EVALUATION OF GENOTYPE® MTBDRPLUS ASSAY AS A SCREENING TOOL FOR MULTI-DRUG RESISTANT TUBERCULOSIS SURVEILLANCE AMONG PATIENTS AT HIGH RISK IN NAIROBI

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Evaluation of genotype MTBDRPLUS
DECLARATION

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

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Date: 2/2/2012

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I would like to dedicate this work to my dear husband Crispin Matere, my daughter Judy Karimi and my sons Antony Mugane and Evans Mugo.
ACKNOWLEDGMENTS

I would like to thank everybody who in different ways made a contribution to this work. I salute my supervisors, Dr. Joseph N. Ngeranwa and Dr. Nicholas K. Gikonyo for their guidance, mentorship and moral support throughout the entire period of this research. You were very patient with me and accommodative whenever I needed your attention. I am grateful to Kenyatta University, School of Public Health and the department of Community Health for all the support I have received including the skills and knowledge acquired throughout the course.

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AFB</td>
<td>Acid Fast Bacilli</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette Guerin</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>CRL</td>
<td>Central Reference Laboratory</td>
</tr>
<tr>
<td>CTRL</td>
<td>Central Tuberculosis Reference Laboratory</td>
</tr>
<tr>
<td>DL/TLD</td>
<td>Division of Leprosy, Tuberculosis and Lung Disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DOTS</td>
<td>Directly Observed Short Course Therapy</td>
</tr>
<tr>
<td>DST</td>
<td>Drug Susceptibility Testing</td>
</tr>
<tr>
<td>GLC</td>
<td>Green Light Committee</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immune Deficiency Virus</td>
</tr>
<tr>
<td>HPF</td>
<td>High Power Field</td>
</tr>
<tr>
<td>INH</td>
<td>Isoniazid</td>
</tr>
<tr>
<td>IUALTD</td>
<td>International Union Against Lung and Tuberculosis Disease</td>
</tr>
<tr>
<td>KatG gene</td>
<td>Gene coding for catalase/peroxidase</td>
</tr>
<tr>
<td>KDHS</td>
<td>Kenya Demographic and Health Survey</td>
</tr>
<tr>
<td>LiPA</td>
<td>Line Probe Assay</td>
</tr>
<tr>
<td>LJ</td>
<td>Lowenstein and Jensen</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>Multi-drug resistant tuberculosis</td>
</tr>
<tr>
<td>MGIT</td>
<td>Mycobacteria Growth Indicator Tube</td>
</tr>
<tr>
<td>MMWR</td>
<td>Morbidity and Mortality Weekly Report</td>
</tr>
<tr>
<td>MTB</td>
<td>Mycobacterium Tuberculosis Bacilli</td>
</tr>
<tr>
<td>MOTT</td>
<td>Mycobacterium Other Than Tuberculosis</td>
</tr>
<tr>
<td>NLTP</td>
<td>National Leprosy and Tuberculosis Control Program</td>
</tr>
<tr>
<td>NTM</td>
<td>Non-tuberculosis Mycobacterium</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PLWA</td>
<td>People Living with HIV and AIDS</td>
</tr>
<tr>
<td>PTB</td>
<td>Pulmonary Tuberculosis</td>
</tr>
<tr>
<td>RIF</td>
<td>Rifampicin</td>
</tr>
<tr>
<td>rpoB gene</td>
<td>Beta subunit of Ribonucleic Acid Polymerase gene</td>
</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>SLD</td>
<td>Second Line Drugs</td>
</tr>
</tbody>
</table>
SOP  Standard Operating Procedures
TB   Tuberculosis
WHO  World Health Organization
X-DR-TB  Extremely- Drug Resistant Tuberculosis
ZN   Ziehl Neelson
OPERATIONAL DEFINITIONS

Accuracy ..........the percentage of correct results obtained by the test under evaluation compared with the results of the gold standard (No. of correct results divided by total number of results X100).

Drug susceptible tuberculosis ..........tuberculosis strain that will be killed by all the first line TB drugs.

Efficacy ..........ability to produce desired effects.


Multi-drug resistant tuberculosis ..........tuberculosis strain that is resistant to at least Isoniazid and Rifampicin.

Negative predictive value ...... the probability that a negative result accurately indicates the absence of infection.

Positive predictive value ......the probability that a positive result accurately indicates the presence of infection.

Sensitivity ..........the probability that patients with the disease as determined by the gold standard test will have a positive test result using the test under evaluation.

Specificity ...... the probability that patients without the disease as determined by the gold standard test will have a negative test result using the test under evaluation.

Patients at high risk of MDR-TB .......... Patients who are clinically or bacteriologically not responding positively to first line anti-TB treatment.
ABSTRACT

Multi-drug resistant tuberculosis (MDR-TB) has emerged as an important global public health threat. The Kenya tuberculosis program has continued to report MDR-TB cases since the year 2005 to date from patients failing first line tuberculosis (TB) treatment. The current MDR-TB diagnostic methods for drug susceptibility testing (DST) are lengthy, take 3 months and are only available in research and reference laboratories in Kenya. This laboratory diagnostic delay has been observed to contribute to spread, increased morbidity and mortality due to MDR-TB. A rapid molecular test such as GenoType®MTBDRplus that would detect the MDR-TB early and in county laboratories needed to be validated in Kenya. This study describes a cross-sectional study that was conducted in Nairobi county from September 2009 to September 2010 where the specimens from TB patients with a history of treatment failure were collected, packed safely and sent for laboratory analysis at Central Tuberculosis Reference Laboratory (CTRL). GenoType®MTBDRplus assay was evaluated against Mycobacteria Growth Indicator Tube (MGIT) DST as the gold standard. Data on patient’s demographics, Human Immunodeficiency Virus (HIV) status and laboratory tests were obtained from the laboratory request forms and worksheets. The data was cleaned and analyzed using Statistical Analysis System (SAS) version 9.1. Chi-square statistic was used to establish association between variables and test study hypothesis. Accuracy of GenoType®MTBDRplus was determined by Kappa statistic, sensitivity, specificity and predictive values. A total of 457 specimens were collected from Kenyatta National Hospital (32), Blue House (21), Eastern Deanery AIDS Relief Program (89), Rhodes chest clinic (57), Kibera South (28) and Kangemi Health Center (10). The rest of the specimens were received from other sites during the study period. Of the 455 specimens with age and gender records, 303 (66.6%) were males and 152 (33.4%) were females. The median age was 32 years, the youngest and the oldest being males of 3 and 67 years respectively. Of the 457 specimens with sputum smear results, 274 (60.0%) were smear positive while the remaining 183 (40.0%) were smear negative. The age group of patients was shown to influence the occurrence of smear positive (P=0.0011). Patients with HIV positive status were 0.27 times likely to be smear positive compared to those with HIV negative status with a 95% confidence interval of 0.14 to 0.50 and a p-value of 0.0001. Of the 451 specimens with TB culture results, 61% had culture confirmed tuberculosis, out of which 22.4% were MDR-TB. Being smear positive was not associated with MDR-TB (P=0.3031). TB and HIV co-infection among the culture confirmed tuberculosis patients was 42% (P<0.0001). There was excellent agreement between MGIT DST and GenoType®MTBDRplus with a kappa statistic of 0.8902 and a confidence interval of 0.7684 to 1.0000 for sputum specimens. A kappa statistic of 0.9312 with confidence interval of 0.8647 to 0.9977 was obtained for the isolates from Lowenstein media. Nairobi county has high rates of MDR-TB among patients with a history of first line anti-TB treatment failure. GenoType®MTBDRplus assay has been validated as a rapid and reliable method for surveillance of MDR-TB in Nairobi, Kenya. The Kenya TB program should consider use of GenoType®MTBDRplus as a rapid test for diagnosing and surveillance of MDR-TB in Nairobi county; accelerate MDR-TB case finding, care and treatment of identified cases. Further research may be needed to explore the cost associated with scaling up this new test to other counties in Kenya.
CHAPTER 1: INTRODUCTION

1.1 Background

Tuberculosis (TB) infection is a major cause of human morbidity and mortality in the world (WHO, 2001). The African region has the highest estimated incidence rate of 345 TB cases per 100,000 populations annually. The most populous countries of Asia harbor the largest number of TB cases: Bangladesh, China, India, Indonesia, and Pakistan together account for half the new cases arising each year (WHO, 2005). In terms of the total estimated number of new TB cases arising annually, about 80% of new cases occur in the top-ranking 22 countries. Kenya is number 13 among 22 high TB burden countries in the world (WHO, 2002). The country had a case notification rate of 320/100,000 of population according to National Leprosy and Tuberculosis Program (NLTP) report in 2004. This is thought to be an underestimation of the actual cases as true yearly rates are likely to be higher due to under-diagnosis and under-reporting in African countries (WHO, 2005).

Of greater concern is the occurrence of multi-drug resistant tuberculosis (MDR- TB) which is resistant to the most potent first line TB drugs (WHO, 2000). Recently, highly lethal outbreaks of extensively drug resistant tuberculosis (XDR-TB) in association with HIV infection have been described in South Africa (Gandhi et al., 2006). The synergistic interaction between Human Immunodeficiency Virus (HIV) and tuberculosis infection has increased the prevalence of both susceptible and multi-drug resistant tuberculosis (Pozniak, 2001) creating a significant challenge in the prevention and control of TB (Kochi et al., 1993). Large patient numbers have led to overcrowding of both TB wards, clinics and general outpatient rendering good nursing care difficult and increasing the risk of nosocomial infection (Andrews et al., 2008).
Tuberculosis drug resistance occurs naturally due to random mutations in the bacterial cell, at rates ranging from $3 \times 10^{-9}$ to $1 \times 10^{-7}$ per cell per generation for first line anti-tuberculosis drugs which include Isoniazid (INH), Rifampicin (RIF), Ethambutol (EMB), Streptomycin (S) and Pyrazinamide (PZA) (Mitchison, 1998). Spontaneous drug resistant mutants are selected whenever inadequate or ineffective chemotherapy is administered. This selection process has been associated to not only expansion of the drug resistant population within the infected individual, but also allows for the transmission of such TB strains, which can then develop further resistances to additional drugs through their own spontaneous mutations, creating a cycle of transmission and amplification of drug resistance. Such events have led to the worldwide development and spread of multi-drug resistant TB, defined as infection with TB resistant to at least Isoniazid and Rifampicin. This form of TB is significantly more difficult to treat than drug susceptible TB, in large part because the necessary second-line drugs are more expensive, must be administered for a longer period of time, are less effective, and are associated with more severe side effects than the standard first line anti-tuberculosis drugs.

In 2004, it was estimated that 424,000 new MDR-TB cases occurred worldwide (Zignol et al., 2006) representing an increase on previous estimations and potentially a trend of increasing numbers of MDR-TB cases globally. Patients at high risk of MDR-TB are those who remain sputum smear positive after five months of TB treatment, those that come up with TB even after completing or getting cured of first infection and those with chronic disease. TB drug susceptibility testing is known to provide an epidemiological indicator to assess the extent of resistant bacteria transmission in the community as well as success of TB program. Potential causes of drug resistance TB include; laboratory delay in identifying infectious cases, inadequate treatment provided by the health services, poor case holding, poor drug supply, poor quality of drugs, non adherence by patients to the prescribed drug regimens and
indiscriminate use of anti-tuberculosis drugs in private sector. In response to the emergence of MDR-TB, the WHO adopted the DOTS-Plus treatment strategy for MDR-TB in 1998 (WHO, 2000). Despite the recommendation of this program and the establishment of the Green Light Committee (GLC), which grants TB programs access to discounted second line anti-tuberculosis drugs (SLD), the management of drug resistant cases of TB and the control of resistance remain serious public health concerns throughout the world. In fact, drug resistant TB was identified in 96% of the countries in the WHO global survey of 1999 and 2002 (WHO/IUALT, 2004). Although Kenya was found to have minimal threat of the problem, NLTP continues to report cases of MDR-TB among patients failing first line TB treatment (NLTP, 2006).

Although well-functioning TB programs are critical in the control of MDR-TB, TB control strategies are hindered by lack of proper laboratory infrastructure and length of time required to diagnose drug resistant TB. Cheap conventional drug susceptibility testing (DST) is a slow process, requiring culture of *Mycobacterium* from clinical specimens, followed by drug susceptibility testing on solid media such as Lowenstein-Jensen (LJ) and is only found in research and reference laboratories. This process can take 2-4 months, during which time a patient is often treated according to the standard protocol for drug susceptible TB, which is not always appropriate for drug resistant TB. The resultant delay in proper treatment may adversely affect treatment outcome and contribute to the transmission of drug resistant TB (Hudson *et al.*, 2000). Evaluation and implementation of near point of care, easy to use rapid tests for the diagnosis of drug resistant TB are essential for long term effective control of TB.

A number of rapid assays have been developed where Rifampicin resistance was used for presumptive diagnosis of MDR-TB. The GenoType®MTBDR*plus* assay has a proven efficacy
under research laboratory conditions, but evaluations in public health laboratory settings have been limited. Information on the evaluation of these tests are required to identify constraints on implementation, confirm the accuracy of the test in these settings, indicate how the test may be best applied and confirm the benefits from implementation.

This study was designed to determine accuracy of the GenoType®MTBDRplus assay under routine TB program laboratory conditions for the rapid and diagnosis and surveillance of MDR-TB among patients at high risk in Nairobi.

1.2 Problem statement

Drug resistant tuberculosis remains a serious public health concern throughout the world. The WHO global survey of 1999 and 2002 (WHO/IUALTD, 2004) did not find significant MDR-TB threat in Kenya. However, the Kenyan TB program continues to report cases of MDR-TB among patients with a history of first line anti-tuberculosis treatment failure (NLTP, 2008 and NLTP, 2009). Treatment success and TB control is heavily hinged on rapid diagnosis. Cheap methods of MDR-TB diagnosis using conventional drug susceptibility testing (DST) are lengthy (3 months) and are only available in research and reference laboratories in Kenya. This laboratory diagnostic delay in diagnosing tuberculosis has been observed to contribute to increased spread, morbidity and mortality (Hudson et al., 2000). Of greater concern is the hospital acquired infections by health care workers as documented in the New York City study where nosocomial outbreaks caused deaths of patients and health care workers, a majority of whom were co-infected with HIV (Agerton et al., 1999). In Africa, the study from Kwazulu Natal found that Health care workers got infected with MDR-TB within the hospitals (O’Donnell et al., 2010).
1.3 Justification

GenoType®MTBDRplus assay shortens the diagnosis MDR-TB from 3 months to two days. This assay can detect MDR-TB from uncultured sputum specimens' making it possible to diagnose MDR-TB in the district laboratories near the point of care, allows early case identification, and contributes to infection prevention and TB control. There is no literature to show that GenoType®MTBDRplus has ever been evaluated in Kenya as a screening tool for surveillance of MDR-TB among patients at high risk.

1.4 Research questions

1. What is the influence of HIV status on tuberculosis laboratory testing outcome?

2. What is the prevalence of MDR-TB among patients seeking medical care in Nairobi TB clinics?

3. What is the accuracy of GenoType®MTBDRplus assay when applied to direct uncultured sputum specimens?

1.5 Null hypothesis (H₀)

GenoType®MTBDRplus assay is not an accurate method for the rapid diagnosis and surveillance of MDR-TB.
1.6 Objectives of the study

1.6.1 General objectives

To determine the accuracy of GenoType®MTBDRplus assay in the diagnosis and surveillance of multi-drug resistant *Mycobacterium tuberculosis* in Nairobi County.

1.6.2 Specific objectives

1. To determine the influence of HIV status on tuberculosis laboratory testing outcome.
2. To investigate the prevalence of MDR-TB among patients seeking medical care in Nairobi TB clinics.
3. To determine the accuracy of GenoType®MTBDRplus assay when applied to direct uncultured sputum specimens.

