Improving methods for genotypic drug resistance testing in *Mycobacterium tuberculosis*

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Declaration

I, Zandile Cleopatra Mlamla, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree

Signature: ..... Date: 1 December 2010

"*Without Him, I am nothing and can do nothing of value.*

He is my only Source."

Summary

An important next step to Tuberculosis control relies on the translation of basic science and modern diagnostic techniques into primary health care clinics. These assays must be rapid, inexpensive, interpretation of results must be easy and they must be simple so that a healthcare worker with limited training can perform the tests under safe conditions. This study consists of four aims. The first aim was to develop a methodology to sterilize sputum specimens for rapid TB diagnosis and drug resistance testing. Candidate bactericides were identified from the literature, and tested for their bactericidal activity in *Mycobacterium tuberculosis*. We identified ultraseptin®*aktiv* as a powerful bactericidal agent which sterilizes sputum specimens for subsequent safe handling prior to light emitting diode microscopy and it also provides a DNA template for PCR-based tests. An algorithm has been proposed for the processing of specimens and rapid diagnosis of TB and drug resistant TB while patients wait for results.

Recently, the World Health Organization has endorsed the MTBDR*plus* test for diagnosis of TB and drug resistant TB. However genotypic tests may have more problems than anticipated. With the HIV pandemic, an increase of non-tuberculous mycobacteria has been reported. The sensitivity of genotypic tests in specimens with underlying non-tuberculous mycobacterial species therefore requires further evaluation. This study therefore also aimed at determining the reliability of the MTBDR*plus* assay for detection of drug resistant TB where non-tuberculous bacterial load is high. Clinically relevant non-tuberculous mycobacterium DNA and DNA from a multi-drug resistant TB isolate were obtained. Ratios of the different NTM with the MDR-TB DNA were made and subjected to the MTBDR*plus* assay. Known mix NTM and TB infected clinical isolates and sputum sediments were also evaluated for TB and drug resistance detection on the MTBDR*plus* assay. Under these conditions, this study provides evidence that the MTBDR*plus* test cannot reliably detect TB and drug resistance TB in specimens with underlying non-tuberculous mycobacteria.

Thirdly, to evaluate the sensitivity of the MTBDR*plus* assay for detecting drug resistance in hetero-resistant isolates, ratios were made using purified DNA from an MDR and pan-susceptible TB isolate. The MTBDR*plus* assay was then performed on the different ratios. We report that the MTBDR*plus* assay can efficiently detect wild type DNA in genes associated with resistance during the early evolution of drug resistance. However, in the later stage during treatment when both the wild type and mutants are present, the detection limit for the mutant DNA was 1:55. Due to these results, the MTBDR*plus* assay should still be further improved or other tests should be developed to address these limitations.

And finally to combat cross amplicon contamination during the final steps of genotypic detection with the MTBDR*plus* assay, a proof of concept for a patentable closed tube line probe device was proposed on the 4th aim. This device can be improved to enable automated drug resistance genotyping of multiple specimens.

The results of this study highlight the need for a sensitive inexpensive point of care drug resistance test that does not require intensive training.

Opsomming

'n Belangrike volgende stap om Tuberkulose te beheer is om basiese wetenskap resultate te gebruik sodat moderne diagnose tegnieke ontwikkel kan word wat in primêre gesondheidsorg klinieke toegepas kan word. Hierdie toetse moet vinnig, goedkoop, en die interpretasie van resultate moet maklik wees. Die toetse moet eenvoudig wees sodat 'n gesondheidswerker met beperkte opleiding die toetse onder veilige omstandighede kan uitvoer. Hierdie studie bestaan uit vier doelwitte, waarvan die eerste was om ‘n metode te ontwikkel vir die sterilisasie van sputum monsters vir vinnige TB diagnose en die toesting van middelweerstandigheid. Kandidaat kiemdodende middels was geïdentifiseer vanaf die literatuur en die middels se kiekdodende aktiviteit was getoets op *Mycobacterium tuberculosis*. Ons het ultraseptin®*aktiv* geïdentifiseer as 'n kragtige kiemdodende middel wat bakteria in sputum monsters steriliseer vir veilige hantering voordat diagnose met n lig uitstralende diode mikroskopie gedoen kan word. Hierdie behandeling met ultraseptin®*aktiv* bied ook 'n DNA templaat vir PCR-gebaseerde toetse. 'n Algoritme is voorgestel vir die hantering van monsters en die vinnige diagnose van sensitiewe- en middel weerstandige Tuberkulose terwyl die pasiënte by die kliniek wag vir die resultate.

Onlangs het die Wêreld Gesondheid Organisasie die genotipiese MTBDR*plus* toets vir die diagnose van Tuberkulose en middel-weerstandige Tuberkulose onderskryf. Hierdie toets word tans op groot skaal in Suid Afrika gebruik. Dit kan egter wees dat genotipiese toetse baie meer probleme kan he as wat aanvanklik verwag is. Die HIV pandemie gaan toenemend gepaard met n toename van nie-tuberkulose mycobacteria. Die sensitiwiteit van genotipiese toetse op monsters met onderliggende nie-tuberkulose mikobakteriese spesies vereis dus verdere evaluasie. Die doel van hierdie studie was ook om die betroubaarheid van die MTBDR*plus*-toets te bepaal vir die opsporing van middelweerstandige TB waar die nie-tuberkulose backteriële lading hoog is. DNA van kliniese relevante nie-tuberkulose mikobakteria en multi-middelweerstige TB isolate was bekom. Verskillende verdunnigs van die spesifieke NTM DNA te same met die van MDR-TB DNA is gemaak en onderwerp aan die MTBDR*plus* toets. Bekende gemengde NTM- en TB geïnfekteerde kliniese isolate en sputum sedimente was ook geevalueer vir die opsporing van TB en middel weerstandigheid met die MTBDR*plus* toets. Hierdie studie verskaf bewyse dat die

MTBDR*plus* toets nie betroubaar is met die diagnose van sensitiewe- en middel weerstandige Tuberkulose in monsters met onderliggende nie-tuberkulose mycobacteria nie.

Verskillende verdunnings van gesuiwerde DNA van MDR en pan-sensitiewe TB isolate is gemaak om die sensitiwiteit van die MTBDR*plus* toets vir die opsporing van middelweerstandigheid te bepaal. Die MDRDR*plus* toets is gebruik met hierdie verdunnings. Resultate in hierdie studie toon dat die MTBDR*plus* toets effektief is met die identifisering van wilde-tipe DNA (dit beteken middel sensitief) in gene wat geassosieer word met middel weerstandigheid gedurende die vroeë ontwikkeling van weerstandigheid. Hier teenoor toon die resultate dat in die later stadium tydens behandeling, wanneer beide die wilde-tipe (sensitief) en mutante DNA (weerstandig) teenwoordig is, is die opsporingslimiet vir die mutante DNA maar 1:55. As gevolg van hierdie resultate raai ons aan dat die MTBDR*plus* toets nog verder verbeter moet word of dat ander toetse ontwikkel moet word om hierdie beperkinge aan te spreek.

Amplikon kruiskontaminasie kan n groot impak hê op die betroubaarheid van enige genotipiese diagnostiese toets. Die finale stappe van MTBDR*plus* toets behels die gebruik van 'n oop sisteem sodat kontaminasie maklik kan plaasvind. In die 4de doewit 'n konsep vir 'n patenteerbare geslotebuis toestel ontwikkel en die resultate het getoon dat kontaminasie suksesvol uitgeskakel kan word. Hierdie toestel kan verbeter na 'n outomatiese apparaat verbeter word sodat die module genotipering van verskeie monsters moontlik kan maak.

Die resultate van hierdie studie beklemtoon die noodsaaklikheid van 'n sensitiewe goedkoop “point of care” diagnostiese toets wat nie intensiewe opleiding benodig nie.

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List of Abbreviations

°C	:	Degree Celsius
µl	:	microlitres
ADC	:	Albumin dextrose catalase
AM	:	Amikacin
bp	:	base pairs
BSA	:	Bovine serum albumin
CAP	:	Capreomycin
CIP	:	Ciprofloxacin
dH2O	:	Distilled water
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
EMB	:	Ethambutol
ETH	:	Ethionamide
EtOH	:	Ethanol
FQ	:	Fluoroquinolone
g	:	Grams
INH	:	Isoniazid
KAN	:	Kanamycin
KCI	:	Potassium chloride
KNO_3	:	Potassium nitrate
LAM	:	Latin-American and Mediterranean
LCC	:	Low Copy Clade
LJ	:	Lowenstein-Jensen
M. abscessus	:	*Mycobacterium abscessus*
M. avium	:	*Mycobacterium avium*
M. bovis	:	*Mycobacterium bovis*
M. chelonae	:	*Mycobacterium chelonae*

M. fortuitum	:	*Mycobacterium fortuitum*
M. intracellulare		*Mycobacterium intracellulare*
M. kansasii	:	*Mycobacterium kansasii*
M. peregrinum	:	*Mycobacterium peregrinum*
M. terrae	:	*Mycobacterium terrae*
M. tuberculosis	:	*Mycobacterium tuberculosis*
MDR	:	Multi Drug Resistant
MIC	:	Minimum Inhibitory Concentration
ml	:	millilitres
mM	:	millimetres
NALC	:	N-acetyl-L-cysteine
NaOCl	:	Sodium hypochlorite
NaOH	:	Sodium hydroxide
OADC	:	Oleic acid/albumin/dextrose/catalase
OFX	:	Ofloxacin
OPA	:	*Ortho*-phthalaldehyde
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
RIF	:	Rifampicin
rpm	:	revolutions per minute
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal RNA
SA	:	South Africa
SDS	:	Sodium dodecyl sulphate
SNP	:	Single nucleotide polymorphism
STR	:	Streptomycin
TB	:	Tuberculosis
Tm	:	Melting temperature
Tris	:	Trishydroxymethylaminomethane
XDR	:	Extensive Drug Resistant
ZN	:	Ziehl-Neelsen

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CHAPTER 1

Introduction

1.1. Background

The World Health Organization (WHO), in an initiative for effective Tuberculosis (TB) /Multi-drug resistant TB (MDR-TB) control, developed and adapted the directly observed treatment, short course (DOTS) scheme (8). The main priority of the DOTS was to prevent the emergence of drug-resistant TB through high cure rates of drug susceptible TB. However, as this strategy did not aim at accelerating early detection and subsequent treatment of drug resistant patients, spread and resurgence of drug resistant TB occurred. In recognition of the deadly threat of virtually untreatable TB, the DOTS is now enforced by the WHO to implement rapid drug resistant TB testing by culture coupled with commercially available line probe systems in countries with a high TB burden (12).

In 2006, after 12 years of the DOTS implementation, South Africa's (SA) cure rate for new smear confirmed cases, re-treatment cases was 74% and 67% respectively, far below the WHO target of 85% (10). With over 14,000 cases of MDR-TB estimated in 2007 to occur annually, South Africa ranks among the top ten countries in the world for drug resistant TB (11).

Mathematical modelling demonstrates that continuance with the current TB control strategies will exacerbate transmission of TB and drug resistance (7) and that implementation of culture and drug susceptibility testing (DST) on 37% of new TB cases and 85% of retreatment cases will save approximately 50,000 lives, preventing nearly 8000 (14%) MDR-TB cases. However, the model also estimates that there would be no impact on incidence of extensive drug resistant (XDR)-TB (3). A subsequent study also suggests that case detection targets above 70% must be pursued if eradication of TB is to be attained. This may be achieved by increasing diagnosis of TB through active rather than passive case finding and utilizing rapid and highly sensitive techniques such as molecular line probe assays (2).

The WHO now recognizes that among response priorities, rapid detection of anti-TB drug resistance, through integration of molecular assays into routine laboratory logarithms as well as monitoring of drug resistance especially MDR/XDR-TB in new patients and its transmission is of paramount importance to curb the disease (11).

1.2. Problem Statement

In 2008 the WHO issued a call for accelerated use of a commercially available PCR based MTBDR*plus* test for detection of isoniazid and/or rifampicin resistant *M. tuberculosis* from smear positive sputum cultures (9). Two tests, the Inno-lipa and the MTBDR*plus* assay were recommended. Evaluation of the genotype MTBDR*plus* test showed a high correlation with routine culture based DST (1). The MTBDR*plus* test is now widely implemented in SA for detection of drug resistant TB. However, successful implementation of this methodology requires a change in the way in which *M. tuberculosis* is inactivated in sputum samples. Prior to microscopy, sputum specimens are currently treated with sodium hypochlorite (NaOCl) to increase the sensitivity of detection of *M. tuberculosis* by microscopy (4,6). This pre-treatment step also sterilizes the specimen and thereby making it safer for laboratory technicians. However, NaOCl compromises mycobacterial deoxyribonucleic acid (DNA) integrity thereby inhibiting subsequent PCR amplification. This suggests that polymerase chain reaction (PCR) based MTBDR*plus* test cannot be performed directly on NaOCl inactivated sputum specimens. Thus an alternative method which will allow sterilization of sputum specimen without compromising mycobacterium cell wall integrity for microscopic detection and genomic DNA such that PCR-based drug resistance genotyping can be performed directly on sputum specimens will enable early detection of TB and drug resistant TB. Furthermore, there is a close association between TB and HIV and many immune compromised patients (such as HIV sero-positive patients) can be infected with non-tuberculous mycobacterium (NTM) (5). Due to the growing HIV epidemic, it is not known whether the presence of NTM's in sputum specimens influence the detection of drug resistant TB by the MTBDR*plus* test.

