RIF), four hundred and ninety were confirmed to be MDR TB. In 2016 almost one million five hundred people advanced to active TB disease and one million seven hundred deaths of which almost four hundred occurred among HIV-AIDS positive people (WHO, 2017). Kenya was among the 14 countries that were listed in all 3 listed 30 high disease burden countries (HBC) categories of high TB burden, high TB/HIV burden, and high MDR-TB burden by WHO for the period 2016 to 2020. The National Division of Leprosy, Tuberculosis and Lung Diseases (DLTLD) policy and algorithm indicates that there is compliance with WHO-recommended rapid diagnostic (WRD) as the first test for all persons believed to have TB but there was no Drug

Susceptibility Testing (DST) provision for all (WHO, 2017). Tuberculosis has continued to be a major cause of morbidity in Kenya and according to the first post-independence survey to estimate the national burden of TB in Kenya the prevalence rate was found to be 558 per 100,000 and was previously underestimated (Ministry of Health, 2016). The country tuberculosis management budget of financial year 2015/2016 was estimated at 62 million US dollars of which 41 % remained unfunded.

MTB variants have appeared on some patients that do not respond to first line tuberculosis treatment and cases of other variants that are difficult to treat with antibiotics reserved for second line treatment. These multidrug resistant (MDR) and extensively drug resistant (XDR) variants present a serious challenge to management of this infection (WHO, 2010). A variant that is not susceptible to rifampin (RIF) and isoniazid (INH) is an MDR-TB while that which is not susceptible to a fluoroquinolone (FQ) and either amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) is an XDR TB (CDC, 2009; Palomino & Martin, 2014; World Health Organization, 2014). It has been found that changes of the nucleotide sequence of MTB is the cause of antibiotic resistance (Maher, Espinal, & Floyd, 2015; Palomino & Martin, 2014; S Ramaswamy & Musser, 1998) and only a few nucleotide changes are responsible for most of the observable failure to respond to antibiotic therapy. Effective management of TB by the Ministry of Health (MoH) relies on timely identification of the causative agent and antibiotic susceptibility testing (Ministry of Public Health and Sanitation, 2009). Traditionally drug resistance in MTB has been done by the tedious culture and susceptibility testing in solid or liquid media which may take up to 2 months to complete. This time is sufficient enough to enhance for transmission of resistant variants to new patients as well as to deteriorate the patient's condition. New procedures that use DNA amplification techniques to detect mutant genes associated with resistance and that can be performed on the same day can be very useful (CDC, 2009; WHO, 2010). The WHO approved Gene Xpert

MTB/RIF real-time PCR assay for sputum-based rapid diagnosis of lung tuberculosis. This technology has been recommended for quick detection and simultaneously testing of rifampin resistant MTB in sputum samples as a proxy for MDR-TB in less than 2 hours (WHO report, 2011). This diagnostic tool has been rolled out in both public health and private facilities in Kenya and has gone along way in improving the fight against TB being a prompt diagnostic aid to disease management. MTB/RIF resistance analysis is depended on establishing the absence of probe hybridization to non-mutant loci, designating the occurrence of mutations in the *rpoB* gene that is the cause of resistance to rifampicin. The challenge of the XpertMTB/RIF is that it only detects RIF resistance and MDR will need to be confirmed by determining results of resistance to both Rifampicin and isoniazid (INH). Pyrosequencing can determine short DNA sequences. This is a method of DNA sequencing that works on the principle of sequencing by synthesis that is performed by detection of nucleotide incorporation by DNA polymerase in real time. Pyrosequencing can be done to identify genes that confer variant strains that are resistant to Streptomycin (SM) Rifampicin (RIF), Isoniazid (INH), Ethambutol (EMB), Amikacin (AMK), and Ofloxacin (OFL) from specimens. Pyrosequencing depends on measurement of the intensity of light in a chain reaction where pyrophosphate is liberated (Fakruddin & Chowdhury, 2012). Pyrosequencing approach in contrast to conventional drugs sensitivity test (DST) shows high sensitivity (94.6%) and specificity (100%) in diagnosis of MDR-TB and also high sensitivity (86.9%) and specificity (99.3%) in the diagnosis of XDR-TB. Pyrosequencing can be useful in reducing the diagnosis to patient treatment time by rapidly providing information on drug susceptibility patterns (Engstrom *et al*, 2012; Grace Lin *et al*., 2013; Rodwell *et al*., 2014). Pyrosequencing (PSQ) testing was incorporated into the testing service by Centers for Disease Control and Prevention (CDC) from June 2012 expanding the testing menu and by incorporating use of DNA technology to give diagnosis of drug resistance (CDC, 2012).

The objective of this study was to determine presence of anti-TB associated mutations in MTB from sputum samples.

1.2 Problem Statement

Antimicrobial resistance of MTB is a danger to world TB management and has worried many countries being costly and difficult to control. The traditional culture based antibiotic sensitivity testing of MTB is tedious and takes weeks to months to obtain results. Prompt identification of antimicrobial susceptibility patterns of TB is important for proper management and to avoid spread of resistant strains. Technologies that help to precisely and quickly identify antimicrobial resistant pathogens are important in successful management of infected persons. Additionally, appropriate planning and commitments by the MoH to use standard antibiotic regiments to manage infections with pulmonary TB has been mired by the occurrence of variations of antimicrobial resistance to both the antibiotics preserved for initial treatment and those designated for second line treatment. The slow growth rate of the MTB in the laboratory means that the traditional ways of generating antibiograms is slow and frequently leads to delays in the administration of the right treatment. Sequencing of the MTB offers the technology that can diagnose mutations that are linked to antimicrobial resistance. This means it is possible to pick MDR-TB by identifying variations in the nucleotide sequences of the genome of MTB associated with drug resistance.

1.3 Justification

In spite newer modalities for diagnosis and treatment of TB, people unfortunately are still suffering. TB has reserved its rank amongst the top ten killer infectious diseases second only to HIV-AIDS. Proper TB management is dependent on precise and prompt chest medical imaging and accurate laboratory analysis coupled with swift test of drug susceptibility patterns (Ministry of Public Health and Sanitation, 2009). Little information is available on

the exact prevalence of resistance to anti-TB drugs in Kenya. DNA pyrosequencing technology used in this study sought to inform on the occurrence of mutations associated with drug resistance of MTB in samples from selected regions of Kenya. The data obtained could be a useful source of reference when planning a suitable TB treatment scheme. This could ultimately mean reduction of treatment time leading to prevention of transmission of resistant strains.

1.4 Research Question

What are the mutations associated with resistance of MTB to commonly used anti- TB drugs in selected county hospitals across Kenya?

1.5 Broad objective

To determine the presence of mutations associated with drug resistance in clinical sputum samples from five counties by pyrosequencing analysis.

1.5.1 Specific objective

1. To determine the presence of mutations associated with first line drug resistance genes on MTB from patients seeking TB diagnosis in selected health facilities in Kenya

2. To determine the presence of mutations associated with second line drug resistance genes on MTB from patients seeking TB diagnosis in health facilities in Kenya

3. To determine the presence of multi-drug and multi-locus mutations on genes coding for anti-TB drug resistance in MTB

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Occurrence of Multidrug-resistant tuberculosis

The occurrence of antimicrobial resistance in MTB is a huge challenge that medical authorities face in TB treatment and management and it is worse when faced with strains that are multidrug resistant (World Health Organisation, 2010). Changes in the genetic makeup of some parts of the genome of MTB is the main reason for the emergence of resistant strains against the antibiotics that are usually used for initial treatment and even those reserved for MDR-TB (S-Y Grace Lin *et al*., 2013; Smit *et al*., 2013). All over the world studies have shown that antimicrobial resistance to TB is extensive and is a great source of alarm to national TB programs in majority of WHO member states (Cucinotta, 2014). Antimicrobial resistance cases usually muddle patient management frequently resulting in poor outcomes especially in people with reduced immunity where it causes suffering and even death. Global estimates of 3.3% of new TB cases and 20% of previously treated cases have MDR-TB, a level that has remained constant through recent years (Maher *et al*., 2015). According to the Ministry of Health statistics in the Kenya, the program reported 1,191 MDR cases between 2006 to 2013 with the highest in the last ten years of 305 reported in 2015 and the trend has been steadily increasing since 2006 when 82 cases, 285 in 2013 and 283 in 2014 were reported (Ministry of Health, 2016). This indicates the numbers are rising and therefore the need for rapid and accurate methods of detection of cases such as pyrosequencing. In this study all smear positive samples were analysed picking new and retreatment cases without discrimination indicating high sensitivity to pick new as well as old cases as indicated (Shou-Yean Grace Lin & Desmond, 2014). The cost of therapeutic agents reserved for MDR-TB cases is much higher than that of first line antibiotics and there is relatively restricted number of therapeutic agents to choose from. Hence, strategies for precise and quick identification of

antimicrobial resistance are essential so as to have swift administration of successful therapeutic policies and reduce the spread of resistant strains.

2.2 Sequencing Protocol

The most widely used technology for antimicrobial drug susceptibility for MTB has been the established observation of growth or metabolic inhibition in a medium containing anti-tuberculosis drug by culture in solid or liquid media. The method has proved to be highly sensitive and specific to most anti-TB drugs. Nevertheless, these procedures were painstaking, long and may not provide efficient turn around time acceptable for clinical use. Therefore rapid molecular diagnostic methods that give same day results have been developed that are beneficial in reducing the detection time and increasing sensitivity and specificity with acceptable cost (Kigozi *et al*., 2018). The WHO at the moment have recommended selected Nucleic acid amplification test methods for specific anti-TB drugs susceptibility by finding gene mutations associated with drug resistance, which include as Xpert MTB/RIF and GeneType MTBDR technologies (CDC Reference Laboratory Division of TB Elimination, 2012; Saglik, Oz, & Kiraz, 2014; Sangsayunh & Chuchothawon, 2014; Vassall *et al*., 2011; World Health Organization, 2012). Pyrosequencing a DNA sequencing technique based on the detection of the pyrophosphate released during DNA synthesis (Cheng, Cui, Li, & Hu, 2014) has been described as well suited for large-scale screening of specific short length DNA fragments. It is poised to be the novel approach for the detection of TB infection and tuberculosis resistance (Kahla, Henry, Boukadida, & Drancourt, 2011; S-Y Grace Lin *et al*., 2014). Mutations were known to be the cause of drug resistance in MTB (da Silva & Palomino, 2011; Sandgren *et al*., 2009). Is has been described that RIF resistance is almost entirely associated with the "hot spot" of was mutation of the 81-bp region of the *rpoB* gene in the RIF resistance determining region (RRDR) (S. Y. G. Lin *et al*., 2014). Mutations of the resistance to INH, EMB, SM, OFL and AMK were principally located in the

katG, embB, rpsl, gyrA and *rrs* genes, respectively (Chan *et al*., 2011). All these resistance-associated mutations were principally found in a short region or a position in MTB chromosomes that can be considered the beacons of molecular drug susceptibility testing. Although the initial cost of acquiring the pyrosequencing technology may be substantial the benefits could overshadow this small one time expense. In one study the researchers used US $60 and took only 6 hours for testing six antimicrobials (Zheng *et al*., 2014). Being a semi-automated procedure and easy to adapt and training can be achieved within a short time. Grace Lin and others have developed a pyrosequencing assay in California that can simultaneous detect MTB and mutations associated with multi-drug and extensively drug resistant agents in bacterial isolates as well as in medical specimens (S-Y Grace Lin *et al*., 2014). This included a target, as a multiplex reaction, to identify MTBC concurrently with drug resistance detection.

2.3 Mutations associated with First Line Anti-TB Drug Resistance First

Several genes have been identified to be associated with resistance to antibiotics designated as a cocktail for treatment at primary diagnosis of TB. Those associated with isoniazid include *ahpC katG, kasA, ndh,* and *inhA,* with rifampin include *rpoB,* with streptomycin include *rpsl, rrs and gidB,* with ethambutol *embB.*

2.3.1 Isoniazid

It seems that Isoniazid (INH) resistance is ordered by a multifaceted set of DNA. Transformation of nucleotide arrangements in any of about five genes can results in mutation associated with resistance to INH. Over sixty five percent of INH resistant MTB have been linked to this kind of mutations (S Ramaswamy & Musser, 1998). The substitution of amino acid Serine to Threonine through the substitution of AGC to ACC at codon 315 of the *katG* gene is the highest globally (Campbell *et al*., 2011; Kigozi *et al*., 2018; Palomino & Martin,

2014). The gene *katG* stands for catalase-peroxidase enzyme activity (S Ramaswamy & Musser, 1998). By itself, INH is inactive until the catalase-peroxidase enzyme *KatG* triggers it (Engström *et al*., 2012). In *MTB* INH main point of action is the enoyl-acyl transporter enzyme reductase InhA, whose function is the production of the bacterial cell wall (Banerjee *et al*., 1994; Miesel, Rozwarski, Sacchettini, & Jacobs, 1998; Quémard *et al*., 1995). Changes in the genetic makeup of *katG*, precisely at codon 315, cause a high-level Isoniazid resistance (S Ramaswamy & Musser, 1998; Y. Zhang, Heym, Allen, Young, & Cole, 1992). A genome change in the promoter region of the *inhA* operon causes low-level resistance against Isoniazid. (Colangeli *et al*., 2007; Larsen *et al*., 2002).

2.3.2 Rifampin

Rifampin (RIF) binds to the beta subunit RNA polymerase a product of *rpoB* gene and so preventing the initial steps of synthesis of proteins (Campbell *et al*., 2011). It has been confirmed that over 90 % of RIF resistance is caused by mutation in a 81 base gene region now commonly referred to as the hot spot or the Rifampin Resistance Determining region (RRDR) (da Silva & Palomino, 2011). Within this region majority of the nucleotide alterations happens on the 531 and 526 at about forty and twenty percent respectively (Campbell *et al*., 2011; S Ramaswamy & Musser, 1998). This is in agreement with another work done in Morocco by Chaoui and others, which showed that the majority of changes happened on the 516, 526 and 531 regions (Chaoui *et al*., 2009).

