Cape Peninsula
University of Technology

Development of a cost-effective drug sensitivity test for multi-drug resistant and extensively drug-resistant tuberculosis

by

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Thesis submitted in fulfilment of the requirements for the degree

Master of Technology: Biomedical Technology

in the Faculty of Health and Wellness Sciences

at the Cape Peninsula University of Technology

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Bellville
September 2010

DECLARATION

I, Fadheela Patel, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed

14 - March - 2011

Date

ABSTRACT

The World Health Organisation estimates that nine million people are infected with tuberculosis (TB) every year of which ninety-five percent live in developing countries. Africa has one of the highest incidences of TB in the world, but few of its countries are equipped to diagnose drug-resistant TB. This study aimed to develop a robust, yet simple and cost-effective assay, which would require minimal sophisticated instrumentation and specialised personnel that would make drug sensitivity screening for multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) accessible to resource-poor high-burden settings.

A four-quadrant colorimetric agar plate method was developed which showed good specificity (97.3%-100%) and sensitivity (77.8%-100%) compared to the polymerase chain reaction (PCR) method used as gold standard. Agreement between the methods, using Simple Kappa Coefficients, ranged between very good and excellent, all with high statistical significance ($P < 0.0001$). The currently used BACTEC MGIT SIRE™ sensitivity assay coupled with the E-test® strip method, as routinely used in the TB reference laboratory, was compared and showed excellent comparison with the newly-developed plate method, for each antibiotic tested, as well as the resultant mono-resistant, MDR- or XDR-TB diagnoses. Moreover, the new method was found to be extremely cost-effective, priced at half the cost of a PCR assay.

These four quadrant plates, with a colorimetric indicator and selected antibiotics, can be considered as an economic alternative or a complimentary method for laboratories wishing to reduce the cost and complexity for TB drug sensitivity testing. Routine diagnostic testing would thus be made more accessible and affordable to laboratories

that are not presently diagnosing drug resistant TB, therefore enhancing case detection and treatment in the resource-poor settings hardest hit by this curable disease.

ACKNOWLEDGEMENTS

I wish to thank:

- my supervisors, Prof Tania Botha and Dr Marthinus Senekal, for their guidance and encouragement;
- Justin Harvey for statistically evaluating the raw data;
- June Solomons for her understanding, support and guidance in assisting me with media preparation and her valuable time which she sacrificed in assisting me whenever I needed her;
- Wendy Brittle my mentor, for the advice and guidance throughout my journey in this career;
- my colleagues at Pathcare TB laboratory and routine Microbiology for their understanding and support; and
- my mother, Salama, sister, Malika and nieces, Ashiqa and Razaan for their patience and love.

The financial assistance of the Cape Peninsula University of Technology (CPUT) and the National Research foundation (NRF) towards this research is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author and are not to be attributed to the CPUT or NRF.

DEDICATION

To my father who passed away 21 years ago

with love and gratitude

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LIST OF ABBREVIATIONS

AFB	acid-fast bacilli
ATCC	American type culture collection
Ak	amikacin
BCG	*Mycobacterium bovis* bacille Calmette-Guèrin
BSA	bovine serum albumin
BSL2	biosafety level two laboratory
Cap	capreomycin
Cip	ciprofloxacin
CDC	Center for Disease Control
CFU	colony-forming unit
CSLI	Clinical and Laboratory Standards Institute
CO_2	carbon dioxide
DOTS	Directly observed therapy short course
DST	drug susceptibility testing
FIND	Foundation for Innovative New Diagnostics
HIV	human immunodeficiency virus
Inh	isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
Kana	kanamycin
LPA	line probe assay
LJ	Löwenstein-Jensen
MDR-TB	multidrug-resistant tuberculosis
MGIT	mycobacteria growth indicator tube
ml	milliliter
NaOH-NALC	sodium hydroxide-N-acetyl-L-cysteine

NTM	nontuberculous mycobacteria
OADC	oleic acid albumin dextrose catalase complex
PANTA	polymyxin B, amphotericin B, naladixic acid, trimethoprim, azlocillin
PCR	polymerase chain reaction
QC	quality control
Rif	rifampicin
STC	2,3-diphenyl-5-thienyl-(2)-tetrazoliumchloride
TB	*Mycobacterium tuberculosis*
TLA	thin layer agar
TTC	2,3,5-triphenyltetrazolium chloride
TTD	time-to-detection
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Robert Koch discovered the tubercle bacillus at the end of the nineteenth century (Gupta *et al.*, 2007), yet tuberculosis currently remains a serious public health problem worldwide (Martin *et al.*, 2009). Tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis,* is a notifiable disease in the developing world. Research has intensified in the last decade in an attempt to gain new understanding of the global TB epidemic.

In 1993, the World Health Organisation (WHO) declared TB a "Global Emergency" (Raviglione *et al.*, 1997). An estimate done by the WHO in 2008 found that globally there were 9.2 million new cases of TB and 1.7 million deaths in 2006 (McGaw *et al.*, 2008). Of the estimated 1.7 million people that succumbed to TB, 14% were actually co-infected with the human immune deficiency virus (HIV) (WHO, 2008).

Prior to 1990, before the establishment of a new TB unit at WHO, TB research was largely neglected (Raviglione, 2003). The declaration by WHO noted the beginning of an upsurge in TB research and led to the ultimate global goal which is to have the disease eliminated by year 2050 (Migliori *et al.*, 2009).

Today TB research is focusing on new generation vaccine and drug development, but also rapidly expanding in the TB diagnostic arena, especially for drug sensitivity testing to assist with rapid multi-drug resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB).

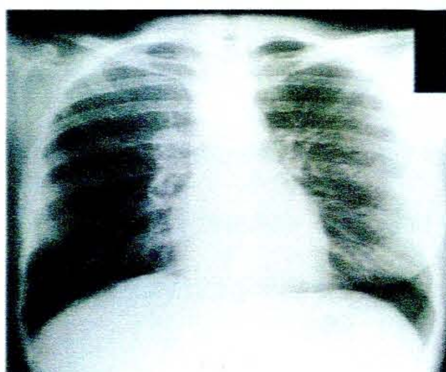
1.2 Literature review

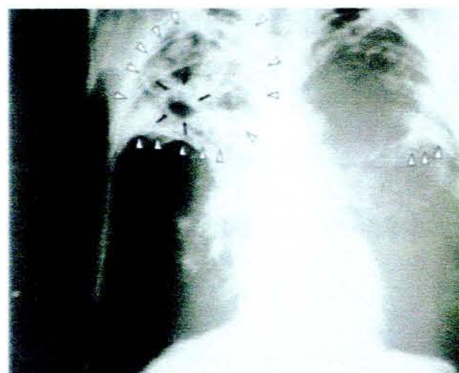
1.2.1 History of tuberculosis

The origin of tuberculosis is unknown; however there is sufficient evidence which suggests that TB has been present since antiquity. It is also unclear whether TB originated in cattle and was then transferred to man (Pearce-Duvet, 2006), but it has been shown that *M.tuberculosis* is not directly related to *M.bovis,* which has more recently evolved (Ernst *et al.,* 2007).

TB was found in Egypt as early as 3700 BC, where tubercular decay was found in ancient skeletal and mummified material of mummies (Zink *et al.,* 2003). At the time of Hippocrates (460 BC), TB was well recognised by its symptoms of coughing up blood, fever and was almost always fatal. Francastoro was the first epidemiologist who recognised the contagious nature of TB and thus the study of tuberculosis only began between 1483 and 1553 during the Renaissance years (Porter & McAdam, 1994). TB was most commonly referred to as either Koch's disease, named after Robert Koch, or consumption, as the disease seemed to consume people within, or *phthisis,* the Greek term for tuberculosis. TB of the abdomen was known as *tabes mesentrica*, wasting disease, white plague, king's evil and Pott's disease. Disseminated TB, also known as miliary TB, occurs when TB bacilli invade the circulatory system and millet-like seeding occurs in the lung and is visible on X-rays (Schaaf & Zumla, 2009).

An anteroposterior X-ray seen in **Figure 1.1 (a)** of a normal chest X-ray and of **(b)** a patient diagnosed with advanced bilateral pulmonary tuberculosis.





(a) **(b)**

Figure 1.1 (a) A normal chest X-ray and (b) an X-ray of advanced pulmonary tuberculosis. This X-ray of the chest reveals the presence of bilateral pulmonary infiltrate (white triangles), and "caving formation" (black arrows) present in the right apical region (Wikipedia, 2010).

1.2.2 Global epidemiology of tuberculosis

When an estimate was carried out by the WHO TB programme in 1991, it was found that a third of the human population, 1.7 billion people, was infected with the tubercle bacillus, although not all presented with clinical TB. Over 95% of the estimated 8 million new cases and 2.9 million deaths were occurring in the developing world (Kochi, 1991; Raviglione, 2003).

Almost a decade later, in 2000, the WHO estimated that there were 8.2 million new TB cases worldwide (Corbett *et al.*, 2003), with the sub-Saharan African countries having the highest incidence rates. The average incidence for this region was at about 300 per 100 000 population (Corbett *et al.*, 2003; Raviglione, 2003). Thus, it was noted that TB was once again rising due to the co-infection with HIV in immunocompromised patients. Recently, it was estimated that 15 million people were

simultaneously infected with HIV and TB (Del Olma *et al.*, 2009), and half a million of the estimated 1.36 million HIV associated deaths in 2007 were directly attributable to TB (WHO, 2009). Now, with the emergence of resistant strains to the current antibiotics used to treat TB, it poses a new threat to TB control programmes.

Multi-drug resistant tuberculosis is defined as mycobacteria resistant to isoniazid and rifampicin and requires long and expensive therapy using second line drugs with a higher toxicity (Zager *et al.,* 2008).

Extensively drug-resistant tuberculosis has been reported worldwide and is classified as resistant to rifampicin, isoniazid and a second line injectable drug such as capreomycin, kanamycin or amikacin and a fluoroquinolone (CDC, 2006; McGaw *et al.,* 2008). XDR-TB is thought to occur due to insufficient case management of MDR-TB cases (Jones *et al.*, 2008; McGaw *et al.*, 2008).

MDR-TB has been documented for several years in publications; however XDR-TB appeared for the first time in literature in March 2006. Fifty two of the fifty three patients died of XDR-TB among the HIV infected patients in KwaZulu-Natal, South Africa and these patients had a median survival time of sixteen days from the date of diagnosis (Gandhi *et al.*, 2006; Barnard *et al.*, 2008; Migliori *et al.*, 2009). The picture has since changed with a higher incidence in drug resistance, with MDR-TB and XDR-TB noted as indicators to control programme failures (Migliori *et al.*, 2009).

Although the number of TB-related deaths has now stabilised at around 2 million deaths per year, the incidence of new infections has increased due to co-infection with HIV, poor resources and resistant mycobacterial strains (Gutierrez-Lugo *et al.*, 2008).

An estimated 440,000 cases of MDR-TB emerged globally in 2008, with India and China carrying the greatest estimated burden of MDR-TB and together accounting for almost 50% of the world's total cases. By 2009, a total of 58 countries had reported at least one case of XDR-TB, with South Africa reporting the largest number of XDR-TB cases (Nathanson *et al.*, 2010), which was largely amongst people co-infected with HIV.

It is feared that there will most likely be an emergence of even more resistant mycobacterial strains in future, which will exhaust the current pool of antibiotics already available. For this reason new classes of anti-TB agents are urgently required (McGaw *et al.*, 2008).

1.2.3 Disease transmission and pathology

Mycobacteria are transmitted via droplet infection. It is transmitted from person to person as illustrated in **Figure 1.2.** When an infected person coughs, sneezes or laughs, mucous containing the micro-organisms are expelled, thus making it an airborne infection. These droplets are then inhaled via the lungs of an individual in close contact, and can lead to pulmonary tuberculosis (Enarson *et al.*, 2000).

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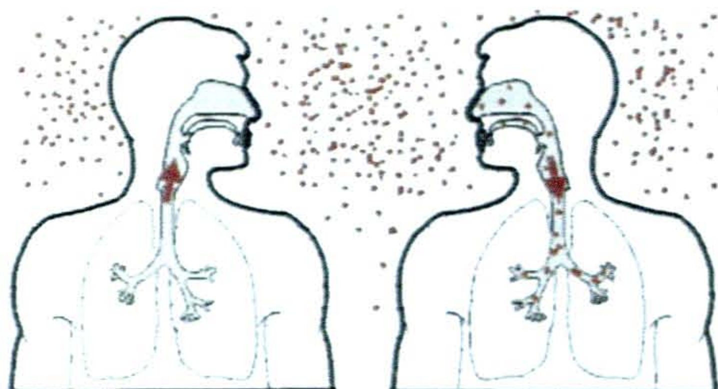


Figure 1.2 Aerosol transmission via droplet infection from a person infected with TB to a healthy individual who inhales the TB bacilli. (Centers for Disease Control and Prevention (CDC), Core curriculum on Tuberculosis, 2000).

Once the bacilli are inhaled, they are engulfed by macrophage cells found in the alveoli. The mycobacteria are able to replicate within the macrophages in two to three weeks before spreading throughout the body. However, in most cases the macrophages are able to contain the bacteria and no apparent disease occurs, but the bacteria may remain dormant for years (WHO, 2009).

Tuberculosis is caused by any of the *M.tuberculosis* complex organisms, which comprises of *M.tuberculosis, M.bovis* BCG, *M.bovis, M.microti* and *M.africanum,* as well as non tuberculous mycobacteria (Lennette *et al.*, 1980; Wayne & Kubica, 1986; Koneman *et al.*, 2006). Severity of the disease depends on the pathogen's ability to persist, the virulence of the organism and the number of organisms inhaled (Marais *et al.*, 2005).

Pulmonary tuberculosis is a contagious bacterial infection which mainly affects the lungs while extrapulmonary TB, also known as miliary TB, occurs when the micro-organisms spread to other parts of the body via the airways, blood stream, lymphatic system or directly to other organs (Enarson *et al.*, 2000).

The immune response, which develops 4-6 weeks after infection, usually retards the multiplication of tubercle bacilli, but a few dormant bacilli may persist. The signs and symptoms of TB as summarised in **Figure 1.3** are predominantly loss of weight, loss of energy, poor appetite, fever, night sweats, a chronic productive cough and haemoptysis (WHO, 2009).

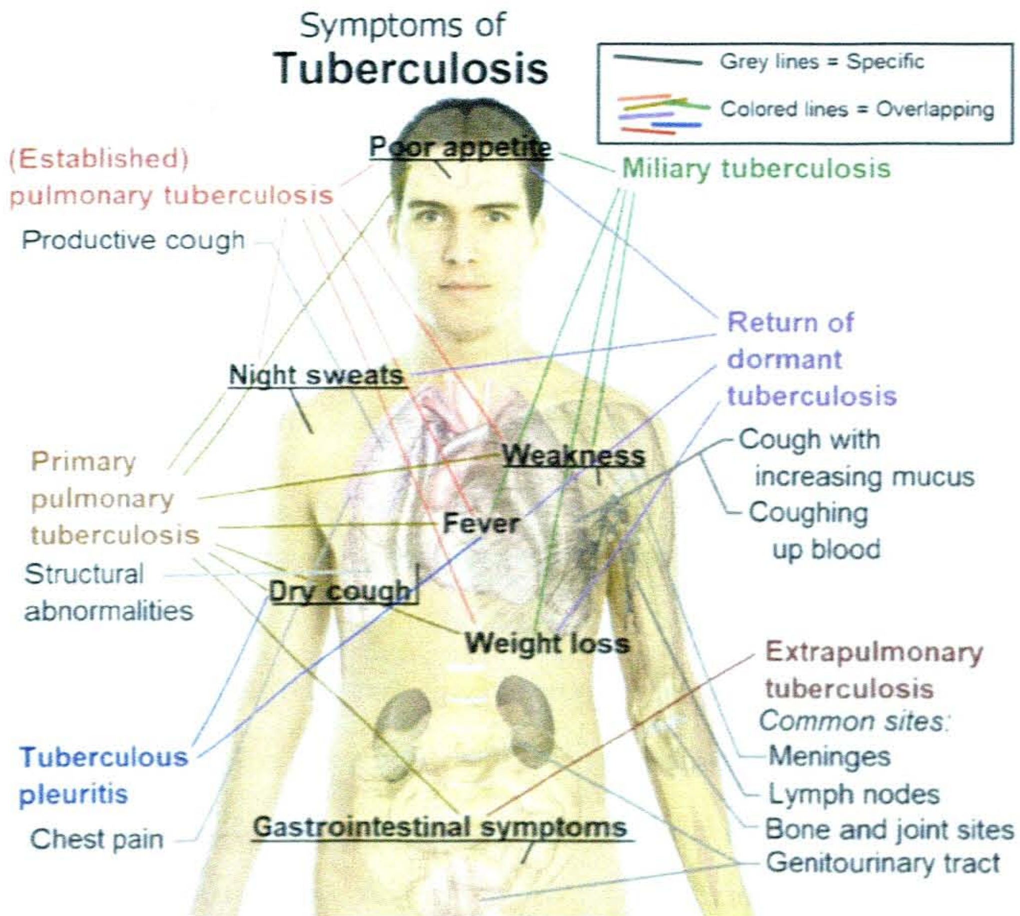


Figure 1.3 Main symptoms of TB with many symptoms overlapping (Wikipedia, 2010).

1.2.4 Treatment of tuberculosis

Each person who has untreated TB may, on average, infect up to ten to fifteen people per year prior to treatment (WHO, 2009), therefore, rapid diagnosis and treatment is essential for TB control.

Streptomycin was the first antibiotic discovered in 1944 for effective treatment of TB (De Souza, 2009). Isoniazid was discovered in 1912, yet was found to be effective against TB only in 1951, whereas rifampicin was introduced in 1967. Isoniazid treatment has been used in conjunction with another drug to minimise resistance occurring. Presently, the treatment for TB is a standard regimen which consists of four first line anti-TB drugs which are isoniazid, rifampicin, pyrazinamide and ethambutol (Migliori *et al.*, 2009). TB that is susceptible to all the first line anti-TB drugs, is defined as pan-susceptible TB (Migliori *et al.*, 2009).

MDR-TB does not respond to the standard six month treatment and can take up to two years to treat with drugs, which are more toxic and more expensive. The management of XDR-TB is challenging as an extended period of treatment is required of up to two years. This poses a problem as there might be a lack of drug accessibility and the expense of the drugs might increase within the period. Also, there might be low adherence due to the toxicity of the second-line drugs, coupled with co-infection of HIV. It is known that it is difficult to co-administer TB therapy with antiretroviral therapy in HIV positive patients (Jones *et al.*, 2008). These are but a few challenges for the effective therapy of XDR-TB patients. Due to the lack of sophisticated monitoring methods available, there are concerns worldwide that the prevalence of drug-resistant TB are at a much higher rate than actually reported (McGaw *et al.*, 2008).

Another concern is that the private sector is virtually universally excluded from the standard TB control practices and collaboration with the governmental services (Raviglione, 2003). Considerable progress in fighting TB has been made by the STOP-TB Partnership and WHO (Zager & McNerney, 2008). The Stop TB Strategy, which supports the Directly Observed Therapy Short course (DOTS) strategy developed by the WHO and the International Union Against TB and Lung Disease (IUATLD), was developed to help reduce the burden of disease (Raviglione & Uplekar, 2006), but its efficiency has recently been questioned (Heller *et al.*, 2006). The global MDR-TB epidemic is estimated at 511,000 new cases and 150,000 deaths annually, and presently there has a case fatality rate of 293.5 per 1000 affected individuals. XDR-TB has 50,000 cases and 30,000 deaths estimated and has a case fatality rate of 600 per 1000 affected cases (Migliori *et al.*, 2009). Statistically this means that 410.9 deaths occur from MDR-TB and 82.2 deaths from XDR-TB daily (Migliori *et al.*, 2009). After reviewing the global statistics of MDR-TB and XDR-TB, it can be safely deduced that the resistant strains of TB pose a threat to achieving the global goal of TB control, which is the elimination of TB by 2050 (Migliori *et al.*, 2009).