1.3. Hypothesis

In this study we hypothesize that an alternative method which will provide a safe sterile concentrated specimen without compromising mycobacterial stainability, genomic DNA

integrity and PCR amplification for genotypic drug DST would significantly improve diagnosis of TB and drug resistant TB. We also hypothesize that a single tube enclosed line probe system will prevent contamination and that the MTBDR*plus* assay will not be able to detect underlying drug resistant TB in sputum samples where NTM bacterial load is high.

1.4. Aims

To improve the MTBDR*plus* assay for genotypic drug resistance testing of *M. tuberculosis* and evaluate the influence of NTM's and hetero-resistance on the performance of this test.

Specific aims

- To develop methods:
    - which will not compromise the ability of inactivated mycobacterium to stain with auramine-O and genomic DNA integrity for PCR amplification.
- To determine the influence of the presence of NTM's on the detection of drug resistant TB.
- To determine the limit of detection of drug resistant *M. tuberculosis* in hetero-resistant isolates.
- To develop a single tube closed line probe system which will prevent contamination

These aims will be discussed separately as different projects:

1.1 Sputum processing
1.2 Influence of NTM's on drug resistance testing
1.3 Hetero-resistance
1.4 Development of a closed line probe assay system

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CHAPTER 2

Literature review

Current methods for drug resistance testing of *Mycobacterium tuberculosis*

2.1. Introduction

Mycobacterium tuberculosis, the bacilli that causes Tuberculosis (TB) remains one of the three deadliest infectious pathogens worldwide despite rigorous attempts to control the epidemic by National TB Control programs under the guidance of World Health Organization (WHO) (241). Surveillance data suggest that interplay of several dynamics work in synergy to aggravate acquisition and transmission within communities fuelling the TB epidemic. These include; 1] emergence of multi-drug resistance (MDR) and extensive drug resistance (XDR) TB strains, 2] Human Immunodeficiency Virus (HIV) (3,18,44,68), 3] lack of systemic monitoring of TB cases, 4] delay in diagnosis of new cases and defaulters and 5] inappropriate therapy due to poor treatment adherence or inadequate drug therapy (9,18,62). In 2006, the TB incidence rate in South Africa (SA) was estimated by the WHO to exceed 900/100 000 population per year (238) with more than 6000 new MDR-TB cases detected each year. In 2008, this number escalated to about 13000 MDR-TB cases per year (240,241). Approximately 9.6% MDR-TB, 10.5% XDR-TB cases with 14.2% fluoroquinolone resistance in SA was reported by the WHO in the fourth world drug resistance surveillance (240).

Drug resistance in SA was extensively highlighted by the KwaZulu Natal province outbreak where 72 patients were diagnosed with MDR-TB, of which 53 had XDR-TB (68). The causal strain, F15/LAM4/KZN was found to have been responsible for cases of MDR-TB since 1994 and XDR-TB from 2001 (170). Subsequently it has been shown that other drug resistant TB strains are widespread in the country (144). Outbreaks of MDR-TB have been described in the Eastern Cape [Atypical Beijing] (206), the gold mines [LAM4] (33) and in the Western Cape [Beijing/W-like (95)], [Low Copy Clade (LCC) (228)], [F11] and [F28] (207). The MDR-TB outbreaks in Western Cape are driven by specific strain lineages, the Beijing R220 cluster and the LCC DRF150 strain (207). These strains are highly transmissible irrespective of the presence of characteristic drug resistance causing mutations (119). It is estimated that the incidence of

drug resistant TB cases in the Western Cape will double by 2.4 every 8 years. The main driving force of this phenomenon is the increase in cases caused by the Beijing strain R220 (228).

Prior to the 1940's, TB drug resistance was thought to emerge only as a result of treatment relapse or inadequate drug therapy, that reactivation of latent infection explained development of disease rather than disease transmission (224). Since the 1950's, it has also been thought that drug resistance strains are less virulent and less transmissible, however recent reports provide evidence that drug resistant strains can develop compensatory mutations which restore fitness (33). Certain drug-resistance mutations have also been shown to incur a very low or no fitness cost (23,24). This suggests that outbreak strains have unique properties which aid in increased transmissibility and drug tolerance, however the influence of HIV cannot be excluded (119). Emerging evidence also suggests that the exponential increase over time of the Beijing lineage may be a reflection of enhanced pathogenicity rather than transmissibility (95).

Members of the same bacterial population can independently acquire distinct drug resistance mutations, suggesting that multiple strains can co-exist within a patient with different susceptibility and fitness profiles (209). This phenomenon is defined as hetero-resistance. Hetero-resistance further complicates diagnostic or treatment outcomes as the one strain can become predominant masking the presence and therefore detection of the other strain (177,235). It is therefore important to establish techniques to rapidly and accurately detect drug susceptible and drug resistant TB.

2.2. Detection of TB by microscopy

Conventional light microscopy remains the primary method for diagnosing pulmonary TB in developing countries regardless of its various limitations (130). These include factors such as 1] its value only in areas with high TB incidence or prevalence where patients are diagnosed with high bacterial loads in sputum (5,000-10,000 bacilli.mL-1) (37), 2] its relatively low sensitivity (20-80%) in extra-pulmonary TB and TB/HIV co-infected patients when compared to traditional culture methods (77,102) and 3] the lack of specificity for *M. tuberculosis*. The problem of the low specificity of smear microscopy has only recently received attention as a result of frequent detection of opportunistic non-tuberculosis mycobacteria (NTM's) in HIV co-infected

individuals (54,139,167,231,233). Detection and diagnosis of NTM's has become particularly important as clinical manifestations of some NTM disease can be indistinguishable from those of TB (46,47). Cases where NTM disease/isolation was misdiagnosed as TB or co-infection with NTM was missed (154) and due to inadequate treatment administration classification of cases as MDR-TB has been documented in resource-limited settings (45,48,101,214). Another major disadvantage of smear microscopy is its limited value for diagnosis in paediatric cases as it is often difficult to get a good quality sputum in sufficient quantities in children (99,152,201).

2.3. Fluorescent light emitting diode (LED) microscopy

Fluorescence microscopy has not gained access to routine diagnostic laboratories in most developing countries despite its higher sensitivity (~10% higher) compared to smear microscopy (37). The cost for routine maintenance as well as running costs (electricity and requirement of specialized mercury vapour light sources) makes fluorescence microscopy less favourable than smear microscopy (140). Several light emitting diode microscopes (LED) which also use fluorescence have been developed (2,140) and recommended to replace fluorescence microscopes and to serve as an alternative to smear microscopy. These microscopes are robust, inexpensive, do not consume too much electricity (124) are portable and can run on batteries (107,140) compared to mercury vapour lights used on fluorescent microscopes. LED microscopes can also be used to view slides at higher magnification (2) and have been shown to reduce time of AFB detection when compared to fluorescence microscopy and are more sensitive than smear microscopy (114,220). Studies evaluating the impact of the implementation of LED microscopes on diagnosis and treatment at point of care sites, as well as combining LED microscopy with novel approaches for early TB and drug resistance case detection are however still required.

Decontamination methods

Strategies to enhance sensitivity of smear microscopy have been assessed. These involve thinning, decontamination and centrifugation of specimens to concentrate bacilli. Numerous decontaminants and digestive reagents such as cetylpyridinium bromide (CPB), cetylpyridium chloride (CDC) have been suggested (239). However N-acetyl-L-cysteine sodium hydroxide

(NALC/NaOH) is the most frequently used decontaminant for respiratory specimen prior to microscopy and mycobacterial cultivation (26,239).

Sterilization of respiratory specimens with sodium hypochlorite (NaOCl) prior to microscopy has also been suggested to be an alternative method to concentrate and provide a relatively safe specimen which is non-infectious to laboratory technicians (80). No significant increase in the sensitivity of microscopy subsequent to decontamination with NALC-NaOH and or NaOCl treatment followed by concentration with centrifugation was indicated (10,36,37). High sensitivities were however reported in clinical specimens from a TB/HIV endemic population on specimens processed with NaOH/NaOCl. NaOCl has gained favour in routine microscopic diagnosis in some laboratories as a useful sedimentation agent (80,173).

2.4. Drug resistance detection

Numerous phenotypic and genotypic methods have been described for DST and only a few are frequently used. Although genotypic methods offer several advantages over culture techniques such as reliability, reproducibility, and a short turnaround time (TAT) for results which may potentially help improve patient management (18), DST by culture remains the mainstay to detect drug resistance. Genotypic methods have become particularly important as they enable determination of the specific gene(s) and mutation(s) causing resistance (86) and coupled with phenotypic methods allow determination of resistance (59,204). This chapter will review the currently available phenotypic and genotypic methods for detection of drug resistance.

2.4.1. Phenotypic methods

One of the most important characteristics of culture based methods is that they take advantage of the critical drug concentration which discriminates drug resistant and susceptible strains. The critical concentration is defined as the drug concentration which completely inhibits growth of actively dividing drug susceptible mycobacteria (34). Table 2.1 summarizes the critical concentrations of drugs in different media which form part of treatment regimens frequently used for the treatment of drug susceptible and resistant TB (239).

2.4.1.1. Solid Medium Based Methods

Four egg or agar based solid culture methods are proposed by the WHO for DST: these are the proportion, resistance ratio, absolute concentration and the micro-dilution method (237). With the proportion method, the minimum inhibitory concentration (MIC) is defined as the drug concentration where <20 colonies (equivalent to 1% critical proportion) form while confluent growth is observed in the drug free media (34). The MIC in the agar dilution method on Middlebrook 7H10 medium is defined as the drug concentration on which fewer colonies were found when compared with the 10^{-4} quadrant together with a confluent growth on the 10^{-2} drug-free quadrants. The laboratory strain, *H37Rv* is included as an internal control to adjust for batch to batch variation of medium on both methods. The absolute concentration method is similar to the resistance ratio method; serial dilutions of carefully controlled inoculums containing 2 x 10^3 to 1 x10^4 CFU of mycobacteria are made (34). These dilutions are then inoculated in media with and without the drug. Resistance is defined as growth that is greater than a certain number of CFU's (usually 20) at a particular drug concentration. In these methods, for a resistant isolate the calculated proportion is higher and for a susceptible strain the calculated proportion is lower than the critical proportion (34,237). The proportion method on Lowenstein-Jensen (LJ) is used worldwide as a reference method as DST critical concentrations for both 1st and 2nd line drugs have been well validated on this media (Table 2.1) (239). A disadvantage of these traditional culture methods is that they take about 4-6 weeks before resistance or susceptibility is confirmed and have limited sensitivity, despite evidence of high specificity (138). Inaccurate or false negative phenotypic results which may result in inappropriate treatment of patients leading to TB related mortalities (176) and the current drug resistant epidemic have also been reported (69).

In-house solid medium based culture techniques have been proposed in an attempt to reduce the time for DST results. These include the E-test, nitrate reductase assay (NRA), thin layer agar (TLA) assay as well as mycobacteriophage based assays.

The E-test

The E-test (AB Biodisk, Solna, Sweden) is a commercial system which detects drug resistance on a plastic strip impregnated with a gradient of the antibiotic. This antibiotic gradient also allows determination of the MIC on the surface of 7H11 agar plates supplemented with OADC. The MIC is defined as the drug concentration at which growth of actively metabolizing

mycobacteria is inhibited. An inoculum equivalent to 3.0 McFarland is recommended with the results available within 5-10 days. Evaluation studies show correlation when the method is tested against the LJ proportion method for the four 1st line drugs (3,64,65,153). A recent study in Uganda for rifampicin (RIF) resistance showed complete agreement between the E-test and the direct BACTEC method when compared to the indirect (DST subsequent to mycobacterial culture) BACTEC 460 assay (162). The E-test system is however criticized for the substantial number of false negatives and positives (Table 2.2) (64,76) although two other subsequent studies reported high specificities and sensitivities (3,162). Further evaluation studies are needed for this test.