2.3.3 Streptomycin

Streptomycin, an aminoglycoside was the first antibiotic with demonstrated antimicrobial activity against TB and has been used to treat TB of the lungs the longest. It works by barring production of proteins in the bacteria by attaching itself to the protein and rRNA precursors. Antimicrobial resistance to streptomycin has mainly been associated with two genes *rpsL* and

rrs. Gene *rpsl* codes for the synthesis of 12S sub-unit protein and mutations takes place the 43rd at codons (A/G, Lys/Arg, Thr) and the 88th codons (A/G/C, Lys/Gln, Arg, Thr). The *rrs* gene on the other hand codes for 16S rRNA with majority of the time mutations taking place on the 912th and 530th codons. Mutations in any of these genes account for over ¾ of SM resistant strains (Mokrousov, Isakova, Valcheva, Aldashev, & Rastogi, 2013; Srinivas V. Ramaswamy *et al*., 2004; Springer *et al*., 2001). The *rpsl* gene changes at codon 43 have been described as the most widespread in China (L. L. Zhao *et al*., 2014).

2.3.4 Ethambutol

Ethambutol (EMB) has been used as a combination therapy as the initial course of antibiotic therapy of pulmonary tuberculosis (Tejada, Walk, & Kharel, 2015). Ethambutol inhibits arabinosyltransferases, programmed by *embCAB* operon, consequently preventing the synthesis of elements needed for buildup of bacterial cell wall (da Silva & Palomino, 2011; Goude, Amin, Chatterjee, & Parish, 2009). Mutations linked to antimicrobial failure of EMB basically sit on the *embB* gene and majority of the changes occurs at codon 306 (da Silva & Palomino, 2011; Ramaswamy & Musser, 1998; Sandgren ., 2009).

2.3.5 Pyrazinamide

The most important milestone in TB management was the ability to reduce the time of the course of treatment from nine months to six months by through the introduction of Pyrazinamide (PZN) use. It is usually administered in the first two of the six months as a combination therapy with EMB, INH, RIF, and SM. It can eradicate MTB hidden in inflammatory locations and is not affected by other antibiotics (Heifets & Lindholm-Levy, 1989; Y. Zhang & Mitchison, 2003). Changes in the gene *pncA* has been linked to pyrazinamide resistance (Campbell *et al*., 2011; Palomino & Martin, 2014; Scorpio *et al*., 1997).

2.4 Mutations associated with second Line Anti-TB Drug Resistance

Genetic mutations have been identified that could be associated with the occurrence of resistance to antibiotics reserved for treatment of patients who have failed treatment to the second line antibiotics. These include *gyrA* and *gyrB* that are associated with fluoroquinolones and *rrs* associated with Amikacin. It therefore means that many antibiotics acquire resistance when genetic alterations build up in a strain of bacteria (Smit ., 2013).

2.4.1 Ofloxacin

Fluoroquinolones are bactericidal antibiotics that have been reserved for use for treating MDR-TB patients and as secondary therapy following relapse or non-compliance to initial therapy. Ciprofloxacin and ofloxacin are the commonly used antibiotics in this group (S Ramaswamy & Musser, 1998). Genetic variations in the part of the chromosomes referred to as quinolone-resistance-determining region (QRDR) have been known to induce antibiotic resistance in MTB that involves *gyrA* and *gyrB.* Work on additional antibiotics in this group, including moxifloxacin and gatifloxacin, have promising potential for use at initial disease diagnosis (Palomino & Martin, 2014).

2.4.2 Amikacin

Amikacin, AMK belongs to a class of antibiotics known as aminoglycosides that prevent protein production therefore preventing the natural functions of the bacterial ribosomes. There is a strong link to A1401G chromosomal change in the *rrs* gene that controls 16S ribosomal RNA (da Silva & Palomino, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

The research was a hospital based cross sectional study.

3.2 Study site

The study was carried out in five Public Heath Hospital Laboratories supported by East Africa Public Health Laboratory Networking Project (EAPHLN) namely Malindi, Kitale, Wajir, Busia and Machakos. These sites were selected on the basis of them being near border towns and which harbours indigenous hard to reach communities across the country. Machakos district hospital is located in high transmission area off Mombasa - Nairobi highway. Wajir district hospital in the north eastern region with large numbers of migrant or refugees; Kitale district hospital is located in high transmission area with possible cross border transmission by large numbers of migrants or refugees from Sudan and Uganda; Busia district hospital sits at the Kenya - Uganda border at the Mombasa Kampala highway which is a high transmission area with large numbers of traders, migrants and refugees; Malindi district hospital lies at the north coast with a large tourist population and business community and proximity to the north eastern migrants from Somalia.

3.3 Sample collection at sites

The samples were collected from the designated sputum collection sites at county hospital laboratories and transported to reference laboratories in Nairobi for molecular analysis. Patients gave sputum samples for routine TB tests of Fluorescence Microscopy and positive samples were reserved for pyrosequencing analysis. Samples were packaged as per the triple packaging standard operating procedures (Appendix I).

3.4 Study Population

Post routinely analyzed TB smear positive sputum samples were collected for sampling in the five county hospital laboratories. Samples that were reported to be smear-positive with Fluorescence Microscopy (FM) of at least 2+ and above were selected for pyrosequencing.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion Conditions

The study-included post routinely analysed TB smear positive sputum samples in the five county referral hospital laboratories. If the sample volume was 2 ml or more, the sample was incorporated in the study.

3.5.2 Exclusion Conditions

All TB smear negative sputum samples were excluded in this study. Samples less than 2 mL were only tested according to standard procedures of the participating laboratory and not included in this study.

3.6 Ethical Consideration

Approval to conduct this study was sought from KNH-UoN Ethical Review Committee (ERC). The DNA to be extracted amplified and sequenced were bacterial and no human genome was involved in the study. All samples were collected and packaged in the district hospitals and transported by courier overnight to the KAVI Institute of Clinical Research (KAVI – ICR) laboratories at the College of Health Sciences University of Nairobi laboratories in Nairobi for molecular analysis. No samples were shipped out of the country. To uphold confidentiality only numbers and no names were used to identify samples throughout the study. Written clearance was also sought from all five district hospitals administrations before inception of the study (Appendix III).

3.7 Sample size determination

Estimation of the sample size was done using fishers exact test formula (Fishers 1998):

$$n = \frac{Z^2 P (1- P) n = 1.96 x 1.96 x 0.242 x (1- 0.242)}{D^2}$$

$$\frac{}{0.0025}$$

n - minimum sample required

z–critical value =1.96

p -prevalence of TB in Kenya 2012 =242 per 100,000

D -margin of error =0.05

A total of 282 TB smears positive sputum samples were analyzed.

3.8 Sampling

National TB Program provided the prevalence rate for each county from which the minimum number of samples for each site was calculated (Table 3.1) (DLTLD annual report, 2013). The sum total prevalence rate for the five counties was 1446 was used as the denominator in as per the fishers exact test formula to determine the sample size per county.

Table 3.1: TB prevalence per 100,000 in the five counties in 2012

Malindi	**Wajir**	**Busia**	**Machakos**	**Kitale**
325	303	120	478	220

Adopted from (DLTLD annual report, 2013).

The study design being a prospective cross sectional study, convenience sampling technique was used. Post routinely analyzed TB smear positive sputum samples were collected until the required sample size was achieved for each county hospital.

The minimum number of samples collected per station was calculated based on prevalence of TB per county in 2012. The denominator used was the total number of the prevalence of the five counties, which was 1446 per 100,000 (Table 3.1).

The total sample size calculated was = 282

Malindi 325/1446*282= **63** smear positive sputum samples.

Wajir 303/1446*282= **60** smear positive sputum samples.

Busia 120/1446*282= **23** smear positive sputum samples.

Machakos 478/1446*282= **93** smear positive sputum samples.

Kitale 220/1446*282= **43** smear positive sputum samples.

Total minimum samples= **282**

The samples sizes were provided to each facility and were asked to provide samples as per the set criteria. At the close of business of the sampling days TB smear positive sputum samples submitted meeting the criteria surpassed the minimum with 63, 62, 26, 94, 43 from Malindi, Wajir, Busia, Machakos, and Kitale respectively. The total number of samples received was therefore 288.

3.10 Infection control measures

Personnel handling the specimens were trained on sample collection, packaging and processing. They were also provided with Personal Protective Equipment (N95 face masks, gloves and laboratory gowns) and all processes of the samples were done in BSL2 Biosafety cabinets to ensure safety to the staff and the environment.

The following Infection prevention precautions were adhered to at all times.

3.11 Sample collection, packaging and postage

Laboratory scientists working in the bacteriology section of the participating laboratories packed and sent the post-analysed smear positive sputum samples. For purposes of this study the samples were coded to protect patients' identity. Screw capped falcon tubes were used for sample collection and were be labeled with the study code, region codes and sample codes consecutively (Table 3.2).

Table 3.2: Sample labelling and identification scheme

Study code	Region codes	Sample codes
RMS	Kit	001 to 043
RMS	Bus	001 to 26
RMS	Mac	001 to 94
RMS	Mal	001 to 63
RMS	Waj	001 to 62

Triple packaging procedure was used (Appendix I) to pack samples sent to the laboratory by courier service. On reaching the laboratory, samples were stored in a refrigerator at $4^{o}C$ and analyzed within 24 hours

3.12 Laboratory Analysis

The TB diagnostic procedures were at two levels FM microscopy at the satellite laboratories and molecular analysis (DNA extraction, DNA amplification and Pyrosequencing) at the reference laboratories. Sputum samples were collected into standard collection cups or tubes. The volume of the samples was visually estimated and an approximate equal volume of

OMNIgene•SPUTUM reagent added to optimize the TB samples by liquefaction and decontamination while preserving viable MTB (Appendix IV). The mixture was then inverted vigorously and incubated at 15ºC to 25ºC for 15 minutes periodically inverting the sample. The sample kept at room temperature ready for transportation within 5 days.

3.12.1 FM Microscopy

Slides were placed on a rack to stain at least a centimeter from each other, and flooded with auramine-rhodamine dye and left to stain for 20 minutes. The dye was washed away with purified deionized water and tipped off to drain. The slides were then flooded with acid-alcohol 1 percent and for two minutes. The acid alcohol in the slide was then washed off with purified water and the slides flooded with 1 percent potassium permanganate for two minutes. The stain was finally washed off with purified water and the slides allowed to dry in a slide rack. The smears were then examined with a fluorescent microscope.

3.12.2 DNA Extraction

At the reference laboratory, sputum samples were centrifuged and the sediment were suspended in sufficient volume of sterile phosphate buffered saline enough to complete the standard laboratory test procedures in a biological safety cabinet. QIAsymphony SP/AS instrument automated extraction system for obtaining the DNA was used after decontaminating the sputa (Appendix V). Samples were homogenized intensely using a tube rotary shaker for 20 minutes. They were then placed in centrifuge cups and span for 20 minutes at 3000 revolutions per minute. The liquid at the top was discarded and the sediments placed in 2 ml cryovial tubes. Specimens were then subjected to a second centrifugation at 12000 revolutions per minute for 10 minutes. The resultant supernatants were discarded and the sediments loaded to the QIAsymphony SP/AS machine for DNA extraction. DNA extraction was automated by use of QIAamp DNA Mini Kit (250) on the QIAsymphony

SP/AS machine (Qiagen Technologies, 2013). An aliquot of 200ul of the test specimen was placed to every well of the testing plate and 400ml Mini Kit and the run identification was in put into the instrument's touch panel as per the instrument manual and the extraction automatically (Append VI).

3.12.3 DNA Amplification

The extracted DNA was then amplified using the Rotor gene Q thermo cycler. Polymerase Chain Reaction (PCR) was done by using 50 microliters µl consisting of 22.75 microliters nuclease free water, 10 microliters of 5X Q-buffer from Qiagen, 5 microliters of 10X buffer, 200µM of dDNTP, 1 microliter forward and reverse primers (10 pmol/µl) 2.5 U of hot star Taq-polymearse from Qiagen and 5 µl of template DNA. Each PCR run had negative controls of master mix with no sample. The PCR was set as 95°C for 15 minutes activation stage, 40 cycles of 30 seconds at 94°C, 2.5 minutes at 60°C, 1 minute at 72°C and 72°C for 5 minutes. The DNA products were then placed in freezer at negative 20^{o}C till ready for pyrosequencing.

3.12.4 Pyrosequencing

Pyrosequencing assays were designed using the latest version of PyroMark assay design software (ADSW) and shipped from Qiagen (see PyroMark® Q24 Advanced user manual Appendix V). One of the primers (opposite sequence primer) was each biotin labeled for immobilization to sepharose beads. Immobilization of the PCR products was done by preparation of the mix for n number of wells + two extra wells (Table 3). The immobilization mix was then added to the PCR strips. 10 µl of PCR product was add to each well plate/strips and agitated at room temp (15–25°C) for 5–10 min at 1400 rpm. Addition of 25 µl of the sequencing primer was done to the corresponding wells of a PyroMark Q24 plate. The plate was then placed on the vacuum tool and transferred through the tool according to instructions

in the pyromark user manual (Appendix V). The PyroMark Q24 plate was heated with the single stranded PCR product and sequencing primer at 80°C for 2 min using a heating block and a pre-warmed PyroMark Q24 plate Holder. The PyroMark Q24 plate was then removed from the plate holder and left on a second plate holder kept at room temperature for 15 minutes.

3.12.5 Loading of Cartridge and starting the run

The cartridge was cleaned before use by "milking" each reagent compartment. The PyroMark Q24 cartridge was loaded with the appropriate volumes of PyroMark Q24 reagents according to the pre run information sheet. The filled PyroMark Q24 cartridge was next placed into the instrument and the USB memory stick (containing the run file) inserted into the USB port at the front of the instrument. The PyroMark Q24 plate was placed into the Q24 instrument and the run started from the USB memory stick by pressing the "Run" key on the instrument (Table 3.3).

Table 3.3: Preparation of streptavidin sepaharose immobilization solution

Component	**Volume/sample (ul)**
PyroMark Binding Buffer	40
Water (H_2O)	29
Streptavidin Sepharose High Performance	1
Total Volume	70

The program automatically generated primer sets that included both PCR and sequencing primers (Table 3.4 and http://jcm.asm.org/content/50/6/2026#supplemental material).