1.7 Limitations of the study

The GenoType®MTBDRplus test kits are very expensive and therefore it was not possible to run all the smear positive specimens and culture isolates. The smear negative specimens were not run on GenoType®MTBDRplus as it was assumed they would turn negative considering the testing cost.
CHAPTER 2: LITERATURE REVIEW

2.1 Tuberculosis causative agent

*Mycobacterium* is a non-spore forming, non-capsulated, straight or slightly curved rod measuring 1-10μm x 0.2-0.6 μm. The *Mycobacterium tuberculosis* complex includes five species: *M. tuberculosis*, *M. bovis*, *M. canetti*, *M. africanum*, and *M. microti*. Within the species complex, most human disease is due to *Mycobacterium tuberculosis* (Kent, 1985). The variants within the species complex differ from each other biochemically and in colony morphology on Lowenstein Jensen (LJ) culture. However, these differences have no known bearing on management or prognosis. The principal exception is *M. bovis*, which accounts for a small fraction of human TB cases, but which is naturally resistant to the drug pyrazinamide.

Human disease can also be caused by species of *Mycobacteria* other than *M. tuberculosis* (MOT), also known as atypical *Mycobacteria* (Cheesbrough, 2000). These organisms are widespread in nature and have been isolated from a variety of sources, including soil, dust, water, milk, animals, and birds. In humans, MOT are low-grade pathogens and usually cause disease only in patients with pre-existing lung disease or immunodeficiency. MOT is still a rare cause of disease in Sub-Saharan Africa. The large majority of patients in Africa who are diagnosed and treated for TB, even those infected with HIV, have disease caused by *M. tuberculosis* (Nunn et al., 1994).

TB bacteria have a gram positive cell wall which is said to stain poorly due to its waxy surface (Murray, 2003). *M. tuberculosis* grows aerobically on protein rich media such as LJ within temperature range of 35-37°C (Cheesbrough, 2000). *Mycobacteria* are acid and alcohol fast, meaning that once stained by an aniline dye, such as carbolufchsin, they resist decolorization with acid and alcohol. *Mycobacteria* are therefore often called "acid-fast bacilli" (AFB). In virtually all other bacteria the dye is removed by the acid-alcohol wash, and the ability of
*Mycobacteria* to retain the aniline dye despite acid and alcohol is probably due to their thick cell wall. This property allows the detection of AFB in specimens by using the simple Ziehl-Neelson (ZN) staining technique, widely used in Sub-Saharan Africa. *Mycobacterium* grows slowly, with generation times measured in hours rather than minutes (Murray, 2003). This means that the normal methods of obtaining cultures from clinical specimens are difficult because of overgrowth by other bacteria. Fortunately, the thick cell wall of *Mycobacteria* also enables them to resist alkalis and detergents, and this property is made use of in culture techniques that use alkalis and special media to reduce contamination.

### 2.2 Tuberculosis disease

Tuberculosis is a bacterial disease caused by *Mycobacterium tuberculosis*. The bacteria usually attack the lungs, but they can also damage other parts of the body. Symptoms of TB in the lungs may include: cough that lasts 3 weeks or longer, weight loss, coughing up blood or mucus, weakness or fatigue, fever and chills and night sweats. People infected with *M. tuberculosis* carry live tubercle bacilli, but the bacilli may be present in small numbers and dormant (latent), in which case there may be no apparent disease. Disease occurs when the bacteria multiply, overcome immune defenses and become numerous enough to cause damage to tissues (Crofton, 2009). Patients with pulmonary tuberculosis (PTB) are the most important source of infection (Harries *et al.*, 2004). Infection occurs by inhaling droplet nuclei, infectious particles of respiratory secretions usually less than five micrometers, which contain tubercle bacilli. These are spread into the air by coughing, sneezing, talking, spitting, and singing, and they can remain suspended in the air for long periods of time. A single cough is said to produce 3,000 infectious droplet nuclei. Direct sunlight kills tubercle bacilli in minutes, but they can survive in dark, unventilated environments for longer periods of time. Droplet
nuclei are so small that they avoid the defenses of the bronchi and penetrate into the terminal alveoli of the lungs, where multiplication and infection begins.

TB is spread by aerosolization of droplet nuclei bearing *Mycobacterium tuberculosis* released from the lungs of patients with pulmonary infection. Infection is established in about one-third of individuals exposed to the tubercle bacilli and of those only 10% ever become symptomatic (Rieder, 1999). In HIV infected population, the risk of TB disease is said to be ten times higher. The risk of infection is determined by the infectiousness of the source case (that is, how many tubercle bacilli are being coughed into the air), the closeness of contact, light and humidity, and the immune status of the host (Rieder, 1999). Patients with sputum smear-positive pulmonary disease are much more infectious than those with smear-negative sputum results (Styblo, 1991). Following infection, the tubercle bacilli multiply in the lungs, spread to the local lymph nodes, and then to the rest of the body. About six weeks after this primary infection, the body develops an immune response to the tubercle bacilli called delayed type of hypersensitivity reaction. In majority of cases, the immune response stops further multiplication of the tubercle bacilli, and the only evidence of infection is a positive response to an immunological test, of which the most commonly used is the tuberculin skin test (Ewer et al., 2003)

### 2.3 Tuberculosis treatment

Despite the availability of drugs to cure tuberculosis since the 1940s, TB remains an important cause of death from an infectious agent, second only to the human immunodeficiency virus (WHO, 2004). Chemotherapy is the most powerful weapon for tuberculosis control. To the individual patient, it reduces morbidity and mortality. The epidemiologic impact is that it breaks the chain of TB transmission and drug resistance when
properly administered (Crofton, 2009). Treatment of TB is given in a combination of drugs. The first line anti-tuberculosis drugs include: Streptomycin, Isoniazid, Rifampicin, Ethambutol and Pyrazinamide. Rifampicin and Isoniazid are the two most important tuberculosis drugs. Isoniazid is said to have early bacteriostatic activity, while Rifampicin has bactericidal and relapse preventing properties. During TB treatment, patients are monitored for clinical and bacteriological response at specific intervals. Patients who remain smear positive at four months following treatment initiation are said to be treatment failures (Chakaya et al., 2009).

The cornerstone of TB control is the prompt treatment of active cases with Short Course Chemotherapy (SCC) using first-line drugs, administered through the Directly Observed Treatment Strategy (DOTS) (WHO, 2002). Many of the 182 National DOTS programs in existence by the end of 2003 have shown that they can achieve high cure rates: the average treatment success rate was 82% (that is, the percentage that were sputum-smear negative at the end of treatment plus the percentage that had completed treatment but for whom cure was not confirmed by sputum smear), not far below the 85% international target (WHO, 2005). Kenya achieved WHO targets of 85% treatment success rates and 70% infectious cases detection rate in 2008 (NLTP, 2009).

2.4 Treatment of MDR-TB

MDR-TB treatment is given to patients who fail the first line anti-TB drugs. Similar to first line TB drugs, MDR-TB treatment is monitored clinically and bacteriologically and patients take their medicines under a DOT supervisor. The drugs are categorized into several groups (WHO, 2005) as shown in Table 2:1.
MDR-TB is more difficult to treat than drug-susceptible strains of TB. The success of treatment depends upon how quickly a case of TB is identified as drug resistant and whether an effective drug therapy is available. The second-line drugs used in cases of MDR-TB are often less effective and more likely to cause side effects as reported in the American Thoracic Society in 2003. Several factors influence the degree of success of treatment programs including duration and complexity of therapy, ease of healthcare access, treatment cost, patient adherence, and drug side effects (Mahmoudi and Iseman, 1993). Treatment for MDR-TB involves drug therapy over 18 to 24 months. Despite the longer course of treatment, the cure rate decreases from over 90% for non-resistant strains of TB to 50% for MDR-TB (American Thoracic Society, 2003).

Table 2:1 Categories of MDR-TB drugs,

<table>
<thead>
<tr>
<th>Category</th>
<th>Examples of drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polypeptides</td>
<td>Capreomycin, Viomycin, Enviomycin</td>
</tr>
<tr>
<td>Aminoglycosides (Injectable drugs)</td>
<td>Kanamycin, Capryomycin, Amikacin</td>
</tr>
<tr>
<td>Floroquinolones</td>
<td>Ciproxacin, Ofloxacin, Levofloxacin</td>
</tr>
<tr>
<td>Thionamides</td>
<td>Ethionamide, Prothionamide,</td>
</tr>
<tr>
<td>Reinforcers</td>
<td>Co-amxiclav, Clofazimine, Clarithromycin</td>
</tr>
</tbody>
</table>

Table 2:1 source: World Health Organization, 2000
2.5 MDR -TB Epidemiology

Tuberculosis is an extensively prevalent, preventable and treatable infectious disease causing great Public Health concern (Corbett et al., 2003). The disease was reported as a major global Health problem according to the World Health Organization (WHO) estimates (Dye et al., 1998). The World Health Organization estimates 8 million cases of tuberculosis occur each year, resulting in 3 million deaths (Sinder and Kochi, 1995). It has been observed that TB is the second highest cause of death due to infectious diseases after HIV and the current trends suggest that TB will still be among the ten leading causes of global disease burden in the year 2020 (Murray and Lopez, 2002). Kenya has reported a steady and substantial increase in the number of new TB cases and deaths since the early 1990s (Currie et al., 2003).

The present era has provoked further concerns with the advent of multi-drug resistant strains of *Mycobacterium tuberculosis*: In recognition of its significance, the World Health Organization declared tuberculosis a global emergency in 1994 (WHO, 2005). The TB epidemiology and the efficacy of control activities have been further complicated by emergence of drug resistant bacilli that is associated with the synergism of TB/HIV co-morbidity. Multi-drug tuberculosis which is defined as bacillary resistance to at least Isoniazid and Rifampicin invitro, is posing significant challenge in TB control worldwide (WHO, 2000). The strains of tuberculosis resistant to anti-TB drugs have been recovered from both immunocompetent and immunocompromised patients’ worldwide (Cohn et al., 1997). A strain of MDR -TB originally develops when a case of drug-susceptible tuberculosis is improperly or incompletely treated. Improper treatment allows individual TB bacilli that have natural resistance to a drug to multiply (Agerton et al., 1999). Once a strain of MDR TB develops it can be transmitted just like a normal drug-susceptible strain. It is estimated that in 2004, 4.3% (424,000) MDR cases occurred worldwide from among new and previously
treated TB patients (Zignol et al., 2006). Nosocomial outbreaks of MDR TB in New York City and Florida were responsible for the deaths of several patients and health care workers, a majority of whom were co-infected with HIV (Agerton et al., 1999). The recent Kwazulu Natal study found that health care workers with MDR-TB were significantly ($P < 0.001$) more likely to report previous treatment of tuberculosis than were non-health care workers (O’Donnell et al., 2010).

### 2.6 Tuberculosis Control

The control of tuberculosis is based on the WHO Directly Observed Treatment (DOT) strategy, whose philosophy is based on accurate and early detection of infectious patients (Ernarson and Jetgens, 1997) and cutting transmission chain by use of adequate and high quality tuberculosis drugs. There are tuberculosis control efforts that target overt disease in childhood through the immunization using Bacille Calmette Guerin (BCG) vaccine (Aronson et al., 2004). Infection prevention in congregate setting and within health care environments is some of the other approaches.
2.7 TB and HIV Interaction

The TB burden in countries with a generalized Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS) epidemic is documented to have increased rapidly over the past decade, especially in the severely affected countries of Eastern and Southern Africa (Corbett et al., 2003). This means that in regions where HIV infection rates are high in the general population, they are also high among patients with TB (Dye, 2005). In Africa, tuberculosis is said to be the first manifestation of HIV infection (Rana et al., 2000).

The TB and HIV guidance from WHO recommends Provider Initiated Testing and Counseling (PITC) of HIV as a standard of care for all patients with signs and symptoms of TB (WHO/UNAIDS, 2007). Kenya developed guidelines on HIV testing in clinical setting in 2005 and quickly scaled up the activities (Odhiambo et al., 2008).

The collaborative TB/HIV activities (WHO, 2003) advocate on the following:

To establish the mechanisms for collaboration by setting up a coordinating body for TB/HIV activities effective at all levels, conducting surveillance of HIV prevalence among tuberculosis patients, carrying out joint TB/HIV planning and conducting monitoring and evaluation.

To decrease the burden of tuberculosis in people living with HIV/AIDS by establishing intensified tuberculosis case-finding, introduction of Isoniazid preventive therapy and ensuring tuberculosis infection control in health care and congregate settings.

To decrease the burden of HIV in tuberculosis patients by providing HIV testing and counseling, introduction of HIV prevention methods, introducing Co-trimoxazole preventive therapy, ensure HIV/AIDS care and support and introduction of antiretroviral therapy.

The fight against HIV among TB patients has made some headway with NLTP reporting over 80% coverage (NLTP, 2008). However, the same reports indicate poor performance in
screening TB among people living with HIV and Aids (PLWA). This is due to laboratory related challenges, particularly for diagnosing MDR-TB. MDR-TB in a HIV infected patient carries a high risk of mortality, especially when diagnosed late (Corbett et al., 2003). It is in the light of these observations that the “Stop TB” strategy of WHO recommended strong MDR-TB surveillance (Stop TB partnership & WHO, 2006).

2.8 Laboratory diagnosis of TB and MDR-TB

The greatest contribution towards tuberculosis elimination is said to come from advances made in applied diagnostics, including new methods that reduce the time needed to detect growth of Mycobacterium tuberculosis in diagnostic specimens (Advisory Council for Elimination of Tuberculosis, 1999). A delayed or missed diagnosis of TB contributes to its transmission, morbidity and mortality due to TB (Lienhardt et al., 2001). Tuberculosis diagnosis’s in resource limited countries rely on clinical judgments, radiological findings as well as sputum smear microscopy and culture (Hale et al., 2001). Laboratory methods for tuberculosis diagnosis consist of three steps: Microscopic examination of smears prepared from sputum specimens, culture and identification and drug susceptibility tests. Smear microscopy is the main diagnostic test because it is cheap, identifies the most infectious patients (Corbett et al., 2003) but has low sensitivity and also cannot distinguish drug susceptible and resistant TB (Behr et al., 1999). Culture is more sensitive than microscopy, has long turnaround time, while molecular methods have both sensitivity and specificity have fast results.
2.8.1 Culture methods of diagnosis MDR-TB

The conventional method for TB culture is Lowenstein Jensen (LJ). Drug susceptibility testing (DST) on LJ allows the precise determination of the proportion of resistant mutants to a certain drug. Several 100-fold serial bacilli dilutions are inoculated into drug-containing and drug-free (control) media. One of these dilutions should produce a number of colonies that is easy to be counted. The number of colonies obtained in the drug-containing and control media are enumerated and the proportion of resistant mutants is then calculated. DST on LJ slopes is first read after 28 days of incubation at 37°C. If the proportion of resistant bacteria is higher than 1% for Isoniazid, Rifampicin and Streptomycin or 10% for the other drugs, the strain is considered resistant and the results are final; otherwise, the test is read again at 42 days of incubation to assess if the strain is susceptible to a certain drug (Heifets, 2000).

The Mycobacterium Growth Indicator Tube (MGIT) from Becton Dickinson, Sparks, medical diagnostics is part of the ‘new generation’ of TB diagnostic tools both in its manual version as well as in its more recently introduced automated format (Pfyffer et al., 1997). It is based on fluorescence detection of Mycobacterial growth in a tube containing a modified Middle brook 7H9 medium together with a fluorescence quenching-based oxygen sensor embedded at the bottom of the tube. Consumption of oxygen in the medium produces fluorescence when illuminated by an ultraviolet lamp. The presence of an orange fluorescence in the drug containing tube at the same time as in the control tube or within two days of positivity in the control is interpreted as resistance to the drug; otherwise, the strain is considered to be susceptible. Since identification of Mycobacterium species resistant to first line TB drugs is too time consuming, there is potential of MDR-TB diagnostic delay. The long delays in TB diagnosis have adverse effects on the patients; their family members, community, as well as TB control in general (Mathur et al., 1994). A study in Australia on diagnostic delay and
transmission of TB in an office concluded that delay in diagnosis was the major factor responsible for the spread of TB (MacIntyre et al., 1995). Introduction of molecular methods could greatly improve the speed and accuracy of TB diagnosis in the Kenyan public health laboratories.