The nitrate reductase assay

The nitrate reductase assay (NRA), is based on the ability of viable *M. tuberculosis* to reduce nitrate to nitrite in standard LJ or modified Middlebrook 7H9 medium incorporated with 100 mg/L of potassium nitrate (KNO_3) (155). Reduction to nitrite causes a reddish/violet colour change on the surface of slants on addition of the Griess reagent (11,171). Clinical specimens or cultured isolates are inoculated in the presence and absence of the drug. Adaptation of NRA method for DST directly on clinical specimens has the advantage of reducing the time for availability of results in comparison to the indirect method and has been shown to yield reliable results with high sensitivities for Isoniazid (INH) and Rifampicin (RIF) (230). Pyrazinamide (PZA) resistance detection by the NRA assay with the drug's analogue nicotinamide which also possesses an anti-tuberculocidal activity has also been described (126). Both PZA and nicotinamide are pro-drugs that are catalyzed by the enzyme pyrazinamidase/nicotinamidase to pyrazinoic acid (POA) and nicotinic acid (246,247). Nicotinamide unlike PZA which requires an acidic pH works well at a neutral pH and a critical concentration of 250 mg/L produced comparative results to the BACTEC 460 system (126) (Table 2.2). Among other limitations, DST for streptomycin (SM) and ethambutol (EMB) has however been reported to be less reliable with low sensitivities and specificities (Table 2.2). The low sensitivity and specificity to these two drugs may be explained by the presence of hetero-resistant isolates (213) or faster deterioration of the drugs in the media (143).

Thin layer agar assay

The thin layer agar (TLA) assay also known as the microcolony method is based on inoculation of an *M. tuberculosis* suspension on either Middlebrook 7H10 or 7H11 TLA and visualization of microcolonies under a microscope (10X magnification) (192). The TLA assay has been evaluated for the four 1st line drugs (INH, RIF, EMB and SM) and for ofloxacin (OFL) and kanamycin (KAN) with reliable results (127,180,192) (Table 2.2). The assay is inexpensive, simple to perform and the results are available faster in comparison to traditional solid medium based assays but slower than with the BACTEC systems. Rigorous safety conditions are however necessary (180) (Table 2.2). MDR-TB can be detected within 13 days on smear positive sputum and within 38 days in smear negative sputa (127). Lower contamination rates in comparison to the BACTEC MGIT 960 system, proportion method and the microscopic-observation drug-susceptibility (MODS) assay were reported (Table 2.2).

2.4.1.2. Mycobacteriophage based methods for DST

Two rapid Mycobacteriophage based assays on solid media for *M. tuberculosis* DST were proposed. The one phage assay is based on Mycobacteriophage replication within *M. tuberculosis*; replication of phage is then determined by counting viral particles on fast-growing *M. smegmatis* after overnight incubation. Growth is indicated by clear plaques which form a turbid lawn on *M. smegmatis* (244). The luciferase reporter format however relies on production of light by recombinant phages on infection into mycobacteria. The recombinant phage contains a luciferase gene which once within viable mycobacteria causes oxidation of luciferin to oxyluciferin and subsequent light emission. Activity of the gene and therefore presence of viable mycobacteria is determined by luminescence in the presence or absence of antibiotics (90). Both formats are based on the replication of phage such as D29, TM4, L5 and Chel2 (16,108,166,187) inside viable mycobacteria.

The Fast*Plaque* response assay also known as the *FastPlaque* TB^{TM}-MDRi (Biotech Laboratories ltd, Ipswich, UK) is a commercially available Mycobacteriophage replication assay and can be used to detect RIF resistance directly from clinical specimens (7). Decontaminated clinical specimens are inoculated in the presence or absence of RIF then in a suspension of phages. After sufficient time has elapsed to allow the mycobacteria to take up the phage, the

remaining extracellular phages not infecting the *M. tuberculosis* are removed with a virucidal solution. The replicating phages are visualized within 2 days as plaques or lysis when plated in non-pathogenic mycobacterium such as *M. smegmatis*. Presence of viable *M. tuberculosis* in the presence of RIF is interpreted as drug resistance to this drug. A major limitation of phage based assays include the generation of inconsistent results in numerous studies (6,7,30,42,66,145,198).

The Luciferase reporter phage assay is a high throughput screening micro-plate bacteriophage assay (66). The assay was shown to be highly sensitive and specific for detection of RIF resistance even in an isolate which did not possess mutations in the 81bp region of the *rpoB* gene suggesting that it could be more sensitive than methods which are limited to the analysis of the hot spot region of the *rpoB* gene (16,17).

Among limitations of phage based assays, the lack of specificity of Mycobacteriophage for *M. tuberculosis*, lack of evaluation studies for drug resistance other than RIF, and its inconvenience in testing a large number of isolates are central (Table 2.2). Phage based assays have also been shown to have a TAT and cost comparative to that most molecular based techniques.

Although the E-test, NRA, TLA and Mycobacteriophage based methods are cheaper, they often are not as simple to perform and require high standards of biosafety and quality control (Table 2.2).

2.4.2. Liquid Based Methods

Numerous liquid culture-based DST methods have been introduced in the past years. Many of these assays are commercially available and rely on the principle that *M. tuberculosis* grows faster in liquid than on solid media. Some of the techniques are manual and require interpretation of results by eye while others are automated.

BACTEC systems

BACTEC system (Becton Dickinson, Franklin Lake, N.J.), the first manual liquid based culture method introduced in the 1970's, utilizes modified 7H9 or 7H12 Middlebrook broth containing a ^{14}C labelled radioactive palmitic acid substrate (196). Growing mycobacteria metabolize palmitic

acid releasing radioactive $^{14}CO_2$. The amount of radioactive $^{14}CO_2$ released is translated into a numerical value designated as the growth index (GI), a GI value higher than 10 is considered as positive (196). The first evaluation on the rapid radiometric DST of *M. tuberculosis* was conducted in 1981 (196,200). A subsequent study reported overall agreement of radiometric results with those obtained by the proportion method with specificities, sensitivities higher than those stated in an earlier study. The BACTEC 460 system has since been the leading rapid culture system for *M. tuberculosis* for the past two decades and provides DST within 4-12 days (69,186,189,200,218). Critical concentrations for both 1st and 2nd line drugs have been validated on this system (Table 2.1). The BACTEC 460 system is also the reference method for DST for PZA resistance as a BACTEC 460 vial is available which provides the necessary acidic medium (pH 5.9) for the activity of the enzyme (92).

The BacT/Alert 3D system

Another addition to the BACTEC system is the BacT/Alert 3D system formerly known as the MB/BacT system (bioMireux, Durham, N.C). It is an automated commercial liquid culture-based system that measures microbial growth every 10 minutes (188). The system uses a modified 7H9 Middlebrook broth containing a pH indicator mixture and incorporates a colometric sensor at the bottom of the vial which measures changes in CO_2 production by the metabolizing mycobacteria. Elevated CO_2 concentrations lower the pH of the medium which in turn produces a colour change in the sensor which is detected by the reflectometric unit of the instrument (236). Change in colour from green to yellow indicates a positive reaction and each vial is continuously monitored inside the apparatus (12,169). Evaluation studies show concordance with reference methods with comparable sensitivities and specificities (Table 2.2).

The BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system

The BACTEC MGIT 960 (Becton Dickinson Microbiology Systems, Sparks, MD) is an automated version of the MGIT system. The systems are based on an oxygen-quenching fluorescence sensor embedded at the bottom of the tube containing enriched Middlebrook 7H9 broth. On the BACTEC MGIT 960 system incubation and reading of fluorescence emitted occurs continuously inside the machine using a predefined algorithm to interpret the fluorescent signal and gives results as either negative or positive (190). Recently included in the BACTEC MGIT

960 system is the MGIT 960 PZA kit containing tubes with modified 7H9 broth which enhances growth of mycobacteria at pH 5.9 which enables detection of PZA resistance (92). In DST, the BACTEC MGIT 960 system interprets the results as susceptible or resistant to the antibiotic under investigation with results available in 8 days. Although this system is used more regularly than the BACTEC TB-460 it poses more danger to laboratory personnel and requires special disposal systems. The BACTEC MGIT 960 system however is reported to be more prone to contamination than in the BACTEC 460 system (Table 2.2) (242). The performance of the BACTEC MGIT 960 system has also not been shown to be superior to that of the BACTEC 460 system (242).

Recently, the focus has shifted to rapid and affordable direct tests in which clinical specimens are directly inoculated in drug-free and drug containing medium (41,74) or amplified for detection of drug resistant-TB (79,104). These assays include the MODS assay, microplate alamar blue assay (MABA), tetrazolium microplate assay (TEMA), resazurin microplate assay (REMA) (53,147,164) and the ESP II system (110).

Microscopic Observation Drug Susceptibility assay (MODS)

MODS is an 'in-house' assay which enables DST by detection of early growth *of M tuberculosis* as 'strings and tangles' of bacterial cells in 7H9 Middlebrook medium (38). The inoculation is performed on 24-well plates with or without antibiotics at 37°C (149). The assay has numerous features which make it suitable for use in resource poor settings; however several aspects also limit its application in these settings (Table 2.2). MODS is not superior to any liquid culture based assay and is also prone to contamination in comparison to other liquid culture systems which necessitates repeated or rigorous decontamination of specimens (57,60,70,150).

The versa TREK system

Previously known as the ESP culture system II (Trek Diagnostic systems, West Lake, OH) this non-radiometric automated method can be used for detection and DST of *M. tuberculosis*. This system along with the BACTEC 460 was the first two broth systems accepted by the Food and Drug Administration for DST. Using an enriched 7H9 broth, the assay detects mycobacterial growth by measuring gas pressure changes inside culture vials caused by mycobacterial

metabolism. The ESP II system can rapidly detect drug resistance to INH and RIF but discrepancies between the system and BACTEC 460 for STR and EMB were observed (20,182). PZA resistance with the BACTEC 460 system as the reference was in concordance (110). No evaluation studies have yet been published for performance of this assay directly on clinical specimens.

Colometric microplate liquid medium based assays

Colometric in-house assays were developed with the aim to overcome the high cost of commercially available techniques (164). These methods rely on colour changes which are observed by eye resulting from oxidation-reduction (redox) reactions. The redox reactions occur between an indicator and O_2, CO_2, NO_3 or drug metabolism in culture medium when *M. tuberculosis* is grown in the presence or absence of an antibiotic. These assays include, methods such as the MABA, TEMA and the REMA (128,158,164,175). The alamar blue, 4, 5-dimethlythiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) and resazurin are used as indicator dyes for MABA, TEMA and REMA assays respectively (116,142,147).

TEMA and MABA have been shown to be highly sensitive and specific for detection of 1st line drugs (Table 2.2) (115,147). Discordances for SM and especially EMB have been reported. These discrepancies have been attributed to differences in the media of the respective assays or degree of degradation of the respective drugs in the media (136). DST for 1st line drugs as well as PZA resistance with nicotinamide has also been determined on the REMA assay (129) (Table 2.2). Nicotinamide at the critical concentration of 250 mg/L on REMA assay produced comparative results to the BACTEC 460 system (129). As colour change can be detected visually, no additional devices are necessary and growth can be detected earlier before colonies become visible (147). REMA is more cost effective compared to MABA and TEMA as resazurin (the main component of alamar blue) is less expensive than alamar blue or MTT (142,147). Although these colometric assays are cost effective, rapid and also allow MIC determination, low sensitivity and specificity for EMB, STR and capreomycin (CAP) is documented (Table 2.2). The need for rigorous safety conditions for lab personnel may also limit their use in resource limited countries.

2.4.3. Drug resistance genotyping

Genotypic methods for DST are based on evidence that acquisition of drug resistance mutations in *M. tuberculosis* occur by chance as a result of imperfect chromosomal replication. Bacteria with drug resistance mutations are positively selected during periods of discontinuous drug therapy (e.g. through non-compliance or inappropriate therapy) (94). Resistance causing mutations occur mainly in regions targeted by antibiotics, in enzymes which activate or deactivate certain essential genes or on the promoter regions upstream of these genes. Resistant bacteria which do not have classical drug resistance mutations suggest unknown mechanism(s) are involved (94,248). This is also supported by the differences observed in MIC's of strains harboring the same mutation (87,94,122).

The mutation rate on the *M. tuberculosis* genome as determined using the Luria-Delbrûk fluctuation analysis demonstrates that *in vitro* INH-resistance emerges at a rate of 3.5×10^{-6} and RIF resistance at a rate of 3.1×10^{-8} mutations per cell division (55). DNA sequencing has shown that 95-98% of RIF-resistant isolates are also resistant to INH, making RIF-resistance a good predictor for MDR-TB (156). More than 90% of *M. tuberculosis* strains phenotypically resistant to both RIF and INH respectively were also shown to harbor point mutations within the 81bp-hot spot region of the *rpoB* gene (codon 507-533). As a result of these findings, detection of RIF resistance conferring mutations within this region forms the foundation for the detection of MDR-TB. More recently, due to the resurgence of XDR-TB, evidence of INH (197) and RIF mono-resistance (207), multiplex PCR assays which incorporate classical mutations causing resistance to both 1st and 2nd line TB drugs have been developed (86). Prior to genotypic drug resistance testing, smear positive sputum specimens are decontaminated followed by mycobacterial cultivation or direct genotyping on sediments. The decontamination step is crucial as it sterilizes the specimen and increases mycobacterial detection.

2.4.3.1. Preparation of samples prior to PCR amplification

The standard protocol for preparation of clinical specimens prior to PCR amplification involves digestion and decontamination of specimens with NALC-NaOH (61). DNA extraction methods by commercial kits, proteinase K phenol-chloroform purification method, carboxypropylbetaine (CB-18) coupled with glass beads homogenation have also been explored (50,100,212,234).