The World Bank has estimated that half the population of Africa averages at an income of less than one dollar a day, yet twenty-five dollars is required for TB treatment (Karekezi, 2002). Clearly this denotes that cheaper methods of identification and sensitivity testing are required, especially in Africa.

1.2.5 New drug and vaccine development

New TB drugs are urgently required as more than forty years have elapsed and still no new TB drugs have been developed or licensed (Migliori *et al.*, 2009). The function of these new TB drugs is aimed at shortening the duration of therapy, improve therapy of the resistant disease, appropriately treat patients co-infected with HIV, and

to shorten the treatment of TB, including treatment of latent TB (Ginsberg *et al.*, 2009). However, the emergence of MDR-TB and XDR-TB has spurred the interests to develop new drugs and drug regimens for improved treatment, such as diarylquinilones (Diacon *et al.*, 2009), nitroimidazole, diamine and pyrrole compounds, which are all undergoing testing in different clinical trial phases (Migloiri *et al.*, 2009). There are also studies which are being conducted for possible adjunctive therapies like vitamin D supplementation, which might be seen as a priority for the future (Wilkinson *et al.*, 2009).

Natural plant products, that have proven to be templates for new drug development (Cragg *et al.*, 1997), have shown interesting biological activities (McGaw *et al.*, 2008). Since South Africa has a high burden of TB incidence and has a rich heritage of plant biodiversity, research here will play a key role in participating in developing new antimycobacterial agents from plant origin (McGaw *et al.*, 2008).

Two French scientists, Albert Calmette and Camille Guèrin, made an attenuated strain from another mycobacterium, namely *M.bovis*. These two renowned bacteriologists cultured the *M.bovis* strain for thirteen years and observed a reduction in the virulence in animals during this period. When infants were inoculated with the vaccine, a 90% reduction in mortality was noted. Since then, the serendipitously discovered Bacille Calmette Guèrin (BCG) vaccine has been used worldwide as a vaccine (Gupta *et al.*, 2007).

However, to completely eradicate TB, most experts believe a cheap and effective vaccine must be developed for complete mass immunisation (Gupta *et al.*, 2007). There are new TB vaccines being developed that are either in, or entering, the first phase of clinical trials (Gupta *et al.*, 2007). Some vaccines, such as inactivated *M.vaccae*, have advanced to higher levels of clinical phase testing, and should offer

exciting prospects for the future (Gupta *et al.*, 2007). Optimistically, a new TB vaccine may be available and licensed by 2015 (Gupta *et al.*, 2007).

1.2.6 Mycobacterial culturing

Robert Koch found the isolation of pure mycobacterial cultures in liquid media problematic and therefore looked for an alternative substrate and method. He experimented with coagulated egg albumin, starch paste and an aseptically cut slice of a potato, as well as meat extracts with gelatin (Lindquist, 2006).

A technician by the name of Fanny Eilshemuis Hesse, who worked in Robert Koch's laboratory, introduced the concept of agar after hearing about its properties from friends and included it in jellies she had made. This solid media became known as agar, a polysaccharide derived from red seaweed that has remarkable properties (Lindquist, 2006). It can melt at high temperatures of 85°C and gels at cooler temperatures between 32 and 42°C. Moreover, agar is much clearer than gelatin and resists digestion by bacterial enzymes (Laboratory news, 2007).

Frederick Loeffler added peptone and salt to Koch's meat extract formulation in 1884. By 1887, Julius Richard Petri, who also worked in Koch's laboratory, modified the flat glass plate by inventing the Petri dish, which is a small round plate with a lid overhanging to keep contamination out (Lindquist, 2006).

In 1931, Löwenstein formulated an egg based medium with congo red and malachite green to culture mycobacteria and partially inhibit other bacteria. This recipe was then modified in 1932 by Jensen to produce the present formula which contains more malachite green and supplements, but no congo red (Difco Laboratories, 1984). Middlebrook and Dubos also developed solid media which contained oleic acid and

albumin to enhance the growth of TB (Difco Laboratories, 1984). From 1958 to 1960 Cohn and Middlebrook improved the formula. Middlebrook 7H10 agar was not affected by the proteolytic organisms and allowed for less contamination as opposed to using the egg based medium by Löwenstein and Jensen (Difco Laboratories, 1984).

Middlebrook 7H11 agar, a modification of Middlebrook 7H10, was introduced with the addition of casein as originally suggested by Cohn and colleagues in 1968 (Kleeberg *et al.*, 1980). Middlebrook 7H10 and 7H11 agar are the solid media of choice for mycobacterial propagation at present.

The need for more rapid methods for diagnostic testing of TB also led to the introduction of liquid culture media. The most commonly used liquid media at present, namely, the Mycobacteria Growth Indicator Tube (MGIT), contains seven milliliters of modified Middlebrook 7H9 broth base and was introduced for diagnostic and sensitivity testing for TB (Siddiqi, 2005). The MGIT™ culturing system has since become a norm in the majority of TB diagnostic laboratories.

1.2.7 *Mycobacterium tuberculosis* complex

To exclude potential contaminants, one should be aware of the *Mycobacterium tuberculosis complex*. The genus *Mycobacterium*, order Actinomycetales, family Mycobacteriaceae, comprises of about fifty acid-fast, aerobic, non-motile, non-sporing bacterial species. Most of these species are environmental saprophytes and are found in soil, water, plants and on mammals and birds (McGaw *et al.*, 2008). The genus is further divided into the saprophytic fast growing species, and the pathogenic slow growers (McGaw *et al.*, 2008). The fast growers, also known as the non-tuberculous group, mycobacteria other than tuberculosis (MOTT) or atypical

mycobacteria, are usually not pathogenic. However, some species may be potentially dangerous and become opportunistic pathogens in both man and animals (Grange *et al.*, 1986; McGaw *et al.*, 2008) and the risk increases in HIV-infected individuals. The pathogenic species belong to the genus *M.tuberculosis* complex, namely *M.tuberculosis, M.bovis, M.africanum, M.microti and M.canetti.* The most common causative agent of tuberculosis in man is *M.tuberculosis. M.africanum* and *M.canetti* are both human pathogens while *M.microti* causes disease in rodents and *M.bovis* mainly infects cattle and their products (McGaw *et al.*, 2008).

The phenotypic characteristics of TB when cultured in the laboratory on solid media are non-pigmented, rough, buff colonies on the Löwenstein Jensen (LJ) slants. These colonies appear from fourteen to twenty eight days when incubated at 37°C. On Middlebrook 7H10 and Middlebrook 7H11, the colonies appear small and white and grow on the media from five to ten days at an incubation temperature of 37°C. Growth in liquid media occurs within seven to fourteen days at 37°C (Difco laboratories, 1984; Siddiqi, 2005).

1.2.8 Diagnostic techniques

There are several extremely sensitive and sophisticated diagnostic techniques available at present, which can be divided into two groups. The one technique is to diagnose *M.tuberculosis* infection and the other diagnoses and evaluates TB disease (Nazish, 2009). A range of these techniques are described below.

1.2.8.1 Mantoux skin test

The test was named after Charles Mantoux, who developed the test in 1908 (Kaufmann *et al.*, 2008). Tuberculin is a glycerol extract of the tubercle bacillus, of which a standard dose is administered intradermally and the result read after 72 hours, by measuring the diameter of the induration as seen in **Figure 1.4**. A positive Mantoux test will read greater than ten millimeters while a negative result is any reading less than ten millimeters (Minodier, *et al.,* 2010).

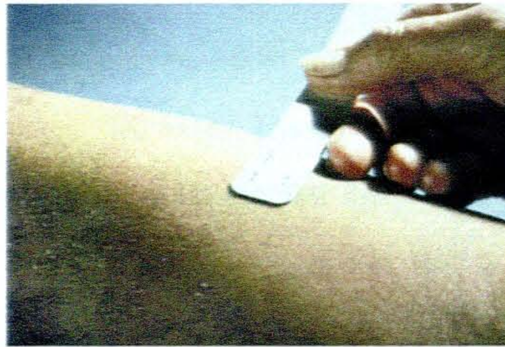


Figure 1.4. A mantoux skin test result and demonstration of how the skin induration is measured. A positive result is ≥10mm while a negative result is ≤10mm. This test is not a very reliable diagnostic tool, but useful as a screening method, as the limitation of this test is the variation of reading a result (Wikipedia, 2010).

1.2.8.2 Ziehl-Neelsen staining

Most laboratories use the acid-fast bacilli (AFB) smear as the diagnostic test for TB, therefore methods to increase the sensitivity of sputum microscopy are being explored.

The microscopic morphology of *M.tuberculosis* on acid fast stains is a thin, slightly curved bacillus with a somewhat beaded appearance that stains red when using the Ziehl-Neelsen (ZN) staining method. The value of the mycobacteriology laboratory lies in diagnosing smear-positive or infectious cases of tuberculosis by examining

clinical specimens microscopically for the presence of micro-organisms (Enarson *et al.*, 2000). This is a rapid and cost-effective method of screening if AFB are present or not. The limitation of this test is that this method does not differentiate between drug-sensitive or drug-resistant TB. It does, however, indicate the morphological difference between *M.tuberculosis* and nontuberculous mycobacteria.

1.2.8.3 Gamma-Interferon assays

There are various gamma-interferon (IFN-γ) assays available, such as the QuantiFERON® TB Gold (Cellestis, USA) assay, which is an enzyme-linked immunosorbent assay and an enzyme-linked immunospot assay, such as the T SPOT® TB (Oxford Immunotec, UK) assay. The IFN-γ level is increased in the blood of patients if mycobacteria are present; the test therefore measures the absolute value of IFN-γ in the blood. The advantage of this test is that the results are obtainable within a single visit and the patient does not have to return in order for the result to be measured and read after three days as is the case for skin tests such as the Mantoux test. Additionally, the variability of the reading of the skin test is also eliminated. The other advantage is that past BCG vaccination does not affect this test, whereas with the Mantoux skin test it does (Nazish, 2009).

1.2.8.4 Culture methods

Another direct method to diagnose TB is through culturing methods, but the disadvantage is that on conventional media the organism takes four weeks to grow. However, new more rapid methods are available, such as microcolony detection on solid media, the Septi-check™ AFB method, Microscopic Observation Drug Susceptibility (MODS) assay, the BACTEC MGIT™ 960 system, the MB/Bact® system and the ESPII culture system, which are all briefly described below.

1.2.8.4.1 Microcolony detection on solid media

This technique is a better technique for culturing TB as it is less expensive and requires approximately half the time needed for conventional culture (Mejia *et al.*, 1999). A thin layer of Middlebrook 7H11 agar is poured into plates and incubated with the organisms. These plates are then microscopically examined on alternate days for the first two days. Approximately within seven days *M.tuberculosis* growth can be detected (Ramachandran & *Paramasivan,* 2003).

1.2.8.4.2 BBL® Septi-check™

The BBL® Septi-check™ AFB method (Becton Dickenson, USA), is a biphasic non-radiometric culture system consisting of modified Middlebrook 7H9 broth with a three-sided paddle containing chocolate, egg-based and modified 7H11 solid agar. One side of the paddle contains non-selective Middlebrook 7H11 agar, while the reverse side is divided into two sections. One section consists of Middlebrook 7H11 agar with *para-nitro-á-acetylamino-β-hydroxypropiophenone* for differentiation of *M.tuberculosis* from other mycobacteria. The other section contains the chocolate agar for the detection of contaminants (Ramachandran & Paramasivan, 2003). The bottle is inverted regularly to inoculate the solid media. Growth is assessed by observing the three sided paddle (Nazish, 2009). This method has the potential to hasten processing and obviate carbon dioxide (CO_2) incubation which results in early detection of positive cultures within three weeks (Ramachandran & Paramasivan, 2003).

1.2.8.4.3 Microscopic observation drug susceptibility

The microscopic observation drug susceptibility (MODS) technique, typically used in endemic high burden countries, requires 7H9 broth, OADC and antimicrobial supplements. *M.tuberculosis* grows faster in liquid medium than in solid medium. The cord formation of TB is microscopically visible at early stages in liquid medium and drug sensitivity testing may also be incorporated. This allows for a faster turn-around-time as the direct drug susceptibility testing and the detection of bacterial growth are simultaneous (Moore *et al., 2006).* Its disadvantages are that it is labour intensive and requires an inverted microscope for reading cultures (Caviedes *et al.*, 2000).

1.2.8.4.4 BACTEC™ 460 radiometric culture system

The BACTEC™ 460 radiometric culture system (Becton Dickenson, USA) consists of culture media containing ^{14}C-labelled palmitic acid. If mycobacteria are present in the broth, it will metabolise the ^{14}C-labelled substrate and release the radioactively labelled $^{14}CO_2$ in the atmosphere, which collects above the broth in the bottle. The instrument withdraws this CO_2 and measures the amount of radioactivity present. Bottles which yield a radioactive index are interpreted as a growth index; a growth index of greater than or equal to ten are considered positive (Nazish, 2009).

1.2.8.4.5 BACTEC MGIT™ 960 system

A BACTEC MGIT™ culture tube contains 7ml of modified Middlebrook 7H9 broth and a fluorescent compound embedded in a silicone sensor at the bottom of each tube. Growth is detected visually using ultraviolet light. The oxygen diminishes the fluorescent output within the tube of the sensor, therefore the consumption of oxygen

by the organism present in the medium is detected by an increased fluorescence (Nazish, 2009).

1.2.8.4.6 MB/Bact ® 3D system

The MB/Bact® 3D system (Biomériuex, France) is based on the colorimetric detection of CO_2, however, it has a slightly longer time for detection of growth than the BACTEC 460 system (Rohner *et al.*, 1997).

1.2.8.4.7 ESP II culture system

The organisms are cultured in a modified Middlebrook 7H9 broth with enrichment and a cellulose sponge to increase the culture's surface area of the ESP II culture system (Difco Laboratories, Michigan, USA). The instrument detects growth by monitoring pressure changes that occur as a result of oxygen (O_2) consumption or gas production by the organisms as they grow. Results are best when used in combination with solid media (Woods *et al.*, 1997).

1.2.8.5 Diagnosis of MDR- and XDR-TB

According to the WHO report, there were 9.27 million new cases of TB in 2007 and among them an estimated 1.37 million were HIV-positive (Martin *et al.*, 2009). This worldwide TB crisis is also driven by the emergence of extensively drug-resistant (XDR) and multi drug-resistant (MDR) strains of *M.tuberculosis* (Jones *et al.*, 2008). In many countries, treatment for MDR-TB and XDR-TB commences after the diagnosis is confirmed, especially as result of HIV co-infection and this is a long process if conventional methods are used (Nathanson *et al.*, 2010). Therefore, patients with MDR and XDR-TB remain in the community untreated for long periods of

time which poses a problem. Thus, rapid diagnosis and treatment is essential to keep case numbers low (Dye, 2009) such as new molecular techniques to strengthen the laboratory services (Nathanson *et al.*, 2010).

1.2.8.5.1 Molecular methods

Nucleic acid amplification tests (NAATs) are mainly used by high income countries and have been in use for many years (Pai *et al.*, 2009). The existing NAATs have high specificity and variable sensitivity especially in smear negative and extrapulmonary TB (Pai *et al.*, 2003). A new fully automated NAAT called the Xpert MTB/RIF assay (Cephoid, Sunnyvale, CA, USA) can detect *M.tuberculosis* and rifampicin resistance simultaneously. This test can be performed outside the reference laboratory as it is a closed system and its main aim is to replace microscopy. The Foundation for Innovative New Diagnostics (FIND) is currently evaluating this test in high burden countries like South Africa (Pai *et al.*, 2009).

Line probe assays (LPAs) such as GenoType® MTBDR*plus* assay which detects resistance in isoniazid and rifampicin and the GenoType® MTBDR*sl* assay which detects resistance in the fluoroquinolones, the injectable antibiotics and ethambutol (Hain Life-science, Nehren, Germany) are highly accurate in smear positive specimens. In 2009, the GenoType MTBDR*sl* was released and this assay together with the GenoType MTBDR*plus* assay allows the simultaneous detection of the *M.tuberculosis* complex and resistance to fluoroquinolones, aminoglycosides or ethambutol from cultured specimens, therefore allowing the rapid detection of MDR-TB and XDR-TB (Pai *et al.*, 2009). In 2008, WHO endorsed the use of LPAs for rapid detection of MDR-TB (Pai *et al.*, 2009).

1.2.8.5.2 E-test® strip method

The E-test® strip (Bioméruex, France) method uses agar plates containing Middlebrook 7H11 and 10% OADC. Four to six week old TB colonies are suspended in Middlebrook 7H9. The surface of the plate is swabbed with the supernatant fluid and one E-test® strip is then placed on each agar plate, each screening for a different antibiotic. Results are obtained after ten days (Wanger *et al.*, 1996).

1.2.8.5.3 BACTEC MGIT SIRE™ sensitivity testing

The BACTEC MGIT SIRE™ sensitivity testing (Becton Dickenson, USA) kit consists of one vial of each lyophilised drug, streptomycin, isoniazid, rifampicin and ethambutol which must be reconstituted with distilled water. A growth control tube is inoculated with every set of antibiotics which is placed in a carrier set and incubated for up to fourteen days in an automated BACTEC machine. Once growth of four hundred growth units is recorded in the growth control tube, the machine will read the other tubes and a result will become available as either sensitive or resistant.

1.3 Financial burden of tuberculosis

In 1991, an estimation done by the World Bank on the population size in Africa was just under 780 million. Then in 1999, the population of the sub-Saharan Africa was estimated to be 642 million (World Energy Council, 1999; World Bank, 2001a).

In 1998 the World Bank estimated that about half the region's population had an average income of below one dollar per day (World Bank, 2001b; Karekezi, 2002), therefore the number of poor people in Africa has grown five times more than the figure for Latin America, and twice for that of Asia (Karekezi, 2002).

Poverty-stricken communities in sub-Saharan African rural areas account for 70% of Africa's population (World Bank, 2001b), and TB is commonly known as a disease of the poor, found mainly in undernourished persons and in high incidence in Africa and Asia (McGaw *et al.*, 2008).

The World Bank issued a report in 1993 calling TB chemotherapy one of the most cost-effective of all interventions in the fight against TB. Data from Malawi, Mozambique and Tanzania showed incremental costs of US$80-110 per cure for ambulatory treatment of TB, with cost per death between 20 and 100 US$. (Raviglione, 2003).

This led to the World Bank endorsing the importance of investing in TB control and thereby influencing financial policies in all countries (Raviglione, 2003). Due to the increasing media attention, the number of US dollars for external financing of TB control, inclusive of aid funds and bank loans, rose from 16 million US$ in 1990 to 50 million US$ in 1996, and eventually 190 million US$ in 2000 (Raviglione, 2003).

It is estimated that at least US$1.2 billion a year are necessary to support TB control efforts worldwide. Sixty-nine per cent of this sum is committed by governments of endemic countries and four per cent is in the form of grants from various donors, thus leaving at least 300 million US$ short which must be mobilised from other sources (Raviglione, 2003). A programme called EXPAND-TB was funded by UNITAID in 2009 and their function was to specifically supply MDR-TB diagnostics to high-burden countries (Pai *et al.*, 2009). A new grant of US$ 61,482,085 led by Global Laboratory Initiative, FIND and the Global Drug Facility, will expand the use of LPAs, a molecular test for rapid MDR-TB diagnosis (Pai *et al.*, 2009).

More recent studies focused on the performance and accuracy of TB diagnosis instead of studies first examining the cost-effectiveness and the impact of new diagnostic tests introduced. However, new TB diagnostic tools should focus on high specificity, affordability and sensitivity for cases missed by using existing diagnostic standards (Dowdy *et al.*, 2008; Pai *et al.*, 2009).

In South Africa, the cost of TB treatment for patients on the six month standard course is approximately three hundred and seventy-seven rand, while MDR-TB treatment for the same period is thirty-one thousand rand and XDR-TB treatment is even more expensive than MDR-TB (Thaver *et al.*, 2006).