3.12.6 Quality Assurance

A repeat of FM microscopy was done at the reference laboratory to confirm the smear results from the satellite laboratory before molecular analysis. Quality assurance was observed at all stages from pre analytical and post analytical stages by ensuring all staff are trained on proper sample labeling, collection and packaging. Prompt transportation and storage of samples was adhered to. Laboratory personnel were trained on standard molecular techniques. The reagents were checked to ensure they met the required standards and were within their expiry period. Internal controls were incorporated at all stages to ensure reliability of the results.

3.12.7 Mutant gene detection

The Primers were designed for the assays with the latest version of PyroMark assay design software and reference to an earlier study (Table 3.4). The mutant genes were tested as per the protocols from the Qiagen manual (Qiagen Technologies, 2013).

3.13 Data analysis

Pyrosequencing, rapid sequencing method based on ‘‘sequencing by synthesis’’ (Bravo, 2009), was used for mutation gene detection. The amplified products were sequenced using the same forward and reverse primers as for the PCR amplification. Nucleotide and amino acid sequences of the amplified fragments were aligned with the corresponding sequences of the reference strain MTB H37Rv as the internal sequencing control using the BLAST 2 software on the website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The mutations found were compared to those included in the TB Drug Resistance Mutation Database (mutation.tbdreamdb.com) (Sandgren., 2009).

Table 3.4: Primers used for detection of mutant genes

Drug	Gene	Primer	Sequences (5'-3')	Annealing temperature (°C)	Size (bp)	Target loci
RIF	*rpoB*	Forward	GTCCGGGAGCGGATGACCACCC	65	204	RRDR
		Reverse*	GCTCACGTGACAGACCGCCG			
		Sequencing 1	GCGATCAAGGAGTTC			
		Sequencing 2	TCATGGACCAGAACAA			
INH	*katG*	Forward	AGATGGGCTTGGGCTGGA	61	133	315
		Reverse*	TAGCCGTACAGGATCTCGAGGA			
		Sequencing	CCGGTAAGGACGCGA			
SM	*rpsl*	Forward*	CAAGGGTCGTCGGGACAAGA	61	299	
		Reverse	TCTTGACACCCTGCGTATCC			
		Sequencing 1	CGCCGAGTTCGGCTT			43
		Sequencing 2	CACACCAGGCAGGTC			88
EMB	*embB*	Forward	ACGACGGCTACATCCTGG	61	110	306
		Reverse*	GTTGTAATACCAGCCGAAGGGA			
		Sequencing	CGACGGCTACATCCTG			
OFL	*gyrA*	Forward	TTCGATTCCGGCTTCCGCCC	61	192	QRDR
		Reverse*	TGGGTCATTGCCTGGCGAGC			
		Sequencing	TACCACCCGCACGGC			
AMK	*rrs*	Forward	TCCTTAAAAGCCGGTCTCAGTTC	61	239	1401
		Reverse*	TCCGGTACGGCTACCTTGTTA			
		Sequencing	CTTGTACACACCGCC			

*: The primer was labelled at the 5-end with biotin.

Adopted from (Zheng *et al*., 2014)

Sequencing of *rpoB, katG, embB gyrA, rpsl* and *rrs* genes, respectively were performed, for mutations and alignment were identified using the NCBI 2 website services. Obtained sequences were then reverse complemented using the online reverse complement tool at https://www.bioinformatics.org/sms/rev_comp.html. The online mapping tool Clustalw at https://www.genome.jp/tools-bin/clustalw was used to map the short reads obtained from the pyro sequencer to the reference gene sequence MTB H37Rv. MEGA software was used to view the alignments. The results obtained were then compared with the known Drug resistant strains in the TB Drug Resistance Mutation Database (Sandgren *et al.*, 2009) (www.tbdreamdb.com).

3.14 Dissemination of results

The results were shared with health care corkers (HCWs) and administrators of the participating hospitals and will be published in peer reviewed journals as well as presentations in scientific conferences. The data obtained from this study was to enable better understanding of mutations associated with TB drug resistance in Kenya and help to improve diagnostic and treatment capacities of TB. The data will also inform the ministry of health division of TB control on diagnostic policy decisions on TB management of multi drug resistant TB.

3.15 Study limitations

Due to limited resources the samples were collected from only five county hospitals. A countrywide study is recommended in future to give the actual picture of the national TB drugs resistance patterns in Kenya.

CHAPTER FOUR

4.0 RESULTS

4.1 Samples assayed

From Busia 26 samples were received, Kitale 43 samples, Machakos 94, Malindi 63 samples and 62 samples from Wajir all 288 sputa (Table 4.1). All 288 sputum samples were subjected to pyrosequencing where 2304 assays to detect resistance in the six drugs.

Table 4.1: Number of samples assayed for each target gene

COUNTY	**AMK**	**EMB**	**INH**	**OFL**	**SM**		**RIF**	
	rrs	*embB*	*katG*	*gyrA*	*rpsl43*	*rpsl88*	*rpoB510*	*rpoB526*
Busia	26	26	26	26	26	26	26	26
Kitale	43	43	43	43	43	43	43	43
Machakos	94	94	94	94	94	94	94	94
Malindi	63	63	63	63	63	63	63	63
Wajir	62	62	62	62	62	62	62	62
Total	288	288	288	288	288	288	288	288
Overall no of assays								2304

Key: AMK- Amikacin, EMB- Ethambutal, INH Isoniazid, OFL- Ofloxacin SM- Streptomycin, RIF- Rifampicin. Gene Primers: *rrs*- 16S rRNA gene, *embB*- arabinosyltransferase B, *katG* - catalase-peroxidase, *gyrA*- DNA gyrase subunit A, *rpsl43*- 30S ribosomal protein S12, rpsl88- *rpsl*- 30S ribosomal protein S12, *rpoB510*- RNA polymerase 510, *rpoB526*- RNA polymerase.

Of the 2304 pyrosequencing assays a total of 43 assays where positive for mutations (Table 4.2). Mutations were detected in 80% (4/5) of the county hospital facilities with no mutations in samples from Kitale. The highest number of mutations were detected in samples from Machakos 44% (12/27); samples from Malindi 30% (8/27); then Busia 15% (4/27) and samples from Wajir had 11% (3/27).

Table 4.2: Mutations per assay

County	Assays with mutations	Assays with negative mutations	Total Assays Done
Busia	5	203	208
Kitale	0	344	344
Machakos	20	732	752
Malindi	15	489	504
Wajir	3	493	496
Grand Total	43	2261	2304

4.1.1 Mutations associated with AMR from TB smear positive samples

Mutations associated with drug resistance genes were reported in 12.2% of the samples. The highest mutation reported was from Rifampicin (8%). There was no resistance to ofloxacin from the samples analysed. A summary of drug resistance is shown on Table 4.3.

Table 4.3: Summary of AMR associated mutations

Drug	*Gene*	Samples with mutations (n=288)	% of mutation
Rif	*rpoB*	23	8.0
INH	*katG*	1	0.3
SM	*Rpsl*	8	2.8
EMB	*embB*	2	0.7
AMK	*rrs*	1	0.3
OFL	*gyrA*	0	0.0
Total		35	12.2

Key: RIF- RIF- Rifampicin INH- Isoniazid, SM- Streptomycin, EMB- Ethambutol AMK- OFL- Ofloxacin, and AMK- Amikacin.

4.1.2 Mutations associated with first line anti-TB drugs

On the RRDR hot spot of the *rpoB* gene, 8% mutation was detected in the samples from codons 511 to 533 multiple (Table 4.4). Codon change CTG/CCG representing amino acid change L511H had the highest frequency of mutations occurring in 12 of the 36 times the mutations were reported on the gene (Table 4.4). The lowest frequency was on the codon

represented by the amino acid change D516V that occurred twice. There was mutations on 2.8% of the samples (8/288) on *rpsl* gene associated with SM, 0.3% (1/288) of the samples on the *katG* gene associated with INH and also mutations 0.7% (2/288) of the samples on the *embB* gene associated with EMB.

Table 4.4: Mutations on *rpoB, embB, katG, rpsl43,* and *rpsl88* genes

Drug	Amino Acid change(s)	Gene/Primer	Codon changes	Frequency
RIF	D518S	*rpob510*	AAC/AGC	9
	D516V	*rpob510*	GAC/GTC	2
	L511P	*rpob510*	CTG/CCG	12
	Q513H	*rpob510*	CAA/CAC	4
	S531L	*rpob510*	TCG/TTG	5
	L533P	*rpob526*	CTG/CCG	4
EMB	M306I	*embB*	ATG/ATC	1
	M306V	*embB*	ATG/GTC	1
INH	S315T	*katG*	AGC/ACC	1
SM	K43R	*rpsl43*	CTT/CCT	8
	K88R	*rpsl88*	CTT/CCT	8

Key: RIF- RIF- Rifampicin EMB- Ethambutol INH- Isoniazid SM- Streptomycin, rpoB- katG- embB- embB- arabinosyltransferase B, rpsl43- 30S ribosomal protein S12, rpsl88- 30S ribosomal protein S12

4.1.3 Mutations associated with second line anti-TB drugs

Mutation on the *rrs* gene associated with AMK was detected on 1 sample (0.3%). There were no mutations associated on *gyrA* gene associated with OFL (Table 4.5).

Table 4.5: Mutations of *rrs* and *gyrA* genes associated with second line anti-MTB

Drug	Amino Acid change(s)	Gene	Codon changes	Frequency
AMK	A1401G	*rrs*	CAC/CGC	1
OFL	None	*gyrA*	None	0

4.1.4 Multi-locus mutations and multidrug directed mutations

Rifampicin and Streptomycin demonstrated multi-locus mutations on the different samples analysed. Eight of the samples had multi-locus mutations of at least two loci on the same

gene *rpoB* and *rpsl* (Table 4.4). Rifampicin showed 4 cases of multiple mutations (Table 4.6) while Streptomycin showed 8 instances of multi-locus mutations (Table 4.4). Two of the four cases with RIF resistant mutations had three codon changes on codons 511, 513 and 518 as well as 513, 516 and 518 respectively (Table 4.7) and (Figure 4.1). Seven samples demonstrated multi drug mutations with 4 samples showing mutations directed towards both RIF and SM (Table 4.6). Two of the samples had multi drug directed mutations towards RIF and AMK; RIF and INH (Table 4.6). One sample demonstrated mutations on 3 genes associated with RIF, EMB and SM (Table 4.6). Two samples had double codon changes on *rpoB* and on *rpsl* (Table 4. 6). The detailed information of sequences was provided in the Appendix VI.

RIF had the highest activity of mutation with several combination of mutations the highest being CTG/CCG and AAC/AGC occurring the highest number of times.

Table 4.6: Count of mutations per samples

County	Sample no	Drug	Count of
Machakos	mac007	RIF	1
	mac008	RIF	1
	mac009	RIF	1
	mac010	RIF	1
		SM	2
	mac013	RIF	2
		EMB	1
		SM	2
	mac014	RIF	2
		SM	2
	mac015	RIF	1
	mac016	RIF	1
	mac017	EMB	1
	mac018	SM	2
	mac020	RIF	1
	mac022	RIF	1
Malindi	mal001	SM	2
		RIF	1
	mal002	SM	2
		RIF	1
	mal004	SM	2
	mal005	SM	2
	mal006	RIF	1
	mal007	RIF	1
	mal008	RIF	1
		AMK	1
	mal009	RIF	1
Wajir	waj001	RIF	1
	waj003	RIF	1
	waj005	RIF	1
Busia	bus001	RIF	1
		INH	1
	bus003	RIF	1
	bus004	RIF	1
	bus005	RIF	1

Table 4.7: Multi-locus mutations of *rpoB* and *rpsl* genes

Drug	Gene	Codon changes	Frequency
RIF	*rpoB*	CTG/CCG + AAC/AGC	5
		CTG/CCG + CAA/CAC+AAC/AGC	1
		CAA/CAC + AAC/AGC	1
		CAA/CAC + GAC/GTC+AAC/AGC	1
		GAC/GTC + AAC/AGC	1
SM	*rpsl* (K43) & (K88)	AAG/AGG + AAG/AGG	8

4.1.5 Alignment of sequences

An alignment of the sequences obtained was compared to the sequences from the NCBI Gene Bank. The standard sequences were downloaded from the Gene bank.

4.1.5.1 Sequence analysis of rpob510

The reference sequence downloaded from the NCBI gene bank compared with the standard DNA used and was used to obtain the alignment to the samples (Figure 4.1). 272 samples had similar sequences as those of the *MTB* H37Rv. Six samples; five and one other samples each had unique mutations (Figure 4.1).

4.1.5.2 Sequence analysis of *rpob526*

Results from the second primer for *rpob* gene showed 279 samples similar to the reference sequence while four and five samples had different mutations (Figure 4.2).

4.1.5.3 Sequence analysis of *katG*

Analysis of the sequences for *katG* indicated 287 samples had similar to the reference sequence only one sample had a single mutation (Figure 4.3).

4.1.5.4 Sequence analysis of *rpsl*

Analysis by both rpsl43 and rpsl88 primers obtained mutations of 8 like samples with multi-locus mutations of the *rpsl* genes (Figure 4.4 and 4.5).