Although these techniques provide purified DNA, multiple steps which are time consuming, labour intensive and expensive are involved (212). The boiling method which involves mycobacterial cell lysis for 5 minutes at 100ºC to release crude DNA and reduce PCR inhibitors is a simple, cost effective and rapid method which has been shown to yield sufficient DNA for PCR (212,217).

Bactericidal reagents which sterilize and simultaneously lyse mycobacterial cells have not yet been explored for PCR based diagnostic techniques. There are commercial kits which use bactericidal reagents coupled with PCR based assays, however the bactericides in these kits are not mentioned (15). Limited data is available on the efficiency of bactericidal reagents against mycobacterial species. Literature reviews focus on bactericides against a variety of microbial organisms but not specifically on *M. tuberculosis*. The most frequently used bactericides are aldehyde based sterilizing reagents such as glutaraldehyde (GTA), NaOCl and peracitic acid ($C_2H_4O_3$) and are mainly used for sterilization of medical equipments and hospitals (71).

More recently *ortho*-phthalaldehyde (OPA), an aromatic dialdehyde has been proposed as a possible alternative to GTA for high-level disinfection (211). It was shown that 0.5% w/v OPA can rapidly and efficiently sterilize a range of NTM's and more importantly GTA-resistant mycobacterial strains (211). Although GTA is a more effective cross-linking agent and its own uptake may be decreased by virtue of its extensive cross-linking nature to amino acid residues at the bacterial cell surface. GTA thus inactivates cells at a slower rate than OPA at the same concentration (63). It is suggested that OPA may induce inactivation by cross linking the active sites of cysteine to neighbouring lysine residues (199).

NaOCl, with hypochlorous (HOCl) acid as the active moiety, is a powerful bactericidal, sporocidal and fungicidal agent which has been demonstrated to be highly effective against *M. tuberculosis* (174). It is relied upon for sterilization of mycobacteria by lysing the mycobacterial cell through interactions with amino acid groups and primarily through progressive oxidation of disulphide resulting in consequent degradation of DNA within minutes of contact (10). HOCL is a potent bactericidal agent which is active at even concentrations below 0.1mg/liter (174). Glucoprotamin is non-volatile, water soluble, non-corrosive, non-mutagenic, and easily

degradable compound (243). It is bactericidal against mycobacteria, fungi and viruses. It is highly active against glutaraldehyde resistant strains of *M. chelonae* (137).

Peracitic acid, an oxidizing agent effective at low temperatures, decomposes to non toxic residues and is effective in the presence of organic matter. The extra oxygen atom is highly reactive and interacts with most cellular components and functions to cause cell death. Peracitic acid inactivates many different critical cell systems and this is the key to its broad spectrum antimicrobial activity even against resistant *M. terrae* and *M. avium* (205,221).

A challenge with bactericide sterilization prior to PCR amplification may be however its implications on DNA integrity or DNA degradation and possible introduction of sequence changes or false positives on genotypic results (15,199). Determination of the time duration for the respective bactericide to penetrate the mycobacterial cell wall, efficiently sterilize the clinical specimen or culture without causing sequence changes or DNA degradation is critical.

2.4.3.2. Nucleic acid based methods

Mutations in one or several genes with different mutation frequencies are implicated in the acquisition of drug resistance in *M. tuberculosis* (Table 2.3). Drug susceptible isolates lack these corresponding gene mutations and this forms the basis of drug resistance genotyping. The inability to detect all mutations conferring resistance remains a major challenge to the successful development of highly sensitive genotypic DST methods. The main reason for this is that the mechanisms of drug resistance to some of the drugs are not fully understood (94). Different geographic regions with different prevalence rates of mutations in the drug resistance conferring genes have been documented and this further complicates the development of these techniques (39,98,184). Region specific mutation screening methods are not yet in place and this may lead to misdiagnosis of drug resistance. Many of these methods are also complicated by the need for downstream processing to enable the detection of genotypes within the amplified PCR product. The complexity and multiple steps of these techniques greatly increase the risk of cross-contamination and thereby misdiagnosis.

Single Strand Conformational Polymorphism (SSCP), Restriction Fragment Length Polymorphism (RFLP), allelic Amplification refractory mutation system (ARMS)-PCR, heteroduplex analysis (HA), pyrosequencing (84) and probe based hybridization methods such dot blot analysis (227) have been described. However, RFLP-PCR based DST has limited use as not all mutations conferring drug resistance results in gain or loss of restriction enzyme cutting sites (229). ARMS, HA and SSCP-PCR (97) are technically challenging, time consuming with limited sensitivity due to high G-C rich regions in mycobacterial genomes which may influence mobility shifts of DNA sequences (157). Pyrosequencing, an alternative sequencing method to direct DNA sequencing is a rapid high throughput technique that relies on real time detection of pyrophosphates (PPi) (89). In contrast to standard sequencing, pyrosequencing does not make use of fluorochromes, radioactivity or need post-reaction processing of PCR-products. Pyrosequencing approach that combines automated real-time (RT) PCR amplification with pyrosequencing has been devised for detection of drug resistance to INH, RIF and fluoroquinolones (27,75). Although pyrosequencing is high throughput similarly to standard sequencing, it is also cumbersome, costly and technically challenging.

The most promising in-house and commercially available molecular methods developed to detect drug resistant TB will be reviewed in the next few sections.

Direct DNA sequencing

For the last decade, this method has been incorporated into the work flow of a number of clinical mycobacteriology laboratories and is the reference method for detection of drug resistance mutations (85). It involves amplification of the gene of interest or a specific region associated with resistance causing mutations and subsequent sequencing of the amplified product to determine the presence or absence of specific mutations (85). Sequencing is not only costly, with the need for expensive equipment, but also requires expertise (227). Continued use of PCR-DNA sequencing for routine DST is therefore impractical in resource poor settings where cost effective, rapid techniques would be more of value (227).

2.4.3.3. Hybridization Based Methods

In these assays, amplified PCR products of genes known to confer drug resistance are reversely hybridized to immobilized nitrocellulose membrane bound allele-specific labelled probes complementary to the wild type or mutant sequence of the gene. Hybridization can be visualized by autoradiography, enhanced chemiluminescence, alkaline phosphatase or other detection systems (111,162). These assays include the commercially available INNO-Lipa RIF-TB test, MTBDR, MTBDR*plus* and the newly developed MTBDR*sl* assay.

There are two commercially available assays recommended by the WHO: the Inno-LiPA Rif.TB (Innogenetics, Belgium) and the Genotype MTBDR*plus* assay (Hain-Lifescience, Nehren, Germany). These assays are based on multiplex PCR amplification of nucleic acid segments and subsequent reverse hybridization of amplicons onto nitrocellulose membrane with immobilized probes specific for *M. tuberculosis* complex and mutations responsible for drug resistance.

Inno-Lipa Rif.TB

The Inno-LiPA Rif.TB (Innogenetics, Ghent, Belgium) targets the 16S-23S ribosomal RNA (rRNA) spacer region for differentiation of the *M. tuberculosis* complex and detects mutations within the hot spot region (codons 507-533) of the *rpoB* gene (91,194). A major limitation of this technique is that it can only detect RIF resistance (91,162,194,203).

Genotype MTBDRplus assay

The MTBDR (Hain Lifescience, Nehren, Germany) assay enables simultaneous detection of the *M. tuberculosis* complex, the most common mutations conferring RIF resistance and mutations in the *katG* for INH resistance. The Genotype MTBDR*plus*, a latter version of the MTBDR includes mutations in the promoter region of the *inhA* gene for INH resistance, which also confers cross resistance to ethionamide (ETH) (88,191). Both versions accurately identified RIF resistance in 98.7% of the cases, when compared to phenotypic DST (18,81,82). Furthermore, the Genotype MTBDR*plus* reported higher sensitivity for INH resistance (Table 2.4). The MTBDR*plus* assay was reported to perform well with significant readability in clinical specimens (160). The assay's reliability however can be hampered by presence of rare mutations and hetero-resistance in clinical specimens (160). Despite these limitations when performed

directly on clinical specimens, the MTBDR*plus* assay has been shown to be a reliable, reproducible line probe PCR-based method which could significantly increase early detection of drug resistant TB (18,31,141,160).

MTBDRsl assay

The MTBDR*sl* assay (Hain Lifescience, Germany), is the latest version of the MTBDR*plus* assay. It is based on the same principle as the MTBDR*plus* except that it detects resistance to 2nd line drugs; fluoroquinolones, aminoglycosides (amikacin and kanamycin) and the cyclic peptide; capreomycin and ETH (104). Three recent studies show sensitivities >75% for aminoglycoside and capreomycin resistance detection and even lower sensitivities for EMB resistance (Table 2.4) (28,83,104). This is currently one of the most promising genotypic assays for rapid detection of 2nd line drug resistance.

MLPA assay

The multiplex ligation-dependent probe amplification (MLPA) assay was initially designed to screen copy number changes and CpG methylation changes in human genomic DNA (161,226) and characterize bacterial genomes. Due to its high probe capacity (can incorporate ~45 probes), this assay was modified to enable simultaneous detection of resistance to RIF, INH, and EMB as well as genotype specific mutations in the *M. tuberculosis* genome for specie identification in a single assay (21). The selected drug resistance markers can detect approximately 70-85% RIF, 65-80% INH and 45-65% EMB resistance (21). *M. tuberculosis* genotype specific speciation with simultaneous drug resistance genotyping may help detect outbreak strains early and may provide valuable information for treatment and prevention of TB transmission. No evaluation studies in large trials have been performed and this needs attention.

Real- time PCR

RT-PCR methods which make use of Molecular Beacon (MB), TaqMan minor groove binder (MGB) and fluorescence resonance energy transfer (FRET) probes have been described (105,181,232,245). The sensitivity and efficiency of MB and TaqMan probe coupled PCR at detecting MDR-TB in DNA extracts with mixed ratios of mutant and sensitive sequences was determined (245). The detection efficiency was shown to be determined first by the amount of

each sequence and then by the ratio of the sequences irrespective of the probe system. The presence of a second allele did not influence specificity of the probe system (245). In this study FRET probes were unable to produce an amplification signal in mixed sequence specimens regardless of the quantity or ratio of the different sequences (245).

Cepheid GeneXpert MTB/RIF assay

The Cepheid GeneXpert MTB/RIF (Sunnyvale, CA) assay a MB RT-PCR based assay has recently been described for detection of RIF resistance (79). This assay is an enclosed, fully automated system which processes, extracts, purifies and amplifies target sequences directly from clinical specimens. The Cepheid GeneXpert MTB/RIF assay makes use of a single use sample-processing cartridge system with the GeneXpert instrument which has an integrated multicolour real-time PCR detection capacity (79,222). It is the first assay which incorporates sample processing, amplification and nucleic acid analysis in one tool (183,222). The assay makes use of 6 MB probes with their respective fluorescent dyes and quenchers for detection of RIF resistance conferring mutations and has been shown to be highly sensitive and specific even on smear negative specimens (26,79). It has also been shown to detect *M. tuberculosis H37Rv* in sputum specimens with mixed NTM species. The GeneXpert has several demonstrated advantages such as its large dynamic range, high specificity and on site diagnosis of MDR-TB while patients wait (79). The authors showed that the amount of aerosols produced are comparative to those generated during smear microscopy and pose insignificant threat to laboratory personnel (15). The sterilizing reagent was reported not to influence the sensitivity of the assay after 3 hours of exposure, however RIF false positive results were reported (15). Although expensive, the GeneXpert MTB/RIF assay is by far one of the most promising recent genotypic tools introduced for DST. Further evaluation of this assay for simplicity, robustness and accuracy for immediate DST performance in health care facilities to enable appropriate drug administration to TB suspects is required (26,79).

2.5. Factors affecting drug resistance genotyping

Numerous factors such as the DNA concentration, presence of PCR inhibitors, silent or excluded mutations and contaminants may affect the specificity and sensitivity of molecular methods; some of the factors are summarized in Table 2.5. Contaminants can be overcome by rigorous

decontamination although this may affect mycobacterial culturability, and DNA concentration methods have been devised but are cumbersome. Advantages of these techniques over culture methods include their sensitivity for detection of low level resistance and hetero-resistance although with a detection limit (28,83,225). The search for bactericides for sterilization of clinical specimens is limited as many chemicals may inhibit PCR or destroy DNA, therefore there is an urgent need to identify chemicals which simultaneously sterilize specimens and lyse mycobacterial cells but leave intact DNA for PCR. An ideal method would be one which is 1) rapid, 2) robust, 3) cheap, and 4) prevents amplicon contamination and 5) overcomes the factors listed in Table 2.5.