### 2.8.2 Genotypic methods of diagnosing MDR-TB

Genotypic methods for drug resistance detect determinants of resistance with two basic steps: nucleic acid amplification such as polymerase chain reaction (PCR), to amplify the sections of the *M. tuberculosis* genome known to be altered in resistant strains; and a second step of assessing the amplified products for specific mutations correlating with drug resistance (Garcia de Viedma, 2003). New deoxyribonucleic acid (DNA) sequence-based techniques for the identification of *Mycobacterium* have been developed in the past ten years, such as DNA sequencing (Cloud et al., 2002), pyrosequencing (Tuohy et al., 2005), restriction fragment length polymorphism assays (Khan and Yadav, 2004), Real-time PCR assays (Shrestha et al., 2003), oligonucleotide assays and commercially available tests such as the AccuProbe from Gen-Probe Inc., San Diego, California. Most of these methods require expensive equipments, elaborate laboratory design, highly skilled expertise and can only identify a limited number of *Mycobacterium* species.

The Line Probe Assay (LiPA) was introduced several years ago and is based on reverse hybridization of amplified DNA from cultured strains to ten probes covering the core region of the *rpoB* gene of *M. tuberculosis*, immobilized on a nitrocellulose strip (De Beenhouwer et al., 1995). From the pattern of hybridization obtained, the presence or absence of mutants or wild regions is visualized by a colorimetric reaction and the strain can be considered as resistant or susceptible to Rifampicin (Rossau et al., 1997). Many studies have been conducted
on the application of the LiPA assay for detection of RIF resistance; most of them have been performed on *M. tuberculosis* isolates and none have applied the test directly on sputum samples (Jureen *et al.*, 2004). Recently WHO recommended the use of molecular line probe assays for rapid screening of MDR-TB in low income settings (WHO, 2009) to enhance TB control.

The GenoType®MTBDRplus is a molecular based test, it detects resistance to INH and RIF in culture samples based on the detection of the most common mutations in the *katG* and *rpoB* genes respectively (Makinen *et al.*, 2006). It also utilizes PCR and reverse hybridization to probes immobilized on a DNA strip. In a recent study that evaluated the GenoType®MTBDRplus assay in 143 *M. tuberculosis* isolates, 99% of the MDR strains were found to have mutations in the *rpoB* gene and 88.4% of strains with mutations in the cordon 315 of the *katG* gene were also correctly identified (Hillemann *et al.*, 2005). There is however no literature suggesting whether a study has been conducted to determine the accuracy of GenoType®MTBDRplus assay in the identification of MDR-TB from direct sputum specimens or isolates obtained from culture in Kenya.
CHAPTER 3: METHODOLOGY

3.1 Study design

This was a cross-sectional study conducted among patients with a history of first-line anti-tuberculosis treatment failure who were seeking medical care within TB clinics in Nairobi County. The cross-sectional study design was chosen because it was saving time and resources.

3.2 Study area

The study was carried out at the TB clinics in Nairobi County during the month of September 2009 to September 2010. Nairobi, occupies 684 square kilometers, has an altitude of 1,795 meters (5,889 ft) above sea level and enjoys a moderate climate. Tuberculosis control activities are structured within the main administrative districts which are Central, Dagoretti, Embakasi, Kasarani, Kibera, Makadara, Pumwani and Westlands. It has been observed that 75% of TB burden in Nairobi is associated with slum settlements and that Nairobi contributes to 20% of the national TB burden (Odhiambo et al., 2002).

Nairobi was chosen because it has a well established integrated public and private TB control program and is home to the Central Tuberculosis Reference Laboratory (CTRL), the only public culture and DST laboratory. The main participating TB clinics included Rhodes’s city council clinic, Kibera South’s community based clinic, Blue House’s clinic, Eastern Deanery AIDS Relief Program of the Catholic Church and Kenyatta National Hospital TB clinic. The laboratory analysis was performed at the CTRL, which performs culture and drug susceptibility testing for tuberculosis patients receiving TB treatment within TB diagnostic
centers in Kenya. CTRL supervises best practices in TB diagnosis at the regions including
decentralization of new TB diagnostic methods.

3.3 Study Population

The study population consisted of all TB patients with a history of first line anti-tuberculosis
treatment failure seeking medical care at the TB clinics in Nairobi County during the study
period, where TB and HIV services were offered at all the TB clinics as part of international
standards of care.

3.4 Sampling methods

All the patients seeking medical services at Nairobi County TB clinics formed the sampling
frame where majority of the patients were from Rhodes, Blue house, Kibera, EDARP and
Kenyatta National Hospital TB clinics. Systematic sampling was used to select every 3rd
patient on the basis of sputum smear positive and smear negative.

3.5 Sample size determination

The sample size was determined using the formula as used by Fisher et al, (1993):

\[ N = \frac{Z^2pq}{D^2} \]

Where,

N was the desired sample size when population >10,000, (the TB program estimates that
more than 10,000 patients failed first line TB treatment in 2008, 50% of whom were smear
positive). Z was the standard normal deviation (1.96) set at 95% Confidence level. The p was
the proportion in the target population with the desired characteristic (smear positive), for this study \( p \) was estimated at 50\%. The \( D \) was the degree of accuracy set at 0.05. Thus \( Q = 1 - p \# (1 - 0.5) = 0.5 \); Therefore, \( N = (1.96)^2 (0.5) (0.5) / (0.05)^2 = 384 \) Patients.

More samples (20\%) patients were added so as to cushion against loss as in indeterminate results, missing specimens and contaminated specimens. The working sample size was 457.

### 3.6 Inclusion criteria

1. TB patients with a history of first line anti-tuberculosis treatment failure who were either sputum smear positive or smear negative and who provided adequate sputum specimens for culture and DST.
2. Patients who consented to participate in the study.
3. Patients whose sputum specimens were submitted to CTRL for culture and DST during the study period.

### 3.7 Exclusion criteria

1. Patients who had no history of first line anti-tuberculosis treatment failure.
2. Patients whose specimens for culture and DST were not submitted to CTRL during the study period.
3. Patients who declined to participate in the study.
4. Patients whose specimens were rejected in the laboratory (leaking, unlabeled or mismatch between the specimen label and the request form).
3.8 Recruitment of patients and specimen collection

The selection of participating patients was carried out by recruiting every third patient from among those found to be sputum smear positive. Similarly, every third smear negative patient was also systematically selected to participate as part of the control group. The patients were educated on study procedures and on the need for drug susceptibility testing to evaluate if the drugs they were taking were working as part of standards of care in addition to routine HIV counseling and testing. Upon understanding of the study process and the benefits, the patients agreed to produce sputum specimen. Each participant was given a sterile container labeled with patient identification in addition to TB culture request form. The patient was advised to collect early morning specimen and how to bring the specimens to the clinic for drug susceptibility testing referral and subsequent further testing. The TB culture request form (Appendix 1) was filled with patient details such as patient TB treatment number, age, attended facility, district, province, HIV status, regimen and duration, type of patient and requesting clinician. Upon reception at the clinics, the specimens were packed and sent to CTRL. The specimens were processed according to routine standard operating procedures for MDR surveillance for culture and susceptibility testing and also evaluation of GenoType® MTBDRplus assay as shown in laboratory workflow in Appendix 2.

3.9 Laboratory methods

3.9.1 Sputum specimen reception

Upon reception at the Central Tuberculosis Reference laboratory, the specimens were unpacked in a biosafety cabinet level II. The specimen identification was matched with those on the request form. Specimens whose labels were not matching with the ones on the request forms were rejected. Similarly, specimens with leaking containers were also rejected. After
ascertaining the correct identity of the specimens, each specimen was given a unique laboratory number. The specimen was then taken through the laboratory process as presented in the algorithm in Figure 3:2.

Figure 3:2 Algorithm for evaluation of GenoType®MTBDRplus assay
3.9.2 Sputum specimen processing

Sputum processing as shown in Appendix 3, involved the treatment of the sputum to digest the mucus so as to release bacteria locked in the mucus, concentration of the specimen and killing of normal flora in the sputum. Equal volume of the sputum specimens and 4% sodium hydroxide was added together in a 50ml graduated centrifuge tube. The mixture was vortexed for two minutes and let to stand at room temperature for 15 minutes. The alkaline specimen was neutralized after 15 minutes with sterile phosphate buffer of pH 6.8 up to the 45th ml mark of the graduated centrifuge tube. The contents were well mixed then concentrated by centrifugation at 3000g relative centrifugal force for 15 minutes according to Petroff’s method (Ballows et al., 1991). The supernatant was poured off and the pellet was re-suspended with 1ml of sterile phosphate buffer pH 6.8. For each specimen, culture was performed on Lowenstein Jensen media and a smear was made from the concentrated deposit and stained using Ziehl Neelsen method. The smear positive specimens were further analyzed using the GenoType®MTBDRplus assay.

3.9.3 Smears microscopy

For each specimen processed, 20μl of the re-suspended pellet was spread on a microscope slide using 10μl disposable loops. The smear were left to dry in the biosafety cabinet for up to four hours. The smears were then heat fixed and stained using ZN method as seen in Appendix 4. The stained smears were then examined using a binocular microscope using 100 X objectives. Smears were graded as negative (0) or positive (+1, +2, and +3) according to WHO/IUALTD guidelines as shown on Table 3:1.
Table 3.1 WHO/IUATLD sputum smear grading scale

<table>
<thead>
<tr>
<th>WHO/IUATLD scale</th>
<th>ZN 1000x, 1 length = 100 HPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0 AFB per length</td>
</tr>
<tr>
<td>Actual numbers</td>
<td>1-9 AFB per length</td>
</tr>
<tr>
<td>1+</td>
<td>10-99 AFB per length</td>
</tr>
<tr>
<td>2+</td>
<td>1-10 AFB per HPF on average</td>
</tr>
<tr>
<td>3+</td>
<td>&gt;10 AFB per HPF on average</td>
</tr>
</tbody>
</table>

High power field (HPF), Acid fast bacilli (AFB)

Table 3.1 source: East African Medical Journal, 2006

3.9.4 Sputum culture

A portion (30μl) of the decontaminated sputum deposit was seeded on two slopes of Lowenstein and Jensen medium (LJ). An additional LJ slope for TB identification containing P-nitrobenzoic acid was inoculated. The slopes were incubated at 37°C at the walk-in incubator where they were examined weekly for evidence of growth until eight weeks as shown on Plate 3:1. The growth was graded as 1+ (20-100 colonies), 2+ (greater than 100 colonies) and 3+ (confluent growth). The Plate 3:2 shows TB colonies on LJ. Culture isolates were identified using characteristic colonial morphology, growth on P-nitro benzoic acid, and also by GenoType®MTBDRplus assay.
Plate 3:1 Lowenstein Jensen slopes incubated in a walk-in incubator

Plate 3:2 shows the different types of *Mycobacterium tuberculosis* (MTB) colonies observed on LJ media. Plate A shows the waxy type of MTB colonies, plate b shows the typical cream colonies and plate c shows the rough type of colonies.
3.9.5 Drug susceptibility testing

Drug susceptibility was performed on liquid media using MGIT 960 instrument SOP as shown in Appendix 5 and Plate 3:3. Isolates from solid media were subjected to growth in the presence of known concentrations of first line TB drugs. A control was also included with no addition of drug. Growth in the control and lack of growth in the drug containing tube was considered susceptible, while presence of growth in the drug containing tube was regarded as resistance. The drugs were reconstituted with sterile distilled water in a clean biosafety cabinet level II. For each clearly labeled drug tube (Streptomycin, Isoniazid, Rifampicin and Ethambutol) 100μl of specific drug concentration was added and mixed well. Colonies from LJ media were emulsified in a bijou bottle containing sterile distilled water and glass beads. The suspension was left to stand for 20 minutes. The supernatant was reconstituted with distilled water to match a turbidity of McFarland # 0.5 standard. The McFarland # 0.5
standard suspension was further diluted 1:5 by adding 1ml to 4mls distilled water to make drug containing tubes inoculum. Some 100μl of the 1:5 dilutions was diluted 1:100 to make growth control inoculum. The tubes were tightened, arranged in susceptibility test set carriers and then loaded onto MGIT 960 instrument. The MGIT instrument was observed daily in accordance with manufacturer’s instructions. The 960 instrument monitored the tubes and interpreted the results as susceptible (S), resistant (R) or invalid (X).

Plate 3:3 MGIT 960 instruments used for DST
Explanation of Plate 3:3

a. Shows MGIT 960 instrument used for DST in this study.
b. Shows typical turbidity observed in an MGIT 960 positive culture
c. Shows typical codes on ZN stain made from a positive MGIT culture
d. Shows the fluorescence indicator at the bottom of the MGIT 960 tubes that is detected by the instrument when the culture is positive

### 3.9.6 GenoType®MTBDRplus assay

The smear positive specimens with smear grading of 1+ to 3+ had the GenoType® MTBDRplus assay performed on the deposits (direct specimens) and a further GenoType® MTBDRplus assay was done on the isolates obtained from culture. A portion (1000μl for pellet) specimen was centrifuged at 14000g in a micro-centrifuge for 15 minutes to further concentrate the specimen. The pellet was re-suspended in 100μl of sterile distilled water, and then heated in a heating block at 95°C for 20 minutes to inactivate the bacteria. For the isolate, 500 μl of culture suspension was heated first at 95°C before it was sonicated. The specimen was then sonicated for 15 minutes at 40°C to extract DNA. The sonicated specimen was then spun at 13000g for five minutes to separate the heavy debris from the lighter DNA. The supernatant was aliquoted into a well labeled cryovial.

Amplification was performed by combining 5μl of the DNA (supernatant) with 45μl of master mix (35μl primer nucleotide mix, 5μl of 10X PCR buffer, 2μl MgCl₂, 3μl molecular grade water and 0.2 HotStar Tag polymerase) and then amplified in a thermocycler (Perkin Elmer 960 thermocycler; Perkin Elmer Corporation, Norwalk, CT) using the test manufacturer’s protocol presented in Appendix 6. The products of amplification were first denatured with
equal volume of the denaturation solution. The denatured amplicons were hybridized onto oligonucleotides probes immobilized onto well labeled test strips. Captured labeled hybrids were detected by colorimetric development. Resistance was determined by detection of missing wild type signals or by presence of mutation signals. Interpretations of results were done using manufacturers guide shown in the Figure 3: 2.

Figure 3:2 GenoType®MTBDRplus assay interpretation chart, Source: Journal of Clinical Microbiology, 2005

Fig 3:2 shows DNA strip patterns obtained from Genotype®MTBDR assay strips. The positions of the oligonucleotide probes are given on the left. The target genes and specific probe lines are shown from top to bottom as follows:

Conjugate control

Universal amplification control (23S rRNA)

*M. tuberculosis* complex-specific control (23S rRNA)

Control of *rpoB* amplification

Five *rpoB* wild-type probes
Four \textit{rpoB} mutant probes with common mutations in codon 516, 526, or 531

Control of \textit{katG} amplification

One \textit{katG} codon 315 wild-type probe

Two \textit{katG} probes with mutations in codon 315

An absence or weak band on the wild-type probe indicates resistance, and presence of the corresponding mutation bands also indicates resistance. Samples with different strip susceptibility patterns are shown as follows:

Strip 1 shows drug susceptible TB since \textit{rpoB} and \textit{katG} wild type is present and no mutations

Strip 2 shows MDR-TB because \textit{rpoB} wild type is missing at position 7 and mutation on probe 2A while \textit{katG} wild probe is missing with presence of probe number 1 mutation

Strip 3 shows INH resistance since no mutation on \textit{rpoB} gene and wild type probes are all present while \textit{katG inhA} wild type probe 1 is missing and \textit{inhA} mutation probe 1 is present

Strip 4 shows MDR because \textit{rpoB} wild type probe 8 is missing and mutation probe 3 is present while \textit{katG} wild type probe is missing, \textit{inhA} wild type probe 2 is missing and mutation probe 3 A is present

Strip 5 shows MDR since \textit{rpoB} wild type probe 7 is missing and \textit{katG} wild type probe is also missing

3.9 Data collection, management and analysis

Each specimen was accompanied by a culture request form which was filled by the attending clinician at the respective TB clinics. Data on patient demographics and clinical history was extracted from the TB culture request forms in Appendix 1. This included age, sex, HIV
status, facility, district, drug regimen, and date of specimen collection, type of patient and clinician. Laboratory data on sputum smear, culture, identification tests, direct GenoType®MTBDRplus assay, and GenoType®MTBDRplus assay on isolates from culture and liquid culture on 960 instrument susceptibility test on first line TB drugs was obtained from the laboratory analysis worksheets shown in Appendix 7. Each GenoType®MTBDRplus test analysis was conducted in a blinded manner without referring to MGIT DST results. All the data was entered into an Excel spread sheet.