4.1.5.5 Sequence analysis of *embB*

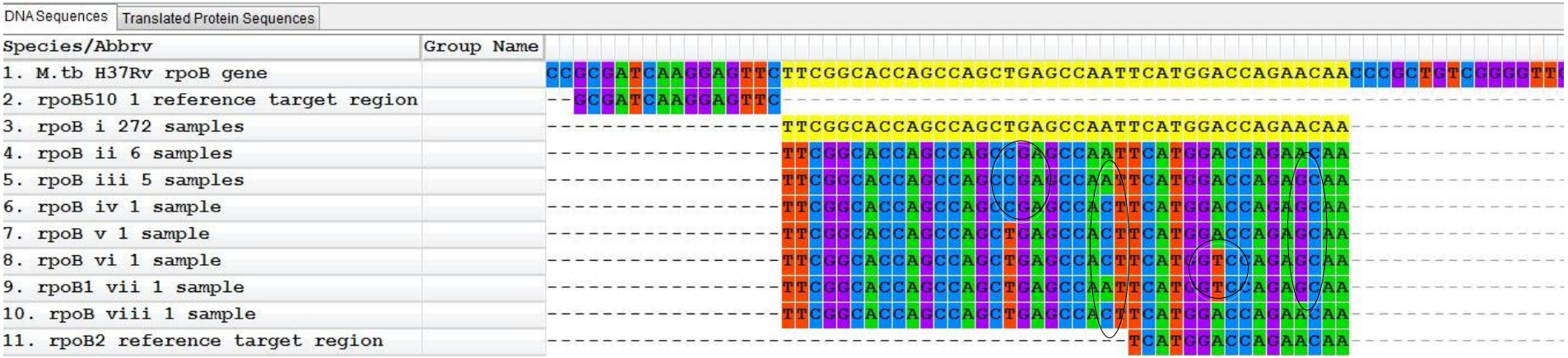
Sequences for *embB* had 287 samples with sequences similar to that of the reference sequence and only one showed a single point mutation (Figure 4.6).

4.1.5.6 Sequence analysis of *rrs*

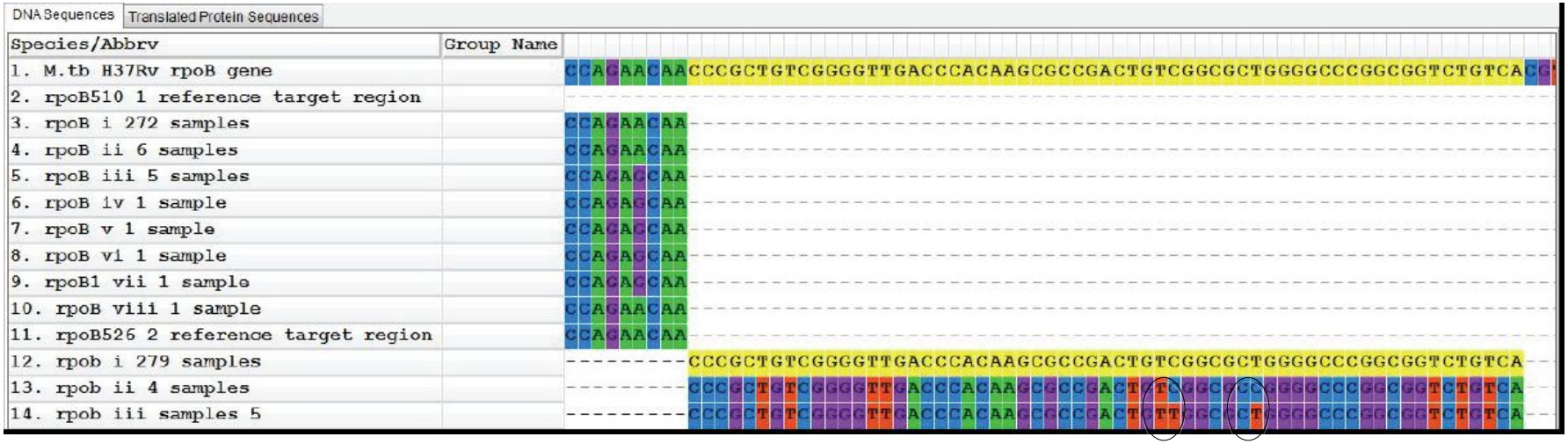
The rrs primers obtained only one sample with different sequence to the reference sequence (Figure 4.7).

4.1.5.7 Sequence analysis of *gyrA*

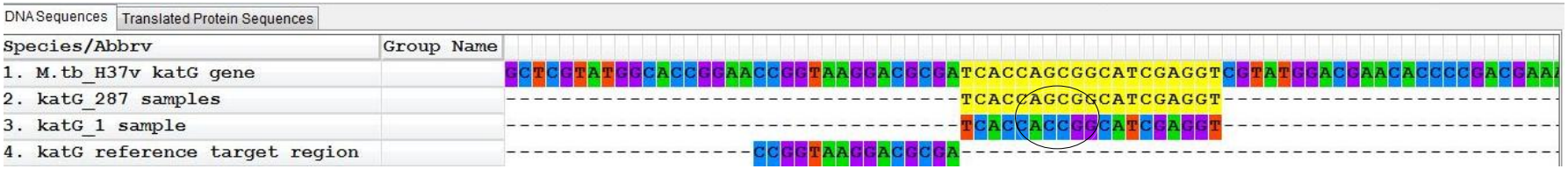
All the samples produced sequences similar to that of the *gyrA* reference sequence. (Figure 4.8).



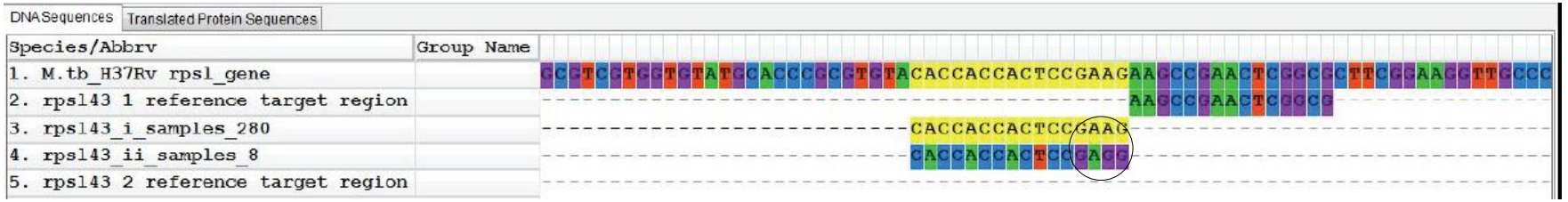
[1] Figure 4.1.Sequence analysis of *rpob510.* Line 1: M. tb H37Rv *rpoB* gene reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: Target reference region for primer rpoB510, line 3 272 samples for with no mutation, lines 4,5&6: samples with CTG/CCG mutation, lines 6,7,8 & 10 CAA/CAC, lines 8 & 9 samples with GAC/GTT mutations, lines 5,6,7,8&9 AAC/AGC mutations



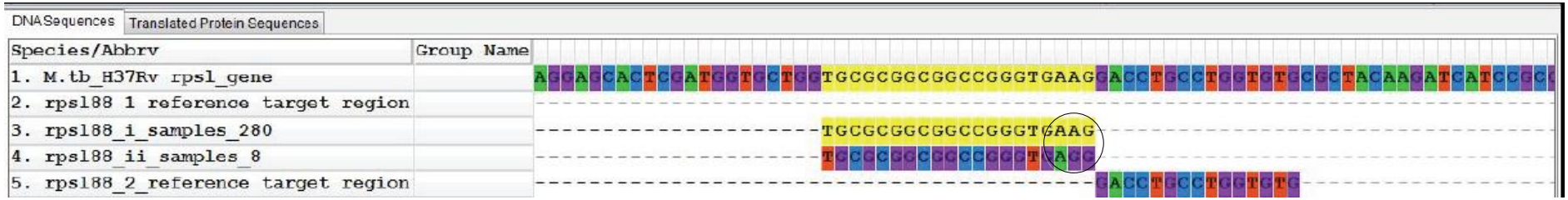
[2] Figure 4.2. Sequence analysis of *rpob526.* Line 12: 279 samples with no mutations, line 13: samples with GTC/GTC mutations, line 14, samples with GCC/GCT mutations



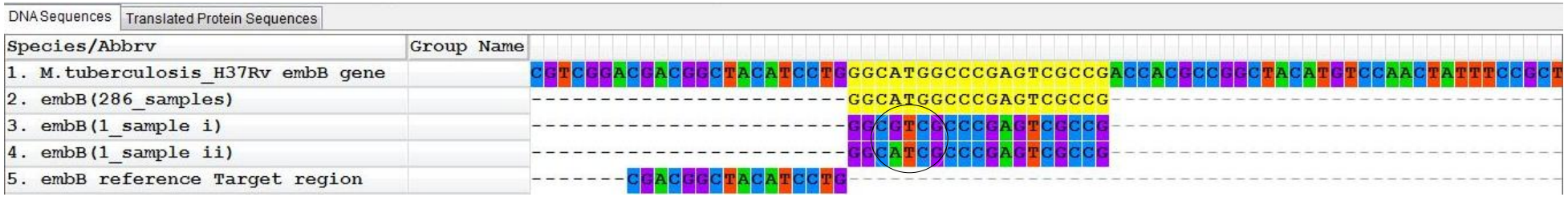
[3] Figure 4.3: Sequence analysis of *katG*. Line 1. Line 1: M. tb *katG* gene reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: sample with AGC/ACC mutations, line 4: *katG* reference target region.



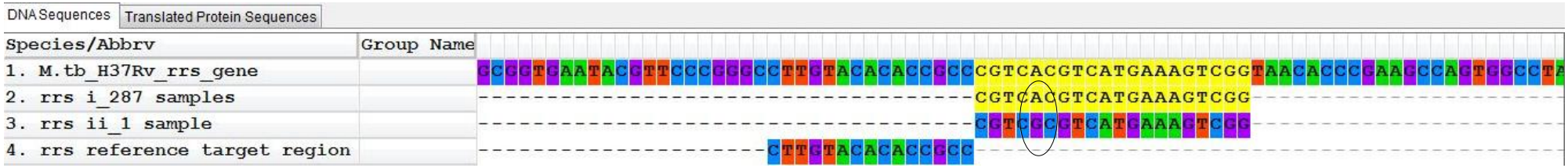
[4]Figure 4.4. Sequence analysis of *rpsl4*. Line 1: M. tb H37Rv *rpsl43* reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: *rpsl* reference target region, line 3 samples with no mutation, line 4: samples with AAG/AGC mutation



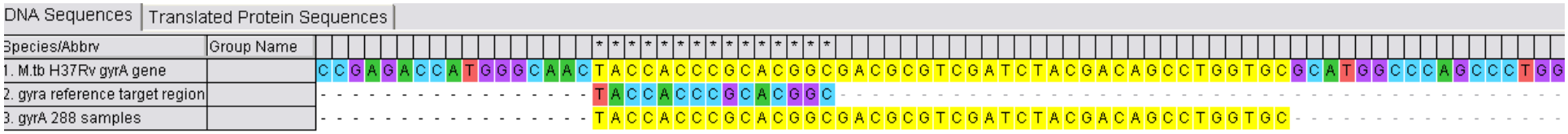
[5]Figure 4. 5. Sequence analysis of *rpsl88.* Line 1: M. tb H37Rv *rpsl88* reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: *rpsl* reference target region, line 3 samples with no mutation, line 4: samples with AAG/AGG mutation



[6] Figure 4.6. Sequence analysis of *embB*. Line 1: M. tb H37Rv *embB* reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: samples with mutation, line 3: sample with ATG/GTC mutations, line 4: samples with ATG/ATC mutations, line 5 *embB* reference target region.



[7]Figure 4.7: Sequence analysis *rrs.* Line 1: M. tb H37Rv *rrs* reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: samples with no mutation, line 3: sample with CAC/CGC mutations, line 4: samples with ATG/ATC mutations, line 5 *embB* reference target region.



[8]Figure 4.8: Sequence analysis *gyrA.* Line 1: M. tb H37Rv *gyrA* reference gene sequence of the standard - *Mycobacterium tuberculosis* (strain ATCC 25618 / H37Rv), line 2: *gyra* reference target region, line 3: all samples with no mutation

CHAPTER FIVE

5.0 DISCUSSION

5.1 Mutations associated with first line antiTB drugs

5.1.1 Mutations associated with RIF

It was observed that there was at least a gene mutation associated with all the four first-line antimicrobial drugs tested in the study. The largest number of mutations was observed in the *rpoB* gene that occurred in the 81-nucleotide rifampicin resistance-determining region (RRDR) considered the "hot spot region" comprising codons 507 to 533. Pyrosequencing was able to pick 36 mutations within the RRDR from codon 511 to 533 of which 75% were on codons from 511 to 518 and 25% on codons 531 and 533 of the *rpoB* gene. These mutations had previously been described in other studies (Ajbani *et al*., 2018; Rodwell *et al*., 2014; Stinear *et al*., 2015; Tan *et al*., 2012; Tang *et al*., 2013)**.** The mutations observed were L511P, Q513H, D516V, S531L, L533P which have previously been encountered among rifampicin resistant isolates worldwide (Fan *et al*., 2003; Tavanaee Sani, Ashna, Kaffash, Khaledi, & Ghazvini, 2018). However a study in Canada identified L511P mutations within the RRDR associated with RIF-susceptible isolates (Jamieson *et al*., 2014) while in another study they were associated with discordant susceptibility (Campbell *et al*., 2011). In this study mutations known to cause discordant results in RIF resistance in a previous study were detected at the extreme ends of the RRDR including codons 533 CTG/CCG representing CTG/CCG and five samples with TCG/TTG mutations coding for amino acid change S531L which would require further studies including counter checking with diagnostic sensitivity testing (DST) by culture. According to a study by Rigouts and others some RIF resistance were detected by pyrosequencing that were missed by liquid culture DST (Rigouts *et al*., 2013).

5.1.2 Mutations associated with SM

Eight samples had mutations on the *rpls* gene associated with SM resistance. Mutations were picked by the two primers rpsl43 and rpsl88 targeting each of the point mutations in codons K43R and K88R of the genes associated with SM resistance. All the samples that had mutations on *rpsl* had multiple mutations on both regions as detected by the two primers in contrast to a study done by Siddiqui and colleagues that showed that rpsl43 picked more mutations than rpsl88 (Siddiqui & Qureshi, 2014). Both mutations are the predominant genetic alterations present and have previously been reported in streptomycin resistance MDR-TB (Cuevas-Córdoba *et al*., 2013; Tudo *et al*., 2010) however in majority of the studies codon K43R is associated with a high frequency of point mutations seen in SM resistant isolates than that exhibited by K88R (Zheng *et al*., 2014).