Table 2-1 Critical concentrations for drug susceptibility testing of *M. tuberculosis* isolates in different media**.**

Drug group	Drug	DST critical concentration (µg/ml)				
		Lowenstein-Jensen	Middlebrook 7H10	Middlebrook 7H11	BACTEC 460	MGIT 960
Group 1	Isoniazid *	0.2	0.2	0.2	0.1	0.1
1st line oral TB regimes	Rifampicin*	40.0	1.0	1.0	2.0	1.0
	Ethambutol*	2.0	5.0	7.5	2.5	5.0
	Pyrazinamide*	-	-	-	100.0	100.0
Group 2[#]	Streptomycin*	4.0	2.0	2.0	2.0	1.0
Injectable TB regimens	Kanamycin	30.0	5.0	6.0	4.0	-
	Amikacin	-	-	-	1.0	1.0
	Capreomycin	40.0	10.0	10.0	1.25	2.5
Group 3	Ciprofloxacin	2.0	2.0	2.0	2.0	1.0
Fluoroquinolones	Ofloxacin[a]	2.0	2.0	2.0	2.0	2.0
	Moxifloxacin[b]	-	-	-	0.5	0.25
Group 4	Ethionamide	40.0	5.0	10.0	2.5	5.0
Oral bacteriostatic 2nd line TB regimens	Prothionamide	40.0	-	-	1.25	2.5

LEGEND TABLE 2.1

The table was adapted from WHO to include only currently used drug regimens in treatment of drug resistant TB in developing countries. - =denotes that critical concentrations of the TB drug is not yet standardized on the specific media or DST system.

Table 2-2 Performance of phenotypic DST methods

	Phenotypic techniques	Advantages	Disadvantages	TB drug	Sensitivity range	Specificity range	References
LIQUID CULTURE METHODS	**BACTEC 460**	• Reference method for PZA • Low contamination rate	• Manual, Expensive • High maintenance • Uses radioactive material which require special disposal systems • Use of glass bottles and syringe	RIF INH	98-100 96-100	99-100 99-100	(92,162,219)
	MGIT & BACTEC MGIT 960	• Automated • Fluorometric • Rapid (8-10 days)	• Expensive, High contamination rates • Highly sensitive to non-homogenous mycobacterial suspensions	RIF INH SM EMB AMI CAP PZA PRO	98-100 100 100 89-100 96-100 92-97.4 100 63-75	100 100 100 95-100 99-100 98-100 96.6 98-99	(14,92,168,190,242)
	BacT/Alert 3D system	• Automated system • Colometric • ~15 days	Expensive	RIF INH SM EMB	92-97 96-100 78-100 100	100 95-100 100 98-100	(12,29,236)
	MODS	• Detects cord formation • Rapid (DST within 7-10 days) • Low cost • Easy to perform	• Biosafety necessity • Cross contamination • Overestimation of growth • Need for high standard quality controls • Need for inverted microscope	INH RIF SM EMB OFL	93.8-100 95-100 51-92 58-95 100	78.4-100 82.9-100 100 100 100	(60,70,74,135,148,149,165)
	ESP II system	• Non-radiometric • Automated, Rapid, Cost effective		PZA	100	100	(110)
	Colometric methods						
	MABA	• Low cost • Rapid (8-14 days) for MABA • Rapid (8-10 days) for TEMA • Rapid (7-10 days) • Read by eye/ fluorometry or spectrophotometer • Multiple tests simultaneously • MIC determination	Biosafety necessity (live cultures)	INH RIF	97-100 93-100	64-97 99-100	(32,43,175)
	TEMA			MDR SM EMB	94-100 92-96 87-94	97-100 93-100 71-96	(147)
	REMA			MDR PZA EMB STR KAN ETH OFL CAP	95-100 100 94 94 100 100 100 84.2	96-100 98 58 89 97-100 97-100 97-100 97 -100	(125,128,129,147,158,179)
SOLID CULTURE BASED METHODS	**TLA assay**	• Rapid • Inexpensive • Low cost • Less prone to contamination than MODS	One plate format	MDR EMB SM KAN OFL	100 100 100 100 100	100 99-100 100 99-100 100	(127,180,192)
	E-test	• Rapid (5-10 days) • Simultaneous determination of MIC's	Expensive (cost per strip and specialized incubator CO_2)	MDR SM	99-100 84-100	100 100	(3,64,162)
	NRA	• Rapid (7-14 days) • Nitrate reductase negative • *M. tuberculosis* is rare (<1)	Most RGM and *M. kansasii* can reduce nitrate to nitrite	INH RIF SM EMB PZA	95-100 96-100 64-100 93-100 94	87-100 93-100 66-100 84-98 98	(1,13,31,115,142,143,146,147,155,195,202,213,230)
	Mycobacteriophage based assay						
	Luciferase reporter system	• Rapid (~3 days) • Simple • More sensitive than BACTEC 460 • Only infection of live bacilli • (treatment monitoring)	• Biosafety necessity • Only live bacilli infected • Non-specificity • High contamination rates High rates of non-interpretable results	RIF INH SM EMB	100 79-100 79-100 33-94	89.2-100 96-98 69-93 95-100	(17,67,78,219)
	Fast*Plaque* assay			INH RIF SM	98-100 94-100 94 -96	93-100 95-100 100	(8,56,66,134,185,219)

RGM=Rapid growing mycobacteria

Table 2-3 Gene(s) associated with drug resistance in *M. tuberculosis*

TB drug	Gene(s)	Prominent mutations	References
Isoniazid	*katG* *inhA* *oxyR, ahpC, furA, ndh*	*katG*-315 -C15T, -24, -16, -8, -17	(103,112,172)
Rifampicin	*rpoB*	*rpoB*-516, -526, -531, -533	(134,172,215)
Pyrazinamide (pH 5.5)	*pncA, IS6110 insertion*		(22,121,193)
Ethambutol	*embCAB*	*embB*-306	(93,172)
Streptomycin	*rpsL* *rrs* *gidB*		(134,163,172)
Amikacin/kanamycin	*rrs*	*rrs*1400	(5,106,216)
Capreomycin	*tlyA*		(131,132,210)
Ofloxacin **Ciprofloxacin**	*gyrA* *gyrB, inhA, ethA, ethR, alr*	*inhA*21, 94, 44 *gyrA*90, 91, 94	(52,72,73)
Ethionamide	*Eta/ethA*		(19,113,151,178)

Table 2-4 Molecular assays with their respective overall accuracies for detection of drug resistance

Genotypic assays	TAT* (hours)	TB drug resistance	Sensitivity range	Specificity range	References
RT-PCR	~3hrs	INH, RIF OFX	83-98% 89-100% 96%	100% 99-100% 100%	(105,181,232,232)
Cepheid GeneXpert System	~2 hrs	RIF	97-100%	99-100%	(26,79)
MLPA assay	~2 hrs	RIF INH EMB	70-85% 65-80% 45-65%	100%	(21)
MTBDR assay	~2 hrs	INH RIF	71-100% 96-99	100 97.8-98	(31,40,82,123),
MTBDR*plus* assay	~2 hrs	RIF INH	95 -100% 82-95.3%	98-100% 99-100%	(18,82,86,118,159)
MTBDR*sl*	~2 hrs	EMB CAP KAN AMI FLQ	57-64.2% 80-100% 77-100% 83-100% 75-90.2%	92-100% 98-100% 100% 100% 96-100%	(28,83,104)
Inno LiPA Rif.TB	~2 hrs	RIF	87-98.6%	87-100%	(91,162,194,203)

*****TAT refers to turn around time to obtain results when performing the assays on decontaminated specimens or crude cultures.

Table 2-5 Factors that negatively impact on drug resistance detection by molecular techniques

Amplification/detection inhibitors	References
Hetero-resistance subpopulations	(28,79,133,180,225)
Mixed mycobacterial species	(44,49,51)
Type, length and position of probes	(117,184,245)
Difficulty to design probes for certain regions, Instability of probes due to repeated thawing High thermal duplex stability of mycobacterial genome due to high GC content	(96,109,223)
Unknown/novel mutations, Unknown /Excluded genes, Silent mutations	(4,28,35,97,120)
Mutation prevalence in different geographic regions	(160)
Loss of DNA integrity following multiple decontamination steps	(25,58,100,208,234)
PCR inhibitors	
Amount of mycobacterial cells in clinical specimens	
DNA concentration and extraction	
Amplicon contamination	

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CHAPTER 3

Materials and methods

Approval for sputum specimen collection and use of clinical mycobacterial isolates as well as ethics clearance was obtained from the Stellenbosch Human Research Committee.

3.1. Experimental approach: Project 1- Sputum processing

3.1.1. Aim

To develop a methodology which will ensure preservation of DNA extracted from sputum for downstream application such as PCR without compromising the ability of the non-viable mycobacterium to stain with the Ziehl-Neelsen or Auramine-O stain. A sputum processing methodology which yields sterile sediments of *M. tuberculosis* is devised. The experimental approach for the bactericide protocol is outlined in *figure 3.1*.

3.1.2. Bactericide selection

Four bactericides were selected based on published literature on their bactericidal nature and activity, low or lack of toxicity or irritation on contact with skin (Table 3.1).

Table 3-1 Selected bactericides

Bactericide	**Active moiety**	**Concentration**	**References**
Sodium Hypochlorite (NaOCL)	Hypochlorous acid (HOCL)	(2% v/v), (5% v/v) and (10% v/v) [final concentration] (1% v/v), (2.5% v/v) and (5% v/v)	(1,8)
Incidin*plus*	Glucoprotamin	(10% v/v) [final concentration] (5% v/v)	(28,32)
Ultraseptin®*aktiv*	Peracitic acid ($C_2H_4O_3$)	(4% w/v) [final concentration] (2% v/v)	(25)
Ortho-phthalaldehyde (OPA)	OPA	(1%w/v) [final concentration] (0.5% v/v)	(6,24,26)
Ethanol C_2H_5OH	C_2H_5OH	[concentration](70% v/v)	(22,33)

3.1.3. Bactericides and BCG Pasteur cells

3.1.3.1. Bactericidal treatment

Prior to determination of bactericidal efficiency on sputa, the bactericides listed in Table 3.1 with the exception of OPA were tested on BCG Pasteur cells to determine the concentration and time duration which would result in effective mycobacterial killing. Bactericidal concentrations and time durations previously reported in the literature for the specified bactericide were investigated (1,6,8,24,25,28). Experimentation with the live BCG Pasteur cell cultures was done inside a Biohazard Class II fume hood. BCG Pasteur cells were inoculated on 7H9 liquid medium (Becton Dickinson, Sparks, MD, USA) supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween-80 and 10% ADC) and incubated at 37°C to mid-log phase (optical density of 0.7-0.8) at a wavelength of 600nm. Culture aliquots (500µl) were made and treated with equal volumes of the respective bactericides at the suggested concentrations (Table 3.1) at different time durations (1, 5, 10, 15, 20, 30 minutes). A positive (no bactericide treatment) and a negative (no BCG Pasteur cells) control were also included. The specimens were then centrifuged for 15 minutes at 3000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in 1.5ml of BACTEC MGIT 960 medium and mixed gently by vortexing for 15-60s.

3.1.3.2. Killing efficacy after treatment

The resuspended pellets (100µl) was inoculated in 7H10 Middlebrook agar medium. Growth was assessed every 2-3 days for 25 days. Only the bactericide(s) which inhibited growth of the BCG cells at time durations no longer than 30 minutes were further investigated. Aliquots (50µl) of the pellet supernatants were made and subjected to PCR amplification of the *rpoB*, *katG*, and the *RV2629* gene (19,21,30,31) (Table 3.2). A 695-bp fragment of the *Rv2629* gene was amplified with the set of primers provided in table 1.3. The PCR reaction was performed under the following temperature profile: 15 min denaturation at 95° C followed by 40 amplification cycles (each cycle: 94°C for 1 min, 62°C for 1 min, 1 min extension at 72°C) and an elongation step of 10 min at 72°C. The PCR protocol for amplification of the *katG* (419-bp) and *rpoB* (437-bp) gene was done as follows; 15 min denaturation at 95°-8 C followed by 40 amplification cycles (each cycle: 94-8°C for 1 min, 62-8°C for 1 min, 1 min extension at 72-°8C) and an elongation step of 10 min at 72-°8C. The PCR products were then electrophoresized on 1.5% agarose gel. Ten microlitres of the same supernatants was used for smear microscopy. Bactericides with

bactericidal concentrations which allowed PCR amplification and did not influence stainability of mycobacteria after treatments were then investigated on *M. tuberculosis* (R439) and sputum specimens containing *M. tuberculosis*.

3.1.4. Bactericide(s) and *M. tuberculosis* strains (R439)

3.1.4.1. Bactericidal treatment

Culture aliquots (500µl) of R439 grown in 7H9 liquid medium (Becton Dickinson, Sparks, MD, USA) supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween-80 and 10% ADC) and incubated at 37°C to mid-log phase (optical density of 0.7-0.8) were made. The aliquots were treated with the candidate bactericides at concentrations and time durations which met the required criteria in BCG Pasteur cells. A positive (no bactericide treatment) and a negative (no R439 cells) control were also included. The specimens were then centrifuged for 15 minutes at 3000 rpm at 4°C. The supernatant was discarded and the pellet was resuspended in 2.5 ml of ddH_2O and mixed gently by vortexing for 15-30s.