The data was examined for accuracy by performing cross tabulation tables. The Statistical Analysis System (SAS) was used to clean and analyze the data from the Excel spread sheet. Summary statistics of the variables were done using contingency tables, frequency tables and graphs. Chi-square was used to establish association between variables and to test study hypothesis. The variables included age, sex, smear results, HIV status, and LJ culture, direct GenoType®MTBDRplus, MGIT DST and TB culture isolate GenoType®MTBDRplus. The accuracy of the GenoType®MTBDRplus assay was assessed by comparing the test under evaluation with the MGIT DST. This was determined by calculating Kappa statistic, sensitivity, specificity, positive predictive value and negative predictive value.
3.10 Ethical and research approval

Authority to carry out this research was granted by Graduate School of Kenyatta University and also by the National Council for Science and Technology Appendix 8 and Appendix 9. Ethical approval was granted by Kenyatta National Hospital/University of Nairobi ethics review committee as shown in Appendix 10. Permission to use CTRL for laboratory work was obtained from the Division of Leprosy, Tuberculosis and Lung Disease (DLTLD) as shown in Appendix 11. The patients were encouraged to participate voluntarily and confidentiality was maintained as no names were used. The patients were given feedback of their drug susceptibility testing through the existing CTRL feedback mechanism.
CHAPTER 4: RESULTS

4.1 Introduction

The study period was September 2009 to September 2010, when a total of 457 specimens were recruited from both public and private TB clinics in Nairobi County. The majority of specimens came from Eastern Deanery Aids Relief program (EDARP), 89 (19.5%), Rhodes chest clinic, 57 (12.5%), Kenyatta National Hospital, 32 (7%), Blue house clinic, 21(4.5%), Kibera South clinic, 28 (6%) and Kangemi clinic, 10 (1%) and others, 217 (49.5%) were from specimens submitted during the study period from other TB clinics in Nairobi. The results are presented as follows: demographic characteristics of the patients, sputum smear outcome, HIV status, culture outcome, DST and performance of GenoType®MTBDRplus.

4.2 Demographic characteristics of the study participants

The study analyzed 457 specimens; two of them did not have records on gender indicated on the request form, while information on age was missing on 11 specimens. Of the 455 specimens with gender recorded on the request form, 303 (66.6%) were males and 152 (33.4%) were females. The youngest and oldest were males of 3 and 67 years respectively. Median age was 32. The ratio of male to female was 2:1. The majority of participants belonged to the age group of 20-39 years.
4.3 Sputum smear outcome

Smear microscopy was performed on each of the 457 specimens received at CTRL according to standard operating procedure in Appendix 4. Of the 457 patients with sputum smear results, 274 (60.0%) and 183 (40.0%) were smear positive and negative respectively. The study compared the participants across gender to check whether sputum smear outcome had any relationship with gender. Of the 455 specimens with gender records on the request form, 187 (41.1%) male participants were smear positive while 116 (25.5%) were smear negative. Similarly, 85 (18.7%) of the female patients had smear positive results, the remaining 67 (14.7%) being smear negative.

The study sought to determine whether age group was associated with being sputum smear positive. The data in Figure 4:1 shows that the age group of the patient influences the occurrence of sputum smear positive TB among the participants (P=0.0011). The study also showed high rates (though not statistically supported) in childhood tuberculosis where 17/23 (73.9%) of children below 15 years who participated in this study were sputum smear positive. The highest sputum smear positive frequencies were observed in the 20-34 years old patients.
The study compared the outcome of sputum smear with the HIV status of the patients as shown in Table 4:1, where there was statistically significant association between sputum smear outcome and HIV status ($P < 0.0001$). The odds ratio also showed that patients with HIV positive status were 0.27 times less likely to be sputum smear positive TB compared with those that had HIV negative status with 95% confidence interval of 0.1417 to 0.5043.
Table 4:1 Comparison of sputum outcome with HIV status

<table>
<thead>
<tr>
<th>HIV Status</th>
<th>Sputum smear</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>%</td>
<td>Negative</td>
<td>%</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>52</td>
<td>26.9</td>
<td>56</td>
<td>29</td>
<td>108</td>
</tr>
<tr>
<td>Negative</td>
<td>66</td>
<td>34.2</td>
<td>19</td>
<td>9.8</td>
<td>85</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>61.1</td>
<td>75</td>
<td>38.9</td>
<td>193</td>
</tr>
</tbody>
</table>

Chi-square statistic of 17.4208 (p < 0.0001), Odds ratio of 0.2673 with a 95% confidence interval of 0.1417 to 0.5043

4.4 HIV status among the study participants

There were 193 participants whose HIV status was recorded in the TB culture request forms; one patient did not have records on gender, therefore the denominator for HIV status analysis was 192 patients. There were 108 (56.2%) HIV positive patients while 84 (43.8%) had HIV negative status according to clinical history recorded in the request forms. The proportion of HIV positive females (69.6%) was higher than that of males (48.8%) although the number of males was more as shown in Table 4:2. The study sought to establish the relationship between HIV positive status and gender, where the data showed significant association between HIV positive status and gender (P=0.0053).
The study also sought to find out if HIV status was associated with age group. The data showed high HIV positive frequencies among patients aged between 20-49 years, however, the study did not find any association between HIV positive status and age group (P=0.0527).

### 4.5 Lowenstein Jensen culture outcome

Of the 457 sputum specimens cultured on LJ media, six (1.3%) specimens had contamination, 278 (60.8%) had growth on LJ and 173 (37.9%) had no growth on LJ as seen in Figure 4:2. Of the 278 specimens with culture growth, 188 (68.1%) were from males and 88 (31.9%) were from females. Approximately 50% of the colonies on LJ were confluent.

<table>
<thead>
<tr>
<th>Gender</th>
<th>HIV status</th>
<th>Total</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>%</td>
<td>Negative</td>
<td>%</td>
<td>Participants</td>
</tr>
<tr>
<td>Female</td>
<td>48</td>
<td>69.6%</td>
<td>21</td>
<td>30.4%</td>
<td>69</td>
</tr>
<tr>
<td>Male</td>
<td>60</td>
<td>48.8%</td>
<td>63</td>
<td>51.2%</td>
<td>123</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>56.3%</td>
<td>84</td>
<td>43.8%</td>
<td>192</td>
</tr>
</tbody>
</table>

Chi-square statistic of 7.7596, (P <0.0053)
The study compared the outcome of culture given sputum positive results. There were 263 (97.0%) smear positive specimens that were culture positive, 15 (8.3%) smear negative specimens had growth on LJ while 8 (2.95%) smear positive specimens did not grow on LJ. The study found excellent agreement between sputum smear positive results and culture on LJ with a Kappa statistic of 0.893 with a 95% confidence interval of 0.8594-0.9356. High accuracy in the sputum smear results was demonstrated in sensitivity (94.6%), specificity (95.4%), positive predictive value (97%) and negative predictive (91.6%) values.

HIV testing among TB patients was performed routinely as part of International Standards of care. The history on patient’s HIV status was recorded on the TB culture request form by the requesting clinician. Some culture request forms did not have the HIV status results. The study compared culture confirmed TB outcome with HIV status. Of the 193 patients with HIV status in this comparison, two patients did not have culture results, so the denominator for this purpose was 191 patients. Among the 191 participants with TB culture and HIV status results, 108 (56.5%) were HIV positive while 83 (43.5%) were HIV negative as shown in Table 4:3.
The results also showed that 50 (42.4%) of the TB patients were also co-infected with HIV and that there was a strong association between TB and HIV status (P <0.0001). The study odds ratio showed that culture confirmed TB patients were 0.1902 times less likely to be HIV positive compared with culture negative tuberculosis patients with a 95% confidence interval of 0.0968 to 0.3735.

Table 4.3 Comparison of TB culture outcome with HIV status

<table>
<thead>
<tr>
<th>HIV status</th>
<th>Culture on Lowenstein Jenson</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>Positive</td>
<td>50</td>
<td>58</td>
<td>42.4%</td>
<td>79.5%</td>
<td>108</td>
</tr>
<tr>
<td>Negative</td>
<td>68</td>
<td>15</td>
<td>57.6%</td>
<td>20.5%</td>
<td>83</td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>73</td>
<td>100%</td>
<td>100%</td>
<td>191</td>
</tr>
</tbody>
</table>

Chi-square 25.2346, (P < 0.0001), Odds ratio of 0.1902 with a 95% confidence interval of 0.0968 to 0.3735.
Culture is often the gold standard test for TB diagnosis. The study compared TB disease burden across age groups among culture confirmed TB patients as represented in Figure 4:3. The data shows that culture confirmed TB rates are higher among patients aged 20-34 years and that there was almost equal proportions of culture confirmed TB and culture negative among patients older than 34 years.

Figure 4:3 Distribution of culture confirmed TB disease by age groups
4.6 Drug susceptibility testing outcome using MGIT 960 instrument

Drug susceptibility testing was performed on MGIT 960 instrument. DST was performed on all the 278 culture positive specimens however, only 246 specimens had DST results available, results of 32 specimens did not have DST results (either missing or contaminated).

The study sought to determine the prevalence of MDR-TB among the study participants. MDR-TB patients are those patients resistant to both Rifampicin and Isoniazid. The data analyzed for this comparison was from 246 of the specimens for which DST was analyzed to determine the specimens that had resistance to both Rifampicin and Isoniazid. MDR-TB was observed on 55 (22.36%), 157 (63.82%) were fully susceptible. There were 33 (13.41%) Isoniazid mono-resistant while 1(0.41%) specimens had Rifampicin mono-resistant as shown in Table 4:4.

Table 4:4 MDR-TB outcome on MGIT

<table>
<thead>
<tr>
<th>MDR-TB results</th>
<th>Frequency</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not MDR-TB</td>
<td>157</td>
<td>63.82</td>
</tr>
<tr>
<td>Yes MDR-TB</td>
<td>55</td>
<td>22.36</td>
</tr>
<tr>
<td>Isoniazid resistant</td>
<td>33</td>
<td>13.41</td>
</tr>
<tr>
<td>Rifampicin resistant</td>
<td>1</td>
<td>0.41</td>
</tr>
<tr>
<td>Total</td>
<td>246</td>
<td>100</td>
</tr>
<tr>
<td>Missing results 32</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The study compared the distribution of MDR-TB across the age groups as shown in Figure 4:4. The data shows that 6 patients diagnosed as MDR-TB by MGIT in the study were children below five years old. The study further shows that higher rates of MDR-TB were observed among relatively younger participants below 35 years old. The study did not find any association between age group and MDR ($P=0.0859$).

![Figure 4:4 Distribution of MDR-TB across age groups](image)

Figure 4:4 Distribution of MDR-TB across age groups
The study further analyzed the data to determine the relationship between MDR-TB and gender as presented in Table 4:5. The study results showed that male TB patients were 1.2910 times likely to be MDR-TB than the females with a 95% confidence interval of 0.6520 to 2.5562. The chi-square statistic (P=0.4630) was in support of the odds ratio outcome regarding lack of association.

Table 4:5 Distribution of MDR-TB by gender

<table>
<thead>
<tr>
<th>Gender</th>
<th>MDR by culture on MGIT 960</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDR</td>
<td>%</td>
<td>Not MDR</td>
<td>%</td>
<td>Total</td>
</tr>
<tr>
<td>Male</td>
<td>38</td>
<td>18.1</td>
<td>104</td>
<td>49.5</td>
<td>142</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>7.1</td>
<td>53</td>
<td>25.2</td>
<td>768</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>25.2</td>
<td>157</td>
<td>74.8</td>
<td>210</td>
</tr>
</tbody>
</table>

Chi-square was 0.5387, P =0.4630, Odds ratio of 1.2910 with a 95% confidence interval of 0.6520 to 2.5562.

The study found it necessary to analyze the data to establish the association between MDR-TB and HIV positive status as shown in Table 4:6. The study findings shows that MDR-TB patients are 0.7448 times likely to be HIV positive compared to first line anti-tuberculosis susceptible patients with a 95% confidence interval of 0.2852-1.9458.
Smear positive TB is believed to be the most infectious form of TB. In fact the first step in MDR-TB surveillance in Kenya is sputum smear microscopy which is routinely performed to monitor treatment progress at months two, four and six of TB treatment initiation. It was therefore important to check if there was any association between MDR-TB and sputum smear status as shown in Table 4:7. The study findings shows that MDR-TB patients are 2.1931 times likely to be sputum smear positive compared to first line anti-tuberculosis susceptible patients with a 95% confidence interval of 0.4751-10.1246. The chi-square statistic (P=0.3031) further supported the lack of association between sputum smear positive and MDR-TB outcome.

<table>
<thead>
<tr>
<th>HIV status</th>
<th>MDR by culture on MGIT 960</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
</tr>
</tbody>
</table>

Chi-square =0.3627, P=0.5470. Odds ratio =0.7448, 95% confidence interval of 0.2852-1.9458
Table 4:7 MDR-TB outcome given smear results

<table>
<thead>
<tr>
<th>MDR-TB</th>
<th>Sputum Smear results</th>
<th>Positive</th>
<th>%</th>
<th>Negative</th>
<th>%</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td></td>
<td>53</td>
<td>25.0</td>
<td>2</td>
<td>0.9</td>
<td>55</td>
<td>25.9</td>
</tr>
<tr>
<td>No</td>
<td></td>
<td>145</td>
<td>68.4</td>
<td>12</td>
<td>5.7</td>
<td>157</td>
<td>74.1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>198</td>
<td>93.4</td>
<td>14</td>
<td>6.6</td>
<td>212</td>
<td>100</td>
</tr>
</tbody>
</table>

Chi-square =1.0603, P = 0.3031, Odds ratio = 2.1931, 95% confidence interval of 0.4751-10.1246

4.7 Performance/accuracy of GenoType®MTBDRplus

The study sought to determine the performance of GenoType®MTBDRplus assay when sputum specimens were used as the biological sample, where MGIT 960 DST was the gold standard. The results of the study showed excellent agreement between the test under evaluation and the gold standard with a Kappa statistic of 0.8902 with a 95% confidence interval of 0.7684-1.0000. The results also showed high negative predictive value of 97.5% as shown in Table 4:8.
Table 4:8 Accuracy of GenoType®MTBDRplus on direct specimens (uncultured specimens)

<table>
<thead>
<tr>
<th>GenoType®MTBDRplus on uncultured specimens</th>
<th>MGIT 960 DST</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDR</td>
<td>Not MDR</td>
<td>Total</td>
</tr>
<tr>
<td>MDR</td>
<td>15</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Not MDR</td>
<td>2</td>
<td>78</td>
<td>80</td>
</tr>
<tr>
<td>Total</td>
<td>17</td>
<td>79</td>
<td>96</td>
</tr>
</tbody>
</table>

Kappa 0.8902; 95% confidence interval of 0.7684 to 1.000

- Sensitivity of GenoType®MTBDRplus on uncultured specimens = 88.2% (15/17 x100).
- Specificity of GenoType®MTBDRplus on uncultured specimens = 98.7% (78/79 x100).
- Positive predictive value of GenoType®MTBDRplus on uncultured specimens = 93.8% (15/16 x100).
- Negative predictive value of GenoType®MTBDRplus on uncultured specimens = 97.5% (78/80 x100)
The study sought to determine the performance of GenoType® MTBDRplus when culture isolates from LJ were used as the biological sample, MGIT 960 DST acting as the gold standard. The results of the study showed excellent agreement between the test under evaluation and the gold standard with a Kappa statistic of 0.9312 with a 95% confidence interval of 0.8647-0.9977. The results also show high negative predictive value = 97.3% as seen in Table 4:9.