1.4 Problem statement

Africa has the highest burden of mycobacterial tuberculosis coupled with HIV infection (Chakaya *et al.*, 2008), however few of its countries are equipped at present to diagnose MDR-TB and XDR-TB. Therefore a rapid, cost-effective and reliable test for the diagnosis of MDR- and XDR-TB is required. There is also a need to identify the different sensitivity patterns of the patient as rapidly as possible. These tests must not be labour-intensive or dependent on expensive equipment for these low cost settings.

1.5 Objective and aim of this study

The main objective of this study was to improve and enhance routine drug susceptibility testing for mono-resistant, MDR- and XDR-TB. It was aimed to improve the time-to-detection of drug susceptibility tests for *M.tuberculosis*, to make routine diagnostic testing more affordable and therefore accessible to countries that are not equipped to diagnose drug-resistant TB and determine drug sensitivity patterns.

The question asked in this study was whether the use of four-quadrant agar plates containing different antibiotics in each segment, namely isoniazid, rifampicin, ciprofloxacin, kanamycin, amikacin and capreomycin, together with a colour growth indicator, would allow for simultaneous detection of MDR- or XDR-TB and show an improvement in the turnaround time and cost-effectiveness of multi-drug resistant and extensive-drug resistant tuberculosis diagnosis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Study setting and laboratory safety

The research for this study was conducted in the diagnostic TB reference laboratory at Pathcare Private Pathology Company, N1 City, Goodwood, South Africa. The company has a fully equipped bio-safety level 2 (BSL2) laboratory for *M.tuberculosis* work. All the equipment required for the proposed research was available within the Pathcare laboratory.

The safety precautions were strictly adhered to according to the standard operating procedures of the laboratory, by processing all infectious specimens, preparing slides and inoculating media in the Class II biological safety cabinet. Negative air pressure was maintained in the laboratory and all surfaces were cleaned before and after working with specimens with 5% phenol, 70% alcohol (Appendix D) and Tri-gene® (Mixmed Sales and Marketing, South Africa). The bio-safety cabinet working area was sterilised between each step as well as before and after processing each batch of specimens. Protective clothing, such as laboratory coats and sterile non-latex gloves as seen in **Figure 2.1**, were worn when processing specimens in this study.

To prevent cross-contamination when working with specimens, equal volumes of TB decontamination fluid (Appendix D) were added to each conical tube before adding a specimen to the tube. Care was also taken to ensure that only one tube was open at a time and that the reagent bottles remained closed between steps, as described before (Carroll *et al.*, 2002).

Figure 2.1 Biological safety cabinet and protective clothing such as coats and gloves were worn to prevent contamination when handling potentially pathogenic specimens. Also note other safety measures such as a sharps container, a container to discard the supernatant of the specimens and a bucket containing 5% phenol solution for other waste items such as orange sticks (Pathcare).

2.2 Ethical considerations

Patient sputum specimens, sent to the Pathcare laboratory for routine TB diagnostic analysis have been used in this study and ethical approval was sought for and granted by the Health and Wellness Sciences Research Ethics Committee, Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology. The ethical approval number was CPUT/HW-REC 2008/15. Personal identifiers were only linked to the patient specimens for the duration of the study and were only accessible to the principal investigator.

2.3 Analysis of patient sputum specimens

2.3.1 Specimen collection

Specimens for this study were sputum samples collected from Pathcare satellite laboratories that were sent to Pathcare TB reference laboratory for routine TB culture and sensitivity testing. Specimens from areas outside of Cape Town were transported at 4°C when delays were expected, according to Pathcare's standard operating procedures, as transport time has been reported to be proportional to the contamination rate of samples (Siddiqi, 2005). Specimens sent to this TB reference laboratory included country-wide national and international designations, such as Namibia, Somalia and Kenya.

2.3.2 Specimen inclusion and exclusion criteria

The criteria required of sputum specimens for inclusion in this study were firstly that they had to be from adult patients between the ages of eighteen and sixty and either be Ziehl-Neelsen (ZN) or Auramine-O positive. Of those ZN or Auramine-O positive specimens, the ones selected were all of those, based on the routine drug sensitivity testing, that were identified as either mono-resistant, MDR-TB or XDR-TB according to WHO criteria (CDC, 2006). Drug-sensitive specimens were excluded. All specimens fulfilling the requirements were selected within a specific time period, from November 2009 until June 2010 and one hundred and twenty specimens in total were selected for this study.

2.4 Specimen processing

Once the specimens were received in the laboratory, they were captured according to the laboratory standard operating procedures. The patient forms were scanned with the specified requisition numbers and details were entered on the computer system. The relevant patient details, type of specimen received, date of birth, laboratory number, date of sample and the specific tests that the clinician requested were captured on the computer. Simultaneously, the specimens were sorted and placed in a specific box for TB diagnostic screening, and were analysed in batches.

2.4.1 Specimen digestion and decontamination procedure

The specimen decontamination method was the standard recommended decontamination procedure used with the BACTEC MGIT™ 960 System (Siddiqi, 2005). Sodium hydroxide was freshly prepared and all specimens were liquefied using a mucolytic agent, N-acetyl-L-cysteine, (NALC) (Merck, Darmstadt, Germany), which was added to the decontaminating fluid (Appendix D) immediately before processing the specimens, as NALC only has a twenty four hour shelf-life period. The purpose of the procedure was to free trapped bacilli from mucous, cells or tissue present in the specimen (Kleeberg *et al.*, 1980; Siddiqi, 2005).

This 4% sodium hydroxide-NALC solution was mixed thoroughly before adding equal volumes of the decontaminating fluid to each labelled conical tube (Amersham, South Africa). After adding the decontaminating fluid to the tubes, each specimen was carefully added to avoid spillages and minimise any cross-contamination. The mixture was vortexed thoroughly and allowed to stand for ten minutes, to preserve any mycobacteria that may be present in the specimen but destroy other contaminating bacteria (Naidoo & Barker, 2010).

Before centrifugation for 20 minutes at 3000 gravity, specimens were placed inside the centrifuge buckets with closed lids to protect the healthcare worker from aerosols emitted from possible broken tubes. After centrifugation, pellet formation was observed at the bottom of each tube and the supernatant was discarded into a container with 5% phenol, which was labelled and dated. This capped container was placed inside a biological safety cabinet as a safety precaution.

Phosphate buffer (Appendix D), at pH 6.8 (Merck, Darmstadt, Germany), was added to neutralise the sodium hydroxide-NALC solution. Specimens were then vortexed and centrifuged again for 20 minutes at 3000 gravity. After centrifugation, the supernatants were gently poured off and the pellets mixed using sterile orange sticks (Laboratory and Scientific Equipment, South Africa). A glass slide was prepared for each specimen for microscopy by combining one drop of fixative (Appendix C), a protein-based serum and one drop of the pellet sediment. This was mixed gently and allowed to air-dry in a safety cabinet. The slides were then heat-fixed to kill any living bacteria and the fixative allowed the sediment to adhere to the glass slide for microscopic observation after staining.

A liquid culture of each specimen was also prepared from the pellet sediment, by combining one millilitre phosphate buffer with the pellet. Half a millilitre of the reconstituted mixture was then transferred to a MGIT™ tube which contained seven milliliters of culture medium and 0.8ml BBL MGIT PANTA™, a host of antibiotics namely, polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin, and incubated in the BACTEC MGIT™ 960 System (Becton Dickinson, Maryland, USA) at 37°C for culturing of mycobacteria. A flow chart (**Figure 2.2**) indicates the different steps of the digestion and decontamination procedures preceding liquid culturing.

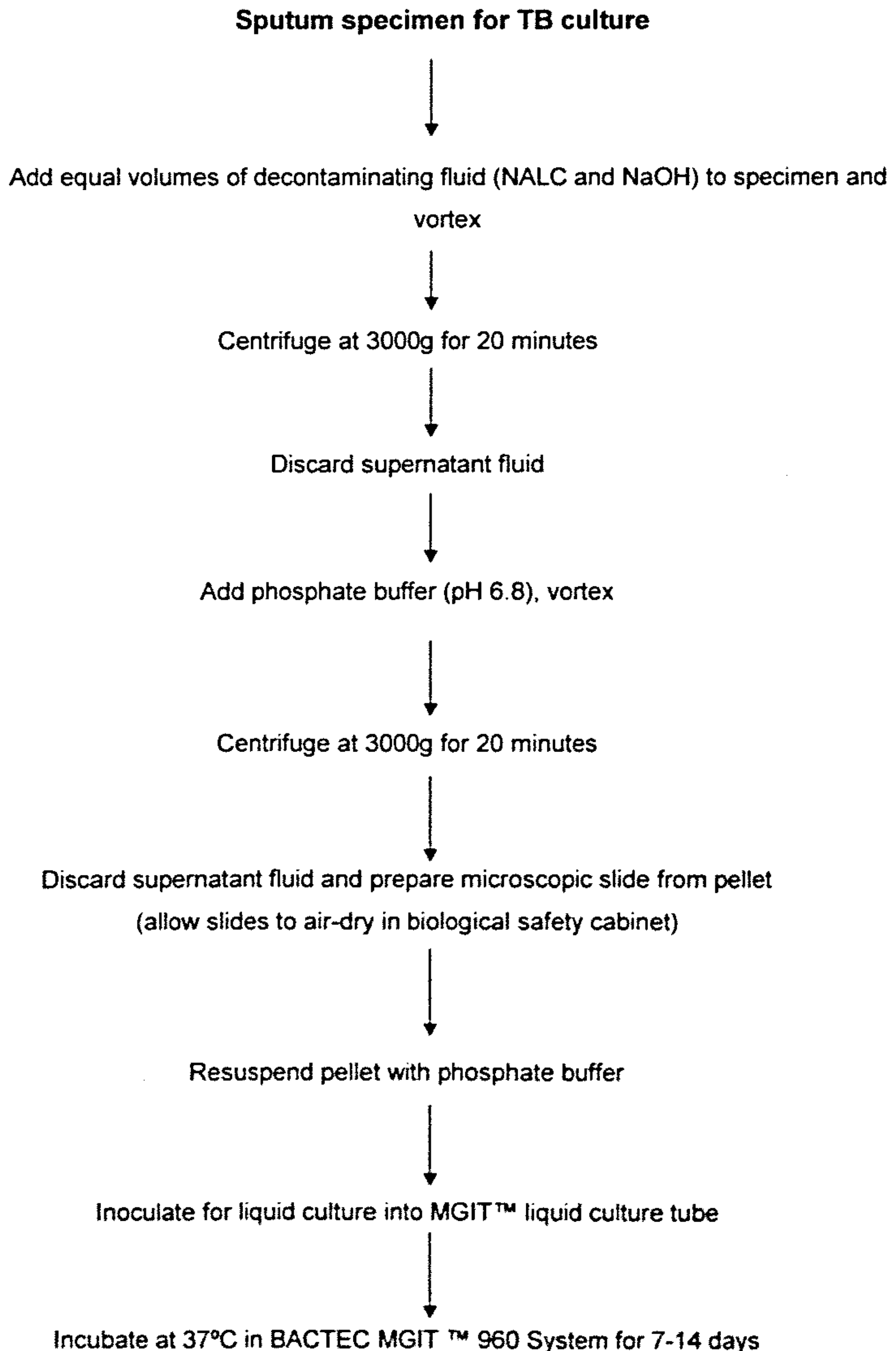


Figure 2.2 Flow chart of the digestion-decontamination method using 4% sodium hydroxide and NALC with phosphate buffer, after which specimens were inoculated and incubated for mycobacterial culturing in the BACTEC MGIT™ 960 System.

2.4.2 Smear microscopy of specimens

The primary tool for the diagnosis of TB in many countries remains smear microscopy (Martin *et al.*, 2009), also in South Africa. In this study both the ZN and Auramine-O staining methods were used as they were specifically requested by the admitting clinicians. The methods for both the ZN staining and Auramine-O staining are described below.

2.4.2.1 Ziehl-Neelsen stain microscopy

A drop of fixative (Appendix C) was mixed with a drop of the concentrated decontaminated specimen on a glass slide (Lasec, South Africa) and allowed to air-dry in a class 2B biological safety cabinet. The prepared smears, of one by two centimetres in size, were then heat-fixed by passing them through a Bunsen burner (Lasec, South Africa) flame to kill any living bacteria, and placed on a staining rack for ZN staining to identify any acid-fast bacilli (AFB) that may be present in the specimen.

The slides were flooded with concentrated Carbol Fuchsin as in Appendix C, heated three times intermittently within a 5 minute period to dissolve the waxy cell wall layer of the AFB and allow the stain to penetrate the bacilli. The slides were then washed with running tap water, rinsed with 3% acid alcohol (Appendix C) until decolourised and counter-stained with Methylene Blue (Appendix C) for 1 minute.

The slides were air dried and examined under a light microscope using a 100X oil immersion magnification lens (Zeiss, Germany). A drop of immersion oil (Merck, Darmstadt, Germany) was placed onto the glass slide it was examined under the

microscope and assessed for the presence of AFB. Positive ZN slides yielded red bacilli against a blue background as demonstrated in **Figure 2.3**.

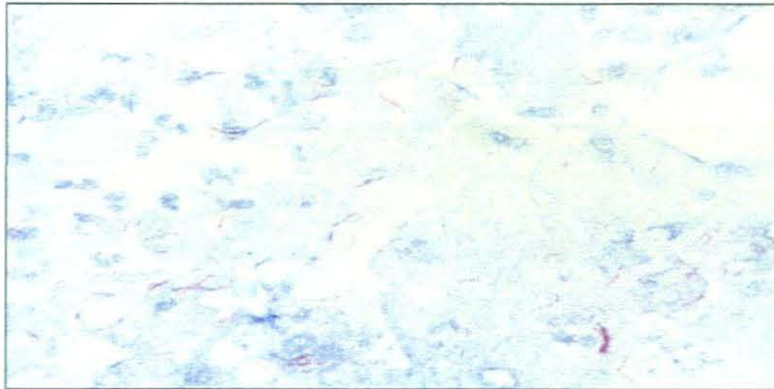


Figure 2.3 Ziehl-Neelsen stain displaying the red acid-fast bacilli that have absorbed the ZN Carbol Fuchsin stain and resisting decolourisation with 3% acid alcohol, against a blue background of the methylene blue counter stain (magnification, 100X).

The slides were examined systematically as illustrated in **Figure 2.4**, and a minimum of 5,000-10,000 acid fast bacilli per millilitre were required before a positive smear was reported, as previously described (Robledo *et al.*, 2006).

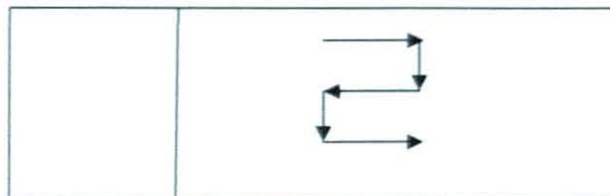


Figure 2.4 A systematic approach was used when evaluating slides for the presence of AFB with a microscope. An area of 2 x 1 cm in size was assessed and each ZN-stained slide was evaluated in the sequence as indicated by the arrows in the diagram.

There is a standard scale, **Table 2.1**, produced by the WHO and the IUATLD which is also used by the Pathcare TB reference laboratory when grading ZN slides (Van Rie *et al.*, 2008). The grading of slides is based on the number of AFB detected per field and assists the clinicians by informing them how infectious a particular patient

potentially is (Van Rie *et al.*, 2008), whereas it gives the laboratory an estimate of the mycobacterial concentration of a patient specimen.

Table 2.1 WHO/ IUATLD quantification scale for acid-fast bacilli found in Ziehl-Neelsen smear microscopy

Number of AFB	Number of fields examined	Reporting
No AFB in 100 fields	100 fields	No AFB detected
1-9 AFB in 100 fields	100 fields	Record exact figure (1-9 AFB per 100 fields)
10-99 AFB in 100 fields	100 fields	1+
1-10 AFB in each field	50 fields	2+
More than 10 AFB in each field	20 fields	3+

AFB – acid fast bacilli

(modified from Van Rie *et al.*, 2008)

2.4.2.2 Auramine–O stain microscopy

Smears made for Auramine-O microscopy were drenched with phenol-ethanol (Appendix B) to ensure that any living bacteria are killed, and allowed to air dry in a BSL 2 safety cabinet. The slides were packed on a staining rack with enough space between them to avoid any carry-over or stain running onto other slides.

The entire smear was flooded with Auramine-O stain (Appendix B) for thirty minutes without heating. This was followed by gentle rinsing of the slide with running tap

water, followed by decolourising with 0.5% acid alcohol (Appendix B) and again gentle rinsing with running tap water. The potassium permanganate counter stain (Appendix B) was added to the slides for four minutes (Siddiqi, 2005) before rinsing off with running tap water and air drying. When the slides were dry they were examined using a microscope equipped with a nonhazardous blue light source (Davies Diagnostics, South Africa).

A positive result, an example of which can be seen in **Figure 2.5**, showed the presence of AFB emitting a bright yellow fluorescence, while a negative result displayed a dark field due to the potassium permanganate counter stain.

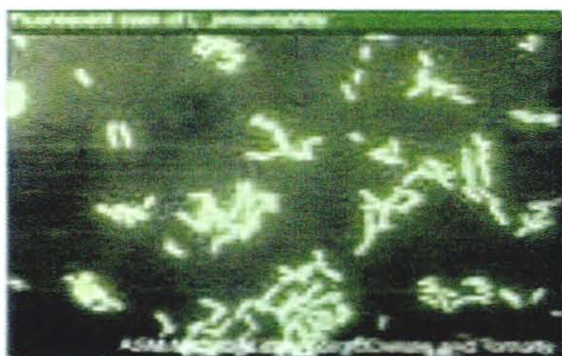


Figure 2.5 Auramine-O fluorescent stain prepared from a sputum sample displaying bright yellow fluorescent acid-fast bacilli against a dark background using a 40X magnification.

2.5 Liquid culturing of *M.tuberculosis*

The following methods described below are methods routinely used in the diagnosis of TB which is currently used in this TB reference laboratory.

The BACTEC MGIT™ 960 System was used for liquid culturing of *M.tuberculosis* as this is an efficient and fast routine system widely used. After the slides were prepared, MGIT™ liquid culture tubes were inoculated with 0.8ml of the BBL MGIT

PANTA antibiotic supplement. The tubes were incubated at 37ºC in a fully automated BACTEC MGIT™ 960 System (Becton Dickinson, Maryland, USA).

The principle of the BACTEC MGIT™ system is that an embedded fluorescent compound in silicone is immobilised at the bottom of each tube, dissolved in liquid media, which consists of 7ml Middlebrook 7H9 broth (Saddiqi, 2005). When a tube is incubated the actively respiring mycobacteria consume oxygen and allow the fluorescence signal to be detected, as the fluorescent compound is sensitive to the presence of oxygen. The automated system monitors the tubes hourly for increasing fluorescence and determines the positivity of a tube in the instrument. A positive MGIT™ tube contains approximately 10^5 to 10^6 colony-forming units (CFU) per ml by the time it is read as positive or a reading of 75 growth units or more on the instrument (Siddiqi, 2005). The colonies of *M.tuberculosis* in these tubes typically resemble the appearance of bread crumbs, as seen in **Figure 2.6**.

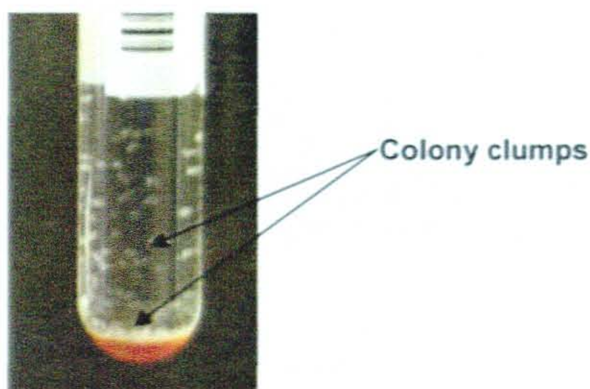


Figure 2.6 Positive MGIT™ tube demonstrating colony clump growth appearing as "bread crumb colonies" within the MGIT™ liquid culture tube. The fluorescent compound within the silicon can be seen at the bottom of the tube.