3.1.4.2. Killing efficacy after treatment

To determine the bactericidal efficacy or post treatment viability, the treated, positive and negative controls were cultured in various media. A volume of 100 µl of the pellet suspension was plated in (i) Middlebrook 7H10 agar, and a volume of 500µl was inoculated in each of the following media (ii) Middlebrook 7H9 (Becton Dickinson, Sparks, MD, USA) supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween-80 and 10% ADC) (iii) BACTEC MGIT 960 vials supplemented with PANTA (BD Diagnostics Systems, Sparks, MD, USA) and incubated at 37°C. Growth was assessed for the different media in the following manner: 7H10 agar plates were examined for growth every 5-7 days for 25 days. Absorbance readings for the 7H9 cultures were measured every 7 days for 28 days. The BACTEC MGIT 960 vials were inoculated up to 42 days.

3.1.5. Bactericide(s) and sputum specimens

3.1.5.1. Sputum collection

Sputum specimens (n=300) were collected from June 2009 until June 2010 from TB suspects at the Tygerberg hospital, Western Cape, South Africa. Smear microscopy identified 216 acid fast

bacilli (AFB) negative and 84 AFB positive specimens. The sputum specimens were graded based on the AFB count system of the International Union against Tuberculosis and Lung Disease (5). The eighty four (n=84) AFB positive and 8 AFB negative sputa were tested (Table 4.2). Sixty five of these (65/84) AFB positive specimens with volumes ≥20 ml were split into two equal portions and liquefied and homogenized with an equal volume of Sputagest activial (4% v/v) (n= 45) or reconstituted NALC-NaOH (4%) (n=20) (*figure 4.1*). Twenty five of the forty five (25/45) sputum specimens liquefied with sputagest activial were exposed to ultraseptin®*aktiv* for 15, 20, and 30 minutes respectively (*figure 4.1*). Twenty of the forty five (20/45) sputum specimens liquefied with sputagest activial and exposed to OPA (*figure 4.1*). Equal proportions (10) of the 20 specimens liquefied with NALC-NaOH were exposed to ultraseptin®*aktiv* and OPA for 15, 20 and 30 minutes respectively (*figure 4.1*). The remaining nineteen specimens from the eighty four (19/84) with volumes <20 ml and bacterial loads varying between 3+ to scanty were not split but only liquefied with sputagest activial (2% v/v) and exposed to ultraseptin®*aktiv* (n=9) and OPA (n=10) for 30 minutes respectively (*figure 4.1*). The 8 AFB negative sputa were also split into 3 portions and exposed to the OPA or ultraseptin®*aktiv* for 15, 20 and 30 minutes respectively and inoculated on BACTEC MGIT 960 along with the above sputum specimens *(figure 4.1*).

Table 3-2 Bacterial load and quality of sputum specimens.

Sputum type	Bacterial load	Number of sputum specimens
Blood stained	-	4
	Scanty	3
	+	3
	++	3
	+++	12
Mucoid	-	2
	Scanty	4
	+	8
	++	8
	+++	23
Watery (saliva)	-	2
	Scanty	3
	+	2
	++	6
	+++	9
TOTAL		92

3.1.5.2. Bactericidal treatment

Smear positive sputum specimens were liquefied with an equal volume of Sputagest Activial or NALC-NaOH and vortexed for 30 to 60s and placed in an orbital shaker (330rpm at 37°C) for 15 minutes according to manufacturer's instructions (Mast Diagnostics, Merseyside, UK). An equal volume of bactericides which met the required conditions in R439 isolates were added, vortexed for 30s and allowed to stand at room temperature. The specimens were then centrifuged for 15 minutes at 3000 rpm at 4°C after which the supernatant was discarded into a container with a phenol based disinfectant (incidin*plus*). The pellet was then resuspended in 1.5 ml of BACTEC MGIT 960 medium and mixed gently by vortexing for 15-30s.

3.1.5.3. Killing efficacy after treatment of sputum specimens

The bactericidal efficacy or post treatment viability of *M. tuberculosis* in sputum specimens was deduced as described on R439 cells. Absorbance readings on sputum specimens treated with OPA were read at 740nm (24).

3.1.5.4. Verification of positive cultures

If growth on the cultures was observed after bactericide treatment, the following was done to rule out contamination; (i) Ziehl-Neelsen (ZN) staining and (ii) Blood agar plating.

ZN staining and blood agar

For ZN staining, 10µl of culture was smeared and heat fixed (heated for 2 hours at 100 °C), stained with carbol-fuchsin (Becton, Dickinson and Company, Maryland, USA), and decolourized with 5% acid alcohol. The smear was then counterstained with methylene blue (Becton, Dickson and Company, Maryland, USA) and read under the microscope for AFB. *M. tuberculosis* is an AFB which retains the carbol-fuchsin dye when heated and treated with acidified organic compounds and it will appear pink in a contrasting background when the ZN test is done. Mycobacterial growth is indicated by formation of mycobacterial strings and tangles. On blood agar, 100µl of the culture was plated and incubated for two days (*M. tuberculosis* does not grow on blood agar within 2 days).

3.1.5.5. Negative cultures after bactericide treatment

After AFB positive specimens flag off as negative on the MGIT 960 (42 days) and no growth is observed on either 7H9 or 7H10 media, the bactericide was considered to be an effective reagent for killing *M. tuberculosis.* Aliquots of 10µl and 50µl of the sediments with negative cultures were further evaluated for mycobacterial DNA integrity by PCR and stainability by ZN and auramine-O staining respectively.

3.1.5.6. Detection of *M. tuberculosis* by microscopy

ZN and Auramine-O stained slides were prepared from the sputum sterilized sediments to check for the stainability of the mycobacteria following treatment with the respective bactericide.

Auramine-O staining

Ten microlitres of culture was smeared onto slides and heat fixed (heated for 2 hours at 100 °C), then stained with auramine-O and decolourized with 5% acid alcohol. The smear was then counterstained with 0.05% potassium permanganate (Becton, Dickson and Company, Maryland, USA) and read under fluorescence microscope. The auramine-O stain contains fluorochromes which have an affinity for the mycolic acid in the cell walls for mycobacteria. Potassium permanganate helps prevent non-specific fluorescence. Under fluorescence microscope, mycobacteria as a result of bound fluorochromes appear as bright yellow, luminous rods against a dark background. Mycobacterial growth is indicated by formation of mycobacterial strings and tangles.

3.1.5.7. Determination of the DNA integrity by PCR

Random genes in the *M. tuberculosis* genome were amplified to confirm that the DNA integrity is not compromised and the bactericide does not influence the PCR reaction. The *rpoB*, *katG*, and *RV2629* genes were amplified with the previously described primer sets (Table 3.2) (14,21). The PCR based MTBDR*plus* assay was also verified on the same set of supernatants and was done according to the manufacturer's instructions (Hain Lifescience, Nehren, Germany).

Table 3-3 Primer sets for gene amplification

Primer	***Sequence***	***Tm***	***Fragment length***	***References***
rpoB gene				(21)
rpoB Forward	TGGTCCGCTTGCACGAGGGTCAGA	72^{o}C	437bp	
rpoB Reverse	CTCAGGGGTTTCGATCGGGCACAT			
katG gene				
RTB 59 Forward	TGGCCGCGGCGGTCGACATT	62°C	419bp	(21)
RTB 38 Reverse	GGTCAGTGGCCAGCATCGTC			
RV2629 gene				
RV2629 Forward	ATGGGCAACAGTGGGTTTG	62°C	695bp	(14)
RV2629 Reverse	AGTTCATTCGGATGGCTTCTT			

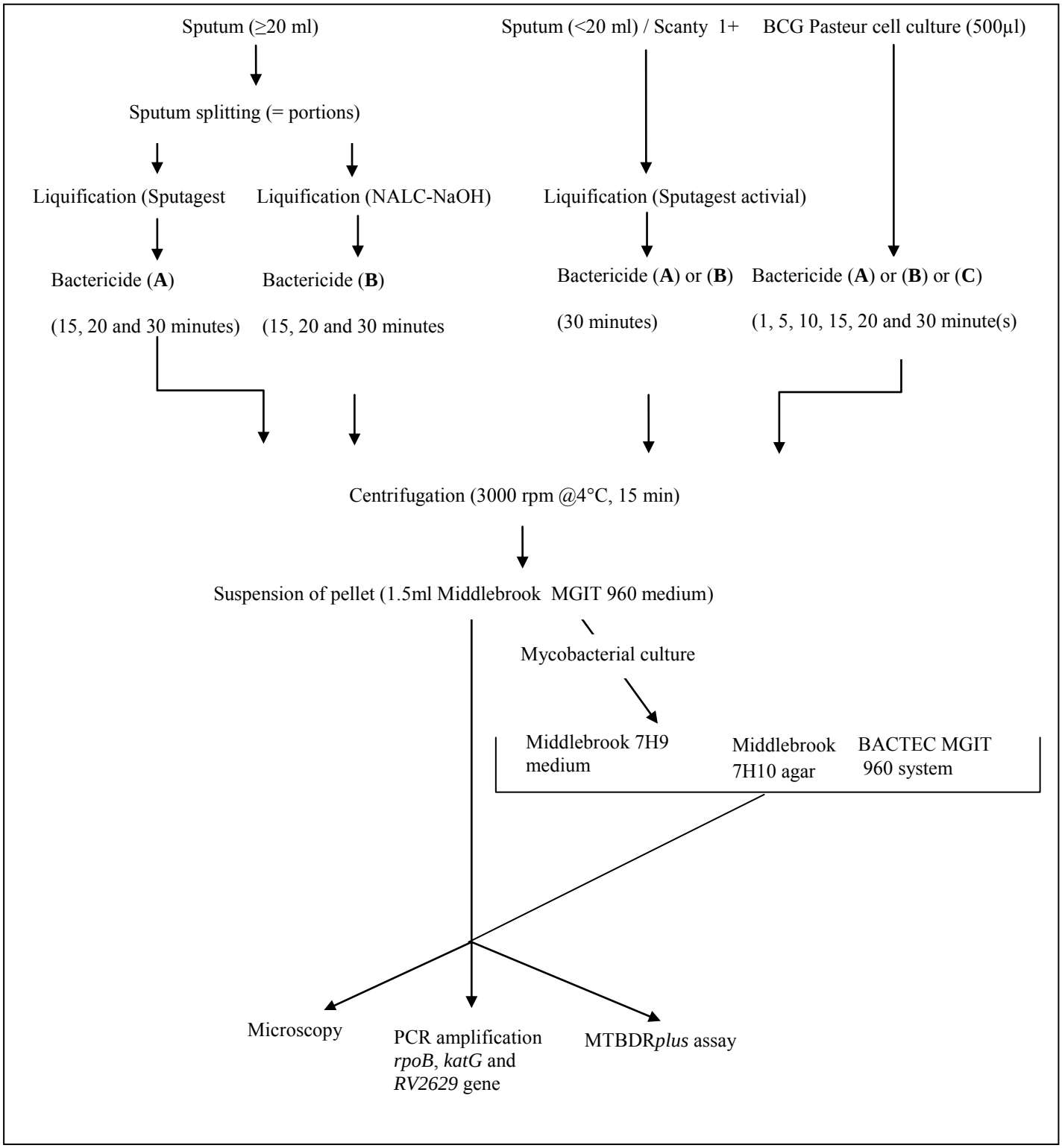

Figure 3-1 Schematic representation of the bactericide protocol.

3.2. Experimental approach: Project 2- Influence of NTM's on drug resistance testing

3.2.1. Aim

To determine the influence of the presence of non tuberculous mycobacterium (NTM's) on the detection of drug resistant TB by the PCR based MTBDR*plus* assay.

3.2.2. Selection of mycobacterial isolates

Ten most prevalent clinically relevant NTM's associated with disease resembling TB (2,3,16,27), isolated from *M. tuberculosis* infected and/or in immunocompromised (HIV/AIDS) patients were selected (Table 3.4). ATTC cultures, clinical isolates and other isolates from the Centre of Disease Control and Prevention (CDC) of the different NTM's were obtained from an existing local sample bank maintained at the Stellenbosch University, Western Cape, South Africa.

Table 3-4 Selected non-tuberculous mycobacterial species and drug resistant isolates.