<table>
<thead>
<tr>
<th>MGIT 960 DST</th>
<th>MDR</th>
<th>Not MDR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GenoType® MTBDRplus on TB isolates from LJ media</td>
<td>37</td>
<td>1</td>
<td>38</td>
</tr>
<tr>
<td>MDR</td>
<td>3</td>
<td>112</td>
<td>115</td>
</tr>
<tr>
<td>Not MDR</td>
<td>40</td>
<td>113</td>
<td>153</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Kappa 0.9312, 95% confidence interval of 0.8647 to 0.9977

- Sensitivity of GenoType® MTBDRplus on TB isolates = 92.5% (37/40x100).
- Specificity of GenoType® MTBDRplus on TB isolates = 99.1% (112/113x100).
- Positive predictive value of GenoType® MTBDRplus on TB isolates =97.4% (37/38x100).
- Negative predictive value of GenoType® MTBDRplus on TB isolates =97.3% (112/115x100).
CHAPTER 5: DISCUSSION

5.1 Demographic characteristics of participants

The study data showed that the age group of a TB patient with a history of treatment failure to first line anti-TB drugs was significantly associated with the occurrence of sputum smear positive TB \( (P=0.0011) \). These findings were consistent with previous studies from developing countries where age group was shown to be associated with occurrence of TB (Kochi et al., 1993).

There were six children below fourteen years who were suffering from MDR-TB. Although this observation was not statistically supported, the data seems to suggest worrying trends of childhood TB and MDR-TB. The World Health Organization, for epidemiological purposes, defines children as patients from 0 to 14 years (Reider, 1999). These children may have been in contact with MDR-TB patients some of whom may have been close relatives. Coming in contact with a known case of tuberculosis was significantly associated with MDR-TB in a case - control study conducted in four countries in Europe (Casal et al., 2005).

5.2 Influence of HIV status on the tuberculosis laboratory test outcome

The study showed that HIV status was significantly associated with sputum smear outcome with a chi-square statistic of 17.4208 and \( P<0.0001 \). This association was explained by interpreting the odds ratio statistic which showed that HIV positive patients were 0.27 times likely to be sputum smear positive compared to HIV negative patients with a 95% confidence interval of 0.1417 to 0.5043. To explain these findings, it is important to note that HIV lowers the ability of the infected patients to form TB cavities which makes HIV patients to have low bacterial load (Corbett et al., 2003). However, it was not possible to determine the WHO stage.
of the HIV disease from the patients. Similar observations were made in the study conducted by De cock and Chaisson, (1992). The data also showed that being HIV positive was significantly associated with gender ($P=0.0053$). Female patients were observed to have higher HIV rates ($69.6\%$). This suggests that the female study participants may have been most at risk of contracting HIV. Similar results were obtained in Kenya Demographic and Health Survey in 2003 where female participants were observed to have higher HIV rates.

The data showed high HIV rates among culture confirmed TB patients. The 191 patients with both HIV status records and culture results, ($56.5\%$) had HIV positive status and that being culture confirmed TB was significantly associated with positive HIV status ($P<0.0001$). The results also showed that $42.4\%$ of the TB patients were also co-infected with HIV. TB is said to be an opportunistic infection among people infected with HIV. This means that these patients may have been initially HIV infected and then contracted TB. It is worth noting that $20\%$ of the National TB burden comes from Nairobi, $75\%$ of which is related to slum settlement (Odhiambo et al., 2002). The study results are consistent with observations made among patients in the private sector in Nairobi where high TB and HIV co-morbidity of $51\%$ was observed by Chakaya et al, (2008).

5.3 Prevalence of MDR-TB among patients seeking medical care in Nairobi TB clinics

The study established that patients with a history of anti-TB treatment failure in Nairobi County had $22.36\%$ MDR-TB rates. Multi-drug resistant tuberculosis rates greater than $2.2\%$ are considered to be high (Yanis et al., 2008). These rates are similar to the findings from South Africa by Marinus et al, (2008), where $19.2\%$ TB patients were MDR. A study
conducted in Uganda showed 11.7% MDR-TB rates among previously treated TB patients. The study by Yanis showed even lower rates of 5.8% MDR-TB in the Democratic Republic of Congo. In interpreting the current study findings, it is worth noting that Nairobi has been using the eight months TB treatment regimen where suspected treatment failures are put on re-treatment regimen. The high MDR-TB rates could have been a result of an amplified resistance effect secondary to adding one drug to an already failing regimen. Re-treatment regimen has been described to promote longer infectiousness, increased drug resistance and increased mortality (Schreiber et al., 2009). This amplifier effect has been described in a study by Mitchison (1998). Given that the study participants may have been living in districts where there was favorable environment to support infection transmission the theory on amplifier effect forms a perfect combination since Nairobi is an urban setting with congestion challenges. The study also showed that the patients within the age groups where TB rates were high, a similarly higher trend of MDR-TB was observed. These observations are consistent with the common knowledge that previous treatment for tuberculosis is a risk factor for developing MDR-TB (Mitchison, 1998).

In this study, HIV positive status was not significantly (P=0.5470) associated with being MDR-TB. These observations differ from those witnessed in the Peru study by Campos et al., (2003) where HIV positive status was a risk factor for MDR-TB. Similarly, this study recorded contrary findings from those observed in a study on four countries in Europe (Casal et al., 2005). In explaining the reason for this difference, it is important to note that these studies were conducted in countries where the laboratory turnaround time for MDR-TB testing was quite short unlike Kenya where DST results take three months. In Kenya, treatment of smear positive TB patients is usually allowed to proceed on treatment and only in the second month when there is evidence of delay of sputum conversion that MDR-TB is suspected. This
may not be the case with HIV positive TB patients who may present with smear negative pulmonary tuberculosis. It has been reported that HIV positive patients co-infected with MDR-TB have extremely high mortality (Ormerod, 2005). This means that an MDR-TB patient suffering from HIV may die within five weeks (Drobniewski et al., 2002) which is shorter period than it takes to diagnose MDR-TB using conventional methods suggesting that many MDR-TB patients die way before they are suspected or even diagnosed. A study in Peru showed that half of the MDR-TB patients who were also HIV infected died in less time than it takes to obtain DST results (Fierer, 2006). In this study, a combination of these factors may have resulted in selective survival of HIV negative MDR-TB patients hence the lack of association. Further studies will need to be conducted to explain this exception from common knowledge.

It has been observed that bacillary load on sputum smear microscopy tends to correlate well with severity of TB disease. Patients with cavitary pulmonary tuberculosis and lung damage have been shown to have a higher bacillary load (Rieder, 1999) which is associated with increased risk of random mutations that result in drug resistance. In this study, sputum smear positive outcome was not significantly associated with MDR-TB (P=0.3031). This observation may be explained by the fact that BCG vaccination was shown to offer MDR-TB protection in adults (Reider, 2002) and considering that BCG vaccination is part of routine immunization in Kenya. It has been previously shown that the presence of a BCG scar among prisoners in Kenya had a protective association with active tuberculosis (Amwayi et al., 2010).
5.4 Accuracy of GenoType®MTBDRPlus assay when applied to direct sputum specimens

The most important element in the diagnostic test is the accuracy of the test in relation to kappa statistic, specificity and sensitivity. The study observed excellent Kappa statistic outcome of 0.8902 with a 95% confidence interval of 0.7684-1.0000 when GenoType® MTBDRplus assay was used on direct sputum specimens. This suggests that about 90% of the times, this test was able to identify patients with MDR-TB from un-cultured sputum specimen. This means that GenoType®MTBDRplus assay is as accurate as MGIT TB culture. The test under evaluation had sensitivity, specificity, negative predictive value and positive predictive value of 88.2%, 98.7%, 97.5% and 93.8% respectively.

When isolates from LJ media were used as the biological sample the Kappa statistic of 0.9312 with a 95 % confidence interval of 0.8647-0.9977 was obtained. The sensitivity, specificity, negative predictive value and positive predictive values were 92.5%, 99.1%, 97.3% and 97.4% respectively. These findings suggest that the accuracy of GenoType® MTBDRplus assay was pretty much the same whether the sputum or culture was used. This means that instead of diagnosing MDR-TB using culture and DST that takes three months, it would be worthwhile using this molecular test as it takes two days.

These findings were consistent with those obtained in a similar study in Uganda where sensitivity, specificity, positive and negative predictive values were 92.3%, 96.2%, 80.0% and 98.7 % respectively, for detection of multidrug-resistance (Heidi et al., 2010). Similar findings were also obtained in Thailand by Rapeepun et al, (2010, where the sensitivity for MDR-TB was 94% when GenoType®MTBDRplus was used on TB isolates. As reported in the Ugandan study, Rifampicin resistance was highly associated with mutation in the 81 base pair region of
the \textit{rpoB} gene. In this study it was observed that mutation was common in the region of \textit{rpoB} 530-533. This mutation was more frequently found in MDR strains than in rifampicin nonresistant strains. This was common with findings in a recent South African study (Barnard \textit{et al.}, 2008).

The study demonstrated a higher culture recovery rates with a positive predictive value of 97\% compared to those observed in the study by Yvonne \textit{et al.}, (2009) from Uganda where the probability of obtaining culture positive given smear positive was 73\%. The smear to culture concordance was excellent with Kappa statistic of 0.89 with a 95\% confidence interval of 0.8594-0.9356. This was attributed to the fact that the specimens were concentrated prior to smear examination. The smear accuracy observed in this study was comparable and consistent with the findings of a study conducted in Dar es Salaam by Basra \textit{et al.}, (2006) but at the same time higher than the results observed in a similar study by Somoskovi and Magyar (1999).
CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The data showed that HIV status was a significant determinant for being sputum smear positive among patients failing first line anti-tuberculosis treatment in Nairobi. Similarly, gender was observed to significantly influence HIV status of the participants. Nairobi has high HIV and TB co-infection. Age group was also observed to significantly influence sputum smear outcome in Nairobi.

2. The study established that patients with a history of anti-TB treatment failure in Nairobi County have high rates (22.36%) of MDR-TB which was not associated with HIV infection and pulmonary tuberculosis.

3. GenoType®MTBDRplus assay is an accurate screening tool for MDR TB, contributes a substantial reduction in laboratory diagnostic delay and that results compared well with those obtained in similar studies.

4. The evidence in this study therefore demands rejection of the null hypothesis which stated that GenoType®MTBDRplus assay was not an accurate method for the rapid diagnosis and surveillance of MDR-TB. The study concluded that GenoType® MTBDRplus assay was an accurate method for rapid diagnosis and surveillance of MDR-TB.

6.2 Recommendations

1. Due to the High MDR-TB rates, TB clinics in Nairobi County are likely to pose high risk of exposure to Health care workers. This study recommends that the TB program should consider appropriate infection prevention measures and accelerated MDR-TB case finding in Nairobi County.
2. This study recommends that TB program may consider GenoType®MTBDRplus as a rapid screening test of MDR-TB diagnosis and surveillance in Nairobi County.

3. Due to the high HIV, TB and HIV co-infection among TB patients in Nairobi, the TB program should consider diagnostic tests superior than sputum smear and also initiate patient management interventions that target TB and HIV diseases.

6.3 Suggestions for further Research

Given the relatively slow progress in implementing molecular testing for MDR-TB, there is need for further research:

1. To determine the risk factors for multi-drug resistant tuberculosis in Nairobi County.

2. To estimate the cost associated with scale up of rapid molecular MDR-TB diagnostic tests in Nairobi.


APPENDIX 1: TB CULTURE REQUEST FORM

Ministry of Health
National Leprosy & Tuberculosis Programme

CULTURE REQUEST FORM

Name:.............................................. Registration No..................................OP/P No..................................
Address:......................................... Age:............................. Sex:..........................
........................................................... Clinic:..................................... Ward:..............................
Facility:................................................
Clinician name:..............................Signature:....................................Date:..........................
Specimen of:......................................Date of collection:...........................

Examination required: Smear O Culture O Sensitivity O

Type of patient: Sputum smear-positive relapse O
Sputum smear-negative relapse O
Returned after defaulting O
Failure 1-st line treatment O
Failure re-treatment O

Previous treatment:
Isoniazid from: date .........................to.........................
Streptomycin from: date .....................to.........................
Rifampicin from: date .......................to.........................
Pyrazinamide from: date ...................to.........................
Ethambutol from: date ......................to.........................
Other (specify) from: date ..................to.........................

SEND SPECIMEN TO CENTRAL REFERENCE LABORATORY: P.O. BOX 20781, NAIROBI
**LABORATORY REPORT**
Central Reference Laboratory

**Direct smear report (WHO/IATLD reporting format)**

<table>
<thead>
<tr>
<th>Count of AFB seen</th>
<th>Description</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0 AFB seen in 300 fields</td>
<td>O</td>
</tr>
<tr>
<td>1 – 9</td>
<td>1 – 9 AFB seen in 300 fields, actual number.</td>
<td>O</td>
</tr>
<tr>
<td>10 – 99</td>
<td>10 – 99 AFB seen in 300 fields</td>
<td>O</td>
</tr>
<tr>
<td>1 – 10</td>
<td>1 – 10 AFB seen in 150 fields</td>
<td>O</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>&gt; 10 AFB seen in 50 fields</td>
<td>O</td>
</tr>
</tbody>
</table>

**Sensitivity testing:**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoniazid (H)</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Streptomycin (S)</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Rifampicin (R)</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Ethambutol (E)</td>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>Pyrazinamide (Z)</td>
<td>O</td>
<td>O</td>
</tr>
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</table>

**Identification test:**

<table>
<thead>
<tr>
<th>Type</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>O</td>
</tr>
<tr>
<td>Bovine</td>
<td>O</td>
</tr>
<tr>
<td>Atypical</td>
<td>O</td>
</tr>
</tbody>
</table>

**Culture report**

<table>
<thead>
<tr>
<th>Mode of culture</th>
<th>MGIT 960</th>
<th>Lowenstein Jensen</th>
<th>Fast Plaque TB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confluent growth</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 100 colonies</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 – 100 colonies</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – 19 colonies</td>
<td>O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contaminated</td>
<td>O</td>
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</table>

**Identification test:**

<table>
<thead>
<tr>
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<th>Code</th>
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<tbody>
<tr>
<td>Human</td>
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<tr>
<td>Bovine</td>
<td>O</td>
</tr>
<tr>
<td>Atypical</td>
<td>O</td>
</tr>
</tbody>
</table>

**Date:**

---

**CENTRAL REFERENCE LABORATORY: P.O. BOX 20781, NAIROBI**

---
APPENDIX 2: FLOW CHART ON LABORATORY METHODS

➢ Systematically recruit every 3rd specimen from a patient with history of treatment failure on basis of sputum smear results

➢ Collect sputum specimens from each patient and transport them to CTRL.

➢ Decontaminate, digest and concentrate the specimens according to Petroff's method, make smears on each specimen and stain using ZN method, seed deposit LJ and perform GenoType®MTBDRplus assay on deposit as well as on growth obtained from LJ slopes. Also perform DST on LJ slopes.

Compare the accuracy (Kappa statistic, sensitivity, specificity and predictive values) of GenoType®MTBDRplus assay performed on direct specimens against the gold standard (MGIT 960) DST.
APPENDIX 3: TB CULTURE ON LJ SPECIMEN PROCESSING SOP

1. Scope

This SOP describes methods of specimen processing and other laboratory procedures for purposes of culturing *Mycobacterium tuberculosis* culture on solid or liquid media.

2. Definitions and abbreviations

BSC: biological safety cabinet
CPC: cetylpyridinium chloride
CSF: cerebrospinal fluid
ID: patient's specimen identification, usually laboratory number
LJ: Löwenstein–Jensen
MW: molecular weight
NALC: N-acetyl L-cysteine
NTP: National tuberculoses programme
RCF: Relative centrifugal force

3. Personnel qualifications

3.1 Medical fitness

In accordance with national laws and practices, arrangements should be made for appropriate health surveillance of TB laboratory workers:

- before enrolment in the TB laboratory;
- at regular intervals thereafter, annually or bi-annually;
- after any biohazard incident;
- at the onset of TB symptoms.

Ideally, individual medical records shall be kept for up to 10 years following the end of occupational exposure.

Laboratory workers should be educated about the symptoms of TB and provided with ready access to free medical care if symptoms arise.
Confidential HIV counselling and testing should be offered to laboratory workers. Options for reassignment of HIV-positive or immuno-suppressed individuals away from the high-risk areas of the TB laboratory should be considered.

All cases of disease or death identified in accordance with national laws and/or practice as resulting from occupational exposure to biological agents shall be notified to the competent authority.