5.1.3 Mutation associated with EMB and INH

In this study two 0.7% (2/288) of the samples were found with EMB resistance associated mutations within the *embB* gene. The two samples expressed amino acid change at codon 306 with different nucleotide base substitution; one sample showed nucleotide alteration from G to C with an amino acid substitution from Methionine to Isoleucine. Additionally the other sample had a double mutation with transition from A to G and G to C with amino acid substitution from Methionine to Valine. Both samples were from one county, samples from the other counties did not show any mutation on the *embB* gene generally indicating regional differences in the rate of mutation within the different counties. *EmbB* gene mutations specifically at codon 306 have been reported in previous studies as well (Hazbón *et al*., 2005; Perdigão, Macedo, Ribeiro, Brum, & Portugal, 2009; Sreevatsan *et al*., 1997). It has been highly considered as a molecular marker to EMB resistance (Cuevas-Córdoba *et al*., 2015; Sreevatsan *et al*., 1997) and as an pointer of possible resistance to other first line antimicrobials including INH and RIF with increased risk to development of MDR-TB

(Guerrero *et al*., n.d., 2013; Hazbón *et al*., 2005; Li *et al*., 2016; Rezaei *et al*., 2016; Shen *et al*., 2007). In this study only one samples had multiple mutations on *embB* in addition to *rpoB* gene and *rpsl gene*. However this may not be in concordance with other studies that have detected *embB* mutation on susceptible and pan-susceptible isolates (Cuevas-Córdoba *et al*., 2015; Mokrousov, Otten, Vyshnevskiy, & Narvskaya, 2002; Van Rie *et al*., 2001) suggesting that the *embB*306 mutants may not be an indicator of EMB resistance but can predispose to occurrence of drug resistant (Safi, Sayers, Hazbón, & Alland, 2008). Other genes, such as *embC* and *embA* might be useful for testing in combination with *embB* for EMB resistance (Brossier *et al*., 2015; Starks, Gumusboga, Plikaytis, Shinnick, & Posey, 2009; Sun *et al*., 2018; L.-L. Zhao *et al*., 2015). However of interest to note is the fact that after sequencing approximately 12 genes that were associated with EMB resistance, Ramaswamy and colleagues found that approximately 1/4 of the analysed isolates did not have a resistance associated mutation suggestive of a naturally occurring genetic mutation that does not confer resistance (Srinivas V Ramaswamy *et al*., 2000). More supportive data from analysis of other loci involved in EMB resistance or additional mutations within the Ethambutol resistance determining region is needed to substantially ascertain which mutations confer EMB resistance. Alternatively combining two tests to confirm resistance of the *embB* mutants will be necessary in such a case to ensure confidence in patient management (Cheng *et al*., 2014; Park *et al*., 2018). In this study 0.3% (1/288) of the samples had mutations associated with INH resistance. The mutation detected was AGC/ACC. This mutation was found in a sample from one a facility right at the Kenya Uganda border, Busia.

5.2 Mutations associated with second line anti-TB drugs

Only one sample exhibited mutation on *rrs* gene associated with AMK, an injectable second line anti-TB drug. This mutant type exhibited mutation in the *rrs* gene encoding the 16S rRNA subunit (Honoré, Marchal, & Cole, 1995). In position 1401 within the *rrs* gene the

amino acid substitution was from A to G. Similar mutation have been reported in previous studies and have shown that this mutation is a marker for high level resistance in AMK as well for KAN resistance (Jugheli *et al*., 2009; Kambli *et al*., 2016; Z. Zhang *et al*., 2014).

The samples analysed in this study were negative for mutations on the *gyrA* gene for codons representing amino acids change from A90V to A94G associated with OFL resistance. The most known mutation is GAC94GGC but this too was not found in the samples analysed confirming that second line drug are still very useful for treatment of TB. However detection of mutation outside the QQRDR region and other genes known to code for FQ resistance is necessary. The other gene known to be associated with development of resistance is *gyrB* gene but this was not tested in this study. However this may be of minimal value since in a study done in Russia mutations in the *gyrA* QRDR were identified in a high proportion of the ofloxacin-resistant strains (Campbell *et al*., 2011). At the same time, *gyrB* mutations were detected in a minor but non-negligible proportion of the strains without *gyrA* mutations (Campbell *et al*., 2011; Mokrousov *et al*., 2008). The low level of mutation observed in these set of antibiotics could be because they are rarely prescribed and are reserved for those who seek re-treatment and thus are less prone to exposure to the MTB and natural selection of resistant strains are less.

5.3 Multi-locus mutations

This study detected multi locus mutations on *rpoB* and *rpsl* genes where at least seven of the samples had multi-locus mutations on more than two loci on the same gene *rpoB* and *rpsl* (Figure 4.1 and 4.2) and (Figure 4.5 and 4.6). Two samples had mutations changes at 3 loci CTG/CCG + CAA/CAC+AAC/AGC and CAA/CAC + GAC/GTC+AAC/AGC. On SM there were simultaneous mutations on both *rpsl43* and *rpsl88* of the seven samples. The study was able to gather data from samples within a short time combined with multi-locus sequencing

of several isolates in parallel that made pyrosequencing an effective method for drug resistance screening of MTB as described in a previous studies (Engström *et al*., 2012). Studies of possible multi-locus mutations have shown that MTB is prone to have multiple loci with resistance related mutations in the same samples, which may exacerbate the chances of treatment failure (Tan ., 2012).

5.4 Multi Drug Resistance

One sample from Busia had a mutation on *rpoB* and on *katG* that fits the definition of an MDR subject to confirmation by culture. None of the samples met the definition of a XDR-TB. Almost 7% of TB cases in Kenya had MDR TB in 2012 and only 0.3% in this study were detected (National Tuberculosis Program Report, 2014). In 2016, NTLTD reported that MDR TB among the repeat treatments and fresh cases was 2.1% and 0.7% respectively (National Tuberculosis Leprosy and Lung Disease Program, 2016). Further, over 400 MDRT cases were reported with 360 picked by GeneXpert as RIF resistant.

5.5 Pyrosequencing technology

Sequencing technology is a rapid molecular technology that enables the detection of resistance conferring mutations. Pyrosequencing takes from DNA extraction to the availability of results, was accomplished within 6 hours (S. Y. G. Lin *et al*., 2014). Based on our analysis the pyrosequencing technique was quite useful in detection of mutations associated with drug resistant strains considering the turn around time in disseminating the results to clinicians. In this context a study in Uganda also recommended the use of this tool in detection of rifampicin/isoniazid-resistant in *M. tuberculosis* isolates (Kigozi *et al*., 2018).

CHAPTER SIX

6.0 CONCLUSION AND RECOMENDATIONS

6.1 Conclusion

Mutations associated with drug resistance were detected in both first and second line anti-TB drugs. Majority of the mutations occurred in the *rpoB* gene coding for rifampicin resistance. Only one mutation was detected on the *rrs* genes associated with amikacin, one of the two-second line antibiotics tested. There was no mutation on the *gyrA* gene coding for ofloxacin in all the samples tested.

6.2 Recommendations

This study demonstrated that pyrosequencing assay is a useful test that could be incorporated in TB research and in reference laboratories for the detection and confirmation of TB drug resistance. This NAAT will be useful in detecting new mutations associated with first line and second line anti TB drugs. Detection of new mutations simultaneously in assays reduces the turnaround time from testing to releasing of results. A countrywide study is recommended in the future to give a more detailed picture of the national MTB mutation associated with drug resistance in Kenya.

REFERENCES

Ajbani, K., Kazi, M., Tornheim, J., Naik, S., Soman, R., Shetty, A., & Rodrigues, C. (2018). Pyrosequencing to resolve discrepant Xpert MTB/RIF and mycobacterial growth indicator tube 960. *Lung India*, *35*(2), 168–170. https://doi.org/10.4103/lungindia.lungindia_71_17

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., … Jacobs, W. R. (1994). inhA, a gene encoding a target for isoniazid and ethionamide in Mycobacterium tuberculosis. *Science*. https://doi.org/10.1126/science.8284673

Brossier, F., Sougakoff, W., Bernard, C., Petrou, M., Adeyema, K., Pham, A., … Veziris, N. (2015). Molecular Analysis of the embCAB Locus and embR Gene Involved in Ethambutol Resistance in Clinical Isolates of Mycobacterium tuberculosis in France. *Antimicrobial Agents and Chemotherapy*, *59*(8), 4800–4808. https://doi.org/10.1128/AAC.00150-15

Campbell, P. J., Morlock, G. P., Sikes, R. D., Dalton, T. L., Metchock, B., Starks, A. M., … Posey, J. E. (2011). Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *55*(5), 2032–2041. https://doi.org/10.1128/AAC.01550-10

CDC. (2009). Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. *MMWR. Morbidity and Mortality Weekly Report*, *58*(1), 7–10. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19145221

CDC Reference Laboratory Division of TB Elimination. (2012). Laboratory User Guide for U.S. Public Health Laboratories: Molecular Detection of Drug Resistance (MDDR) in Mycobacterium tuberculosis Complex by DNA Sequencing (Version 2.0), 1–10. Retrieved from https://www.cdc.gov/tb/topic/laboratory/mddrusersguide.pdf

Chan, T., Huang, H., Jou, R., Yang, Y., Lu, P., Huang, S., & Chang, T. (2011). Detection of gene mutations causing antibiotic resistance in Mycobacterium tuberculosis complex by an array. *Clinical Microbiology and Infection*.

Chaoui, I., Sabouni, R., Kourout, M., Jordaan, A. M., Lahlou, O., Elouad, R., … El Mzibri, M. (2009). Analysis of Isoniazid, Streptomycin and Ethambutol resistance in Mycobacterium tuberculosis isolates from Morocco. *Journal of Infection in Developing Countries*, *3*(4), 278–284.

Cheng, S., Cui, Z., Li, Y., & Hu, Z. (2014). Diagnostic accuracy of a molecular drug susceptibility testing method for the antituberculosis drug ethambutol: a systematic review and meta-analysis. *Journal of Clinical Microbiology*, *52*(8), 2913–2924. https://doi.org/10.1128/JCM.00560-14

Colangeli, R., Helb, D., Vilchèze, C., Hazbón, M. H., Lee, C. G., Safi, H., … Alland, D. (2007). Transcriptional regulation of multi-drug tolerance and antibiotic-induced responses by the histone-like protein Lsr2 in M. tuberculosis. *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.0030087

Cucinotta, F. A. (2014). Article @ Journals.Plos.Org. *Plos One*. Retrieved from http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0031409

Cuevas-Córdoba, B., Cuellar-Sánchez, A., Pasissi-Crivelli, A., Santana-Álvarez, C. A., Hernández-Illezcas, J., & Zenteno-Cuevas, R. (2013). rrs and rpsL mutations in streptomycin-resistant isolates of Mycobacterium tuberculosis from Mexico. *Journal of Microbiology, Immunology and Infection*, *46*(1), 30–34. https://doi.org/10.1016/J.JMII.2012.08.020

Cuevas-Córdoba, B., Juárez-Eusebio, D. M., Almaraz-Velasco, R., Muñiz-Salazar, R., Laniado-Laborin, R., & Zenteno-Cuevas, R. (2015). Mutation at embB codon 306, a potential marker for the identification of multidrug resistance associated with ethambutol in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *59*(9), 5455–5462. https://doi.org/10.1128/AAC.00117-15

da Silva, P. E. A., & Palomino, J. C. (2011). Molecular basis and mechanisms of drug resistance in Mycobacterium tuberculosis: Classical and new drugs. *Journal of Antimicrobial Chemotherapy*, *66*(7), 1417–1430. https://doi.org/10.1093/jac/dkr173

DLTLD annual report. (2013). *Division of Leprosy, Tuberculosis and Lung Disease Annual report 2012*. Nairobi.

Engström, A., Morcillo, N., Imperiale, B., Hoffner, S. E., & Juréen, P. (2012). Detection of first- and second-line drug resistance in Mycobacterium tuberculosis clinical isolates by pyrosequencing. *Journal of Clinical Microbiology*, *50*(6), 2026–2033. https://doi.org/10.1128/JCM.06664-11

Fakruddin, M., & Chowdhury, A. (2012). Pyrosequencing-an alternative to traditional Sanger sequencing. *American Journal of Biochemistry and Biotechnology*, *8*(1), 14–20. https://doi.org/10.3844/ajbbsp.2012.14.20

Fan, X.-Y., Hu, Z.-Y., Xu, F.-H., Yan, Z.-Q., Guo, S.-Q., & Li, Z.-M. (2003). Rapid detection of rpoB gene mutations in rifampin-resistant Mycobacterium tuberculosis isolates in shanghai by using the amplification refractory mutation system. *Journal of Clinical Microbiology*, *41*(3), 993–997. https://doi.org/10.1128/JCM.41.3.993-997.2003

Goude, R., Amin, A. G., Chatterjee, D., & Parish, T. (2009). The arabinosyltransferase EmbC is inhibited by ethambutol in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *53*(10), 4138–4146. https://doi.org/10.1128/AAC.00162-09

Guerrero, E., Lemus, D., Yzquierdo, S., Vílchez, G., Muñoz, M., Montoro, E., & Takiff, H. (n.d.). Association between embB mutations and ethambutol resistance in Mycobacterium tuberculosis isolates from Cuba and the Dominican Republic: reproducible patterns and problems. *Revista Argentina de Microbiologia*, *45*(1), 21–26.

Guerrero, E., Vílchez, G., Muñoz, M., Takiff, H., Lemus, D., Yzquierdo, S., & Montoro, E. (2013). Association between embb mutations and ethambutol resistance in mycobacterium tuberculosis isolates from cuba and the dominican republic: Reproducible patterns and problems. *Revista Argentina de Microbiologia*.