Mycobacterial species	Isolates origin	References
M. avium	TMC724	(13,18)
***M. terrae* 2209**	Ravensmead/Uitsig [a], SA	(7,23)
M. intracellulare	ATCC 15985	(9)
M. kansasii	CDC Jun 2005	(2,4,15)
M. fortuitum	ATCC 6841	(10)
M. bovis	ATCC 19210	(17)
M. xenopi	CDC 2004	(7)
M. chelonae	CDC Jan 2008	(7)
***M. peregrinum* 2708/2111**	Ravensmead/Uitsig [a], SA	(7)
M. abscessus	CDC Jan 2007	(29)
Beijing R439	George [a], SA	(20)

LEGEND TO TABLE 3.4

CDC= Centre for Disease Control and Prevention, TMC724 =Trudeau Mycobacterial Culture Collection,

[a] = Clinical strains, SA= South Africa

The NTM's were confirmed by *16SrRNA*, *gyrB* sequencing as well as with a speciation assay (Genotype Mycobacterium CM) (Hain Lifescience, Nehren, Germany) (11,12). Segments of the *16SrRNA* and *gyrB* gene were amplified using primer sets provided in Table 3.4. The amplification reaction was subjected to 35 cycles with a denaturation step at 95°C for 1 min, an annealing step at 63°C for 1 min, and an extension step at 72°C for 2 min. PCR amplification of a region (approximately 577 bp) of the *16SrRNA* gene was performed. 5 ml of extracted DNA was added to 45 ml of the PCR amplification mixture, with the final 50-ml volume containing 1.25 U of *Taq* DNA polymerase (Promega Corporation,Madison, Wis.), 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 0.1%Triton X-100, deoxyribonucleotides, each at a concentration of 150 mM (Pharmacia,San Francisco, Calif.), 30 pmol of each of the two primers, and 50 mg of 8-methoxypsoralen (Sigma Chemical Company Ltd., Dorset, United Kingdom) per ml, and the mixture was overlaid with light mineral oil (Sigma Chemical Company Ltd.). The PCR amplifications were performed in a thermocycler (Perkin-Elmer Corporation, Norwalk, Conn.), and the amplification mixtures were subjected to 4 min of denaturation at 94°C and 40 cycles of 94°C for 1 min, 60°C for 2 min, and 72°C for 2 min, followed by a final extension period of 72°C for 7 min and refrigeration. The PCR products were then sent for sequencind at the Central Analytic Facility at Stellenbosch University, Cape Town, South Africa.

Table 3-5 Primer sets for gene amplification

Primer	***Sequence***	***Tm***	***Fragment length***	***References***
16SrRNA gene				
16SrRNA Forward	AGA GTT TGA TCC TGG CTC AG	60 °C	577bp	(12)
16SrRNA Reverse	GCG ACA AAC CAC CTA CGA G			
gyrB gene				
gyrBspec-Forward	TCG GAC GCG TAT GCG ATA TC	60 °C	1020bp	(12)
gyrBspec-Reverse	ACA TAC AGT TCG GAC TTG CG			

Pure and crude DNA from a well characterized clinical isolate (R439) of *M. tuberculosis* was selected from the same sample bank (Table 3.4). The clinical isolate was selected based on its possession of mutations in the *katG* 315 ACA (INH resistance) and *rpoB* 531 TTG (RIF resistance) gene. Only mycobacterial DNA without any background of other mycobacterial

species based on genotypic pattern on the Genotype Mycobacterium CM was used.

The DNA concentration of the respective NTM's and *M. tuberculosis* isolates was determined by spectrometry. The DNA concentration necessary was determined as the minimum amount of DNA required for standard PCR. Dilution of drug resistant *M. tuberculosis* clinical isolate with increasing concentrations of NTM was made. The ratios ranged from 1:1 to 1:200 with increasing NTM DNA and the *M. tuberculosis* DNA kept constant.

3.2.3. Known mixed NTM and *M. tuberculosis* in clinical isolates

Results from a study conducted in our laboratory identified 4 clinical isolates with mixed NTM and *M. tuberculosis* by analyzing their *16SrRNA* and *GyrB* gene sequences (Table 3.5). The clinical isolates (6865, 7426, 7690, and 7618) were LJ cultures from sputum samples obtained from TB suspects at Ravensmead /Uitsig residents near Tygerberg hospital (Table 3.6).

Table 3-6 Mixed NTM and *M. tuberculosis* clinical isolates

Clinical isolate reference nr	*16S rRNA* sequencing	*GyrB* sequencing
6865	*M. kumamotonense*	*M. tuberculosis*
7426	*M. triviale*	*M. tuberculosis*
7618	*M. terrae*	*M. tuberculosis*
7690	*M. acapulcensis*	*M. tuberculosis*

To determine whether the PCR-based assays (MTBDR*plus*) will be able to detect the *M. tuberculosis* in the NTM/TB mixed isolates. The Genotype Mycobacterium CM and MTBDR*plus* assay were performed on the four clinical isolates (Table 3.6). The results were then compared with those obtained from *16S rRNA* and *GyrB* sequencing (Table 3.5).

3.2.3.1. MTBDR*plus* assay on sputum specimens

Randomly selected sputum specimen sediments from Project 1 were subjected to the MTBDR*plus* assay. The sputum specimens were speciated by *16SrRNA*, *gryB* sequencing and with the Mycobacterium Genotype CM. These results were then compared to those of the MTBDR*plus* assay.

3.3. Experimental approach: Project 3- Hetero-resistance detection with the MTBDR*plus* assay

3.3.1. Aim

To determine the detection limit of the MTBDR*plus* assay for identifying drug resistance in hetero-resistant isolates. Hetero-resistance is defined by presence of wild type and drug resistant isolates in the same sample.

Pure DNA of MDR (R2126) and a drug susceptible (212) TB isolates was obtained from the South African Western Cape (SAWC) study. The MDR-TB isolate has known *katG* 315 ACA (INH resistance) and *rpoB* gene 531 TTG mutations (RIF resistance) whereas the susceptible isolate has wild type sequences for both RIF and INH sequences. Their DNA concentration was determined and the mixed MDR and drug susceptible ratio's was made as described previously

in the study for NTM's. The ratios ranged from 1:1 to 1:200 with the MDR-TB isolate ratio kept constant. The MTBDR*plus* assay was then done on the specimens with the respective ratios according to the manufacturer's instructions.

3.4. Experimental Approach: Project 4- Development of a closed line probe assay system

3.4.1. Aim

To develop a single tube closed line probe device for the MTBDR*plus* assay which will prevent contamination

3.4.2. Development of a device which will prevent contamination during the MTBDR*plus* assay

A challenge with the 12-blot line-probe based MTBDR*plus* assay is to prevent cross contamination which may occur during the multiple manual steps done before obtaining results. An automated 48-blot system which allows 48 samples or less to be genotyped simultaneously is less prone to cross contamination than the manual system. However, for implementation at health care clinics where less than 20 TB suspects a day may present at the clinic, the 48 blot system is less advantageous. To overcome the challenges with PCR amplicon contamination, a single tube closed line probe system would be an advanced step forward.

The device that was developed consists of wire clumps, rubber tubes, plastic reservoir, plastic 4-way connector and modified 7ml plastic tubes (*figure 3.2*). The rubber tubes are divided into 7 compartments by wire clumps. Each compartment contains the reagents used in the different steps followed during the MTBDR*plus* assay (*figure 3.3*). These compartments will respectively contain the hybridization buffer, stringent wash solution, conjugant, 2 sections for the rinse solution, ddH_2O and finally the substrate. A single strip of the MTBDR*plus* membrane is inserted inside the device and closed by the wire clumps. The denaturing reagent (20µl) is first mixed with the PCR amplicons (20µl) into a 1ml tube before introduction of the two into the 1st chamber. After the hybridization buffer is allowed to mix with the membrane, the denatured amplicons (40µl) are injected with a 1ml syringe into the rubber tube on the compartment with the membrane. The different reagents are pushed through the wire clumps to the compartment containing the membrane. The single tube device is placed onto a shaking oven at 45ºC during

the hybridization and stringent wash step; the rest of the assay is performed in a biosafety level II cabinet at room temperature. This is a completely sealed device and no amplicons will leak out of the system. This system is only a prototype for proof of concept and in the long run, it may offer prospects for automation for patenting.

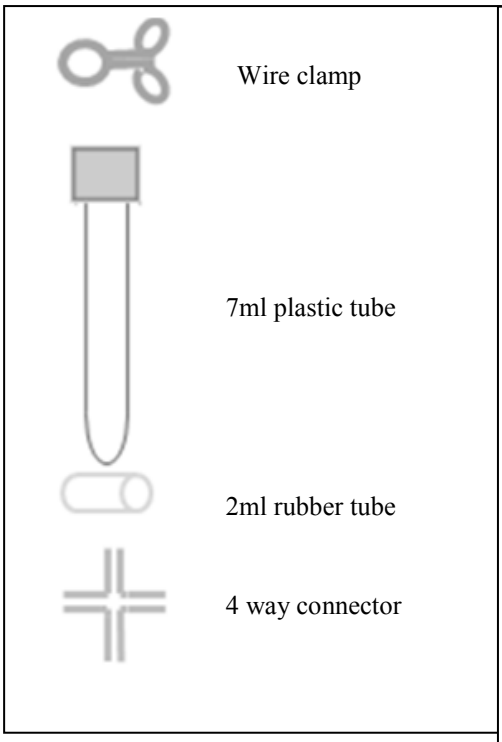

Figure 3-3 Material used to construct the single tube line probe prototype

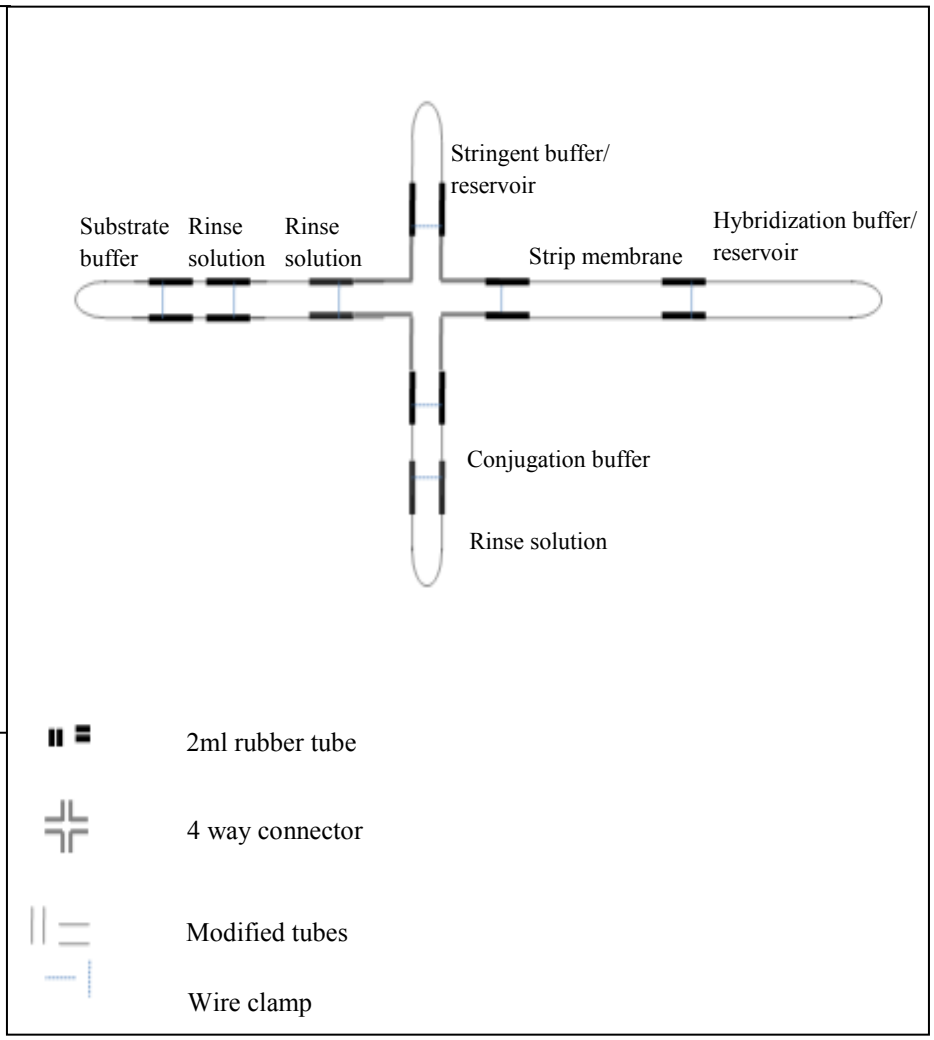

Figure 3-2 Single tube line probe assay prototype

All the materials were bought from local suppliers. The following samples (blank, MDR-TB and susceptible clinical isolates) were tested on the single tube enclosed method.