3.2 Education and training

Basic education and training must be given on the following topics:

- potential risks to health (symptoms of TB disease and transmission);
- precautions to be taken to minimize aerosol formation and prevent exposure;
- hygiene requirements;
- wearing and use of protective equipment and clothing;
- handling of potentially infectious materials;
- laboratory design, including airflow conditions;
- use of biological safety cabinet, microscope, balance, centrifuge, autoclave, pipetting aids (operation, identification of malfunctions, maintenance);
- prevention of incidents and steps to be taken by workers in the case of incidents (biohazard incidents, chemical, electrical and fire hazards);
- good laboratory practice and good microbiological techniques;
- organization of work flow;
- procedures;
- waste management;
- importance of laboratory results for patient management;
- Importance of laboratory results for the national TB programme.

The training shall be:

- given during orientation before a staff member is deployed to the work bench
- strictly supervised;
- adapted to take account of new or changed conditions; and
- repeated periodically, preferably every year.
4. Procedure

4.1 Principle

Culture examination detects fewer bacilli than microscopy and increases the number of TB cases found by 20–50%, depending on local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and allow the detection of drug resistance.

**DLTLD policy for culture targets patients on TB re-retreatment, failure cases, MDR-TB and MDR-TB contacts.**

Specimens for isolation of tubercle bacilli contain associated bacterial and/or fungal flora which have to be eliminated before the specimen is inoculated onto culture media.

4.2 Samples

Refer to SOP “Sample conditions and transport for culture procedure” for checking the quality of specimens. The following specimens should not be processed:

- dried swabs
- Un-labeled
- Miss-match of form and specimen label
- saliva
- specimens in broken containers
- Specimens collected more than 7 days previously.

*Note: DLTLD recommend that saliva be processed but that an additional specimen be requested and processed.*

4.3 Equipment and materials

BSC, class I or II, annually certified
Slides
Slide warmer
Refrigerated centrifuge with safety shield, a minimum RCF of 3000g, operated at 8–10 °C
Centrifuge tubes, preferably 50-ml capacity, clear plastic, with screw-caps, resistant to RCF of >3000g RCF.
Rack for tubes
Balance
Pasteur pipettes for 3.0 ml (with graduation), sterile, single-use, plastic (non-sterile pipettes must be sterilized on site before use)
Pipetting aids
Mini-blender (for biopsy)
Disinfectants (see relevant SOP)
Separate waste containers, autoclavable, for pipettes and disposals
Autoclave
Buckets, stainless steel or polypropylene
Vortex mixer
Timer
Incubator
General laboratory glassware
Refrigerator
Decontamination reagents and solutions (refer to SOP “Preparation of reagents for specimen processing for culture”).

4.4 Reagents and solutions

Tubes/vials for culture media: for culture on solid media use egg-based media, Lowenstein–Jensen or commercially available liquid media (MGIT); preparation of media is described in SOP "Preparation of plain egg-media" and SOP "Preparation of reagents for specimen processing for culture”).

The reagents and solutions needed are indicated for each method below.
Avoid using reagent bottles that have already been opened: Use aliquoted stock solutions (see SOP “Preparation of reagents for specimen processing for culture”).

4.5 Detailed instructions

**Important points about specimen processing procedures**

- Process clinical specimens as soon as possible.
- Properly label the media to be inoculated to avoid any mix-up of the specimens.
- Minimize aerosol production by opening specimen containers slowly, letting the tubes stand for a few minutes after shaking and before opening, and avoiding expulsion of the last drop from the pipette.
- Process only one specimen at each time. Do not allow open containers or open centrifuge tubes in the BSC. Use aliquots of buffer and decontamination solutions. Use a fresh pipette at every step to avoid transfer of bacilli from one specimen to the other.
- Aseptic technique is important to avoid contamination by bacteria other than tubercle bacilli and especially cross-contamination by tubercle bacilli from other specimens.
- Remember that most techniques require exposure time to disinfectant to be strictly controlled.
- If liquid media are used, it is recommended that solid media are also inoculated to provide back-up cultures in case of contamination of liquid media or in case of malfunction problem if an automated system is used.
- If solid media are used, it is recommended that liquid media are also inoculated to increase the sensitivity of recovery – especially for tissue biopsy, CSF or other small volume of aseptically collected body fluid.
- Prepare smears for staining *after* all media have been inoculated.
- If *M. bovis* is suspected, inoculate one slope of LJ containing pyruvate medium in addition to other inoculated LJ slopes.

*A. Sputum processing*
Except for the simple culture method, specimens must be processed in centrifuge tubes. If collected in standard containers, sputa must be transferred into centrifuge tubes, which increase the risk of cross-contamination and labeling error. It is thus practical to use 50-ml centrifuge tubes to collect specimens for culture.

Sputa should not be processed in sets of more than 8–12 because the methods described here are strictly time-dependent. Larger sets cannot be handled in time (except in the trisodium phosphate and CPC procedures). The first step of these less time-dependent methods can be performed in microscopy laboratories, outside a BSC. Transport to a biosafety level 2 laboratory must be organized within one day for the trisodium phosphate method and within one week for the CPC method.

4.5.1 Sodium hydroxide (modified Petroff) method

Sodium hydroxide is toxic, both for contaminants and for tubercle bacilli; strict adherence to the indicated timings is therefore essential.

Reagents

Sodium hydroxide (NaOH) solution, 4%
Phosphate buffer 0.067 mol/litre, pH 6.8

Procedure

1. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of 4% NaOH and tighten the screw-cap.
2. Vortex to digest.
3. Allow to stand for 15 minutes at room temperature.
4. Fill the tube to the 5-ml mark on the tube with phosphate buffer.
5. Centrifuge at 3000g for 15 minutes.
6. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other Mycobacterial disinfectant.
7. Re-suspend the deposit in approximately 1 ml phosphate buffer.
8. Inoculate deposit on two slopes of egg-based medium labeled with the ID number. Use a pipette to inoculate each slope with 3–4 drops (approximately 0.1–0.15 ml).
9. Smear one drop on a slide, marked with the ID number, for microscopic examination.
4.6 Reading, interpretation, recording and reporting

4.6.1 Incubation of cultures

Incubate tubes/vials at 36 ±1 °C.

For solid media, tubes should be incubated in a slanted position, with screw-caps loose, for at least 1 week to ensure even distribution and absorption of inoculum. After 1 week of incubation, caps are tightened to minimize evaporation and drying of the media. Tubes may then stand upright to save space in incubators.

4.6.2 Reading, interpretation, recording and reporting

Reading and interpretation using solid media

Check colony formation every week, preferably twice within the first week, to allow rapid detection of contamination and a timely request for another specimen if necessary.

Contaminated cultures and rapidly growing Mycobacteria (colonies apparent in less than 7 days) are removed. Report results immediately and ask for another specimen.

*M. tuberculosis* colonies should be well developed within 3–4 weeks. Report results immediately after detection and identification (refer to SOP "Identification of *M. tuberculosis*".

Cultures should be kept for up to 8 weeks before being reported as negative.

Laboratory register

Record in the laboratory register:

- the date of detection of growth and the colony characteristics of positive cultures;
- negative and contaminated tubes – individually entered at the end of the recommended incubation time or when detected;
- The reporting date.

Reporting

Results should be reported in accordance with qualitative and quantitative criteria.

Fill out an individual form for each positive patient for whom diagnostic specimens were submitted (see Annex).
4.7 Quality control

4.7.1 Sensitivity of plain egg-based medium

The quality of commercially available egg-based media should be certified by the manufacturer. However, storage conditions – especially during long-distance transportation – may not be optimal. It is therefore good practice to check new batches medium used in the laboratory.

Within the laboratory network, the sensitivity of medium batches should be checked by the laboratory producing the medium and not rechecked by users. It is the responsibility of the network to organize the transportation of media under appropriate conditions. Egg-based media are robust and retain their sensitivity unless exposed to direct sunlight or prolonged high temperatures.

Serious problems affecting the sensitivity of culture medium, i.e. its capacity to sustain consistent growth of tubercle bacilli, can be detected by seeding a 1/10 000 dilution of a suspension of *M. tuberculosis* calibrated to McFarland No. 1 (equivalent to a bacterial suspension containing 1 mg/ml of tubercle bacilli – refer to the relevant SOP "Preparation of MacFarland standard suspensions"):  

- Prepare a McFarland No. 1 suspension with a *M. tuberculosis* reference strain.
- Dilute the suspension with 10-fold dilutions to the $10^{-4}$ dilution.
- Inoculate five tubes of a previous batch of medium and five tubes of the new batch of medium with 0.2 ml of the $10^{-4}$ diluted suspension.
- Incubate at $36 \pm 1 \degree C$.
- Read and interpret as usual, following instructions given above in section 4.6.1.
- If the number of colonies obtained on the recently prepared or purchased batch of medium is significantly lower than that on the reference batch, the sensitivity of the new medium, whether prepared or purchased, is not adequate.

4.7.3 Specimen handling

Reception

Make sure that the samples received are clearly identified and are accompanied by a completed NTP request form. It is critical for the laboratory to be able to differentiate specimens received for diagnostic purposes from those received for control of treatment. This
will allow proper interpretation of results and guide the sequence of necessary bacteriological studies for each patient. Lack of this information rules out the use of monitoring of bacteriological results as a method of internal quality control.

Make sure also that the laboratory register shows the date on which the sample was cultured as well as all the above-mentioned information and any relevant details concerning the processing of the specimen (e.g. decontamination of aseptically collected specimens).

Organization of work

The routine of laboratory work will determine the maximum possible number of days per week that can be allocated to specimen processing. Each day of delay in inoculation of the specimen on growth media diminishes culture positivity rate. Do not delay the processing of the specimens beyond a period of 7 days following collection. Use commonly recommended methods of preservation, such as the CPC/NaCl method, when transportation delays are expected. The processing of gastric washings from children should be expedited as much as possible.

Maintain a systematic and traceable sequence of processing the specimens during the work day and process smear-positive specimens (potential sources of cross-contamination) last. Do not process culture isolates alongside clinical specimens.

Decontamination and cross-contamination

Take all precautions to avoid the transfer of bacilli from one specimen to another:

- use aliquoted reagent solutions;
- do not reuse the reagent solution aliquots opened during the work day;
- do not open a specimen container or a tube before capping the previous one;
- dispense solutions without touching the neck of the tubes with the pipettes or dispensers; and
- carefully decant supernatants into a flask containing a 5% phenol aqueous solution to avoid splashing (e.g. along a funnel).

The total contact time (15 minutes) of the sample with the decontaminant must be strictly controlled when using the methods in sections 4.5.1, 4.5.2, 4.5.4 and 4.5.6. Too short a time results in high contamination rates, too long a time causes loss of viability of the bacilli.
Note that some manufacturers of commercially available liquid media based on modified Middle brook media recommended the NALC decontamination method only.

Concentration by centrifugation

When using a centrifuge (for all methods applied to specimens except the Kudoh method), verify that the rotor reaches and maintains the required RCF of 3000g for 15 minutes in order to obtain good recovery of the Mycobacteria. Non-refrigerated centrifuges are not suitable because temperatures reached during centrifugation typically exceed 37 °C and will affect the viability of the bacilli. However, using CPC for decontamination/digestion of sputa, centrifugation must be done at room temperature because CPC precipitates at lower temperatures.

Incubation

Record the temperature of the incubator daily with a thermometer accurate to ±1 °C. The temperature should not fluctuate beyond the 35–37 °C range. When an incubation chamber is used, place thermometers at different and easily visible places to ascertain temperature uniformity. Maximum–minimum thermometers provide more information on temperature variation.

Whenever the temperature exceeds 38 °C or falls below 35 °C, the event should be entered in the laboratory registry. Excessive temperature will invalidate culture-negative reports for the specimens that were incubated during the occurrence. New specimens should be obtained from these patients for retesting.

When using automated systems for liquid cultures, refer to the manufacturer’s manual.

Reading

• Solid media

Make sure that cultures are checked at regular intervals:

➢ at 3 days of incubation to detect and record early contamination;
➢ Weekly to detect growth as early as possible.

Confirm that new specimens have been requested whenever smear-positive specimens turn out to be culture-negative or when all inoculated tubes/vials are contaminated.
• Liquid media

Check every day or refer to the manufacturer's instructions when using automated systems.

Recording and reporting of results

Make sure that:

• the date of detection of growth and the colony characteristics of positive cultures are entered in the laboratory register;

• for liquid media, the date and number of days to detection of growth are recorded;

• negative results are entered at the end of the recommended incubation time;

• contaminated tubes are individually entered when detected;

• the laboratory register also records the reporting date;

• results are reported to clinicians as soon as they are available;

• results are reported according to qualitative and quantitative criteria.

Using liquid media, contaminated or mixed cultures may be interpreted as positive. A preliminary check for the presence of Mycobacteria is carried out by microscopic examination of a smear prepared from the growing organisms at the bottom of the liquid culture (do not mix as the growth is mainly at the bottom of the tube). The purity of a positive liquid culture can be verified by inoculating a blood agar or chocolate agar plate with the liquid culture. Different types of colonies on solid media contribute to the detection of possible mixed Mycobacterial cultures, whereas mixed cultures are difficult to detect in liquid media and may require the use of a molecular method that can reveal the presence of other Mycobacterial species in addition to tubercle bacilli.

Fill out an individual form for each patient for whom specimens for diagnosis were submitted. Future results of all bacteriological testing will be added to the form. This facilitates the detection of anomalies, e.g. patients who systematically show smear-positivity and culture-negativity, non-reproducible reports, patients positive after the third month of treatment. It also allows the determination of performance indicators, such as the contribution of the culture to diagnosis.

The supervisor, who oversees all technical aspects of TB laboratory work, will monitor monthly the compliance with guidelines in the preparation of reagents, media, handling of samples, recording and reporting etc. The supervisor will also check records of pH readings of
the salt solutions used in the preparation of egg-based media and sterilization records obtained with temperature monitoring devices.

4.7.3 Daily monitoring routines

Daily monitoring allows early correction of errors. The following paragraphs deal with "alarm signals" that require attention.

Smear-positive/culture-negative specimens

Find out whether the specimen was collected for control of treatment. If this is the case, a negative culture could simply reflect the fact that the patient is shedding dead bacilli and that treatment is effective.

If the sample was collected for diagnostic purposes, watch for a recurrence of this type of result.

Ensure that:

- the concentration of the decontaminating solution and the time of contact with the specimens are those recommended in the technical guidelines;
- the temperature of the incubator did not exceed acceptable limits;
- the sensitivity of the culture batch being used has been thoroughly checked.

Contaminated tubes/specimens

Find out whether the time that elapsed between specimen collection and specimen processing was too long. If so, corrective measures will have to be introduced in the specimen transportation system, in the laboratory work routine or in both.

Recurrent contamination

Recurrent contamination can occur in specimens processed during a particular day or in decontaminated specimens or in specimens collected in one particular place. In such cases, the sterility of the decontamination reagent solutions, of the whole decontamination process or of the specimen collection/transportation system will have to be checked; if errors are detected, immediate remedial action must be implemented. If the problem is traced to the technologist performing the procedure, he or she should be immediately retrained.
If contamination of specimens from the same patient recurs, a harsher decontamination procedure may have to be used for further specimens from the patient. Increase the reagent concentration, not the time of exposure. Use two volumes of decontaminant solution to one volume of specimen. Do not apply the modified procedure to all specimens – only to contaminated specimens.

**Clustering of culture-positive specimens**

Cross-contamination between specimens from epidemiologically unrelated patients can cause a sequence of positive culture isolations in a short interval of time. The occurrence of cross-contamination should be investigated to rule out false-positive culture diagnoses. The following circumstances will be investigated:

- some of the patients involved do not have clinical symptoms compatible with tuberculosis;
- other specimens from the same patient are not culture-positive;
- one or several specimens involved, which yielded cultures with very few colonies, were processed immediately after a highly smear-positive specimen.

If, in laboratories processing numerous extra pulmonary, supposedly sterile specimens, it is found that the positive cultures were derived from decontaminated specimens only, this would strongly imply that the transfer occurred via the decontamination solutions or the laboratory equipment. If cross-contamination is suspected in such circumstances, the cultures involved in the contamination episode should be submitted to a reference laboratory for genotyping.