Once a MGIT™ tube was flagged positive on the BACTEC MGIT™ 960 System it was removed from the instrument for further processing. A second ZN smear was prepared from a vortexed MGIT™ tube by combining a drop of fixative and a drop of the mixed positive culture on a glass slide and a blood agar plate was also prepared

as a purity check, as per example in **Figure 2.7**. The second ZN was done to confirm the presence of mycobacteria and not other contaminating bacteria or yeasts within the MGIT™ tube.

If the ZN slide was found to be negative the tube was decontaminated and discarded according to the standard operating procedure. If cording was observed in a MGIT™ tube, where many mycobacterial bacilli grouped together, the tube was kept for further testing. Positive growth on a blood agar plate after eighteen hours of incubation at 37°C indicated contamination, as bacterial contaminants would grow much faster compared to *M.tuberculosis*.

Figure 2.7 A blood agar plate and a second ZN smear were prepared from a positive MGIT™ tube to check for purity and confirm *M.tuberculosis* in positive culture tubes.

2.6 Diagnostic methods for MDR-TB and XDR-TB screening

2.6.1 MGIT SIRE™ system

Once the second ZN stain was positive, which was considered the confirmation slide, and the blood agar plate was found to be sterile, five MGIT™ tubes were labelled with a growth control and the following antibiotics streptomycin, isoniazid, rifampicin and

ethambutol, respectively as shown in **Figure 2.8**. This was prepared from a commercially available BACTEC MGIT SIRE™ kit (Becton Dickinson, Maryland, USA).

Figure 2.8 Antibiotic sensitivity testing using the MGIT SIRE™ system. The five labelled tubes, a growth control suspension tube and the four lyophilised antibiotics were required for this drug sensitivity screening assay.

An antibiotic sensitivity screening set was prepared as follows. A growth control tube was prepared by combining 10ml sterile saline and 0.1 ml of the growth suspension from a positive MGIT™ tube to obtain a 1:100 growth control suspension. This was mixed thoroughly and 0.5 ml of the 1:100 growth control suspension was inoculated into a growth control tube for each set of four antibiotic-containing MGIT SIRE™ tubes per sample.

The four MGIT SIRE™ tubes were prepared by adding 0.8 ml of the BACTEC MGIT SIRE™ supplement (Becton Dickinson, Maryland, USA) seen in **Figure 2.9**.

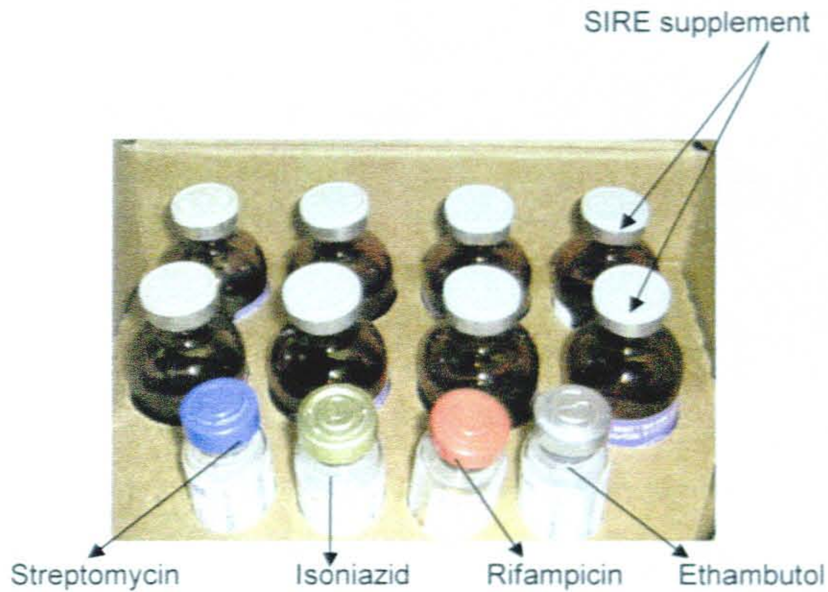


Figure 2.9 MGIT SIRE™ sensitivity kit consisting of the four antibiotics indicated and eight bottles of the SIRE supplement used during the drug sensitivity screening procedure.

To the appropriately labelled MGIT™ tube, 0.1ml of each drug was aseptically pipetted, after which 0.5 ml of the mycobacterial suspension was aseptically added to each drug-containing tube. The tubes were tightly recapped and mixed by gentle inversion. The antimicrobial sensitivity testing carrier set was then scanned into a BACTEC MGIT™ 960 instrument as seen in **Figure 2.10**, and incubated until the growth control tube read 400 growth units. At this point the four tubes cultured with the respective antibiotics could be read by the machine as either sensitive (S) or resistant (R) to the respective antibiotics. When resistance was obtained with one or more of these antibiotics tested, an E-test® strip was performed for second line sensitivity testing.

Figure 2.10 Loading of a MGIT SIRE™ carrier set, consisting of a sample growth control and four MGIT SIRE™ tubes, each containing a different antibiotic, streptomycin, isoniazid, rifampicin and ethambutol.

2.6.2 E-test® strip method

To determine the second line drug sensitivity profile, 0.1ml of a culture positive MGIT™ tube was plated and grown onto a 90mm Middlebrook 7H10 agar plate at 37°C for 3-4 weeks. When sufficient growth was visible between 3 to 4 weeks, an E-test® strip (BioMérieux, France) for antibiotic screening of amikacin, kanamycin, ciprofloxacin, and ethionamide, was performed by scraping off all colonies and suspending them in 3 ml Middlebrook 7H9 broth with four sterile glass beads added to the suspension. This was vortexed vigourously for 3 minutes and the tube was allowed to stand at room temperature for twenty minutes. A 3.0 McFarland standard (Wanger & Mills, 1996) had to be obtained for the test to work effectively. The entire surface of the 90mm Petri plate was then swabbed in three directions and the plate was pre-incubated at 37°C in 7-10% CO_2 and an E-test® strip was applied to each agar plate. The plates were then sealed with shrink seal (Davies Diagnostics, South Africa) and incubated at 37°C for ten days.

2.6.3 Molecular methods

The molecular LPA used was the rapid GenoType® MTBDR*plus* test (Hain Lifescience, Nehran, Germany), which is based on DNA strip technology. This comprised of three steps which were DNA extraction, multiplex polymerase chain reaction (PCR) amplification and reverse hybridisation.

Approximately 0.5ml decontaminated reconstituted pellet from each specimen was used for the DNA extraction step, after which an aliquot of the specimen was inoculated into a MGIT™ tube for further culturing. The 60 minute DNA extraction process included heating, sonification and centrifugation, which were done following the manufacturer's instructions. A master mix was prepared and used in the 60 minute PCR amplification step. All these steps were carried out in separate rooms with restricted access to avoid cross-contamination. Thereafter, hybridisation was performed on the automated hybridisation machine GT Blot 48 (Hain Lifescience, Germany), as seen in **Figure 2.11**. The strips were then washed, air-dried and pasted on paper for record purposes (Barnard *et al.*, 2007).

Figure 2.11 Line probe assay instrument (Hain Lifesciences) where DNA hybridisation was performed on the automated GT Blot 48 hybridisation machine.

This Hain molecular genetic assay is based on DNA-STRIP® technology which is used for the molecular genetic identification of the *M.tuberculosis (*Hain Lifescience, 2010*)*. The strip detects the resistance to rifampicin and or isoniazid from pulmonary smear positive clinical samples or cultured samples. The kit has the ability to detect the most significant mutations of the *rpoB* gene, coding for the beta-sub-unit of the *M.tuberculosis* RNA polymerase, and this would indicate a rifampicin resistance result.

For high-level isoniazid resistance the *katG* gene (coding for the catalase peroxidase) was examined and for low-level isoniazid resistance, the promoter region of the *inhA* gene (coding for the NADH-enoyl ACP reductase). If resistance to one or both drugs were observed further molecular testing was done using the MTBDR*sl* kit (Hain Lifescience, 2010) as illustrated in **Figure 2.12**. The MTBDR*sl* kit also allowed for the molecular identification of the MTB complex and its resistance to the fluoroquinolone antibiotic group, such as ciprofloxacin and moxifloxicin, and for aminoglycosides, which are the injectable antibiotics such as amikacin, capreomycin and kanamycin and ethambutol (Hain Lifescience, 2010).

Figure 2.12 Components of the GenoType®MTBDR*sl* kit, which are used for testing the fluoroquinolones, aminoglycosides and ethambutol.

For a result to be resistant to the fluoroquinolones the *gyrA* gene had to be present, which is coding for DNA gyrase. When resistance for the aminoglycosides was detected the 16S rRNA gene *rrs* was used. Lastly, for the detection of resistance to ethambutol, the *embB* gene, which together with the gene *mAMC*, coding for arabinosyl transferase, were examined.

2.7 Development of a four-quadrant plate method

A polystyrene four-quadrant Petri plate (Lasec, South Africa) as illustrated in **Figure 2.13**, was used as the basis for developing a simple, cost-effective MDR- and XDR-TB screening method. It is a single Petri plate, which consists of dividers within the plate with four quadrants available, which were used to test for the various drug sensitivity profiles that would enable classification of MDR- and XDR-TB in patient specimens.

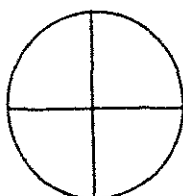


Figure 2.13 An illustration of a single four-quadrant plate with four different segments which is commercially available.

2.7.1 Optimising the media

A pilot study was done to see how *M.tuberculosis* would grow on Middlebrook 7H10 compared to Middlebrook 7H11 solid media in the four-quadrant plates. Casein hydrolysate is an added ingredient found in the Middlebrook 7H11, which is a modification of the Middlebrook 7H10 agar (Difco Laboratories, Detroit, Michigan). It also had to be determined which volume of the Middlebrook media should be added to the four-quadrant plate sectors for the optimal growth of *M.tuberculosis*.

The volumes assessed were *1ml, 2ml, 3ml, 4ml, 5ml, 6ml and 7ml of Middlebrook* agar per quadrant, and it had to be established what volume would support the fastest growth of *M.tuberculosis,* without the plates being too moist or drying out during the prolonged incubation period. All the tests were done in triplicate.

2.7.2 Optimising the colour growth indicator solution

A study where 2,3-diphenyl-5-(2)-tetrazolium chloride (STC) was used as an indicator in agar (Herrera *et al.*, 2007) inspired the incorporation of a colour growth indicator for mycobacterial growth detection in this study, however, STC is exorbitantly expensive and would have resulted in a single test plate set costing in excess of R4,000 if it were to be used. Therefore, other colour growth indicators were experimented with such as the related 2,3,5-triphenyltetrazolium chloride (TTC) (Merck, Darmstadt, Germany) and potassium permanganate (Merck, Darmstadt, Germany). Potassium permanganate was used at different concentrations in agar, ranging from 0.5% to 2.0% in 200 ml but there were no growth noted on all the plates whereas TTC showed promising result at 1%.

Thus, the colorimetric indicator then used was TTC (Merck, Darmstadt, Germany) and was used in all the agar quadrants, except for the growth control sectors, at a final concentration of 2 ml of a 1% TTC stock (Appendix D) solution using 200 ml agar.

2.7.3 Optimising the antibiotics for MDR- and XDR-TB screening

The antibiotics of choice for this research study were isoniazid and rifampicin to screen for MDR-TB and in addition to this, ciprofloxacin, amikacin, kanamycin and capreomycin for XDR-TB screening. A stock solution was made of the different lyophilised antibiotics in the appropriate solvents to make up a working solution of each antibiotic as seen in Appendix E. The appropriate volumes of the working solutions were pipetted into the Middlebrook 7H10 agar medium after it was cooled to 50-56°C in a water bath following autoclaving. This mixture was also supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Maryland, USA) to obtain the final concentration of each antibiotic and of TTC, also incorporated within the agar medium. The final concentration of isoniazid was 0.1µg/ml, rifampicin 1.0µg/ml, kanamycin 5.0µg/ml, amikacin 5.0µg/ml, capreomycin 10.0µg/ml and ciprofloxacin 2.0µg/ml. The general antibiotic solvent used was sterile distilled water, but rifampicin was first dissolved in dimethyl sulfoxide (DMSO) (Merck, Germany) and ciprofloxacin was added to 0.1 Molar sodium hydroxide before combining with sterile distilled water, according to CLSI guidelines, 2008 (CLSI, 2008). The control quadrant contained no antibiotics, only the Middlebrook 7H10 agar supplemented with OADC.

2.7.4 Optimising the specimen suspension and plating method

Different volumes of mycobacterial suspension from positive MGIT™ tubes, to be plated onto four-quadrant plates, were tested, ranging from 0.05 ml to 1.0 ml per plate quadrant. For each patient specimen screened for MDR- and XDR-TB, a set of two four-quadrant plates were prepared with Middlebrook agar, a colour growth indicator and the appropriate antibiotics in the different plate quadrants. Another alternative explored, was to first inoculate the mycobacterial suspension of a positive MGIT™ tube onto a 90mm Petri plate, which was incubated for 3 to 4 weeks at 37°C in the

presence of 5% CO_2, prior to inoculating a set of four-quadrant plates for drug sensitivity testing. When sufficient growth was visible on the 90mm Petri plate after 3 to 4 weeks, all the colonies were picked off using a sterile loop (Progen, South Africa), which were placed in a capped tube containing 3 ml Middlebrook 7H9 broth with four glass beads added, after which it was suspended by vortexing for 3 minutes. This solution was then allowed to stand for twenty minutes before inoculating the optimal volume of mycobacterial suspension onto each quadrant of a set of four-quadrant plates. The four-quadrant plates were then incubated at 37°C in the presence of 5% CO_2 for ten days.

A flow chart of the different methods used in this study, as well as the relative time lines is illustrated in **Figure 2.14**.

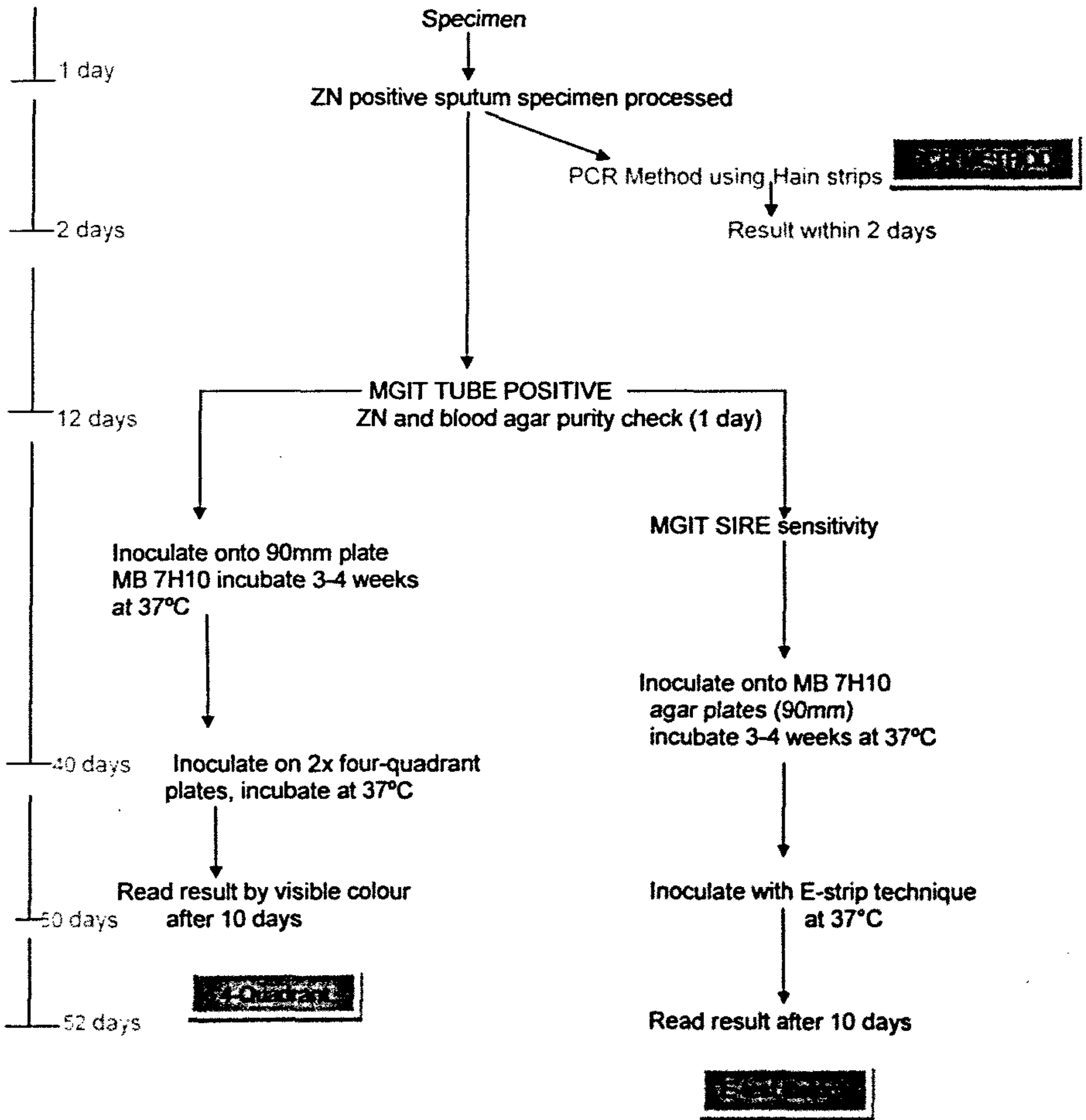


Figure 2.14 Methodology flow chart with time line on the side.

2.8 Statistical analysis

One hundred and twenty TB-positive sputum specimens were obtained during the sample collection period of this research study, which were all evaluated for drug sensitivity by the two established currently used methods in this laboratory, namely PCR and the E-test® strip method. In addition, the drug sensitivity profiles of these samples were also obtained with the newly-developed four-quadrant plate method.

Agreement between the different methods, namely PCR, the E-test® strip method and the four-quadrant plate method, was determined by the Simple Kappa Coefficient, using SAS version 9. The agreement and statistical significance between the actual diagnosis of MDR- or XDR-TB and the predicted diagnosis using the new method were established. In addition, agreement was determined for each of the antibiotics assayed using the different methods.

Specificity and sensitivity of the methods were also determined for each antibiotic sensitivity assay done and for the different diagnoses. Lastly, statistical significance of the contamination rate of the E-test® strip and the four-quadrant plate method was determined.

CHAPTER THREE

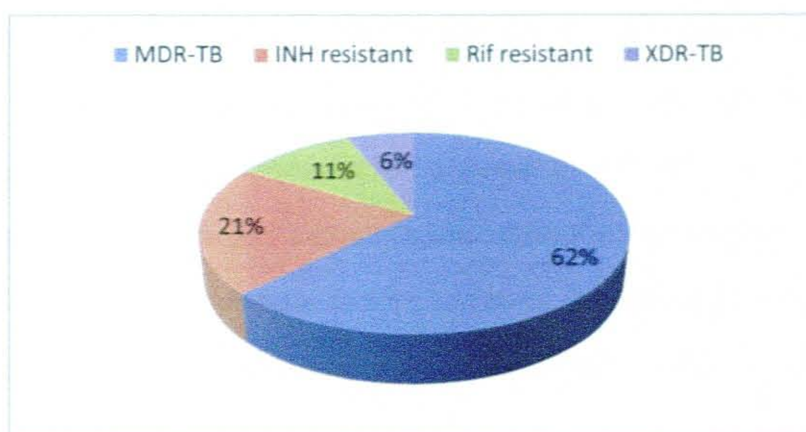
RESULTS

3.1 Sample distribution

The samples for this study were collected from Pathcare satellite laboratories which were sent to the reference laboratory for standard TB culture and drug sensitivity testing. The inclusion criteria for the sample selection were a positive microscopic AFB result, patient ages between eighteen and sixty, and the drug sensitivity pattern had to either be drug mono-resistant, MDR- or XDR-TB.

The total sample population comprised of one hundred and twenty samples, sub-divided into seventy five MDR-TB patient samples, seven XDR-TB samples and thirty eight mono-resistant samples. The latter group was further divided into twenty five isoniazid-resistant and thirteen rifampicin-resistant samples, as depicted in **Figure 3.1**.