Hazbón, M. H., Bobadilla del Valle, M., Guerrero, M. I., Varma-Basil, M., Filliol, I., Cavatore, M., … Alland, D. (2005). Role of embB codon 306 mutations in Mycobacterium tuberculosis revisited: a novel association with broad drug resistance and IS6110 clustering rather than ethambutol resistance. *Antimicrobial Agents and Chemotherapy*, *49*(9), 3794–3802. https://doi.org/10.1128/AAC.49.9.3794-3802.2005

Heifets, L., & Lindholm-Levy, P. (1989). Comparison of bactericidal activities of streptomycin, amikacin, kanamycin, and capreomycin against Mycobacterium avium and M. tuberculosis. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.33.8.1298

Honoré, N., Marchal, G., & Cole, S. T. (1995). Novel mutation in 16S rRNA associated with streptomycin dependence in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *39*(3), 769–770.

Jamieson, F. B., Guthrie, J. L., Neemuchwala, A., Lastovetska, O., Melano, R. G., & Mehaffy, C. (2014). Profiling of rpoB mutations and MICs for rifampin and rifabutin in Mycobacterium tuberculosis. *Journal of Clinical Microbiology*, *52*(6), 2157–2162. https://doi.org/10.1128/JCM.00691-14

Jugheli, L., Bzekalava, N., Rijk, P. de, Fissette, K., Portaels, F., & Rigouts, L. (2009). High Level of Cross-Resistance between Kanamycin, Amikacin, and Capreomycin among Mycobacterium tuberculosis Isolates from Georgia and a Close Relation with Mutations in the rrs Gene. *Antimicrobial Agents and Chemotherapy*, *53*(12), 5064. https://doi.org/10.1128/AAC.00851-09

Kahla, I. B., Henry, M., Boukadida, J., & Drancourt, M. (2011). Pyrosequencing assay for rapid identification of Mycobacterium tuberculosis complex species. *BMC Research Notes*, *4*(1), 423. https://doi.org/10.1186/1756-0500-4-423

Kambli, P., Ajbani, K., Nikam, C., Sadani, M., Shetty, A., Udwadia, Z., … Rodrigues, C. (2016). Correlating rrs and eis promoter mutations in clinical isolates of Mycobacterium tuberculosis with phenotypic susceptibility levels to the second-line injectables. *International Journal of Mycobacteriology*, *5*(1), 1–6. https://doi.org/10.1016/j.ijmyco.2015.09.001

Kigozi, E., Kasule, G. W., Musisi, K., Lukoye, D., Kyobe, S., Katabazi, F. A., … Kateete, D. P. (2018). Prevalence and patterns of rifampicin and isoniazid resistance conferring mutations in Mycobacterium tuberculosis isolates from Uganda. *PLoS ONE*, *13*(5), 1–17. https://doi.org/10.1371/journal.pone.0198091

Larsen, M. H., Vilchèze, C., Kremer, L., Besra, G. S., Parsons, L., Salfinger, M., … Jacobs, W. R. (2002). Overexpression of inhA, but not kasA, confers resistance to isoniazid and ethionamide in mycobacterium smegmatis, M. bovis BCG and M. tuberculosis. *Molecular Microbiology*. https://doi.org/10.1046/j.1365-2958.2002.03162.x

Li, Y., Wang, Y., Zhang, Z., Gao, H., Wang, H., Cao, J., … Dai, E. (2016). Association between embB Codon 306 Mutations, Phenotypic Resistance Profiles, and Genotypic Characterization in Clinical Mycobacterium tuberculosis Isolates from Hebei, China. *Antimicrobial Agents and Chemotherapy*, *60*(12), 7295–7302. https://doi.org/10.1128/AAC.00532-16

Lin, S-Y Grace, Rodwell, Rodwell, T. C., Victor, T. C., Rider, E. C., Pham, L., … Desmond, E. P. (2014). Pyrosequencing for rapid detection of extensively drug-resistant Mycobacterium tuberculosis in clinical isolates and clinical specimens. *Journal of Clinical Microbiology*, *52*(2), 475–482. https://doi.org/10.1128/JCM.01821-13

Lin, S-Y Grace, Rodwell, T. C., Victor, T. C., Rider, E. C., Pham, L., Catanzaro, A., & Desmond, E. P. (2013). Pyrosequencing for Rapid Detection of Extensively Drug-Resistant Tuberculosis in Clinical Isolates and Clinical Specimens. *Journal of Clinical Microbiology*, *52*(2), JCM.01821-13-. https://doi.org/10.1128/JCM.01821-13

Lin, S. Y. G., Rodwell, T. C., Victor, T. C., Rider, E. C., Pham, L., Catanzaro, A., & Desmond, E. P. (2014). Pyrosequencing for rapid detection of extensively drug-resistant Mycobacterium tuberculosis in clinical isolates and clinical specimens. *Journal of Clinical Microbiology*, *52*(2), 475–482. https://doi.org/10.1128/JCM.01821-13

Lin, Shou-Yean Grace, & Desmond, E. P. (2014). Molecular diagnosis of tuberculosis and drug resistance. *Clinics in Laboratory Medicine*, *34*(2), 297–314. https://doi.org/10.1016/j.cll.2014.02.005

Maher, D., Espinal, M., & Floyd, K. (2015). The Global Plan to Stop TB ,. *Tuberculosis: A Comprehensive Clinical Reference*, 949–956. https://doi.org/10.1016/B978-1-4160-3988-4.00107-4

Miesel, L., Rozwarski, D. A., Sacchettini, J. C., & Jacobs, W. R. (1998). Mechanisms for isoniazid action and resistance. *Novartis Foundation Symposium*. https://doi.org/10.1002/0470846526.ch15

Ministry of Health, N.-P. (2016). *Kenya Tuberculosis Prevalence Survey*.

Ministry of Public Health and Sanitation. (2009). *DLTLD Guidelines on Management of Leprosy and Tuberculosis.* Retrieved from http://www.nltp.co.ke/docs/DLTLD_Treatment_Guidelines.pdf

Mokrousov, I., Isakova, J., Valcheva, V., Aldashev, A., & Rastogi, N. (2013). Molecular snapshot of Mycobacterium tuberculosis population structure and drug-resistance in Kyrgyzstan. *Tuberculosis*. https://doi.org/10.1016/j.tube.2013.05.008

Mokrousov, I., Otten, T., Manicheva, O., Potapova, Y., Vishnevsky, B., Narvskaya, O., & Rastogi, N. (2008). Molecular characterization of ofloxacin-resistant Mycobacterium tuberculosis strains from Russia. *Antimicrobial Agents and Chemotherapy*, *52*(8), 2937–2939. https://doi.org/10.1128/AAC.00036-08

Mokrousov, I., Otten, T., Vyshnevskiy, B., & Narvskaya, O. (2002). Detection of embB306 mutations in ethambutol-susceptible clinical isolates of Mycobacterium tuberculosis from Northwestern Russia: implications for genotypic resistance testing. *Journal of Clinical Microbiology*, *40*(10), 3810–3813. https://doi.org/10.1128/JCM.40.10.3810-3813.2002

National Tuberculosis, L. & L. D. U. (2014). Programmatic management of drug resistant turberculosis (pmdt)-Kenya, (Third Edition). Retrieved from http://apps.who.int/medicinedocs/documents/s21976en/s21976en.pdf

National Tuberculosis Leprosy and Lung Disease Program. (2016). Kenya TB Prevalence Survey. Retrieved from http://www.nltp.co.ke/tb-prevalence-survey-2015-2016/

Palomino, J., & Martin, A. (2014). Drug Resistance Mechanisms in Mycobacterium tuberculosis. *Antibiotics*, *3*(3), 317–340. https://doi.org/10.3390/antibiotics3030317

Park, J., Shin, S. Y., Kim, K., Park, K., Shin, S., & Ihm, C. (2018). Determining Genotypic Drug Resistance by Ion Semiconductor Sequencing With the Ion AmpliSeq^{TM} TB Panel in Multidrug-Resistant *Mycobacterium tuberculosis* Isolates. *Annals of Laboratory Medicine*, *38*(4), 316. https://doi.org/10.3343/alm.2018.38.4.316

Perdigão, J., Macedo, R., Ribeiro, A., Brum, L., & Portugal, I. (2009). Genetic characterisation of the ethambutol resistance-determining region in Mycobacterium tuberculosis: prevalence and significance of embB306 mutations. *International Journal of Antimicrobial Agents*, *33*(4), 334–338. https://doi.org/10.1016/j.ijantimicag.2008.09.021

Quémard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., & Blanchard, J. S. (1995). Enzymatic Characterization of the Target for Isoniazid in Mycobacterium tuberculosis. *Biochemistry*. https://doi.org/10.1021/bi00026a004

Ramaswamy, S, & Musser, J. M. (1998). Molecular genetic basis of antimicrobial agent resistance in Mycobacterium tuberculosis: 1998 update. *Tuber Lung Dis*, *79*(1), 3–29. https://doi.org/10.1054/tuld.1998.0002\rS0962-8479(98)90002-8 [pii]

Ramaswamy, Srinivas V., Dou, S. J., Rendon, A., Yang, Z., Cave, M. D., & Graviss, E. A. (2004). Genotypic analysis of multidrug-resistant Mycobacterium tuberculosis isolates from Monterrey, Mexico. *Journal of Medical Microbiology*. https://doi.org/10.1099/jmm.0.05343-0

Ramaswamy, Srinivas V, Amin, A. G., Göksel, S., Stager, C. E., Dou, S. J., El Sahly, H., … Musser, J. M. (2000). Molecular genetic analysis of nucleotide polymorphisms associated with ethambutol resistance in human isolates of Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *44*(2), 326–336. https://doi.org/10.1128/AAC.44.2.326-336.2000

Rezaei, F., Haeili, M., Mohajeri, P., Hashemi Shahraki, A., Imani Fooladi, A. A., Zahednamazi, F., & Feizabadi, M. M. (2016). Frequency of mutational changes in the embB among the ethambutol-resistant strains of Mycobacterium tuberculosis in Iran. *The Journal of Infection in Developing Countries*, *10*(04), 363. https://doi.org/10.3855/jidc.7215

Rigouts, L., Gumusboga, M., De Rijk, W. B., Nduwamahoro, E., Uwizeye, C., De Jong, B., & Van Deun, A. (2013). Rifampin resistance missed in automated liquid culture system for Mycobacterium tuberculosis isolates with specific rpoB mutations. *Journal of Clinical Microbiology*, *51*(8), 2641–2645. https://doi.org/10.1128/JCM.02741-12

Rodwell, T. C., Valafar, F., Douglas, J., Qian, L., Garfein, R. S., Chawla, A., … Catanzaro, A. (2014). Predicting extensively drug-resistant Mycobacterium tuberculosis phenotypes with genetic mutations. *Journal of Clinical Microbiology*, *52*(3), 781–789. https://doi.org/10.1128/JCM.02701-13

Safi, H., Sayers, B., Hazbón, M. H., & Alland, D. (2008). Transfer of embB codon 306 mutations into clinical Mycobacterium tuberculosis strains alters susceptibility to ethambutol, isoniazid, and rifampin. *Antimicrobial Agents and Chemotherapy*, *52*(6), 2027–2034. https://doi.org/10.1128/AAC.01486-07

Saglik, I., Oz, Y., & Kiraz, N. (2014). Evaluation of the GenoType MTBDR assay for detection of rifampicin and isoniazid resistance in Mycobacterium tuberculosis complex isolates. *Indian Journal of Medical Microbiology*. https://doi.org/10.4103/0255-0857.136587

Sandgren, A., Strong, M., Muthukrishnan, P., Weiner, B. K., Church, G. M., & Murray, M. B. (2009). Tuberculosis drug resistance mutation database. *PLoS Medicine*, *6*(2), 0132–0136. https://doi.org/10.1371/journal.pmed.1000002

Sangsayunh, P., & Chuchothawon, C. (2014). Genotype MTBDR plus (Hain) test in suspected MDR-TB patients. *Journal of the Medical Association of Thailand*.

Scorpio, A., Lindholm-Levy, P., Heifets, L., Gilman, R., Siddiqi, S., Cynamon, M., & Zhang, Y. (1997). Characterization of pncA mutations in pyrazinamide-resistant Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/aac.41.3.540

Shen, X., Shen, G., Wu, J., Gui, X., Li, X., Mei, J., … Gao, Q. (2007). Association between embB codon 306 mutations and drug resistance in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*, *51*(7), 2618–2620. https://doi.org/10.1128/AAC.01516-06

Siddiqui, R. T., & Qureshi, J. A. (2014). The prevalence of common mutations in rpsL gene associated with resistance to streptomycin in random and multiple drug resistant isolates of M. tuberculosis from Pakistan. *Pakistan Journal of Zoology*, *46*(4), 1163–1166.

Smit, P. W., Haanperä, M., Rantala, P., Couvin, D., Lyytikäinen, O., Rastogi, N., … Soini, H. (2013). Molecular epidemiology of tuberculosis in Finland, 2008-2011. *PLoS ONE*, *8*(12). https://doi.org/10.1371/journal.pone.0085027

Springer, B., Kidan, Y. G., Prammananan, T., Ellrott, K., Böttger, E. C., & Sander, P. (2001). Mechanisms of streptomycin resistance: Selection of mutations in the 16S rRNA gene conferring resistance. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.45.10.2877-2884.2001

Sreevatsan, S., Stockbauer, K. E., Pan, X. I., Kreiswirth, B. N., Moghazeh, S. L., Jacobs, W. R., … Musser, J. M. (1997). *Ethambutol Resistance in Mycobacterium tuberculosis: Critical Role of embB Mutations* (Vol. 41).