If cross-contamination cannot be ruled out, check that the following precautions are being respected:

- solutions are dispensed without touching the necks of the tubes;
- aliquoted reagent solutions are being discarded after single use;
- the processing sequence of specimens is maintained, i.e. smear positive specimens are processed last;
- tubes are not uncapped simultaneously or immediately after being taken from the centrifuge;
- supernatants are discarded carefully;
- gloves, if worn, are frequently changed and never reused.
In busy laboratories in high-incidence settings, it may be advisable to have a BSC dedicated to processing of smear-positive specimens since the probability of cross-contamination increases with the number of smear-positive specimens.

4.7.4 Periodic monitoring routine

Depending on the workload and the prevalence of bacteriologically positive cases, analysis of the results obtained during a month, a quarter or a semester allows the detection of systematic errors. These analyses are key to the quality control of diagnostic cultures of pulmonary TB in adults (but do not apply to follow-up).

Classification of specimens from adult pulmonary TB patients investigated for diagnosis:

- **a** smear-positive and culture-positive
- **b** smear-positive and culture not done
- **c** smear-negative and culture-positive
- **d** smear-positive and culture-negative
- **e** smear-positive and culture contaminated
- **f** smear not done and culture-positive

From this classification, calculate the following indicators:

- Contribution of culture to diagnosis for a given setting

  \[
  \frac{c + f}{a + b + c + d + e + f} \times 100
  \]

- Contribution of culture to diagnosis over microscopy

  \[
  \frac{c}{a + c + d + e} \times 100
  \]

Culture is more sensitive than smear microscopy and is expected to contribute at least 20% to the bacteriological confirmation of adult pulmonary TB cases.

- Percentage of smear-positive and culture-negative diagnostic cases
This percentage should be very low, typically around 2-3%. Exceptionally, patients are found with persistent smear-positive and culture-negative diagnostic specimens. These are usually undisclosed treatment control specimens. Higher percentages could be the result of decontamination procedures that are too harsh or of transport delays.

### 4.7.5 Determination of the contamination rate

The contamination rate is a valuable indicator of the efficiency of procedures used for specimen processing. It is calculated as the percentage of contaminated tubes among all inoculated tubes or vials and not as the percentage of samples. It should be within the range 2-5% and not exceed 5% if the Petroff decontamination method is used.

Using liquid media, the percentage contamination is usually higher but should not exceed 10%, otherwise the contribution of culture to diagnosis is not cost-effective.

When available, computer databases should be preferred to hard-copy forms for registering and monitoring results of positive patients and culture quality indicators.

### 4.7.6 Alarm signals

The indicators in the following table are valid for specimens from adult pulmonary TB patients investigated for diagnosis (but do not apply to follow-up):

<table>
<thead>
<tr>
<th>Indicators of culture performance</th>
<th>Normal value (%)</th>
<th>Much higher: investigate</th>
<th>Much lower: investigate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contribution of culture to bacteriological diagnosis of tuberculosis</td>
<td>20</td>
<td>A</td>
<td>B and C</td>
</tr>
<tr>
<td>Percentage of smear positive/culture negative specimens</td>
<td>2–3</td>
<td>C and D</td>
<td>Not a problem</td>
</tr>
<tr>
<td>Percentage of contaminated tubes</td>
<td>2–4</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>with liquid media</td>
<td>6–8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Smear microscopy reading errors: false-negatives”
- A high percentage of incipient pulmonary TB and paediatric TB cases are being tested (not a problem)

**B**

- Inadequate use of culture: patients who are not TB suspects are being examined, rather than incipient TB cases

**C**

- Excessive delay between specimen collection and specimen processing
- Over-harsh specimen decontamination procedures (excessive concentration and/or too long a contact time with the decontaminant)
- Low relative centrifugal force or overheating of centrifuge
- Low culture media sensitivity (lack of homogeneity, overheating during inspissations, too much malachite green, too acidic a pH)
- Incubation at too high or too variable a temperature
- Misclassification of a follow-up specimen

**D**

- Smear microscopy reading errors: false-positives

**E**

- Un-refrigerated storage of specimens
- Excessive delay between collection and processing of specimens
- Low decontaminant concentration
- Too short a contact time between decontaminant and specimen
- Deficiency in the sterilization procedure
- Careless use of the Bunsen burner, heavy people movement in the work area, generation of air draughts by fans or air-conditioning systems, etc,

**F**

- Too high a concentration of decontaminant
- Too long a contact time of the specimen with the decontaminant
• Poor specimen neutralization
• Too high a concentration of malachite green in the culture medium
• Incubation at too high or too variable a temperature

4.7.7 Delay in the delivery of reports

Culture procedures for tuberculosis bacteriology are notoriously time-consuming, often taking weeks or months to complete. For this reason, interim reports should be issued. The following schedule is recommended:
• If the cultures have been contaminated, a report should be sent out immediately and a repeat specimen requested
• If cultures are positive and growth has been identified as *M. tuberculosis* a report should be sent out immediately
• At four weeks an interim report (optional) could be sent out on all negative specimens, stating that another report will be issued in the event of the specimen becoming positive later on
• At eight weeks a final report should be issued containing all the data previously reported so that earlier interim reports can be destroyed and only the final report retained in the patients’ file

4.8 Waste disposal

All inoculated tubes and vials, whether negative or contaminated, should be autoclaved as potentially infectious material.

5. Related documents


**APPENDIX 4: ZIEHL NEELSON STAINING SOP**

Kenya Ministry of Public Health, NPHLS

<table>
<thead>
<tr>
<th>Procedure Number: Issue Number:</th>
</tr>
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<tbody>
<tr>
<td>SOP-----------------</td>
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**Procedure Title:**

The Detection of Acid Fast Bacilli by Microscopy - Ziehl-Neelsen staining Method

**Amendment History**

**Description of changes**

**Date of Issue: February 6th 2009** | **Total Pages: 4**

**Purpose:**

Acid-fast stains can be divided into two types, fluorescent stains and carbol fuchsin based stains. While fluorescent stains give greater sensitivity when screening initial specimens, carbol fuchsin stains are preferred for staining smears from cultures as they exhibit better morphological features.

**Reagents**

**Carbol fuchsin 1%**

**Fuchsin**

- Basic fuchsin 10g
- 95% ethanol (technical grade) 100ml

Dissolve basic fuchsin in ethanol Solution 1

**Phenol**

- Phenol crystals 5g
- Distilled water 100ml

Dissolve phenol crystals in distilled water (gentle heat may be required) Solution 2

**Working solution**

Combine 10ml of solution 1 with 90ml of solution 2 and store in an amber bottle. Label bottle with name of reagent as well as preparation and expiry dates. Can be stored at room temperature for six to twelve months and filter before use.
Decolourising agent solutions

3% Acid-alcohol solution

95% ethanol (technical grade) 970 ml
Concentrated hydrochloric acid (technical grade) 30 ml

Carefully add concentrated hydrochloric acid to 95% ethanol. **Always add acid slowly to alcohol, not vice versa.** The mixture will heat up. Store in an amber bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

Or, when alcohol is unavailable:

25% aqueous sulphuric acid solution

Distilled water 750 ml
Concentrated sulphuric acid (technical grade) 250 ml

Carefully add concentrated sulphuric acid to water. **Always add acid slowly to water, not vice versa.** The mixture will heat up. Store in an amber bottle, but if clear bottles are used, keep stocks of reagents in a closed cabinet. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

Methylene blue counterstaining solution 0.3%

Methylene blue chloride 0.3 g
Distilled water up to 100 ml

Dissolve methylene blue chloride in distilled water and store in an amber bottle. If clear bottles are used, keep stocks of reagents in a closed cabinet. Label bottle with name of reagent
and dates of preparation and expiry. Can be stored at room temperature for six to twelve months.

Quality Control:
Let the freshly prepared reagents stand until quality control procedures have been performed. After these reagents have passed quality control, pour the solutions into clean bottles and label them. If bottles are reused, clean them thoroughly. Carbol fuchsin crystals stick to the bottom and are hard to remove, use acid alcohol and a bottlebrush to remove this residue. On the label of the bottle, clearly print the reagent name, concentration and the preparation date.

Carbol fuchsin staining reagent can be filtered in the laboratory that prepared it, but this is not sufficient since it may precipitate again. Filter carbol fuchsin again during the process of staining, using a funnel with filter paper. The other staining reagents do not need to be filtered.

A positive and negative control slide is included with each run of stains. This will verify the correct performance of the procedure as well as the staining intensity of the acid-fast organisms.

Staining procedure

Fixing
Place slides on the staining rack in serial order, smeared side up. Slides should be separated by a 1 cm gap, and should never touch one another. Fix dried smears by passing the flame 5 times under each smear. Do not heat-fix moist slides, and do not overheat.

Staining
1. Cover slides individually with filtered 1% Ziehl's carbol fuchsin working solution.
2. Heat slides from underneath with the flame of a Bunsen burner, an alcohol lamp or an alcohol soaked cotton swab until vapor starts to rise. Staining solution should never be allowed to boil. Do not allow the stain to dry.
3. Keep slides covered with hot, steaming carbolfuchsin for 5 minutes by re-flaming as needed. Rinse slides gently with water to remove excess carbolfuchsin.
4. Drain off excess rinsing water from slides.
5. Cover slides with 25% sulfuric acid or acid-alcohol solution and allow to stand for 3 minutes, after which the red colour should have almost completely disappeared. If needed, repeat sequences until the red colour disappears, but do not overdecolourise.
6. Gently wash away the sulfuric acid or acid alcohol and the excess stain with water. Drain off excess rinsing water from slides. Rinse slides individually with water.
7. Cover slides individually with 0.3% methylene blue counterstaining solution and allow to stand for 1 minute. Rinse slides individually with water.
8. Drain water off the slides, which are then allowed to air dry.

Microscopic examination of smears

Acid-fast bacilli appear bright red or pink against the blue counterstained background. They vary greatly in shape, from short, coccoid to elongated filaments; they can be uniformly or unevenly stained, and can even appear granular. They occur singly or in variable sized clumps, and typically appear as long, slender curved rods.

The microscopic examination must be systematic and standardised. It can start at the left end of the smear. The reading begins at the periphery of the field and ends at the centre. When the field is read, the slide is moved longitudinally to examine adjacent fields. The slide can be moved vertically so that a second length can be read from right to left. There are about 100 immersion fields in the 2 cm long axis of a smear.

Results

WHO/IUATLD Grading

Reporting scale

Follow the scale when reporting smears:

- If no AFB is seen in at least 100 fields, report as negative for AFB.
- If 1–9 AFB are seen in 100 fields, report actual number of AFB seen.
- If 10–99 AFB are seen in 100 fields, report as (+).
- If 1–10 AFB/field in at least 50 fields report as (++).
- If more than 10 AFB/field in at least 20 fields, report as (+++).

The microscopist should initial the smear result as well as other result entries in the laboratory register.

The microscopist should take at least 5 minutes to read 100 fields, and should never be expected to process and read more than 25 ZN-stained sputum specimens per day when working full time. No more than 10 to 12 specimens should be processed at one time.
Drug Susceptibility Testing, BACTEC MGIT 960 Method

1. Purpose.

In order to guide appropriate therapy for the treatment of tuberculosis, cultures of *M. tuberculosis* (MTB) isolated from patient specimens are tested for susceptibility to each of the five first line anti-TB drugs, streptomycin, rifampin, Isoniazid, ethambutol and pyrazinamide. Multi-drug resistant MTB, (MDRTB), has recently become a serious public health problem. Drug susceptibility testing (DST) identifies patients who have MDRTB or other resistance patterns and thus is a vital guide to appropriate patient therapy.

2. Principle of the Procedure

The method of proportion (MOP) is used. Resistance to a drug is detected when 1% or more of the bacterial population is resistant to the drug at the critical concentration. MTB has a low level of intrinsic resistance to anti-tuberculous drugs. By using the MOP, this low level resistance is eliminated and only significant (>1%) resistance is detected.

The critical concentration is defined as the lowest concentration of drug that inhibits 95% of wild strains of MTB that have never been exposed to the drug, while at the same time allowing growth of MTB that have been isolated from patients who are not responding to therapy (resistant). An isolate is determined to be resistant if 1% or more of the test population grows in the presence of the critical concentration of the drug.

3. Principle of the BACTEC 960/MGIT DST system
The BBL MGIT 7mL Mycobacteria Growth Indicator Tube (MGIT) is a tube containing a modified Middlebrook 7H9 Broth which supports the growth and detection of Mycobacteria. The MGIT tube contains a fluorescent compound embedded in silicone at the bottom of a round-bottom tube. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Actively growing microorganisms consume the oxygen which allows the compound to fluoresce. The DST tests are based on growth of the *Mycobacterium tuberculosis* isolate in a drug-containing tube compared to a drug-free tube (Growth Control).

The BACTEC MGIT 960 instrument (BT 960) monitors hourly the tubes for increased fluorescence. Analysis of fluorescence in the drug-containing tube compared to the fluorescence of the Growth Control tube is used by the instrument to determine susceptibility results. The BT 960 instrument automatically interprets these results and reports a susceptible or resistant result.

4. **Samples Used.**

Pure cultures of MTB are used for DST. Contaminated or mixed cultures must be purified before testing. Cultures for DST can be grown on solid or in liquid (MGIT) media.

5. **Equipment, Supplies and Reagents**

**Equipment**

- Biosafety Cabinet (Certified annually)
- BACTEC 960 instrument (with AST feature enabled), and printer
- Refrigerator
- Freezer (-70 to -80 deg C)
- Autoclave
- Vortex mixer

**Supplies**

- Pipettes
- Sterile pipette tips, 100μl (plugged for culture material, unplugged for drugs)
- Plastic, disposable graduated 1ml pipettes
DST carriers (2, 3, 4, 5, 8 tubes capacity)
DST MGIT racks
Screw capped glass bottles or tubes with caps, sterile
tubes, 2mL for freezing drugs
Disinfectant (5% phenol or 1% bleach)
Discard containers
Autoclave bags
Paper towels
Racks for holding LJ

Reagents
Sterile distilled water
BD SIRE DST kit: Streptomycin (STR), Isoniazid (INH), Rifampin (RIF),
Ethambutol (EMB), SIRE Supplement
BD PZA DST kit: Pyrazinamide (PZA), PZA supplement
(Optional :BD INH 0.4 mg/L kit)
PZA test media
MGIT tubes
OADC /PANTA MGIT supplement (for sub-cultures only)
Sterile distilled water aliquoted in 4ml tubes with glass beads, autoclaved
Blood agar plates (BAPs)
LJ slants

6. Calibration procedure:
The BT960 instrument contains calibrator tubes at the end of each row, in each drawer. These
tubes must be changed before their expiry date. Failure of any calibrator tube will be flagged
by the instrument as Error EO5. The instrument calibration is automatic


A. Drug Preparation (S,I,R,E)

BD BT 960 SIRE Drug kit – On receipt, store the lyophilized drug vials at 2 – 8 deg C.
Aseptically reconstitute S, I, R, E drugs with 4mL of sterile distilled water
Final concentrations: S 1.0, INH 0.1 mg/L, RIF 1.0 mg/L, EMB 5.0 mg/L
(Optional) High level INH: reconstitute with 2mL of sterile distilled water, final concentration 0.4 mg/L

Quality Control: See 13. New Drug Lot #s, when reconstituted, must meet QC requirements before being released for use.

Once reconstituted, the antibiotic solutions are aliquoted in labeled cryovials, frozen and stored for up to six months, not to exceed the original expiration date. Once thawed, use immediately. Discard unused portions.

B. Preparation of the Inoculum from Solid Media Growth (should be < 3 weeks old from when colonies first appear)

Label Dilution Bottles with the culture number

- Bottle A – 8-10 glass beads & 4 mL distilled water
- Tube B - empty to use for supernatant of Tube A.
- Tube C - empty to use for supernatant of Tube B.
- Tube D - 4.0 mL sterile saline
- Tube E - 9.9 mL sterile saline

Prepare Suspension

- Harvest growth with a loop, place in tube A. Vortex Tube A several times
- Allow Tube A to stand for 20 minutes for clumps to settle
- Transfer supernatant from Tube A to Tube B
- Allow Tube B to stand for 15 minutes
- Transfer supernatant from Tube B to Tube C
- Adjust Tube C suspension to a 0.5 McFarland Standard
- Transfer 1mL of Tube C to 4ml of Tube D. *This is the inoculum source for the drug containing tubes (1:5)
• Transfer 0.1 mL of Tube D to 9.9 mL of Tube E. *This is the inoculum for the growth control tube only. (1:100)

C. Inoculation Procedure for MGIT 960 SIRE Susceptibility Test. (from LJ or MGIT inoculum)

1. Label five 7 mL MGIT tubes for each test isolate with the drug name and the culture number.

2. Label as: C (Growth Control), S (STR), I (INH), R (RIF), and E (EMB.)

3. Add supplement: aseptically add 0.8 mL of MGIT SIRE Supplement to each tube. (Only use the supplement supplied with the DST kit)

4. Add drugs: aseptically pipet 100 μL working drug concentrations to each of the appropriately labeled MGIT tubes. No antibiotics are added to the MGIT Control tube.