The different methods used to determine the drug-sensitivity profiles of samples in this study were the newly developed four-quadrant plate method and the MGIT SIRE™ coupled with the E-test® strip plate method, which were compared to the PCR gold standard method.



Mono-resistant TB		MDR-TB	XDR-TB
Isoniazid-resistant	**Rifampicin-resistant**		
25	13	75	7

Figure 3.1. Classification of patient samples (n=120) collected for this study, 62% (n=75) of the samples were MDR-TB resistance, 6% (n=7) were XDR-TB resistant (n=25), 21% samples accounted for isoniazid mono-resistance and 11% (n=13) for rifampicin mono-resistance.

3.2 Development of a four-quadrant plate method

In an attempt to develop a robust, fast and cost-effective method for screening TB specimens for their drug sensitivity profile, a four-quadrant plate method was developed, based on a presentation that was delivered by Peruvian research group at a meeting held in Cape Town in 2007 at the Médecins Sans Frontiéres One Day Symposium on TB field diagnostics (Herrera *et al.*, 2007).

It was therefore the aim to develop a four-quadrant Petri plate method and screen TB positive specimens for selected antibiotic sensitivities simultaneously, thus to be able to classify a patient specimen as either drug-sensitive, mono-resistant, MDR or XDR, with the resultant drug-sensitivity profile of each specimen, in a single assay.

In order to achieve this, the choice of solid growth medium and volume had to be optimised, the nature and plating method of the patient specimen had to be determined, the choice and concentration of a colour growth indicator had to be optimised, as well as the choice of antibiotics and optimal concentrations added to the separate plate quadrants. All these factors had to be determined and were done in triplicate before optimising the four-quadrant plate test method for the study.

3.2.1 Optimising the mycobacterial solid growth media

Initially, mycobacteria were cultured on solid Middlebrook 7H11 agar in plates, but growth of colonies appeared scant after incubation for 12 – 15 days. In an attempt to improve growth, mycobacterial suspensions were grown on solid Middlebrook 7H10 media, which resulted in faster growth and a higher yield in colonies after incubation for 8 – 10 days. It was therefore decided to use Middlebrook 7H10 agar for the four-quadrant plate method. The differences in appearance of agar and mycobacterial growth between Middlebrook 7H11 and Middlebrook 7H10 are listed below in **Table 3.1**. The autoclaving conditions of these batches were identical.

Table 3.1 Differences between Middlebrook 7H11 and Middlebrook 7H10 agar appearance and mycobacterial growth characteristics

	Middlebrook 7H10 agar	**Middlebrook 7H11 agar**
Days to positivity	8 – 10 days	12 – 15 days
Growth	Moderate to heavy	Scanty to moderate
Colour of agar	Darker	Lighter

Next it had to be determined what the most optimal volume of the Middlebrook 7H10 agar per quadrant of the four-quadrant plate would be. It was aimed to have sufficient agar to promote and support fast growth of mycobacteria, while neither causing a moisture problem nor dry out during the extended periods of incubation. Agar

volumes ranging from 1ml to 7ml per quadrant were used and observations of each presented in **Table 3.2**.

Table 3.2 The volumes of Middlebrook 7H10 agar used to optimise the four-quadrant plate method and resulting observations were captured to arrive at a recommended optimal volume

Agar volume per plate quadrant	Description of observations
1ml	Agar volume was too little to even fill a quadrant of the four-quadrant plate
2ml	Agar volume still insufficient to cover a quadrant of the four-quadrant plate
3ml	Agar barely covered the quadrant of the four-quadrant plate, however, it was very thin
4ml	Agar covered the quadrant of the four-quadrant plate but the agar layer was thin
5ml	Agar covered the quadrant of the four-quadrant plate and looked evenly spread
6ml	Agar covered the quadrant of the four-quadrant plate, was evenly spread but started to appear quite thick
7ml	Agar covered the quadrant of the four-quadrant plate, but the plate was heavy as a result of the thick agar. It was difficult to pour the 7ml per quadrant in a time-efficient manner

The optimal Middlebrook 7H10 agar volume decided upon was 5 ml per quadrant, which showed an appropriate yield of mycobacterial colonies with normal morphology while not drying out or becoming too moist.

The optimal mycobacterial inoculum and plating method had to be established, and this was done by assessing growth on Middlebrook 7H10 agar after plating varying volumes of the same mycobacterial liquid suspension from a MGIT™ positive tube that was first grown on a Middlebrook 7H10 plate and incubated for 4 weeks. The colonies were picked off the plate and resuspended using 3ml of Middlebrook 7H9 broth base. These mycobacterial suspensions were then plated in incremental volumes on Middlebrook 7H10 agar by swirling on four-quadrant plates, after which the plates were incubated at 37°C and observations recorded after 3 weeks. A summary of the different volumes and the growth assessment on solid media is shown in **Table 3.3**.

Table 3.3 Optimising mycobacterial specimen suspension volume *on the* different agar volumes. Mycobacterial growth and plate moisture levels were assessed.

	Mycobacterial inoculation volume per plate quadrant				
Agar volume per plate quadrant	0.05ml	0.1ml	0.2ml	0.3ml	0.5ml
4ml	NG	G	HG	HG	HG+wet
5ml	NG	G	G	HG	HG+wet
6ml	NG	G	G	HG	HG+wet
7ml	NG	G	G	HG	HG+wet

NG – No growth
G – Growth
HG – Heavy growth
HG+wet – Heavy growth but quadrant still wet

As seen in the **Table 3.3**, mycobacterial growth was similar on the different agar volumes on the plate sectors and with varying volumes of mycobacterial suspension, and it was decided to consistently use 0.1ml specimen suspension and 5ml of Middlebrook 7H10 agar per quadrant of the four-quadrant plate for all experiments.

Only the *4ml to 7ml agar per quadrant were tested as the 1ml to 3ml were not used* when optimising the agar volumes as the plates dried out. Inoculation of 0.05ml resulted in no growth after 3 weeks, irrespective of the agar volumes, whereas 0.2ml showed variations of growth on the different volumes, 0.3ml showed heavy growth and 0.5ml resulted in heavy growth but the plates were still wet.

The rationale for selecting 0.1ml specimen suspension and not a higher volume was that the total volume of specimen suspension was not always enough for all experimental procedures, as tests were done in duplicate.

3.2.2 Optimising the colour growth indicator

Based on the preliminary findings of Herrera *et al.* (2007), using STC as colour growth indicator, of which the chemical structure can be seen in **Figure 3.2**, it was decided to incorporate a similar colour growth indicator for easier and faster detection of mycobacterial growth on the four-quadrant plates. As the colour indicator of choice, STC, as used by Herrera's group was exorbitantly expensive, and as the aim was to develop a more cost-effective method, cheaper redox colour indicators were then explored as an alternative.

Potassium permanganate and TTC were selected, undergoing colour change during oxidation or reduction. However, no growth occurred on the plates which had potassium permanganate added to the agar, even at lower concentrations than suggested. Plates containing the related TTC indicator, of which the chemical structure during the redox reaction is shown in **Figure 3.3**, on the other hand, showed growth by the same time growth was observed on control plates.

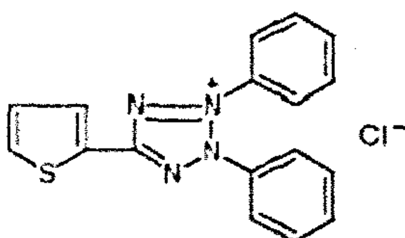


Figure 3.2 Chemical structure of STC (TCI America Product information, 2010).

Figure 3.3 Chemical structure of TTC, which is a redox indicator. The white compound is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activity of various dehydrogenases during oxidation of organic compounds (Wikipedia, 2010).

However, the colonies were red in colour but not the agar in which it was diffused, as was anticipated. The appearance of the mycobacterial colonies taking up the red TTC colour growth indicator is depicted in **Figure 3.4**.

Figure 3.4 A set of two four-quadrant plates with red TTC-incorporated colonies of *M.tuberculosis* growing on the different sectors of the four-quadrant plate, containing 5ml of Middlebrook 7H10 agar media per quadrant, the respective antibiotic and TTC colour growth indicator, with the control quadrants containing only agar.

3.2.3 Optimising antibiotics

The respective antibiotic concentrations which were used within the respective sectors of the four-quadrant plates, were done according to CSLI and WHO guidelines (CLSI, 2008; WHO 2008). The rationale for the choice of antibiotics within the quadrants was to find an alternative method for the current methods used for drug sensitivity testing in the routine reference TB laboratory, and to ensure that a full antibiotic screening set per patient sample would enable diagnosis of mono-resistant TB, MDR- and XDR-TB in a set of two four-quadrant plates, as depicted in **Figure 3.5**.

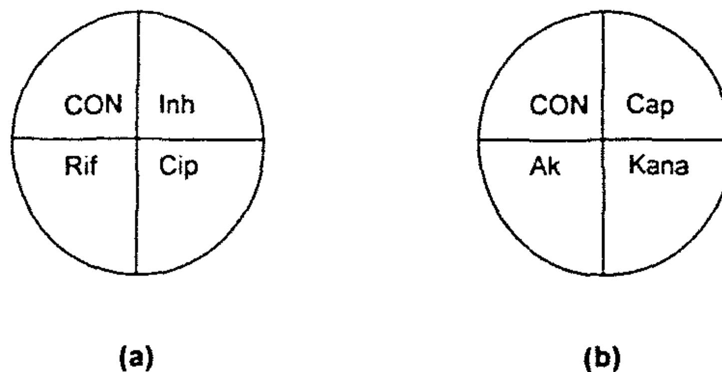


Figure 3.5 A full antibiotic screening set per patient specimen, comprising of two four-quadrant plates with quadrants containing **(a)** isoniazid (Inh), rifampicin (Rif), ciprofloxacin (Cip) and a control (CON) quadrant in one plate, and **(b)** the control (CON), amikacin (Ak), capreomycin (Cap) and kanamycin (Kana), each at its optimal concentration.

Therefore, a choice of six antibiotics was made to be able to screen for mono-resistant, MDR- and XDR-TB within a single plate set. The first quadrant of each plate was an antibiotic- and colour indicator-free growth control of a patient sample. Isoniazid and rifampicin were used at final concentrations of 0.2µg/ml and 1.0µg/ml, respectively, and represented the screen for MDR-TB according to the WHO criteria (WHO, 2004). The rest of the quadrants contained 2.0µg/ml ciprofloxacin, 5.0µg/ml amikacin, 5.0µg/ml kanamycin and 10.0µg/ml capreomycin, which enabled that a specimen could be screened for XDR-TB, according to WHO criteria (CDC, 2006).

3.3 Agreement with existing methods

As the aim of this study was to find a robust and more cost-effective method to screen for MDR- and XDR-TB within a single test, the newly develop four-quadrant plate method was compared to the gold standard PCR method, as well as to the E-test® strip method currently in use in this routine diagnostic laboratory. As little comparative data are available, the PCR and E-test® strip methods were also compared to each other. This was done at the level of each comparative antibiotic individually, as well as the resulting diagnoses of each of isoniazid or rifampicin mono-resistant TB, MDR-TB or XDR-TB.

Growth profiles were scored after ten days of inoculating the four-quadrant plates at 37°C and were indicated as either resistant, indicated by mycobacterial growth in respective quadrants, or sensitive, indicated by no growth for each antibiotic-containing quadrant, provided the growth control sectors were positive and plates were free of contamination. Raw data of these analyses are provided in Appendix F. These tests were all done in duplicate and both sets had to be contamination-free to be interpreted for results.

Table 3.4 The number of specimens diagnosed with the different methods with the PCR method as gold standard

Resistance diagnosis of patient specimens analysed	**Number of samples diagnosed with the different methods used**		
(n=120)	**PCR**	**E-test®**	**4-Q**
Number of specimens diagnosed for isoniazid monoresistance	25	23	25
Number of specimens diagnosed for rifampicin monoresistance	13	13	10
Number of specimens diagnosed for MDR-TB	75	72	71
Number of specimens diagnosed for XDR-TB	7	7	8
Inconclusive results	0	5	5

Of the 120 patient sputum specimens analysed for drug sensitivity using the three different methods, the actual numbers of patients diagnosed with MDR- and XDR-TB are listed in **Table 3.4**. Inconclusive results were those that were either contaminated, or that yielded a drug resistance profile that could not be classified as either of the drug resistance patterns according to the WHO criteria (WHO, 2004; CDC, 2006).

Agreement for each antibiotic result was compared between the three methods, and was statistically analysed by calculating the Simple Kappa Coefficient for each as presented in **Table 3.5**.

Table 3.5 Agreement for the antibiotics screened by Simple Kappa Coefficients with P values (at a 95% confidence interval) using the different drug sensitivity screening methods, with PCR as the gold standard method. Interpretation of the level of agreement is stated below the table.

Antibiotic tested		**PCR vs 4-Q**	**ES vs 4-Q**	**PCR vs ES**
Isoniazid	Kappa (K)	0.8480	0.8470	0.9581
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*
Rifampicin	Kappa (K)	0.8824	0.9010	1.0000
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*
Ciprofloxacin	Kappa (K)	0.7143	0.8661	0.8661
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*
Amikacin	Kappa (K)	0.8824	0.8824	0.8476
	P (95%)	*<.00001*	*<0.0001*	*<0.0001*
Kanamycin	Kappa (K)	0.9191	0.9136	0.8384
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*
Streptomycin	Kappa (K)	–	–	0.7394
	P (95%)	–	–	*<0.0001*
Ethambutol	Kappa (K)	–	–	0.6534
	P (95%)	–	–	*<0.0001*

KEY:

K range	*Interpretation*	*Colour coding*
0.81-1.00	*Excellent agreement*	
0.81-0.92	*Very good agreement*	
0.61-0.80	*Good agreement*	
0.41-0.60	*Fair agreement*	
0.21-0.40	*Slight agreement*	
0.01-0.20	*Poor agreement*	
0	*No agreement*	

The kappa values have been interpreted with the following approximate ranges according to Byrt (1996), where the level of agreement between the methods compared is graded according to the Simple Kappa Coefficients.

When agreement was determined between the various antibiotics tested for resistance or sensitivity to *M.tuberculosis*, the new four-quadrant method and the E-test® strip assay for each was compared to the PCR gold standard using the Simple Kappa Coefficient. In addition, the agreement between the E-test® strip and four-quadrant plate methods were determined for each antibiotic.

For the 120 patient sputum specimens analysed for drug sensitivity using the three different methods, the four-quadrant method compared very well for the MDR-TB screen with 94% of the specimens having the same outcome as the PCR method, with slightly higher (96%) comparison with the E-test® strip. For the XDR-TB screen the E-test® strip method correlated 100% with the PCR result, whereas with the four-quadrant plate method an extra specimen was diagnosed with XDR-TB.

Diagnosis of isoniazid mono-resistance resulted in 92% of samples corresponding between the E-test® strip method and the PCR method, while there was 100% correspondence when comparing the four-quadrant plate results with PCR results. For diagnosing rifampicin mono-resistant specimens, the E-test® strip method showed 100% correlation with the PCR method, and the four-quadrant plate method lower correspondence at 76% compared with the PCR method, of which five specimens were false positives as compared to the PCR gold standard.

For both isoniazid and rifampicin, there was excellent agreement between the PCR and E-test® strip methods (Simple Kappa Coefficients of 0.9581 and 1.000, respectively), whereas it was considered very good between the PCR and the four-quadrant plate method (0.8480 and 0.8824), and the E-test® strip and four-quadrant methods (0.8470 and 0.9010), accordingly to the interpretation by Byrt (1996). In all instances the results were highly significant, with $P < 0.0001$.

For the assayed antibiotics denoting extensively drug resistant tuberculosis, ciprofloxacin, amikacin and kanamycin, the agreement between the methods were as follows. The Simple Kappa Coefficient for ciprofloxacin was interpreted as very good agreement for both the PCR versus E-test® strip method (0.8661) and the E-test® strip method versus the four-quadrant plate method, at 0.8661, and for the PCR versus the four-quadrant plate method it was 0.7143, which was considered good agreement.

All the methods used to test for amikacin resistance was in very good agreement and the Simple Kappa Coefficient was ranging between 0.8476 and 0.8824, while kanamycin also showed very good agreement for all combinations of methods with a Simple Kappa Coefficient of 0.8384 for the PCR method versus the E-test® strip method, 0.9191 for the PCR method versus the E-test® strip method and 0.9136 for the E-test® strip method versus the four-quadrant plate method, all with $P < 0.0001$.

Streptomycin was not included in the four-quadrant plate method, but only used in the PCR and E-test® strip method, the latter which showed good agreement with a Simple Kappa Coefficient of 0.7394 compared to the PCR gold standard. The Simple Kappa Coefficient for the ethambutol E-test® strip assay was 0.6534 and was in good agreement with the PCR method. Ethambutol was also not included in the four-quadrant plate method. No comparisons could be made to capreomycin, as it was only used in the four-quadrant plate method for diagnosis of XDR-TB.

The agreement between the actual diagnoses of MDR- and XDR-TB was also statistically compared by looking at the level of agreement between the three methods using the Simple Kappa Coefficients, as in **Table 3.6** for MDR-TB and XDR-TB.

Table 3.6 Agreement by Simple Kappa Coefficients with P values (at a 95% confidence interval) for the different methods used to arrive at a MDR-TB or XDR-TB diagnosis

Diagnosis made		**PCR vs 4-Q**	**ES vs 4-Q**	**PCR vs ES**
MDR-TB	Kappa (K)	0.9441	0.9609	1.0000
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*
XDR-TB	Kappa (K)	0.9300	1.0000	1.0000
	P (95%)	*<0.0001*	*<0.0001*	*<0.0001*

KEY:

K range	***Interpretation***	***Colour coding***
0.81-1.00	*Excellent agreement*	
0.81-0.92	*Very good agreement*	
0.61-0.80	*Good agreement*	
0.41-0.60	*Fair agreement*	
0.21-0.40	*Slight agreement*	
0.01-0.20	*Poor agreement*	
0	*No agreement*	

As indicated in **Table 3.6** all three methods compared were in excellent agreement with each other, with Kappa coefficients between 0.94 and 1.00 for MDR-TB, and Kappa coefficients between 0.93 and 1.00 for XDR-TB, all results being highly significant with $P < 0.001$.

3.4 Sensitivity and specificity

The specificity and sensitivity of the currently used E-test® strip method and the newly developed four-quadrant plate method were compared to each other as benchmarked against the PCR assay as gold standard. The specificity was a measure of the proportion of negatives which were correctly identified as negatives (drug sensitive), while the sensitivity was a measure of the proportion of actual positives which were correctly identified (drug resistant).

Both the specificity and sensitivity correlated very well for isoniazid (100%; 96.3%) and rifampicin (100%; 99.0%) when comparing the four-quadrant plate method and

the E-test® strip method to PCR, as indicated in **Table 3.7**. For rifampicin, specificity was excellent, with 100% for both the four-quadrant plate and the E-test® strip methods, however, the sensitivity for the four-quadrant plate method was a little lower (94.7%) compared to the E-test® strip method (100%).

The methods also correlated well when ciprofloxacin was assayed, with specificity high at 97.3% for the four-quadrant plate and 100% for the E-test® strip methods. In terms of sensitivity, the methods were identical in relation to PCR, although both appeared to have the lowest sensitivity (77.8%) to this antibiotic in the selected drug sensitivity profile.

Amikacin compared very well with a specificity of 99.1% and 98.1% for the four-quadrant plate and E-test® strip methods, respectively, whereas sensitivity was at 86.7% for both assays.

Specificity for the kanamycin assay was higher for the four-quadrant plate method, at 100%, than the E-test® strip method, at 99.1%, and sensitivity was much higher for the four-quadrant plate method compared to the E-test® strip method, 86.7% versus 80.0%.

Streptomycin and ethambutol could only be measured as E-test® strip method against PCR, and gave high levels of specificity (95.1%) and sensitivity (85.7%) for streptomycin. Ethambutol gave high specificity at 95.4%, but the sensitivity compared to PCR was very low, at 68.0%.