Starks, A. M., Gumusboga, A., Plikaytis, B. B., Shinnick, T. M., & Posey, J. E. (2009). Mutations at embB codon 306 are an important molecular indicator of ethambutol resistance in mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy*. https://doi.org/10.1128/AAC.01357-08

Stinear, T. P., Denholm, J., Globan, M., Leslie, D., Seemann, T., Meumann, E. M., … Fyfe, J. A. M. (2015). Genome sequence comparisons of serial multi-drug-resistant Mycobacterium tuberculosis isolates over 21 years of infection in a single patient. *Microbial Genomics*, *1*(5). https://doi.org/10.1099/mgen.0.000037

Sun, Q., Xiao, T.-Y., Liu, H.-C., Zhao, X.-Q., Liu, Z.-G., Li, Y.-N., … Wan, K.-L. (2018). Mutations within embCAB Are Associated with Variable Level of Ethambutol Resistance in Mycobacterium tuberculosis Isolates from China. *Antimicrobial Agents and Chemotherapy*, *62*(1), e01279-17. https://doi.org/10.1128/AAC.01279-17

Tan, Y., Hu, Z., Zhao, Y., Cai, X., Luo, C., Zou, C., & Liu, X. (2012). The beginning of the rpoB gene in addition to the rifampin resistance determination region might be needed for identifying rifampin/rifabutin cross-resistance in multidrug-resistant Mycobacterium tuberculosis isolates from Southern China. *Journal of Clinical Microbiology*, *50*(1), 81–85. https://doi.org/10.1128/JCM.05092-11

Tang, K., Sun, H., Zhao, Y., Guo, J., Zhang, C., Feng, Q., … Sun, Q. (2013). Characterization of rifampin-resistant isolates of Mycobacterium tuberculosis from Sichuan in China. *Tuberculosis*, *93*(1), 89–95. https://doi.org/10.1016/j.tube.2012.10.009

Tavanaee Sani, A., Ashna, H., Kaffash, A., Khaledi, A., & Ghazvini, K. (2018). Mutations of rpob Gene Associated with Rifampin Resistance among Mycobacterium Tuberculosis Isolated in Tuberculosis Regional Reference Laboratory in Northeast of Iran during 2015-2016. *Ethiopian Journal of Health Sciences*, *28*(3), 299–304. https://doi.org/10.4314/ejhs.v28i3.7

Technologies, A. (2013). February 2013 PyroMark ® Q24 Advanced User Manual Sample & Assay Technologies, (February).

Tejada, F. R., Walk, A. R., & Kharel, M. K. (2015). Drugs Used in Tuberculosis and Leprosy. In *Side Effects of Drugs Annual*. https://doi.org/10.1016/bs.seda.2015.06.009

Tudo, G., Rey, E., Borrell, S., Alcaide, F., Codina, G., Coll, P., … Gonzalez-Martin, J. (2010). Characterization of mutations in streptomycin-resistant Mycobacterium tuberculosis clinical isolates in the area of Barcelona. *Journal of Antimicrobial Chemotherapy*, *65*(11), 2341–2346. https://doi.org/10.1093/jac/dkq322

Van Rie, A., Warren, R., Mshanga, I., Jordaan, A. M., van der Spuy, G. D., Richardson, M., … Victor, T. C. (2001). Analysis for a limited number of gene codons can predict drug resistance of Mycobacterium tuberculosis in a high-incidence community. *Journal of Clinical Microbiology*, *39*(2), 636–641. https://doi.org/10.1128/JCM.39.2.636-641.2001

Vassall, A., van Kampen, S., Sohn, H., Michael, J. S., John, K. R., den Boon, S., … Cobelens, F. (2011). Rapid diagnosis of tuberculosis with the Xpert MTB/RIF assay in high burden countries: A cost-effectiveness analysis. *PLoS Medicine*. https://doi.org/10.1371/journal.pmed.1001120

Who. (2010). Global Tuberculosis Control. WHO 2010. *Control*, *WHO/HTM/TB*, 218. https://doi.org/WHO/HTM/TB 2010.7

WHO. (2016). Global Tuberculosis Report 2016. *Cdc 2016*, (Global TB Report 2016), 214. https://doi.org/ISBN 978 92 4 156539 4

WHO. (2017). *Global Tuberculosis Report 2017*. https://doi.org/WHO/HTM/TB/2017.23

WHO report. (2011). Towards universal access to diagnosis and treatment of multidrug-resistant and extensively drug-resistant tuberculosis by 2015. *Who Progress Report*, 1–127. Retrieved from http://publication/uuid/54933732-F89D-4D1D-BBF0-89DB96995D0A

WHO, W. H. O. (2010). *Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response*. *Geneva: World Health Organization*. Retrieved from http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Multidrug+and+extensively+drug-resistant+TB+(M/XDR-TB):+2010+global+report+on+surveillance+and+response.#0

World Health Organisation. (2010). Multidrug and extensively drug-resistant TB. *The Indian Journal of Tuberculosis*, *57*(4), 180–191. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21141336

World Health Organization. (2012). Global tuberculosis report 2012. *WHO*, 258. https://doi.org/978 92 4 156450 2

World Health Organization. (2014). *Companion handbook*. https://doi.org/WHO/HTM/TB/2014.11

Zhang, Y., Heym, B., Allen, B., Young, D., & Cole, S. (1992). The catalase - Peroxidase gene and isoniazid resistance of Mycobacterium tuberculosis. *Nature*. https://doi.org/10.1038/358591a0

Zhang, Y., & Mitchison, D. (2003). The curious characteristics of pyrazinamide: A review. *International Journal of Tuberculosis and Lung Disease*.

Zhang, Z., Liu, M., Wang, Y., Pang, Y., Kam, K. M., & Zhao, Y. (2014). Molecular and phenotypic characterization of multidrug-resistant Mycobacterium tuberculosis isolates resistant to kanamycin, amikacin, and capreomycin in China. *European Journal of Clinical Microbiology & Infectious Diseases*, *33*(11), 1959–1966. https://doi.org/10.1007/s10096-014-2144-5

Zhao, L.-L., Sun, Q., Liu, H.-C., Wu, X.-C., Xiao, T.-Y., Zhao, X.-Q., … Wan, K.-L. (2015). Analysis of embCAB mutations associated with ethambutol resistance in multidrug-resistant mycobacterium tuberculosis isolates from China. *Antimicrobial Agents and Chemotherapy*, *59*(4), 2045–2050. https://doi.org/10.1128/AAC.04933-14

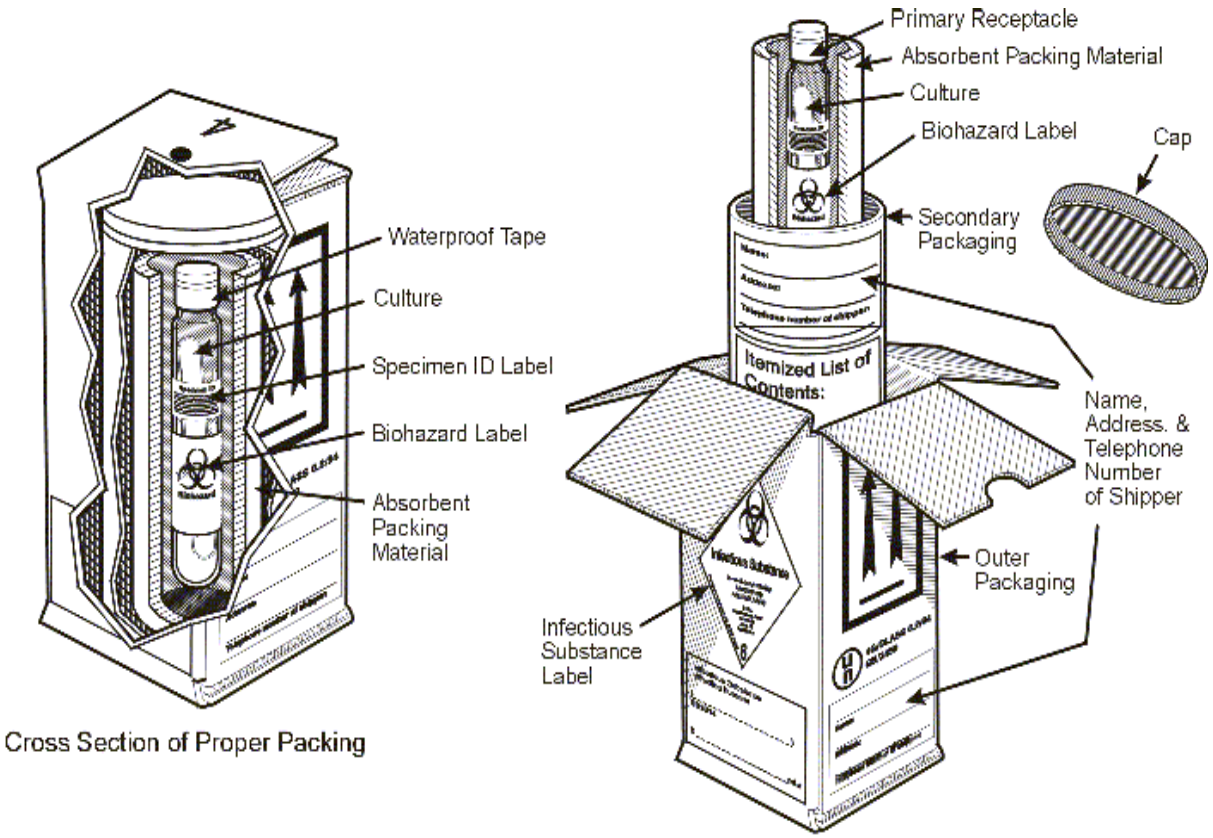
Zhao, L. L., Chen, Y., Chen, Z. N., Liu, H. C., Hu, P. L., Sun, Q., … Wan, K. L. (2014). Prevalence and molecular characteristics of drug-resistant mycobacterium tuberculosis in hunan, china. *Antimicrobial Agents and Chemotherapy*, *58*(6), 3475–3480. https://doi.org/10.1128/AAC.02426-14

Zheng, R., Zhu, C., Guo, Q., Qin, L., Wang, J., Lu, J., … Hu, Z. (2014). Pyrosequencing for rapid detection of tuberculosis resistance in clinical isolates and sputum samples from re-treatment pulmonary tuberculosis patients. *BMC Infectious Diseases*, *14*(1), 200. https://doi.org/10.1186/1471-2334-14-200

APPENDICES

Appendix I: Packaging criteria

Triple Packaging of sputum samples for transport



Cross Section of Proper Packing

Packing and Labeling of Infectious Substances

Appendix II: Laboratory Results Form

	Study site……. **Sample code…….**				
	Drug	*Gene*	No of Isolates tested	No of isolates with indicated genotype	Nucleotide change
1	**Isoniazid**	*katG*			
2	**Rifampicin**	*rpoB,*			
3	**Streptomycin**	*rpsL,*			
4	**Ethambutol**	*embB*			
5	**Amikacin**	*Rrs*			
6	**Ofloxacin**	*gyrA*			

Appendix III: Authority to collect samples

REPUBLIC OF KENYA

COUNTY GOVERNMENT OF TRANS NZOIA
STATE DEPARTMENT OF HEALTH

From the Office of the County Director
P. O. Box 4287 - 30200,
KITALE.

Our Ref: CGTN/MH/96 Date: 29th May 2015

To:

KNH/UON-Ethics and Research Committee
Kenyatta National Hospital
P O Box 20723
Code 00202
NAIROBI

Dear Sir/Madam,

RE: DATA COLLECTION AUTHORIZATION SUPPORT FOR JOSHUA K. ROTICH

The above named is a Medical Microbiologist working for the NPHLS and is currently pursuing studies at the University of Nairobi School of medicine.

He intends to collect post-analyzed sputum for molecular analysis. There are no objections and will be authorized to go ahead upon production of a letter of approval from the KNH/UON ERC.

Yours truly,

COUNTY DIRECTOR OF HEALTH
TRANS-NZOIA
29 MAY 2015
P .O. Box 4287-30200,
KITALE.

Dr. Philip Mbithi,
County Director of Health,
TRANS NZOIA COUNTY.

Appendix IV: Transport Media

OMNIgene·SPUTUM

Reagent for optimizing TB samples

REF OM-SPD

Product handbook

The following handbook is a method for the collection and preparation of sputum samples for use in tuberculosis testing using OMNIgene®·SPUTUM.

Note: TB bacteria will remain viable in the OM-SPD reagent.

15°C / 25°C

Wash with water if liquid solution comes in contact with eyes or skin. Do NOT ingest. See MSDS at www.dnagenotek.com.

Patent (www.dnagenotek.com/legalnotices)

For research use only,
not for use in diagnostic procedures.
Not available for clinical diagnostic use in the United States.

DNA genotek

www.dnagenotek.com

CAUTION: Clinical specimens which may contain *Mycobacterium tuberculosis* (TB) should be considered infectious and handled with appropriate biosafety precautions and standards (follow local and/or federal regulations as appropriate).

Note: TB bacteria will remain viable in the OM-SPD reagent.

Intended use

OMNIgene•SPUTUM optimizing reagent (OM-SPD) is intended for liquefaction and decontamination of fresh or frozen sputum samples while preserving viable *Mycobacterium tuberculosis.*

The liquid-based reagent enables room temperature shipping and storage of sputum for up to 5 days.

For research use only, not for use in diagnostic procedures. Not available for clinical diagnostic use in the United States.

Storage

OM-SPD should be stored at room temperature (15°C-25°C) and is stable until the expiration date indicated on the bottle label.

Safety information

For further safety information on OM-SPD, refer to the appropriate material safety data sheet (MSDS) available at www.dnagenotek.com.

Laboratory protocol for preparation of OMNIgene•SPUTUM samples for tuberculosis diagnostics (Smear, culture, Cepheid GeneXpert® and molecular diagnostics)

The following protocol is for the preparation of sputum samples in OMNIgene•SPUTUM (OM-SPD) optimizing reagent.

Equipment and reagent supplied by user

- Centrifuge that can accommodate 50 mL tubes and is capable of generating 3,800 × *g*
- 50 mL conical polypropylene tubes (e.g., Sarstedt #62.547.205)
- Sterile phosphate buffered saline (PBS) or sterile water
- Appropriate biosafety equipment and personal protective equipment as required by your institution and/or biosafety committee.

Procedure

Sample preparation steps	Notes
1. Vortex for 15–20 seconds or invert 10–20 times to mix.	
2. Spin OM-SPD specimen at 3,800 × *g* for 15–20 minutes to obtain a sediment.	Transfer sample to a 50 mL conical tube if needed.
3. Gently pour off supernatant into appropriate waste container without disturbing the sediment. **Do NOT discard sediment.**	The sediment contains viable *Mycobacterium tuberculosis.*

(next page)

(continued)

Sample preparation steps	Notes
4. Resuspend the sediment in sufficient volume of sterile phosphate buffered saline or sterile water in order to complete standard laboratory test procedures.	
5. Aliquots may be removed for: a) Smear b) Culture testing (including BBL MGIT culture system) c) Cepheid GeneXpert® MTB/RIF testing d) Molecular diagnostics	a) Follow laboratory SOPs for smear. b) Follow laboratory SOPs for culture. c) Follow manufacturer's recommended protocol for Cepheid GeneXpert® MTB/RIF d) For molecular diagnostics, follow DNA Genotek's prepIT®•MAX for TB purification protocol or other extraction systems.