5. MGIT inoculums: If using a MGIT Day 3-5, dilute 1:5, using 1.0 mL of well mixed MGIT culture and 4.0 mL of sterile saline. This is now the inoculum. MGIT tubes Day 1 and 2 are used undiluted as the inoculum.

6. Control tube (1:100 of DST inoculum), preparation and inoculation: from MGIT inoculums (see 5), aseptically pipette 0.1 mL into 9.9 mL of sterile saline to prepare the 1:100 Growth Control inoculum. Mix thoroughly. For LJ inoculum use Tube E (7B). Inoculate 0.5 mL into the MGIT tube labeled “C.”

7. Drug-containing tube inoculation: aseptically pipet 0.5 mL of the MGIT inoculum or the LJ Tube D inoculum (7B), into each of the drug tubes (S, I, R, E)

8. Tightly recap the tubes. Mix tubes thoroughly by gentle inversion 3 to 4 times

9. Wipe the tubes with disinfectant and leave inside the BSC for 10mins before removing
10. Load tubes into the appropriate AST carrier (5 place). Enter the AST set into the BT 960 using the AST set entry feature (refer to MGIT 960 User’s Manual, AST Instructions). Ensure that the order of the tubes in the AST Set Carrier is C, S, I, R, E.

D. Entry of all DST Tubes to BT 960

Scan DST set carrier into the BACTEC MGIT 960 instrument using the barcode on the carrier set. See Manufacturer’s manual for full instructions.

E. Monitoring of Tests in Progress

All AST tests in progress in the BT 960 instrument are monitored daily by checking for Growth Units (GU’s). A rise in GU will give an early indication of any developing resistance or contamination so that intervention or repeat testing can be initiated.

Any test for drug resistance must be checked to eliminate the possibility of false resistance due to contamination.

Check the resistant tubes for any sign of turbidity. Make a smear and stain by ZN to look for the presence of non-AFB. A blood agar plate may be inoculated as a purity plate. Any tube showing contamination cannot be reported.

8. Acceptable Parameters for the Test

Test completion within 4-13 days for SIRE tests and 4-21 days for PZA tests.

Over or under-inoculation of tests that result in completion of the test outside these limits will be rejected by the instrument as invalid. These tests must be repeated.

9. Quality Control: A SIREP test is set up using the pan-susceptible MTB strain H37Rv, with each batch of DST set up. The QC test follows the same procedure and interpretation as used for patient tests except that the QC strain of MTB is used instead of the patient strain.

The QC test must show a susceptible result for all drugs to be acceptable. If the QC test is unacceptable, the clinical DSTs cannot be reported. QC for new Lots of drugs: use the above method to QC new drug Lots after reconstitution.

10. Limitations. AST tests can only be carried out on pure, viable cultures of MTBC.
11. Reporting of results:

DST results are recorded in the register and on the patient report form. Results are reported as susceptible or resistant for each drug tested. The report sheet from the BT960 for DST is printed and filed.

12. Laboratory interpretation:

- The BT960 instrument will interpret results between days 4-13 for SIRE tests and days 4-21 for PZA

- Susceptible results: When the test strain is susceptible to all drugs tested. This will occur when on the day that the Control tube reaches a GU of 400 the drug tubes have GUs of <100, and the time is within the acceptable parameters for the test.

- Drug Resistance: This will occur when there is a rise in GU to GU =/> 100, in a drug test tube(s), and growth in the Control tube, GU =400, within the acceptable time parameters
APPENDIX 6: GENOTYPE®MTBDRPLUS ASSAY SOP

DNA EXTRACTION
(N/B: always work with decontaminated sputum samples)

A. Material

- Biosafety cabinet class II
- Heat block
- Centrifuge
- Vortex
- Centrifuge
- Ultra sonic bath
- Heat block
- Timer

B. Reagents (include all reagents per these steps below)

Mechanical extraction
- Distilled water

C. Consumables

- 1.5 ml screw capped tubes
- Pipettes and corresponding tips
- Tube falcon (50ml)
- Gloves- powder free
- Disinfectant (0.5% bleach)
- Lab coat
- Test kits
N.B. Clean and decontaminate all the materials before and after the use. Clean and Remove all used material from and clean BSC, turn off lights in the and Wash hands immediately. Always close doors behind you when leaving or entering the room. Leave used lab coat on the hanger outside the lab and wash hands again.

I. DNA EXTRACTION BY MECHANICAL METHOD

II.1. SPUTUM
(N/B: always work with decontaminated sputum samples)

- Take 1000 µl of decontaminated sample and transfer it into 1.5 ml screw capped tube
- Centrifuge at 14,000 x g for 15 minutes.
- Discard the supernatant and re-suspend pellet in 100 µl of sterile distilled and vortex
- Inactivate in heat block at 95°C for 20 minutes.
- Incubate in ultrasonic bath for 15 minutes at 45°C
- Centrifuge at 13,000 x g for 5 minutes
- Use 5 to 10µl of supernatant for PCR.

The supernatant of the DNA extract is transferred into a new tube and stored at 2-8°C for 2 weeks; or at -20°C for a longer period.

N.B.: - For DNA extraction from sputum a positive control may not be included, but a negative control must be included.
- Remember to decontaminate materials and the BSC before and after use.

II.2. Culture

- Take a few loops (2-3 colonies) of the culture from LJ slant and transfer into 500 µl of sterile distilled water in 1.5 screw capped tube.
- Re-suspend bacterial colonies by short vortexing
- Heat inactivate at 95°C for 20 minutes.
- Incubate in ultrasonic bath for 15 minutes
- Centrifuge at 14,000 x g for 5 minutes
- Transfer the supernatant containing DNA into a new tube. DNA solution can be used immediately or stored at +4 to -20°C.

_N.B. Remember to include a negative and a positive control each time._

**PRE-PCR STATION**

This step deals with preparation of Master Mix which will be used for amplification.

**Reagents**

- PNM
- PCR buffer
- MgCl2
- H2O
- Taq polymerase

**Material and consumables**

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

**Biosafety precautions**

- First put on new gloves, then lab coat
- Decontaminate work area with freshly diluted 0.5% Sodium hypochlorite solution.
- Use fresh aliquots of molecular biology grade water
- Use new PCR tubes
- Close all vials in between pipetting steps to avoid pipetting and handling above open vials.

**Procedure**

1. Determine the number of samples to be processed add controls and a correction volume.

2. Using master mix per one reaction, calculate the total amplification mix for all reactions to be done.

Example for calculating the master mix reagents per sample. The following reagents and their volumes are required for one reaction as stated in the table below:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>One reactions (µl)</th>
<th>24 reactions (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNM</td>
<td>35</td>
<td>840</td>
</tr>
<tr>
<td>10 x PCR Buffer</td>
<td>5</td>
<td>120</td>
</tr>
<tr>
<td>25 x MgCl₂</td>
<td>2</td>
<td>48</td>
</tr>
<tr>
<td>H₂O</td>
<td>3</td>
<td>72</td>
</tr>
<tr>
<td>Taq polymerase (= 1 unit)</td>
<td>0.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

**Calculation of total reactions**

3. The total reactions is given by the total number of specimens to be processed, plus one positive control (if culture), one negative control, 2 PCR controls (both negative and positive) and one extra correction volume. Example: Suppose you are requested to analyse/process 6 specimens from culture, the total reactions will be 11 reactions and calculation of the volume to be used for each reagent is calculated as below:

N.B.: If you are requested to process specimens from sputum, there will not be a positive control. Therefore, use the lab strain H37Rv, from culture as a positive control.

4. In 1.5 ml micro-tube, pipette the volume as calculated above.
Master Mix aliquoting into PCR reaction tubes

5. Aliquot 45 μl mixture of the master mix in a PCR tube.
6. Note: The following step will be done in the BSC or UV hood in the Pre-PCR room.

AMPLIFICATION (PCR-AREA)

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

Material and consumables

- Thermocycler
- Micropipettes (20-200 μl, 100-1000 μl)
- Pipette tips (20-200 μl, 100-1000 μl)
- Gloves powder free

Reagents

- DNA from extraction taken on supernatant

Precautions

Decontaminate all instruments, racks, bench space with freshly prepared 0.5% of hypochlorite solution.

Procedure

7. Transfer 5 μl of DNA in 45 μl of master mix
8. Using a thermocycler, select Hain user and the required program (hot 30 if specimens are from culture or Hot 40 if specimens are from sputum).
9. The thermocycler will amplify the target DNA in thousands DNA of interest to be used for hybridization and detection.

HYBRIDIZATION

A. Material and consumables

- Tray for hybridization
- Twincubator
- Water bath
- Timer
- Micropipettes (20 μl, 200 μl, 1000 μl)
- Pipettes tips
- Waste biohazard bag
- Waste container containing 10% bleach
- White paper
- Permanent tube Fine Markers
- DNA strip markers
- Set of Tweezers
- Gloves

B. REAGENTS

- DNA strips
- Amplicons or DNA amplification product
- DNA Denaturation reagent (DEN)
- Hybridization reagent (HYB)
- STR Solution
- RIN solution
- Conjugate concentrate (CON C)
- Conjugate Diluent
- Concentrated Substrate concentrate (SUB C)
- Substrate Diluent
- Distilled water
- Bleach solution

C. SCOPE AND OBJECTIVE OF THE TEST

The hybridization of the amplified DNA is a start to check the quality of the amplified DNA, the conjugate and the substrate will help to visualize/detect the presence or not of the target DNA in the amplicon.

Procedures

First put on new gloves and a lab coat.
Decontaminate working area with freshly prepared 5% Sodium hypochlorite.
Prewarm HYB and STR at 45°C to dissolve all precipitates
Bring to room temperature RIN solution and distilled water.
Freshly dilute CON-C and SUB-C; 1:100 in the respective diluents provided in the kit and protect them from light.

Denaturation

- Put 20 µl of denaturation solution (DEN) in each tray
- Add 20 µl of DNA PCR products in each tray
- Incubate at room temperature for 5 min. (at the bench).

Hybridization
- Add 1ml of hybridization solution (HYB) to each tray containing DEN + amplified DNA.
- Mix the solution by moving the pellet up and down.
- Label the strips according to the samples labelling.
- Add the labelled strips to each tray corresponding to each sample (the well contains already the mixture DEN, DNA, HYB).
- Incubate for 30 min. at 45°C in the Twincubator.
- Aspirate the whole quantity of HYB completely.
- Add 1 ml of STR (fixing and washing solution) to each tray.
- Incubate for 15 min. in the Twincubator.
- Remove the whole quantity of STR completely.
- Add 1 ml of RIN (rinsing solution) to each tray.
- Incubate for 1 min. at room temperature in the Twincubator.
- Remove the whole quantity of RIN.

**Detection/Visualization**

- Add 1 ml of the diluted conjugate. For dilution: (10μl Con-c + 1ml Con-D).
- Incubate for 30 minutes at room temperature in the Twincubator.
- Remove the conjugate completely.
- Add 1ml of RIN (rinsing solution) to each tray.
- Incubate for 1 min. at RT in the Twincubator.
- Remove the whole quantity of RIN completely.
- Rinse once with water for 1 min.
- Add 1 ml of substrate. Dilution: (10μl SUB-c + 1ml SUB-D) to each tray.
- Incubate for 2-10 minutes at RT in the Twincubator.
- Remove the substrate completely.
- Stop the reaction by rinsing twice with water for 1 minute.
- Remove DNA strips from the tray.
- Air Dry DNA strips on absorbent paper.
- Stick dried strips to result sheet and interpret.
# APPENDIX 7: TB CULTURE WORK SHEET

<table>
<thead>
<tr>
<th>TB Culture and Identification Worksheet</th>
<th>Lowensen Jensen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WEEKS</strong></td>
<td></td>
</tr>
<tr>
<td>Date Processed</td>
<td>Lab No.</td>
</tr>
<tr>
<td>Patient Identification</td>
<td>Primary Smear</td>
</tr>
<tr>
<td></td>
<td>1   2    3    4</td>
</tr>
<tr>
<td></td>
<td>LJ Smear</td>
</tr>
</tbody>
</table>

| # Positive | # Contaminated | # Negative | Total Number | Reviewed |

---

104
Internal Memo

FROM: Dean, Graduate School        DATE: 12th February 2010

TO: Margaret C. Mburu        REF: 157/OL/0256/04
C/o Public Health Dept.

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

This is to inform you that your M.P.H Research Proposal was approved on 1st February 2010.

Thank you.

JOHN M. ODONGI
FOR: DEAN, GRADUATE SCHOOL

cc. Chairman, Public Health Department
Supervisors:
1. Dr. Joseph Ngerenwa
   C/o Department of Biochemistry & Biotechnology
2. Dr. Kamindu Gikonyo
   C/o Department of Pharmacy and Complementary/Alternative Medicine
Ms. Margaret Chiina Mburu
Kenyatta University
P. O. Box 43844
NAIROBI

Dear Madam,

RE: RESEARCH AUTHORIZATION

Following your application for authority to carry out research on “Evaluation of Genotype R MTBDR – plus assay as a screening tool for Multi-drug-resistant Tuberculosis surveillance in High HIV prevalence population in Nairobi” I am pleased to inform you that you have been authorized to undertake research in Nairobi Province for a period ending 31st December, 2010.

You are advised to report the Provincial Commissioner, the Provincial Director of Education Nairobi Province, the Medical Officer of Health, Nairobi and the Officer In Charge of the selected Clinics before embarking on the research project.

On completion of the research, you are expected to submit two copies of the research report/thesis to our office.

P. N. NYAKUNDI
FOR: SECRETARY/CEO

Date 30th April, 2010
APPENDIX 10: ETHICAL APPROVAL BY KENYATTA HOSPITAL REVIEW COMMITTEE

Margaret C. Mburu
157/01/0256/04
Kenyatta University

Dear Margaret

Research Proposal: “Evaluation of Geno Type® MTBDRplus assay as a screening tool for Multi·drug·Resistant tuberculosis surveillance in High HIV prevalence population in Nairobi” (P80/3/2010)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and approved your above revised research proposal for the period 5th August 2010 to 4th August 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

c.c. Prof. K. M. Bhatt, Chairperson, KNH/UON-ERC
The Deputy Director CS, KNH
The HOD, Records, KNH
Supervisors: Dr. Ngeranwa J. J. N, Kenyatta University
Dr. N. Kamindu Gikonyo, Kenyatta University

Ref: KNH-ERC/ A/547

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 20723, Nairobi.
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP”, Nairobi.
Email: KNHplan@Ken.Healthnet.org

5th August 2010

Margaret C. Mburu
157/01/0256/04
Kenyatta University

Dear Margaret

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Yours sincerely

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Dr. N. Kamindu Gikonyo, Kenyatta University

Ref: KNH-ERC/ A/547

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Telegrams: MEDSUP”, Nairobi.
Email: KNHplan@Ken.Healthnet.org

5th August 2010
INTERNAL MEMO

Ref: NLTP /DC/8/15(16) 12th August 2010

THE LABORATORY MANAGER
CENTRAL REFERENCE LABORATORY

RE: Research authorization, Ms. Margaret C. Mburu

I write to introduce Ms. Mburu who is a postgraduate student at Kenyatta University, department of Public Health. She intends to conduct research for a thesis project entitled, "Evaluation of GenoType® MTBDRplus assay as a screening tool for Multi-drug-Resistant tuberculosis surveillance in High HIV prevalence population in Nairobi.

Kindly give her the necessary assistance.

Yours faithfully,

Dr. Joseph Sitienei
Head, DLTLD