Table 3.7 The sensitivity and specificity for each antibiotic assayed comparing the new four-quadrant plate method and the E-test® strip method to PCR as gold standard, with 95% confidence intervals, except for (*)one-sided 97.5% confidence intervals

Antibiotic assessed		**4-Q**	**95% Confidence interval**	**ES**	**95% Confidence interval**
Isoniazid	Specificity (%)	100	*(97.8-100)**	100	*(97.8-100)**
	Sensitivity (%)	96.3	*(92.6-99.9)*	99.0	*(97.0-100)*
Rifampicin	Specificity (%)	100	*(97.8-100)**	100	*(97.8-100)**
	Sensitivity (%)	94.7	*(90.3-99.1)*	100	*(97.8-100)**
Ciprofloxacin	Specificity (%)	97.3	*(94.1-100)*	100	*(97.8-100)**
	Sensitivity (%)	77.8	*(69.7-85.9)*	77.8	*(69.7-85.9)*
Amikacin	Specificity (%)	99.1	*(97.2-100)*	98.1	*(95.4-100)*
	Sensitivity (%)	86.7	*(80.0-93.4)*	86.7	*(80.0-93.4)*
Kanamycin	Specificity (%)	100	*(97.8-100)**	99.1	*(97.3-100)*
	Sensitivity (%)	86.7	*(80.0-93.4)*	80.0	*(72.2-87.8)*
Streptomycin	Specificity (%)	–	–	95.1	*(90.1-99.3)*
	Sensitivity (%)	–	–	85.7	*(78.8-92.6)*
Ethambutol	Specificity (%)	–	–	95.4	*(91.3-99.5)*
	Sensitivity (%)	–	–	68.0	*(58.9-77.1)*

The four-quadrant plate method was in excellent correlation with the currently used E-test® strip method for specificity to isoniazid, rifampicin, ciprofloxacin, amikacin and kanamycin, as illustrated in **Figure 3.6**, with the lowest specificity at 97.3% for ciprofloxacin. The specificity values for the E-test® strip method compared to PCR for streptomycin and ethambutol were the lowest overall levels, at 95.1% and 95.4%, respectively. These two antibiotics were not included in the four-quadrant plate method.

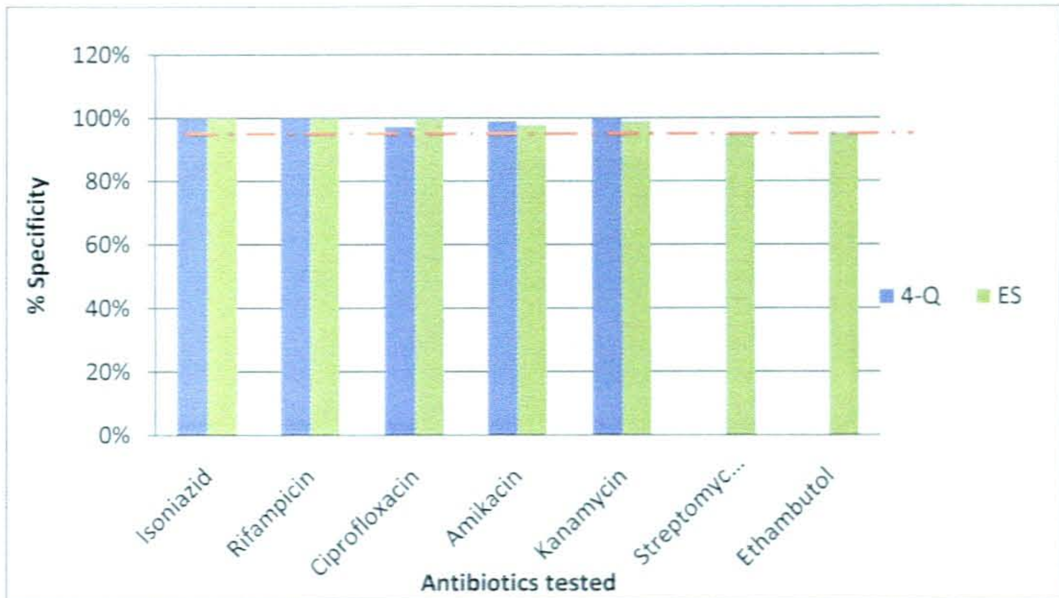


Figure 3.6 Percentage specificity of the four-quadrant plate and E-test® strip methods compared to PCR for the individual antibiotics assayed. The red dashed line indicates the cut-off for the lowest level of specificity obtained.

As is illustrated in **Figure 3.7**, the percentage sensitivity for isoniazid and rifampicin for the methods tested were markedly higher than for the other antibiotics tested. The correlation between the four-quadrant plate method and E-test® strip method were excellent for ciprofloxacin and amikacin, with the new method showing much higher sensitivity in the kanamycin assay.

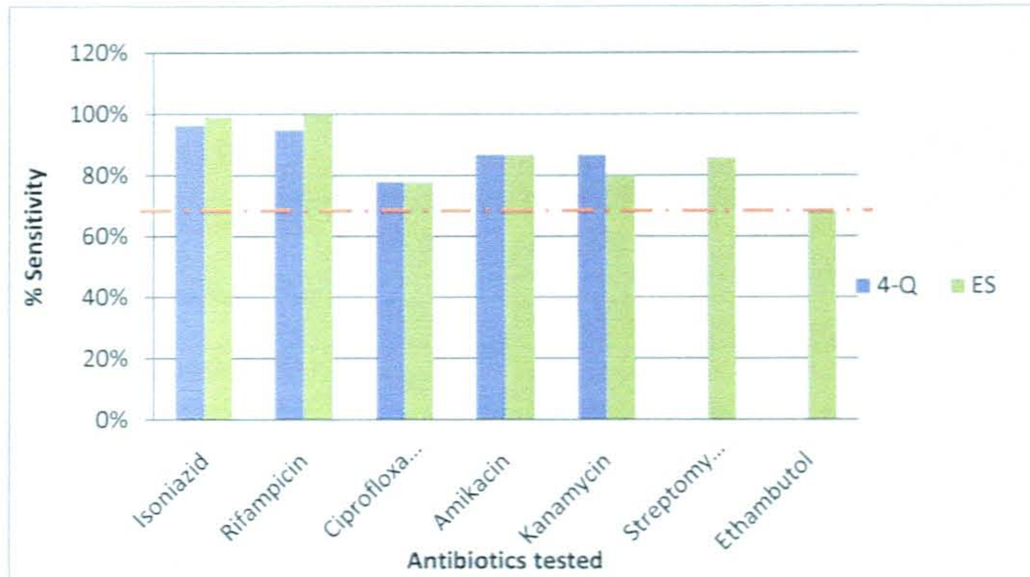


Figure 3.7 Percentage sensitivity of the four-quadrant plate and E-test® strip methods compared to PCR for the individual antibiotics assayed. The red dashed line indicates the cut-off for the lowest level of sensitivity obtained.

The specificity and sensitivity between the four-quadrant plate method and the E-test® strip method were also assessed for the actual MDR- and XDR-TB diagnoses, as compared to the diagnosis for each using the PCR method. As can be seen in **Table 3.8**, both specificity and sensitivity correlated extremely well at the diagnosis level in both categories, with the new four-quadrant plate method showing slightly less specificity for MDR-TB diagnosis, and marginally less sensitivity for XDR-TB diagnosis.

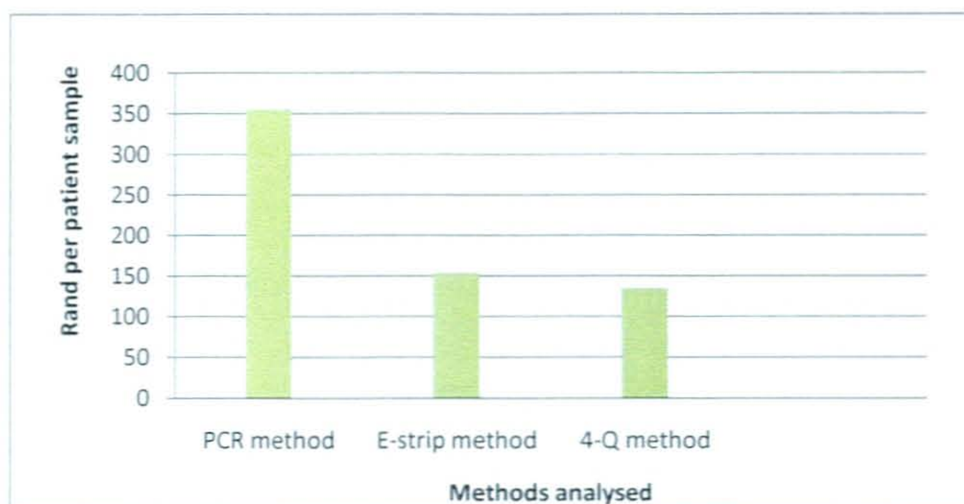
Table 3.8 Sensitivity and specificity for the diagnosis of MDR- and XDR-TB of the new four-quadrant plate method and the E-test® strip method compared to PCR, with 95% confidence intervals, except for (*)one-sided 97.5% confidence intervals

Diagnosis outcome		**4-Q**	**95% Confidence interval**	**ES**	**95% Confidence interval**
MDR-TB	Specificity (%)	96.0	*(92.2-99.8)*	100	*(97.8-100)**
	Sensitivity (%)	100	*(97.8-100)**	100	*(97.8-100)**
XDR-TB	Specificity (%)	100	*(97.8-100)**	100	*(97.8-100)**
	Sensitivity (%)	99.1	*(97.3-100)*	100	*(97.8-100)**

3.5 Cost-effectiveness

The cost per sample for the identification of MDR-TB and XDR-TB drug-resistance profiles was calculated for each diagnostic screen separately and also collectively.

The actual cost for a MDR-TB screen per patient sample for the different test methods in this study is seen in **Figure 3.8**. For the PCR method, the monetary value was three hundred and fifty six rand, while for the E-test® strip method it was one hundred and fifty five rand and for the four-quadrant plate method was one hundred and thirty six rand. These costs were totals of consumables for each method and exclusive of labour and sophisticated equipment but inclusive of 14% VAT.

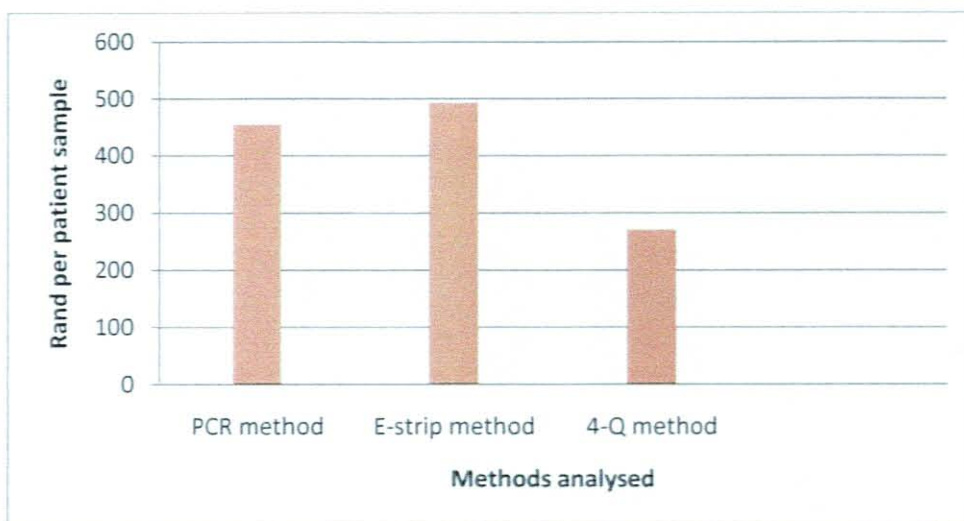


PCR method	ES method	4-Q method
R356,00	R155,00	R136,00

Figure 3.8 Cost per patient sample for MDR-TB screening for the different methods used in this study. The PCR method was over R350, the E-test® strip method over R150 and four-quadrant plate method below R140.

There was a marked difference in cost between the PCR method and the other two assays used. The E-test® strip method cost 43% and the four-quadrant plate method 38% of the PCR method, respectively.

Surprisingly, the additional cost for an XDR-TB screen per patient sample with the E-test® strip method was more expensive than the PCR method, which is commonly known as the most expensive option, as shown in **Figure 3.9**. The E-test® strip method was just under five hundred rand, while the PCR method was costed at four hundred and fifty six rand and the four-quadrant plate method just over two hundred and seventy rand.

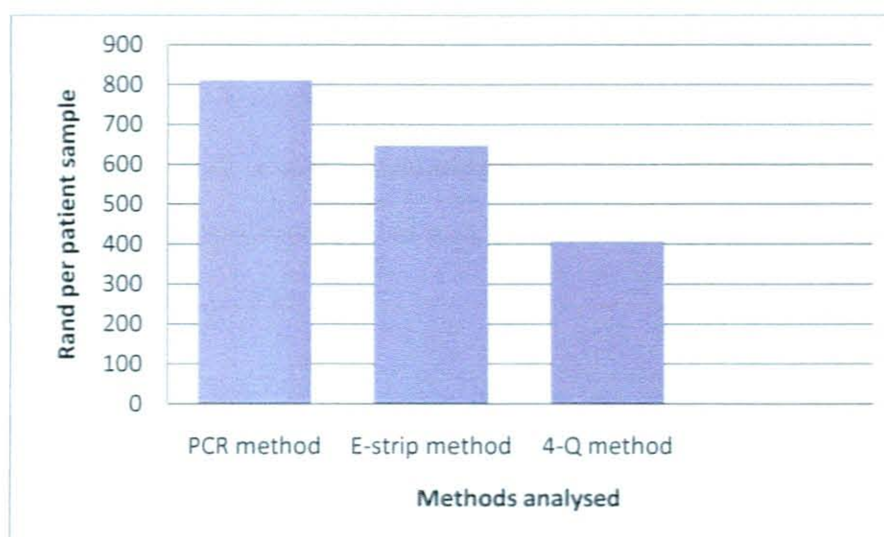


PCR method	ES method	4-Q method
R456,00	R494,00	R272,00

Figure 3.9 Additional cost per patient sample for XDR-TB screening for the different methods used in this study. The PCR method costed at R456, the E-test® strip method just under R500 and the four-quadrant plate method R272.

Here, both the PCR and E-test® strip methods were much more expensive than the four-quadrant plate method, with PCR being 40% more expensive and the E-test® strip method being 45% more expensive than the newly developed plate method.

Taking a drug-resistance screen per sample in its entirety, based on the three methods used in this study, the total cost per sample for the PCR method was eight hundred and twelve rand, the E-test® strip method at six hundred and forty nine rand and the four-quadrant plate method four hundred and eight rand, as illustrated in **Figure 3.10**.



PCR method	ES method	4-Q method
R812.00	R649,00	R408,00

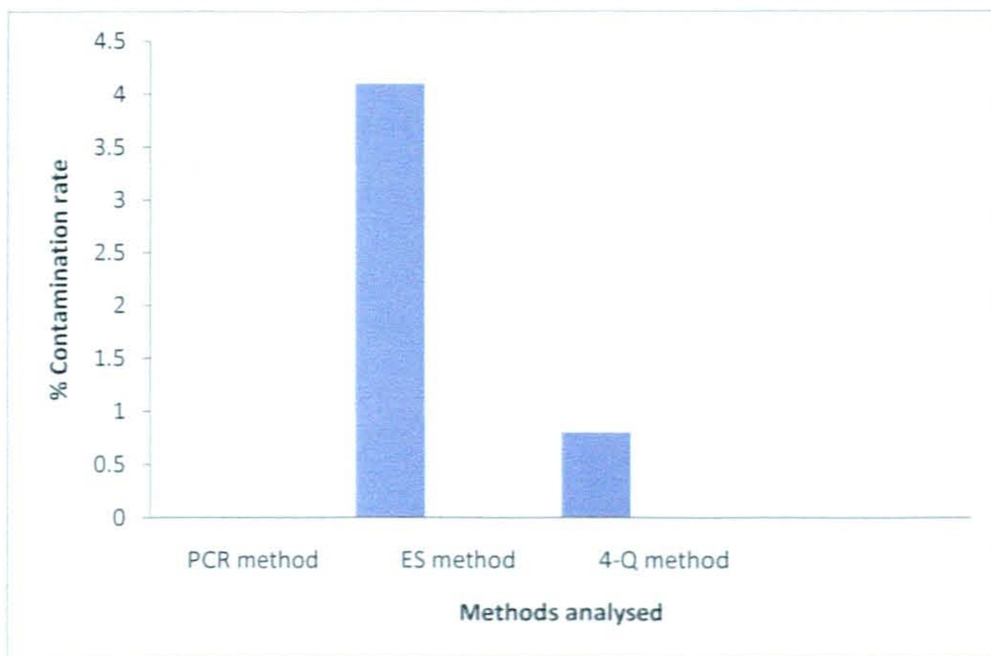
Figure 3.10 Total cost per patient sample to identify the combined MDR- and XDR-TB drug sensitivity profile where the four-quadrant plate method was found to be the most cost-effective method by far and PCR method the most expensive method as used to determine the drug-resistance profile for diagnosis of both MDR- and XDR-TB.

What was clearly evident from this data, was that the four-quadrant plate method cost half of what the PCR method amounted to per sample and that the E-test® strip method cost only 20% less than the PCR method, but almost double that (37%) more than the four-quadrant plate method.

3.6 Quality control and contamination

In order for a sample to be classified as contaminated, at least one of the duplicates had to be contaminated and the results of such a sample was not included for analysis in this study, but reported as inconclusive. For all patient sputum samples

processed and analysed in this study (n = 120), there was no contamination recorded using the PCR method, while the E-test® strip method, which included the BD MGIT SIRE™ liquid method, had a 4.1% contamination rate (n = 5/120). The newly developed four-quadrant plate method had a much lower contamination rate, 0.8% (n = 1/120), as shown in **Figure 3.11**.



Contamination rate (n =120)	PCR method	ES method	4-Q method
Number of samples contaminated	0	5	1
Contamination rate	0%	4.1%	0.8%
		P = 0.0981	

Figure 3.11 Comparison of contamination rate of samples using the three different methods. The PCR method showed no contamination, whereas the E-test® strip method had a 4.1% contamination rate and the four-quadrant plate method had a 0.8% contamination rate.

Although the contamination constituted relatively low numbers, the difference between the contamination rate of the new four-quadrant plate method and the E-test® strip method was not statistically significant ($P = 0.0981$), although the four-quadrant plate method results appeared to be more reliable in this regard.

CHAPTER FOUR

DISCUSSION

4.1 Introduction

Little information is available on non-molecular diagnostic methods available for a comprehensive mycobacterial drug sensitivity screen against the major antibiotics, which would enable classification and diagnosis of mono-resistant, MDR- or XDR-TB, within the same assay. Most previous studies only evaluated isoniazid and rifampicin drug susceptibility and suggest that their methods could also be extended to second and third line susceptibility testing for TB, including the more resistant TB strains (Mohammadzadeh *et al.*, 2006). Therefore this study aimed to develop and evaluate a robust, cost-effective agar-based plate method which could be used routinely to screen for MDR-TB and XDR-TB simultaneously, even in relatively resource-poor settings.

4.2 Specimens used in this study

The place where the research study was conducted was the TB reference laboratory at Pathcare Private Pathology Company at N1 City. All sputa specimens sent to this laboratory for routine TB diagnostic and drug sensitivity testing, were in parallel tested using the newly developed four-quadrant plate method and drug sensitivities and resultant diagnostic outcomes compared.

Sample collection was based on a fixed time period of collection, during which all microscopically-screened adult sputum specimens positive for the presence of *M.tuberculosis* but resistant to at least one TB drug, were included in this study. Smear microscopy methods used for the initial *M.tuberculosis* screening, were both ZN staining and the Auramine-O staining methods, as the laboratory was in a

transition phase, adopting the Auramine-O method as staining method of choice. Therefore, microscopy was done according to the methods as requested by clinicians. Part of the reason why the Auramine-O method is becoming the method of choice, is that the light emitting diode lamp is more cost effective and lasts longer than the standard mercury vapour lamp still used in laboratories for ZN microscopy (Marais *et al.*, 2008).