Shipping

Note: TB bacteria will remain viable in OM-SPD reagent.

Samples optimized using OM-SPD reagent should be considered infectious/dangerous goods and travel as UN3373, Biological Substance, Category B.

Packaging and shipment of specimens in OM-SPD reagent must be done in accordance with local regulations and/or IATA guidelines for the shipment of biohazardous/infectious specimens.

Technical support is available Monday to Friday (9h00 to 17h00 EST):

- Toll-free (North America): 1.866.813.6354, option 6
- All other countries: 613.723.5757, option 6
- Email: support@dnagenotek.com

Some DNA Genotek products may not be available in all geographic regions.
OMNIgene is a registered trademark of DNA Genotek Inc.
All other brands and names contained herein are the property of their respective owners.
All DNA Genotek protocols, white papers and application notes are available in the support section of our website at www.dnagenotek.com.

Label legend:

REF	Catalog number
⚠	Caution, consult instructions for use
15°C 25°C	Storage instructions
	Manufacturer

Made in Canada
DNA Genotek Inc.
Ottawa, ON, Canada K2K 1L1
Subsidiary of OraSure Technologies, Inc.

PD-PR-00421 Issue 3/2015-05

Laboratory protocol for preparation of OMNIgene•SPUTUM samples for tuberculosis diagnostics (Smear, culture, Cepheid GeneXpert® and molecular diagnostics)

The following protocol is for the preparation of sputum samples in OMNIgene•SPUTUM (OM-SPD) optimizing reagent.

Equipment and reagent supplied by user

- Centrifuge that can accommodate 50 mL tubes and is capable of generating 3,800 × *g*
- 50 mL conical polypropylene tubes (e.g., Sarstedt #62.547.205)
- Sterile phosphate buffered saline (PBS) or sterile water
- Appropriate biosafety equipment and personal protective equipment as required by your institution and/or biosafety committee.

Procedure

Sample preparation steps	Notes
1. Vortex for 15–20 seconds or invert 10–20 times to mix.	
2. Spin OM-SPD specimen at 3,800 × *g* for 15–20 minutes to obtain a sediment.	Transfer sample to a 50 mL conical tube if needed.
3. Gently pour off supernatant into appropriate waste container without disturbing the sediment. **Do NOT discard sediment.**	The sediment contains viable *Mycobacterium tuberculosis.*

(next page)

Protocol for collecting sputum in OMNIgene•SPUTUM reagent (OM-SPD)

Reagent included

- OM-SPD

Equipment supplied by user

- Standard collection cup for sputum
- Pipettes and pipette tips

Procedure

Collection steps	Notes
1. Collect sputum into standard collection cup or tube.	
2. Visually estimate volume of sputum collected.	
3. Add approximately an equal volume of OMNIgene•SPUTUM reagent.	
4. Recap collection cup tightly.	
5. Vigorously invert specimen 10 times to mix.	
6. Incubate specimen at room temperature (15°C-25°C) for a minimum of 15 minutes. Periodic mixing (by inversion or vortexing) will facilitate liquefaction.	Highly mucoid specimens may require longer hold times or the addition of NALC to ensure full liquefaction is achieved. Samples may remain at room temperature (15°C-25°C) for up to 5 days before proceeding with the next step.

Laboratory protocol for preparation of OMNIgene•SPUTUM samples for tuberculosis diagnostics (Smear, culture, Cepheid GeneXpert® and molecular diagnostics)

The following protocol is for the preparation of sputum samples in OMNIgene•SPUTUM (OM-SPD) optimizing reagent.

Equipment and reagent supplied by user

- Centrifuge that can accommodate 50 mL tubes and is capable of generating 3,800 × *g*
- 50 mL conical polypropylene tubes (e.g., Sarstedt #62.547.205)
- Sterile phosphate buffered saline (PBS) or sterile water
- Appropriate biosafety equipment and personal protective equipment as required by your institution and/or biosafety committee.

Procedure

Sample preparation steps	Notes
1. Vortex for 15–20 seconds or invert 10–20 times to mix.	
2. Spin OM-SPD specimen at 3,800 × *g* for 15–20 minutes to obtain a sediment.	Transfer sample to a 50 mL conical tube if needed.
3. Gently pour off supernatant into appropriate waste container without disturbing the sediment. **Do NOT discard sediment.**	The sediment contains viable *Mycobacterium tuberculosis*.

(next page)

(continued)

Sample preparation steps	Notes
4. Resuspend the sediment in sufficient volume of sterile phosphate buffered saline or sterile water in order to complete standard laboratory test procedures.	
5. Aliquots may be removed for: a) Smear b) Culture testing (including BBL MGIT culture system) c) Cepheid GeneXpert® MTB/RIF testing d) Molecular diagnostics	a) Follow laboratory SOPs for smear. b) Follow laboratory SOPs for culture. c) Follow manufacturer's recommended protocol for Cepheid GeneXpert® MTB/RIF d) For molecular diagnostics, follow DNA Genotek's prepIT®•MAX for TB purification protocol or other extraction systems.

Shipping

Note: TB bacteria will remain viable in OM-SPD reagent.

Samples optimized using OM-SPD reagent should be considered infectious/dangerous goods and travel as UN3373, Biological Substance, Category B.

Packaging and shipment of specimens in OM-SPD reagent must be done in accordance with local regulations and/or IATA guidelines for the shipment of biohazardous/infectious specimens.

August 2015

QIAsymphony® DSP DNA Instructions for Use (Handbook)

Σ 192 (cat. no. 937236)

Σ 96 (cat. no. 937255)

Version 1

IVD

For in vitro diagnostic use

QIAsymphony DSP DNA Mini Kit

QIAsymphony DSP DNA Midi Kit

CE

REF 937236, 937255

QIAGEN GmbH
QIAGEN Strasse 1
40724 Hilden GERMANY

R4 MAT 1069185EN

Sample to Insight

QIAGEN

Kit components

QIAsymphony DSP DNA Kits contain ready-to-use proteinase K solution that can be stored at room temperature.

Do not store reagent cartridges (RC) at temperatures below 15°C.

Partially used reagent cartridges (RC) can be stored for a maximum of 4 weeks, enabling cost-efficient reuse of reagents and more flexible sample processing. If a reagent cartridge (RC) is partially used, replace the cover of the trough containing the magnetic particles, and seal the reagent cartridge (RC) with the provided Reuse Seal Strips immediately after the end of the protocol run to avoid evaporation.

To avoid reagent evaporation, the reagent cartridge (RC) should be open for a maximum of 15 hours (including run times) at a maximum environmental temperature of 30°C.

Running batches with low sample numbers (<24) will increase both the time that the reagent cartridge (RC) is open and the required buffer volumes, potentially reducing the total number of sample preparations possible per cartridge.

Avoid exposure of the reagent cartridges (RC) to UV light (e.g., used for decontamination) as exposure may cause accelerated aging of the reagent cartridges (RC) and buffers.

Specimen Collection and Preparation

Prevent formation of foam in or on the samples. Depending on the starting material, sample pretreatment may be required.

Samples should be equilibrated to room temperature (15–25°C) before starting the run.

For more information about the automated procedure (including information about sample tubes that can be used with specific protocols) and specific sample pretreatments, see the relevant protocol sheet, available at **www.qiagen.com/goto/dspdnakits**.

Procedure

Automated purification on QIAsymphony SP

The QIAsymphony SP makes automated sample preparation easy and convenient. Samples, reagents and consumables, and eluates are separated in different drawers. Simply load samples, reagents provided in special cartridges, and preracked consumables in the appropriate drawer before a run. Start the protocol and remove purified DNA from the "Eluate" drawer after processing. Refer to the user manuals supplied with your instrument for operating instructions.

Note: Optional maintenance is not mandatory for instrument function, but is highly recommended to reduce risk of contamination.

The range of protocols available is continually expanding, and additional QIAGEN protocols can be downloaded free of charge at **www.qiagen.com/goto/dspdnakits**.

Loading reagent cartridges (RC) into the "Reagents and Consumables" drawer

Reagents for purification of DNA are contained in an innovative reagent cartridge (RC) (Figure 2, page 13). Each trough of the reagent cartridge (RC) contains a particular reagent, such as magnetic particles, lysis buffer, wash buffer, or elution buffer. Partially used reagent cartridges (RC) can be reclosed with Reuse Seal Strips (RSS) for later reuse, which avoids generation of waste due to leftover reagents at the end of the purification procedure.

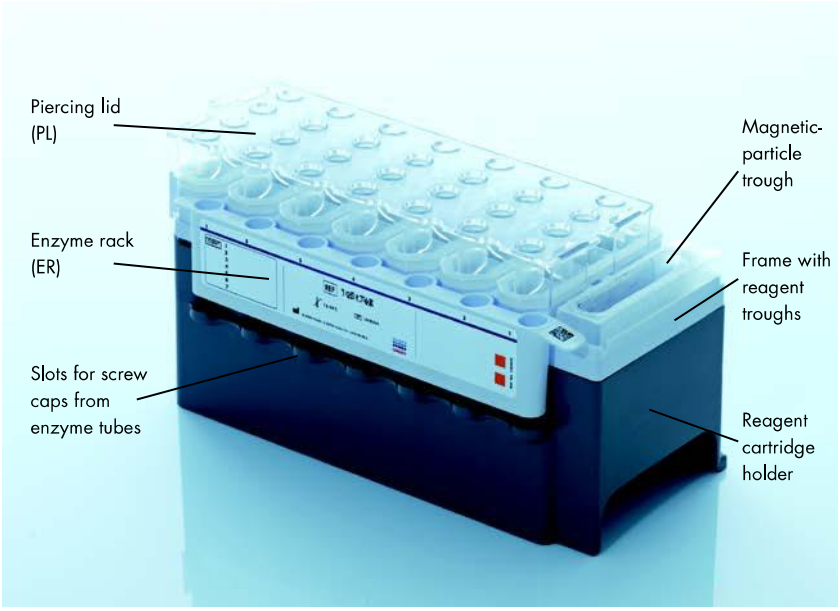


Figure 2. QIAsymphony reagent cartridge (RC). The reagent cartridge (RC) contains all reagents required for the protocol run.

Before starting the procedure, ensure that the magnetic particles are fully resuspended. Remove the magnetic-particle trough from the reagent cartridge frame, vortex it vigorously for at least 3 minutes, and replace it in the reagent cartridge frame before the first use. Place the reagent cartridge (RC) into the reagent cartridge holder. Place the enzyme rack (ER) into the reagent cartridge holder. Before using a reagent cartridge (RC) for the first time, place the piercing lid (PL) on top of the reagent cartridge (RC) (Figure 2, above).

Note: The piercing lid (PL) is sharp. Take care when placing it onto the reagent cartridge (RC). Make sure to place the piercing lid (PL) onto the reagent cartridge (RC) in the correct orientation.

Appendix VI: Individual Sequences

1) Rifampicin

a)rpoB Primer 1

i) *rpoB* 510 (272 samples)
TTCGGCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTG
TCGGGGTTGA

ii) *rpoB* 510 (6 samples)
TTCGGCACCAGCCAGCCGAGCCAATTCATGGACCAGAACAACCCGCTG
TCGGGGTTGA

iii) *rpoB* 510 (5 samples)
TTCGGCACCAGCCAGCCGAGCCAATTCATGGACCAGAGCAACCCGCTG
TCGGGGTTGA

iv) *rpoB* 510(1 sample)
TTCGGCACCAGCCAGCCGAGCCACTTCATGGACCAGAGCAACCCGCTG
TCGGGGTTGA

v) *rpoB* 510(1 sample)
TTCGGCACCAGCCAGCTGAGCCACTTCATGGACCAGAGCAACCCGCTG
TCGGGGTTGA

vi) *rpoB* 510(1 sample)
TTCGGCACCAGCCAGCTGAGCCACTTCATGGTCCAGAGCAACCCGCTG
TCGGGGTTGA

vii) *rpoB* 510(1 sample)
TTCGGCACCAGCCAGCTGAGCCAATTCATGGTCCAGAGCAACCCGCTG
TCGGGGTTGA

viii)*rpoB* 510(1 sample)
TTCGGCACCAGCCAGCTGAGCCACTTCATGGACCAGAACAACCCGCTG
TCGGGGTTGA

b) rpoB primer 2

i) *rpoB* 526(279 samples)
CCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTGGGGCC
CGGCGGTCTGTCA

ii) *rpoB* 526(4 samples)
CCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCCGGGGCC
CGGCGGTCTGTCA

iii) (*rpoB* 526(5 samples)
CCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTTGGCGCTGGGGCC
CGGCGGTCTGTCA

2) Isoniazide

a) Gene *katG*

i) *katG* (287samples) TCACCAGCGGCATCGAGGT
ii) *katG* (1 sample) TCACCACCGGCATCGAGGT

3) Ethambutol

a)Gene *embB*

i) (286 samples) GGCATGGCCCGAGTCGCCG
ii) (1 sample) GGCGTCGCCCGAGTCGCCG
iii) (1 sample) GGCATCGCCCGAGTCGCCG

4) **Ofloxacin**

a) Gene *gyrA*

i) (All of the 288 samples) GACGCGTCGATCTACGACAGCCTGGTGC

5) **Streptomycin**

a) Gene *rpsl88*

i) Samples) CTTCACCCGGCCGCCGCGCA

ii) (8 samples) CCTCACCCGGCCGCCGCGCA

Gene *rpsl43*

iii) (280 samples) CTTCGGAGTGGTGGTG

iv) (8 samples) CCTCGGAGTGGTGGTG

6) **Amikacin**

a) Gene *rrs*

i) 1 All 288 samples) CGTCACGTCATGAAAGTCGG