Further criteria required that these specimens had to be resistant to one or more of the standard antituberculous antibiotics tested. The drug-susceptibility information was obtained from the PCR method as routinely used in this laboratory, as this method only took two days for results to be known.

This resulted in the total study population comprising one hundred and twenty specimens of which twenty-five samples were isoniazid mono-resistant, thirteen were rifampicin mono-resistant, seventy-five had a MDR-TB and seven had a XDR-TB drug-resistant profile. The number for XDR-TB was low as this form of TB is only now being tested for routinely in laboratories. Furthermore, a figure of almost six percent of routine TB-resistant specimens being XDR-TB would be considered an alarming rate in most countries, but a troublesome norm in South Africa and neighbouring countries.

4.3 Diagnostic methods currently used for drug sensitivity testing

The WHO had recommended that countries expand their capacity for the culture-based drug-susceptibility testing and consider new molecular-based assays for diagnosing drug resistance (Stop TB Department, 2007; WHO 2008).

The molecular avenue is one thrust and is constantly evolving, with newer assays that have been developed to detect resistance faster by using genotype rather than phenotype (Hilleman, *et al.*, 2005; Barnard *et al.*, 2008; Anek-Vorapong *et al.*, 2010).

The GenoType® MTBRDR*plus* assay is a deoxyribonucleic strip method that makes use of PCR and hybridisation to detect mutations in the *inhA, katG* and *rpoB* genes that identify isoniazid and rifampicin resistance (Hilleman, *et al.*, 2005; Barnard *et al.*, 2008; Anek-Vorapong *et al.*, 2010). The GenoType® MTBRDR*sl* assay detects resistance to the fluoroquinolone antibiotic group, such as ciprofloxacin and moxifloxicin, and aminoglycosides, which are the injectable antibiotics such as amikacin, capreomycin and kanamycin and then a first line antibiotic ethambutol.

A study done by Anek-Vorapong demonstrated MDR-TB, isoniazid resistance as well as rifampicin resistance with a high sensitivity and 100% specificity in the compared methods which they used and also demonstrated the reduced turn-around time (Anek-Vorapong *et al.*, 2010). Also in a study done in 2008 in South Africa, using the GenoType® MTBRDR*plus* assay but with a larger volume of specimens, a sensitivity of 99% was achieved for both MDR-TB resistance and rifampicin resistance, while the sensitivity for the detection of isoniazid resistance was 94%. The specificity was 100% for both MDR-TB and isoniazid resistance, while rifampicin specificity was almost as high at 99% (Barnard *et al.*, 2008).

However, the disadvantage of this PCR assay is that it does not work on smear negative specimens (Barnard *et al.*, 2008). A weakness of the GenoType® MTBDR*plus* assay, on the other hand, was that only certain isoniazid resistance-related mutations were observed when compared to the reference BACTEC system and DNA sequencing analysis (Neonakies *et al.*, 2008). It thus appears that one avenue for improved drug sensitivity testing is to increase the sophistication of molecular techniques, which is receiving its due attention, but the main disadvantage is the high cost of these assays.

In this study a few methods were used to determine the drug sensitivity profiles of patient sputum specimens. Two methods are used routinely in this TB reference

laboratory for this purpose, the first being the GenoType® MTBRDR*plus* and GenoType® MTBDR*sl* PCR assays. The other method also used, was the liquid MGIT SIRE™ assay coupled with the E-test® strip method.

The aim of this study was therefore to take drug sensitivity testing assays in an opposite direction compared to new molecular developments, by aiming to simplify a technique in terms of sophistication, interpretation and cost-effectiveness. The idea was that this assay should enable drug sensitivity testing of patient sputum specimens not only in TB reference or large diagnostic laboratories, but also in relatively unsophisticated resource-poor laboratories, even at rural primary health care stations. This lead to the development of the four-quadrant plate method, based on an idea presented by a Peruvian group visiting South Africa a few years ago (Harrera *et al.*, 2007).

4.4 Mycobacterial media suitable for drug sensitivity testing

Egg-based media like LJ and Ogawa media are unsuitable for drug susceptibility testing (Inderlied & Pfyffer, 2003). The reason for not making use of the above mentioned media is due to the phospholipids, proteins and certain amino acids present in the medium that affect some of the drug sensitivities (Inderlied & Pfyffer, 2003). *M.tuberculosis* is a slow-growing bacterium which requires specialised culture media (Gradwohl, 1970; Lennette *et al.*, 1980; Koneman *et al.*, 2006), of which liquid-based mycobacterial detection systems such as the BACTEC MGIT 960 system allow for improved sensitivity and reduced time-to-detection in comparison with conventional solid LJ media (Hanna *et al.*, 1999; Tortoli *et al.*, 1999; Siddiqi, 2005). However, Middlebrook 7H10 is the reference agar method which is proposed by the CDC and CSLI (Woods, 2000). In this research study Middlebrook 7H10 agar was

used as the medium of choice for drug susceptibility testing on solid media, which was superior compared to Middlebrook 7H11 agar in our hands.

4.5 Establishment of a colorimetric four-quadrant plate method for drug sensitivity testing

A colorimetric method has been reported to be used for easy diagnosis as well as the determination of the susceptibility for *M.tuberculosis* (Lee *et al;* 2006). Various colorimetric methods used previously, have been proposed for the detection of drug susceptibility testing, such as Alamar blue, 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MMT), 2,3-bis(2-methoxy-4-nitro-5-sulphophenol)-2*H*-tetrazolium-5-carboxanilide (XXT), 2,3-diphenyl-5-thienyl-(2)-tetrazoliumchloride (STC) and 2,3,5-triphenyltetrazolium chloride (TTC) (Mohammadzadeh *et al.*, 2006). According to Yamane *et al.* (1996), STC was used was used on Ogawa medium for susceptibility testing which produced highly reliable results. As one of the aims in this study was to keep the cost of this assay to a minimum, TTC was the colour indicator of choice.

Our results using TTC-containing four-quadrant plates was in good agreement with the study of Mohammadzadeh *et al.*, 2006, although the latter study was done using liquid Middlebrook 7H9 media. Even the serendipitous observation of the red-coloured mycobacterial colonies when they grew on these plates, instead of the agar undergoing a colour transition, was welcomed, as this too enabled enhanced visual observation of antibiotic resistance, probably more clearly and faster than with normal mycobacterial growth.

A major limitation of previous studies was that they only tested first line drug susceptibilities of isoniazid and rifampicin. Very few studies included second line susceptibility testing which incorporated the identification of XDR-TB, which is

presently an emerging threat. The antibiotics incorporated in the four-quadrant plates in this study were isoniazid, rifampicin, ciprofloxacin, amikacin, kanamycin and capreomycin. These were at optimal concentrations in six of the eight sectors of a four-quadrant plate, with one growth control sector per plate. The rationale for including these antibiotics was to enable a multi-screen with antibiotics from all classes that would enable diagnosis of not only mono-resistant TB, but also MDR- and XDR-TB within the same assay. It was also aimed to keep the cost low, and therefore 6 antibiotics were chosen to represent a thorough patient profile in a set of two four-quadrant plates. It should be achievable, as the need arises, to replace one of the growth control sectors of a set of plates with another antibiotic, or even replace some antibiotics with alternate ones.

4.6 Comparison of the four-quadrant plate method to the BACTEC MGIT SIRE™ and E-test® strip methods

The drug sensitivities done using the BACTEC MGIT SIRE™ kit were isoniazid, rifampicin, streptomycin and ethambutol, enabling MDR-TB detection, while the E-test® strip method was used in conjunction, testing for kanamycin, amikacin and ciprofloxacin, for XDR-TB detection. PCR, as gold standard assay for benchmarking the others, were used to evaluate the same antibiotics as used in the BACTEC MGIT SIRE™ and E-test® strip method combination.

In terms of agreement between the methods, high Simple Kappa Coefficients were obtained overall, with excellent to good agreement between all the methods tested, which were all statistically highly significant ($P < 0.0001$), according to the interpretation scale as proposed by Byrt (1996). The antibiotic which gave the poorest result compared between the four-quadrant plate method and PCR was ciprofloxacin, although it was still classified as having good agreement. This was a very positive result and showed that the four-quadrant plate method could very well be used as

replacement assay to determine TB drug susceptibilities for these antibiotics in resource-poor settings.

Comparison of the sensitivity and specificity of the four-quadrant plate method also yielded very promising results, with consistently high levels of specificity for isoniazid, rifampicin, ciprofloxacin, amikacin and kanamycin, against the PCR method. As it was thought valuable to also compare the BACTEC MGIT SIRE™ and E-test® strip method combination with PCR, both of which are used within the TB reference laboratory, both sensitivity and specificity results were extremely similar as found with the four-quadrant plate method. This included the antibiotics which showed the lowest sensitivity, amikacin (86.7%; 86.7%), kanamycin (80.0%; 86.7%) and ciprofloxacin (77.8%; 77.8%). What was interesting was that the four-quadrant plate method gave equally low or even better results, as with kanamycin, 86.7% versus 80.0% for the E-test® strip method.

Findings in previous studies like that of Mohammadzadeh and co-workers (2006), using TTC in a liquid culture, showed sensitivity and specificity for both isoniazid and rifampicin of 100% and 92% respectively. Other studies done by Syre *et al.* used nitrate reductase-based antibiotic susceptibility methods and obtained results of 100% sensitivity and a specificity of 95% for isoniazid, and for rifampicin a 94% sensitivity and a 100% specificity (Syre *et al.*, 2003). This data validates that the proposed four-quadrant plate method would be a suitable replacement of equally high sensitivity and specificity to PCR, if not better.

4.7 Arriving at the diagnoses of MDR-TB and XDR-TB

The agreement of drug-resistant diagnoses between the BACTEC MGIT SIRE™ and E-test® strip combination methods, and the four-quadrant plate method compared with PCR respectively, was excellent. Simple Kappa Coefficients for all were showing

excellent agreement and all with highly significant P values (<0.0001), the lowest value at XDR-TB diagnosis level between the new plate method and PCR gold standard, but still with a very high Kappa value of 0.9300.

Sensitivity and specificity between these methods were excellent, with 100% sensitivity and specificity comparing the E-test® strip method with PCR, whereas the specificity and sensitivity of the four-quadrant plate method ranged from 96% to 100% compared to PCR. These data validated that this method, in its simplicity, compared exceptionally well with the PCR gold standard and other currently used methods.

The number of actual specimens correctly diagnosed in terms of drug resistance profiles was compared. The four-quadrant plate method came up better than the E-test® strip method for accuracy for isoniazid drug resistance, whereas the latter method proved better with rifampicin mono-resistance diagnosis. Both the four-quadrant plate method and E-test® strip method missed some MDR-TB specimens that were detected with PCR, 5% and 4% respectively, but all cases of XDR-TB were detected by both these methods, however, an extra sample was identified as XDR-TB using the four-quadrant plate method.

4.8 Time-to-detection

Rapid diagnosis and patient treatment is essential to keep case numbers of TB low, therefore it is the aim of new diagnostic techniques to yield a TB drug sensitivity profile as fast as possible. One of the aims of this study was to develop a rapid method to achieve this, but reduction of the cost per test was a priority. PCR is a benchmark for rapid diagnosis, with results available within a day or two, however, the cost is also one of the highest of currently available methods and its sophistication may limit its use in resource-poor laboratories.

The BACTEC MGIT SIRE™ sensitivity kit coupled with the E-test® strip method gave a final result within fifty-four days and the four-quadrant plate method demonstrated a final result within fifty days in this study. This could however not be statistically evaluated, as the plates for the newly-developed four-quadrant plate method were analysed in batches after an incubation period of 10 days for the last step, and not as soon as colonies were visible. This means that it would be possible to further reduce the time-to-detection for this method, and by analysing early colony growth using an inverted light microscope, it could even further be reduced. The fact that a colour growth indicator is used in this assay eases detection of drug-resistant colonies on these plates and should be beneficial for early detection.

There was an attempt made to obtain faster yet reliable results using this method, by plating directly from a positive MGIT™ culture tube onto a four-quadrant plate set, but the results obtained during the pilot phase were somewhat discrepant between the duplicate plates. This resulted in the inclusion of an extra step, adding at least 21 days to the time-to-detection, by first propagating the positive MGIT™ tube culture onto a 7H10 Middlebrook agar plate until sufficient growth was visible, for 3 to 4 weeks. Once enough growth was present these colonies were suspended in Middlebrook 7H9 broth before it was inoculated onto four-quadrant plates.

Compared to the 10 days for the last step as in this study, Syre and co-workers (2003) obtained results within 5 days for their liquid culture-based nitrate reduction assay, and it was the same for the Alamar blue study as was done before (Franzblau *et al.*, 1998). However, liquid culture methods appear to be more susceptible to contamination, as will be discussed later.

The time-to-detection was therefore not dramatically shortened using the new plate method as compared to the E-test® strip method as was originally hypothesised, but it is still possible to reduce this time period and simultaneously identify drug-

resistance patterns to both first and second line TB drugs. A study conducted by Martin *et al.* (2003), using resazurin microtiter assay plate testing had results available within eight days. In another study done by Abate *et al.* (2004),results were available within two weeks by adding rifampicin to decontaminated sputum specimens followed by the colorimetric indicator namely, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide thus proving that a reduction in time is possible with this method.

4.9 Cost-effectiveness and sophistication

TB is commonly known as a disease of the poor and is found mainly in undernourished persons, with a very high incidence in Africa and Asia (McGaw *et al.*, 2008). New TB diagnostic tools should focus on high specificity and sensitivity to prevent that cases are missed, but a very important factor, which was one of the main aims in this study, was to keep the diagnostic assay cost as low as possible to thus improve accessibility.

For a MDR-TB screen using the PCR method the consumable costs were calculated as R356 per sample, whereas for the XDR-TB it was calculated at R456, therefore a total of R812 for the combined MDR-TB and XDR-TB screen. This is not even a reflection of the real cost, which would include labour, the expensive equipment required and the laboratory infrastructure and running costs. Moreover, the requirements for a good quality-controlled PCR facility involves separate rooms for the different steps, away from the routine TB laboratory, with limited access to avoid contamination (Barnard *et al.*, 2008). The PCR method also requires the use of a suitably trained molecular biologist to accurately interpret the results and troubleshoot problem cases. To implement this method in low-income countries definitely present overwhelming challenges, and may be prohibitive in most laboratories in need of a MDR- and XDR-TB diagnostic facility.

The E-test® strip method was much cheaper than the PCR method for a MDR-TB screen, but slightly more than 10% more expensive than the four-quadrant plate method. However, the cost of a XDR-TB screen was exceptionally expensive using the E-test® strip method, even more expensive than PCR for the assayed drugs. When the total cost for the entire MDR- and XDR-screens was calculated, the new plate method was by far the cheapest alternative in consumable costs, working out to about half the cost of a PCR screen, and more than 1.5 times cheaper than the E-test® strip method. The MGIT SIRE™ sensitivity kit along with the E-test® test method appears to be cost-effective compared to PCR, as no costly instrumentation is required, but the simplicity and cost of the four-quadrant plate method speaks for itself.

The newly developed four-quadrant plate method did not require specialized personnel or any further sophisticated equipment, but rather standard laboratory instruments such as a balance and an autoclave for media preparation. Should the availability of even the basic diagnostic laboratory equipment pose a problem, these four-quadrant plates could be pre-prepared and delivered to more remote laboratories as alternative solution. Moreover, the results of this method doesn't require specialised skills to record or interpret, thus adequately-trained staff or primary healthcare workers will also be able to assess the results.

4.10 Specimen contamination

In this study the four-quadrant plate method had a contamination rate of 0.8% and the liquid culture had a higher contamination rate at 4.1% ($P = 0.0981$) while the PCR method wasn't influenced by contamination (Barnard *et al.*, 2008), which makes this the superior method when contamination of specimens should be considered. The four-quadrant plate method also appears to be much less influenced by contamination compared to liquid culture methods, which have been reported to typically have a normal range of contamination between 3 and 7% (Siddiqi, 2005).

Due to the fact that the contamination rate was low using the four-quadrant method coupled with its cost-effectiveness and remarkable simplicity, it is promising as method to be implemented in low-income, resource-poor areas where tuberculosis is often seen as a fatal disease.

4.11 Limitations of this study

When comparing results it was observed that there were some false negative results using the four-quadrant method compared to PCR, which even after replating in duplicate, was sensitive to the relevant antibiotics tested. Further testing and optimisation may give answers to these types of discrepancies.

The number of specimens analysed in this study, even though results were statistically significant, were relatively low, especially the XDR-TB specimen numbers. It is therefore suggested that further samples are evaluated prior to considering its implementation as drug-sensitivity diagnostic tool.

This study also addressed very specific research questions, therefore didn't look at related facets. Typically, the results of such a study raise more questions that may have to be addressed in further studies.

CHAPTER FIVE

CONCLUSION

In 2009 a total of 58 countries reported at least one case of XDR-TB with the largest number of XDR-TB reported from South Africa, 10.5% of all cases of MDR-TB, largely due to the rapid spread amongst people co-infected with HIV (Nathanson *et al.*, 2010).

Few countries in sub-Saharan Africa perform culture and drug sensitivity testing for routine diagnosis as costs are limiting and maintaining adequate quality-assured laboratories are challenging (Hafkin *et al.*, 2010). Liquid culturing techniques have been introduced to these areas, however they have proven to be problematic as result of cost and contamination issues in resource-poor settings, therefore molecular techniques offer solutions to the current crisis; however they come at a large cost and may not be sustainable (Hafkin *et al.*, 2010).

This study sought to address some of these issues by developing a robust, yet sensitive and specific four-quadrant agar-based plate method that has the potential to be routinely used to screen for mono-resistant, MDR- and XDR-TB. Although it still needs some refinement and should be validated using larger numbers of specimens to verify findings from this study, the four-quadrant agar plate method with colorimetric growth indicator should be considered as an economic and simple alternative or a complementary method for a laboratory wishing to increase cost-effectiveness for routine TB drug sensitivity screens and thereby making diagnosis and treatment more accessible to the people who most likely need it.

APPENDICES

APPENDIX A

Materials

1. 10 ml polypropylene centrifuge tubes — Amersham, SA.
2. 25 ml sterile plastic pipettes — Lasec SA (Pty) Ltd.
3. Frosted glass slides — Lasec SA (Pty) Ltd.
4. Gilson pipette — Lasec SA (Pty) Ltd.
5. Pipette tips — Lasec SA (Pty) Ltd.
6. 90mm Petri-plate — Lasec SA (Pty) Ltd.
7. four-quadrant plate — Lasec SA (Pty) Ltd.
8. 50 ml polypropylene centrifuge tubes — Amersham, SA.
9. Glass beads — Davies Diagnostics, SA.
10. Orange sticks — Laboratory & Scientific Equipment, SA.

Equipment

1. Biological Safety Cabinet — Lab&Air
2. Scientific Series 9000 Incubator (37°C) — Labotec, SA.
3. Automated pipettor aid — Lasec SA (Pty) Ltd.
4. Centrifuge — Adcock Ingram, SA.
5. Light/Fluorescent microscope — Zeiss, Germany
6. LED fluorescent lamp — Davies Diagnostic
7. BACTEC MGIT 960 System — Becton Dickinson, Maryland, USA
8. Bunsen Burner — Lasec SA (Pty) Ltd.
9. Line Probe Assay instrument — Hain Life Sciences, Germany
10. Balance — Lasec SA (Pty) Ltd.
11. Autoclave — Labotec, SA
12. 36L Waterbath — Scientific Manufacturing

Reagents

1. Phosphate Buffer — Merck, Darmstadt, Germany
2. NaLC — Merck, Darmstadt, Germany
3. Sodium hydroxide — Merck, Darmstadt, Germany
4. Sodium citrate — Merck, Darmstadt, Germany
5. Trigene — Mixmed Sales and Marketing, SA
6. PANTA — Becton Dickinson, Maryland, USA
7. MGIT SIRE sensitivity kit — Becton Dickinson, Maryland, USA
8. DMSO — Merck, Darmstadt, Germany
9. OADC — Becton Dickinson, Maryland, USA
10. TTC — Becton Dickinson, Maryland, USA
11. Kanamycin E-test — BioMérieux, France
12. Ciprofloxacin E-test — BioMérieux, France
13. Amikacin E-test — BioMérieux, France
14. Ethionamide — BioMérieux, France
15. Microscope immersion oil — Merck, Darmstadt, Germany
16. 0.2% BSA fixative — Pathcare, N1 City
17. ZN Carbol Fuchsin — Pathcare, N1 City

18. Acid alcohol	Pathcare, N1 City
19. Methylene Blue counter stain	Pathcare, N1 City
20. Auramine-O stain	Pathcare, N1 City
21. Potassium permanganate	Pathcare, N1 City
22. 70% ethanol	Pathcare, N1 City
23. GenoType MTBDR*plus*	Hain Life Sciences, Germany
24. GenoType MTBDR*sl*	Hain Life Sciences, Germany
25. Kanamycin	Sigma-Aldrich, USA
26. Amikacin	Sigma-Aldrich, USA
27. Ciprofloxacin	Sigma-Aldrich, USA
28. Isoniazid	Sigma-Aldrich, USA
29. Capreomycin	Sigma-Aldrich, USA

APPENDIX B

Auramine-O fluorochrome stain

Reagents

1. **Auramine (slides) fixative**

Phenol crystals	5 g
95% Ethanol	100 ml

The phenol crystals are added to the 95% ethanol. Mix well to allow crystals to dissolve.

2. **Auramine-O (to make 5L)**

Auramine-O	5 g
Alcohol	475 ml
Phenol crystals	150 g
Distilled water	4375 ml

Dissolve the auramine-O and alcohol. Mix the phenol crystals with distilled water, and then mix all ingredients before storing in a brown bottle away from heat and light.

3. **3% acid alcohol decolourising solution**

Methanol	5 L
Concentrated hydrochloric acid	150 ml

4. **Potassium Permanganate counter stain**

Potassium permanganate	20 g
Distilled water	2 L

Mix until the potassium permanganate is completely dissolved. Filter the stain before use and transfer in a brown bottle.

APPENDIX C

Ziehl-Neelsen stain

Reagents

1. Smear fixing solution

Thimersol	0.04 g
Distilled water	160 ml
Phenol	1.0 ml
Horse serum	40 ml

Dissolve thoroughly and add horse serum, Mix well and keep in a brown sealed bottle with a rubber cap so that the solution can be taken out with a syringe. This solution may be used for many months, however do not use if turbid or obviously contaminated.

2. Carbol Fuchsin

Basic Fuchsin powder	40 g
Phenol crystals	200 g
Methanol	400 ml
Distilled water	4 L

Dissolve the phenol crystals in some of the methanol and add the basic fuschin powder and mix well. Add the rest of the methanol and mix well until dissolved. Add the water and swirl. Filter the stain before use and transfer to a clean leak proof bottle and mix well.

3. Decolourising solution: Acid alcohol

Ethanol, 95% conc.	97, 0 ml
Concentrated hydrochloric acid	3, 0 ml

4. Methylene blue counter stain

Methylene Blue	40 g
Distilled water	4 L

Mix until the methylene blue is completely dissolved. Filter stain before use and transfer to a clean leak-proof bottle and mix well.

APPENDIX D

Reagent preparation

1 Middlebrook 7H11

Middlebrook 7H11	20 g
Demineralised water	900 ml
Glycerol	5 ml
OADC	100 ml

Suspend the Middlebrook's medium in water containing the glycerol. Heat to boiling with agitation to completely dissolve. Sterilise by autoclaving at 121°C for 15 minutes and cool to 45-50°C and add 100ml OADC enrichment.

2 Middlebrook 7H10

Middlebrook 7H11	20 g
Demineralised water	900 ml
Glycerol	5 ml
OADC	100 ml

Suspend the Middlebrook's medium in water containing the glycerol. Heat to boiling with agitation to completely dissolve. Sterilise by autoclaving at 121°C for 15 minutes and cool to 45-50°C and add 100ml OADC enrichment.

3 Middlebrook 7H9

Middlebrook 7H9	4.7 g
Demineralised water	900 ml
Glycerol	5 ml
OADC	100 ml

Suspend the Middlebrook's medium in water containing the glycerol. Heat to boiling with agitation to completely dissolve. Sterilise by autoclaving at 121°C for 15 minutes and cool to 45-50°C and add 100ml OADC enrichment.

4 TTC –colorimetric indicator 1% Stock solution

TTC	1 g
Distilled water	100 ml

5. NALC-NaOH

Prepare 4% NaOH solution by dissolving 20g NaOH pellets into 500 ml distilled water. Concentration of NaOH may be varied (2-5%).
Prepare 2.9% sodium citrate solution by dissolving 14.7g sodium citrate in 500 ml distilled water. Prior to use, mix equal quantities of NaOH and sodium citrate solution. Prepare only as much volume as can be used in a day (200ml). Sterilize by autoclaving at 121°C for 15 minutes. Add NALC powder to achieve a final concentration of 0.5% (1g NALC/200ml NaOH-sodium citrate solution). Mix well and use the same day. NALC activity is lost if left standing for more than 18 hours.

NaOH Solution:
This solution can be stored and used for decontamination of contaminated cultures and specimens (non-mucoid).

6. TB PHOSPHATE BUFFER

anhydrous di-sodium phosphate	4.7 g / 500 ml
monopotassium phosphate	4.5 g / 500 ml

Dissolve 4.7g of anhydrous di-sodium phosphate (Na_2HPO_4) in 500 ml distilled water, using a volumentric flask.
Dissolve 4.5g monopotassium phosphate (KH_2PO_4) in 500 ml distilled water, using a volumetric flask.
Mix equal quantities of the two solutions. Check the pH. Adding more of solution A will raise the pH; more of B will lower the pH.

7. 70% Alcohol

Alcohol	700 ml
Distilled water	300 ml

Mix together and dissolve

8. 5% PHENOL

Phenol	5 g
Distilled water	100 ml

Mix together and dissolve

APPENDIX E

Antibiotic chart for the final concentrations:

Antibiotic	Solvent used	Stock solution	Working solution	Volume to 200ml 7H10	Final concentration
Isoniazid	Sterile distilled water	10 000µg/ml	1000	400µl	0.1µg/ml
Rifampicin	DMSO and Sterile distilled water to 10ml	10 000µg/ml	1000	200µl	1.0µg/ml
Kanamycin	Sterile distilled water	10 000µg/ml	1000	1.0ml	5.0µg/ml
Amikacin	Sterile distilled water	10 000µg/ml	1000	1.0ml	5.0µg/ml
Capreomycin	Sterile distilled water	10 000µg/ml	1000	2.0ml	10.0µg/ml
Ciprofloxicin	Sterile distilled water and 0.1N NaOH	10 000µg/ml	1000	400ul	2.0µg/ml

APPENDIX F

Raw data of drug resistance and sensitivity profiles using the different methods:

PCR							
Sample no:	INH	RIF	CIP	AMIK	KANA	*STREP*	*ETHAM*
1	R	R	S	S	S	S	R
2	R	S	S	S	S	S	S
3	R	R	S	S	S	S	R
4	R	R	S	S	S	S	R
5	S	R	S	S	S	S	R
6	R	R	S	R	R	R	S
7	R	R	S	S	S	S	S
8	R	R	S	S	S	S	S
9	R	S	S	S	S	S	S
10	R	R	R	R	R	R	R
11	R	R	S	S	S	S	R
12	R	R	S	S	S	S	R
13	S	R	S	S	S	S	S
14	R	S	S	S	S	S	S
15	R	R	S	S	S	S	R
16	R	R	S	S	S	S	R
17	R	R	S	S	S	S	S
18	R	S	S	S	S	S	S
19	R	R	S	S	S	S	S
20	S	R	S	S	S	S	S
21	R	R	S	S	S	S	S
22	R	R	S	S	S	S	S
23	R	R	R	R	R	R	R
24	R	R	S	R	R	R	S
25	R	R	S	R	R	R	R
26	R	R	S	R	R	R	R
27	R	R	S	S	S	S	R
28	R	R	S	S	S	S	S
29	R	R	R	R	R	R	S
30	R	R	S	S	S	S	S
31	S	R	S	S	S	S	S
32	R	R	S	S	S	S	R
33	S	R	S	S	S	S	S
34	S	R	S	S	S	S	S
35	R	S	S	S	S	S	S
36	R	S	S	S	S	S	S
37	R	R	R	R	R	R	R
38	R	R	S	S	S	S	S

39	S	R	S	S	S	S	S
40	R	R	S	S	S	S	R
41	R	R	S	S	S	S	R
42	R	S	S	S	S	S	R
43	R	R	S	S	S	S	S
44	R	R	S	S	S	S	R
45	R	R	S	S	S	S	S
46	R	S	S	S	S	S	S
47	R	R	R	R	R	R	R
48	R	R	S	R	R	R	R
49	R	R	S	S	S	S	R
50	R	R	S	S	S	S	R
51	R	S	S	S	S	S	S
52	R	R	R	R	R	R	R
53	R	R	S	S	S	S	S
54	R	R	S	R	R	R	R
55	R	R	S	R	R	R	R
56	R	S	S	S	S	S	S
57	R	S	S	S	S	S	S
58	R	R	S	S	S	S	S
59	R	S	S	S	S	S	S
60	R	R	S	S	S	S	S
61	R	S	S	S	S	S	S
62	R	S	S	S	S	S	S
63	R	S	S	S	S	S	S
64	R	R	S	S	S	S	R
65	S	R	S	S	S	S	R
66	R	R	S	S	S	S	R
67	R	R	S	S	S	S	R
68	R	S	S	S	S	S	S
69	R	S	S	S	S	S	S
70	S	R	S	S	S	S	S
71	R	R	R	S	S	S	R
72	R	R	S	R	R	R	R
73	R	R	R	S	S	S	R
74	R	S	S	S	S	S	S
75	R	R	R	R	R	R	R
76	R	S	S	S	S	S	S
77	R	S	S	S	S	S	S
78	R	R	S	S	S	S	S
79	R	R	S	S	S	S	R
80	R	R	S	S	S	S	R
81	S	R	S	S	S	S	S
82	R	S	S	S	S	S	S
83	R	R	S	S	S	S	R

84	S	R	S	S	S	S	S
85	R	R	S	S	S	S	R
86	R	R	S	S	S	S	R
87	R	R	S	S	S	S	R
88	R	R	S	S	S	S	S
89	R	R	S	S	S	S	R
90	R	R	S	S	S	S	S
91	R	R	S	S	S	S	S
92	R	R	S	S	S	S	R
93	R	R	S	S	S	S	S
94	R	R	S	S	S	S	R
95	R	R	S	S	S	S	S
96	R	R	S	S	S	S	S
97	R	R	S	S	S	S	S
98	R	S	S	S	S	S	R
99	R	R	S	S	S	S	S
100	R	S	S	S	S	S	S
101	R	R	S	S	S	S	R
102	S	R	S	S	S	S	S
103	R	R	S	S	S	S	S
104	R	R	S	S	S	S	S
105	R	R	S	S	S	S	S
106	R	R	S	S	S	S	S
107	R	R	S	S	S	S	S
108	R	R	S	S	S	S	S
109	R	R	S	S	S	S	R
110	R	R	S	S	S	S	S
111	R	R	S	S	S	S	R
112	R	S	S	S	S	S	S
113	R	R	S	S	S	S	R
114	R	S	S	S	S	S	S
115	R	R	S	S	S	S	S
116	R	R	S	S	S	S	R
117	R	R	S	S	S	S	R
118	R	R	S	S	S	S	R
119	R	R	S	S	S	S	R
120	S	R	S	S	S	S	S

BD INH & RIF AND E-STRIPS								
Sample no:	INH	RIF	CIP	AMIK	KANA	*STREP*	*ETHAM*	*ETHIO*
1	R	R	S	S	S	S	R	S
2	R	S	S	S	S	S	S	S
3	R	R	S	S	S	S	S	S
4	R	R	S	S	S	S	S	S
5	S	R	S	S	S	S	S	S
6	R	R	S	R	R	R	S	S
7	R	R	S	S	S	S	S	S
8	R	R	S	S	S	S	S	S
9	R	S	S	S	S	S	S	S
10	R	R	R	R	R	R	R	R
11	R	R	S	S	S	S	R	S
12	R	R	S	S	S	S	R	S
13	S	R	S	S	S	S	S	S
14	R	S	S	S	S	S	S	S
15	R	R	S	S	S	S	R	S
16	R	R	S	S	S	S	R	S
17	R	R	S	S	S	S	S	S
18	R	S	S	S	S	S	S	S
19	R	R	S	S	S	S	S	S
20	S	R	S	S	S	S	S	S
21	R	R	S	S	S	S	R	S
22	R	R	S	S	S	S	R	S
23	R	R	R	R	R	R	R	R
24	R	R	S	R	R	R	S	R
25	R	R	S	R	R	R	R	R
26	R	R	S	R	R	R	R	S
27	R	R	S	R	S	R	S	R
28	R	R	S	R	R	S	S	R
29	R	R	R	R	R	R	R	R
30	R	R	S	S	S	S	S	S
31	S	R	S	S	S	S	S	S
32	R	R	S	S	S	S	R	S
33	S	R	S	S	S	S	S	S
34	S	R	S	S	S	S	S	S
35	R	S	S	S	S	S	S	S
36	R	S	S	S	S	S	S	S
37	R	R	R	R	R	R	R	S
38	R	R	S	S	S	S	S	S
39	S	R	S	S	S	S	S	S
40	R	R	S	S	S	R	S	S

41	R	R	S	S	S	R	S	S
42	R	S	S	S	S	S	S	S
43	R	R	S	S	S	S	S	S
44	R	R	S	S	S	S	R	S
45	R	R	S	S	S	S	S	S
46	R	S	S	S	S	S	S	S
47	R	R	R	R	R	R	R	R
48	R	R	S	S	S	S	R	S
49	R	R	S	S	S	S	S	S
50	CONTAM	CONTAM	S	S	S	CONTAM	CONTAM	S
51	R	S	S	S	S	S	S	S
52	R	R	R	R	R	R	R	R
53	R	R	S	S	S	S	S	S
54	R	R	S	R	S	R	R	R
55	CONTAM	CONTAM	CONTAM	R	R	CONTAM	CONTAM	S
56	R	S	S	S	S	CONTAM	CONTAM	S
57	R	S	S	S	S	S	S	S
58	R	R	S	S	S	S	S	S
59	R	S	S	S	S	S	S	S
60	R	R	S	S	S	S	S	S
61	R	S	S	S	S	S	S	S
62	R	S	S	S	S	S	S	S
63	S	S	S	S	S	S	S	S
64	R	R	S	S	S	S	R	S
65	S	R	S	S	S	S	R	S
66	R	R	S	S	S	S	R	S
67	R	R	S	S	S	S	R	S
68	R	S	S	S	S	S	S	S
69	R	S	S	S	S	S	S	S
70	S	R	S	S	S	S	S	S
71	R	R	S	S	S	S	S	S
72	R	R	S	S	S	S	S	S
73	R	R	S	S	S	S	R	S
74	R	S	S	S	S	S	S	S
75	R	R	R	R	R	R	R	R
76	R	S	S	S	S	S	S	S
77	CONTAM	CONTAM	S	S	S	CONTAM	CONTAM	S
78	R	R	S	S	S	S	S	S
79	R	R	S	S	S	R	S	S
80	R	R	S	S	S	R	S	S
81	S	R	S	S	S	S	S	S
82	R	S	S	S	S	S	S	S
83	R	R	S	S	S	S	R	S
84	S	R	S	S	S	S	S	S
85	R	R	S	S	S	S	R	S

86	R	R	S	S	S	S	R	S
87	R	R	S	S	S	S	R	S
88	R	R	S	S	S	S	S	S
89	R	R	S	S	S	S	S	S
90	R	R	S	S	S	S	S	S
91	CONTAM	CONTAM	S	S	S	CONTAM	CONTAM	S
92	R	R	S	S	S	S	R	S
93	R	R	S	S	S	S	S	S
94	R	R	S	S	S	S	R	S
95	R	R	S	S	S	S	S	S
96	R	R	S	S	S	S	S	S
97	R	R	S	S	S	S	S	S
98	R	S	S	S	S	S	R	S
99	R	R	S	S	S	S	S	S
100	R	S	S	S	S	S	S	S
101	R	R	S	S	S	S	S	S
102	S	R	S	S	S	S	S	S
103	R	R	S	S	S	S	S	S
104	R	R	S	S	S	S	S	S
105	R	R	S	S	S	S	S	S
106	R	R	S	S	S	S	S	S
107	R	R	S	S	S	S	S	S
108	R	R	S	S	S	S	S	S
109	R	R	S	S	S	S	R	S
110	R	R	S	S	S	S	S	S
111	R	R	S	S	S	S	S	S
112	R	S	S	S	S	S	S	S
113	R	R	S	S	S	S	S	S
114	R	S	S	S	S	S	S	S
115	R	R	S	S	S	S	S	S
116	R	R	S	S	S	S	R	S
117	R	R	S	S	S	S	R	S
118	R	R	S	S	S	S	R	S
119	R	R	S	S	S	S	R	S
120	S	R	S	S	S	S	S	S

PLATE METHOD (FOUR QUADRANT)						
Sample no:	INH	RIF	CIP	AMIK	*CAP*	KANA
1	R	R	S	S	S	S
2	R	S	S	S	S	S
3	R	R	S	S	S	S
4	R	R	S	S	S	S
5	S	R	S	S	S	S
6	R	R	S	R	S	R
7	R	R	S	S	S	S
8	R	R	S	S	S	S
9	R	S	S	S	S	S
10	R	R	R	R	R	R
11	R	R	S	S	S	S
12	R	R	S	S	S	S
13	S	R	S	S	S	S
14	R	S	S	S	S	S
15	R	R	S	S	S	S
16	R	R	S	S	S	S
17	R	R	S	S	S	S
18	R	S	S	S	S	S
19	R	R	S	S	S	S
20	S	R	S	S	S	S
21	R	R	S	S	S	S
22	R	R	S	S	S	S
23	R	R	R	R	R	R
24	R	R	S	R	S	R
25	R	R	S	R	R	R
26	R	R	S	R	S	R
27	R	R	S	S	S	S
28	R	R	R	S	S	S
29	R	R	R	R	R	R
30	R	R	S	S	S	S
31	S	R	S	S	S	S
32	R	R	S	S	S	S
33	S	R	S	S	S	S
34	S	R	S	S	S	S
35	R	S	S	S	S	S
36	R	S	S	S	S	S
37	R	R	R	R	R	R
38	R	R	S	S	S	S
39	S	R	S	S	S	S
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51	R	S	S	S	S	S
52	R	R	R	R	R	R
53	R	R	S	S	S	S
54	R	R	R	R	S	R
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56	R	S	S	S	S	S
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58	R	R	S	S	S	S
59	R	S	S	S	S	S
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67	R	R	S	S	S	S
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77	R	S	S	S	S	S
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79	R	R	S	S	S	S
80	R	R	S	S	S	S
81	S	R	S	S	S	S
82	R	S	S	S	S	S
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85	R	R	S	S	S	S
86	R	S	S	S	S	S
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88	R	R	S	S	S	S
89	R	R	S	S	S	S
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91	S	S	S	S	S	S
92	R	R	S	S	S	S
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100	R	S	S	S	S	S
101	R	R	S	S	S	S
102	S	R	S	S	S	S
103	R	R	S	S	S	S
104	R	R	S	S	S	S
105	R	R	S	S	S	S
106	R	R	S	S	S	S
107	R	R	S	S	S	S
108	R	R	S	S	S	S
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110	R	R	S	S	S	S
111	R	R	S	S	S	S
112	R	S	S	S	S	S
113	R	R	S	S	S	S
114	R	S	S	S	S	S
115	R	R	S	S	S	S
116	R	R	S	S	S	S
117	R	R	S	S	S	S
118	R	R	S	S	S	S
119	R	R	S	S	S	S
120	S	R	S	S	S	S

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