MATERIALS

Acid alcohol (5%)	Hydrochloric acid (5ml) Ethanol (100ml)
Carbol-fuchsin ZN stain Methylene blue Potassium permanganate (0.05%)	
Sputagest Activial	Dithiothreitol (DTT) (0.1g) Sodium chloride (0.78g) Potassium chloride (0.02g) Disodium hydrogen phosphate (0.112g) Potassium dihydrogen phosphate (0.02g) dH_2O to make up 100ml of sputum digestant (pH of 7.4 ± 0.2)
Sodium hypochlorite working stock	Commercial Sodium Hypochlorite solution (10%) dH_2O (90%)
Ortho-pthalaldehyde (1% w/v)	*Ortho*-pthalaldehyde (1g) dH_2O (100 ml)
Ultraseptin®*Aktiv* (4%)	Ultraseptin®*Aktiv* (20g) dH_2O (500 ml)
Incidin*plus* (10%)	Concentrated Incidin*plus* (10%) dH_2O (90%)
Ethanol (70%)	Absolute Ethanol (70%) dH_2O (30%)

Sodium bisulphite (1% w/v)	Sodium bisulphite (1g) dH_2O (100ml)
Middlebrook 7H9 medium:	7H9 medium (4.7g) dH_2O (900ml) Glycerol (2ml) Polysorbate (Tween-80) (0.5ml)
ADC:	BSA (25g) Glucose (10g) Catalase (0.75ml) dH_2O to make up 500ml
Middlebrook 7H10 medium:	7H10 medium (19g) dH_2O (900ml) Glycerol (5ml)
OADC: Catalase (0.03g)	Bovine Albumin (Fraction V) (50.0g) Dextrose (20.0g) Oleic acid (0.6 ml) Sodium Chloride (8.5g) dH_2O to make up 1L

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CHAPTER 4

Results

This chapter will be divided into the following sections: (i) identification of an efficient bactericide agent against *M. tuberculosis* which does not compromise the mycobacterial cell wall for staining and DNA integrity for PCR amplification (project 1 in Materials and Methods), (ii) identification of factors which may influence the reliability of the MTBDR*plus* assay (project 2-4 in Materials and Methods).

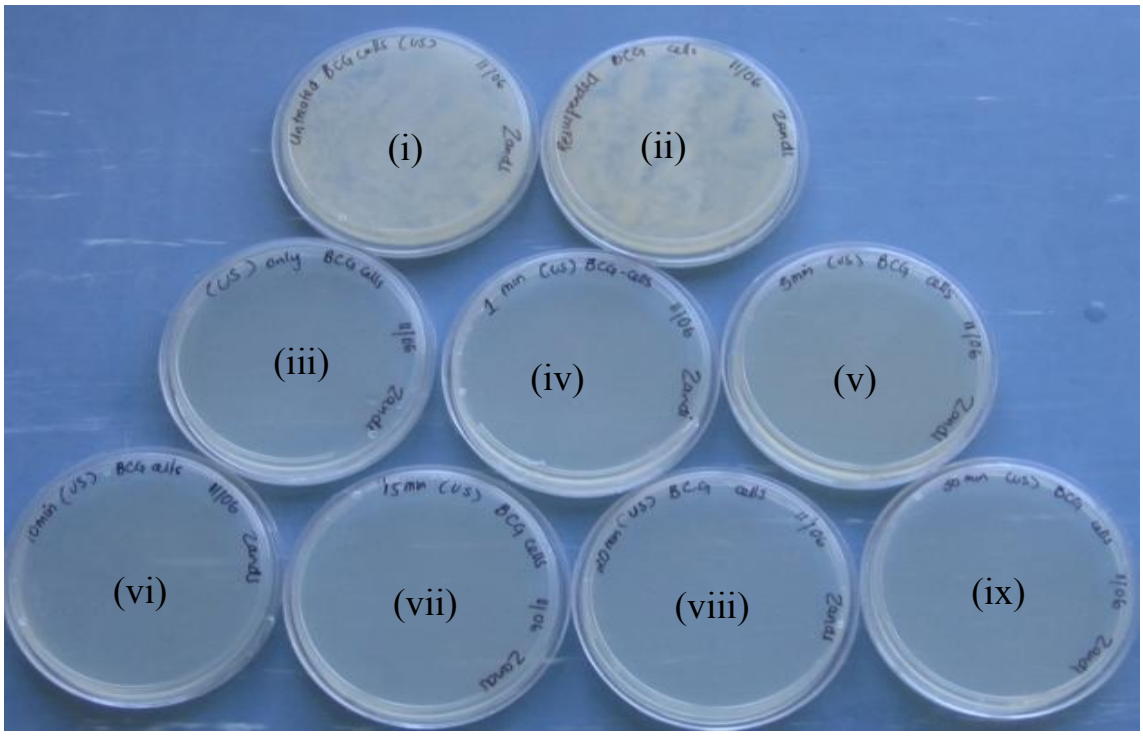
4.1. Bactericidal efficacy

4.1.1. Bactericidal efficacy on BCG Pasteur cells

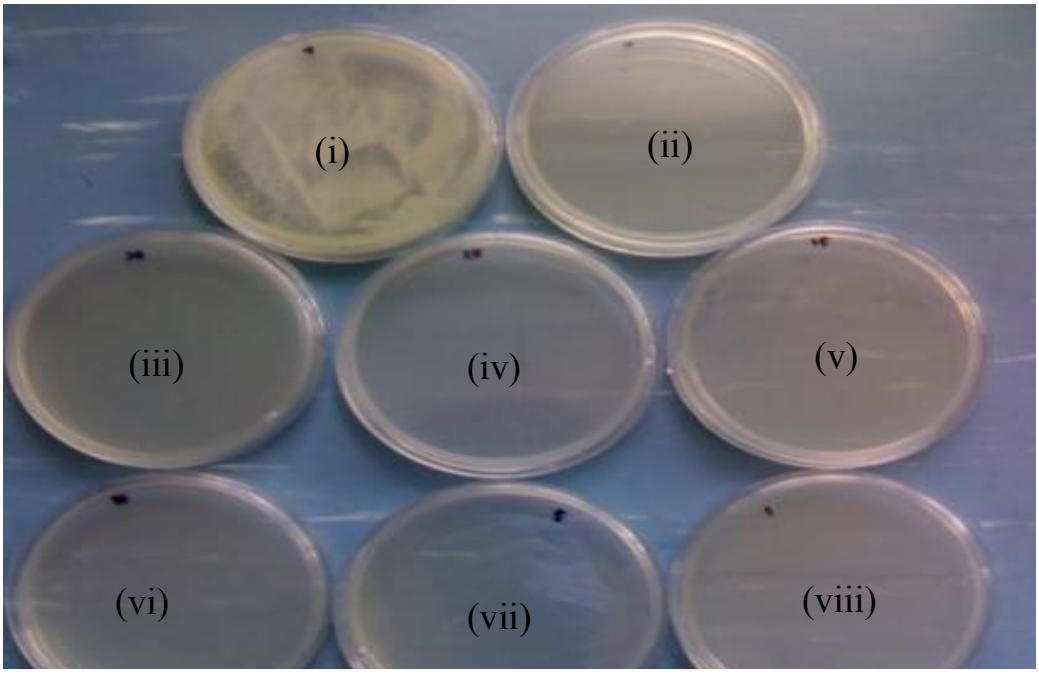
In an attempt to identify an efficient bactericide against *M. tuberculosis*, BCG Pasteur cells were exposed to candidate bactericides (sodium hypochlorite (NaOCl), 70% ethanol (EtOH), incidin*plus* and ultraseptin®*aktiv*) at different concentrations and time durations followed by culture and PCR amplification.

Figure 4.1 shows Middlebrook 7H10 agar plates of BCG Pasteur cells exposed to 2% w/v of ultraseptin®*aktiv* **(A)**, 5% v/v of incidin*plus* **(B)** and 1% v/v, 2.5% v/v and 5% v/v of NaOCl **(C)** [final concentrations] for different time durations (30, 20, 15, 10, 5, 1 minute(s) respectively. Complete growth inhibition was observed on cells exposed to either ultraseptin®*aktiv* (2%) (**A**), incidin*plus* (5%) (**B**) and NaOCl (5%) (**C**) within 1 minute of contact up to 30 minutes. NaOCl at a concentration of 1% v/v did not inhibit growth of the cells exposed for 1 minute to 30 minutes. Partial growth inhibition was observed following 2.5% NaOCl exposure for 15 minutes and after 30 minutes complete growth inhibition occurred [data not shown]. Also included in *figure 4.1A* is an agar plate of BCG Pasteur cells which grew even after exposure to 70% EtOH (ii) for 30 minutes. With each bactericide tested, positive and negative controls were also included. The positive and negative controls were untreated BCG Pasteur cells and media inoculated without BCG Pasteur cells respectively. As expected, growth was observed on the positive controls and not on the negative controls.

(A) Ultraseptin®*aktiv* (2%)



(B) Incidin*plus* (5%)



(C) NaOCl (5%)

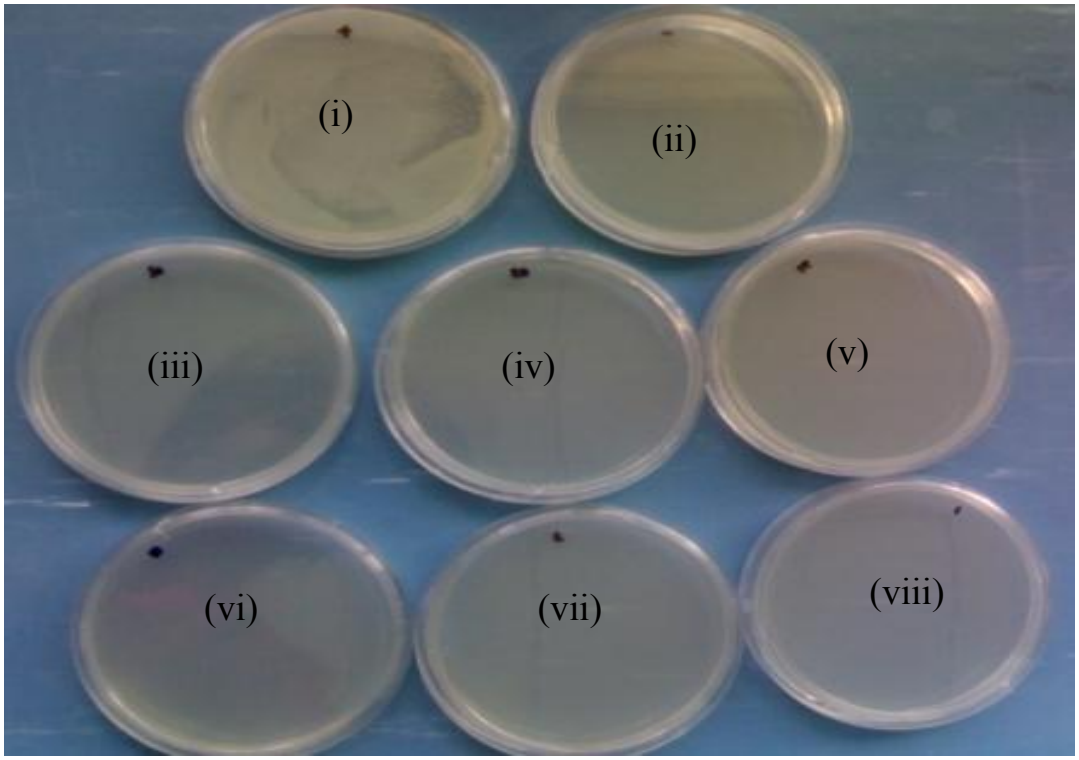


Figure 4-1 BCG Pasteur cells exposed to bactericides.

LEGEND TO FIGURE 4.1

Figure 4.1A. Agar plates with (i) untreated BCG cells (positive control), (ii) BCG cells exposed to 70% EtOH for 30 minutes, BCG cells treated with ultraseptin®*aktiv* for (iii) 30, (iv) 20, (v) 15, (vi) 10, (vii) 5 and (viii) 1 minute(s) and an agar plate with (ix) no BCG cells inoculated. ***Figure 4.1B.*** agar plate with (i) untreated BCG cells, (ii) BCG cells exposed to incidin*plus* for (iii) 30, (iv) 20, (v) 15, (vi) 10, (vii) 5 and (viii) 1 minute(s) and an agar plate with (ix) no BCG cells inoculated. ***Figure 4.1C.*** Agar plates with (i) untreated BCG cells, (ii) BCG cells exposed to NaOCl for (iii) 30, (iv) 20, (v) 15, (vi) 10, (vii) 5 and (viii) 1 minute(s) and an agar plate with (ix) no BCG cells inoculated.

4.1.2. DNA integrity

To investigate whether the integrity of mycobacterial DNA was retained in BCG Pasteur cells following inhibition of growth due to exposure to various bactericides, (Ultraseptin®*aktiv* (2% w/v), NaOCl (5% v/v) and Incidin*plus* (5% v/v) [final concentrations]) for 1,5 ,10, 15, and 30 minute(s), three genes (*rpoB*, *katG* and *RV2629*) were amplified.

Figure 4.2 shows representative agarose gel pictures of PCR products of the *rpoB* gene after exposure to ultraseptin®*aktiv*, incidin*plus* and NaOCl. Both NaOCl (*figure 4.2A*) and incidin*plus* (*figure 4.2B*) completely damages the DNA even after 1 minute exposure. From *figure 4.2C,* it is evident that ultraseptin®*aktiv* does not damage the DNA integrity even after 30 minutes of treatment.

Of the three candidate bactericides evaluated ultraseptin®*aktiv* was the most promising as it enabled sterilization of BCG Pasteur cells and amplification of the candidate genes [only *rpoB* gene results shown].

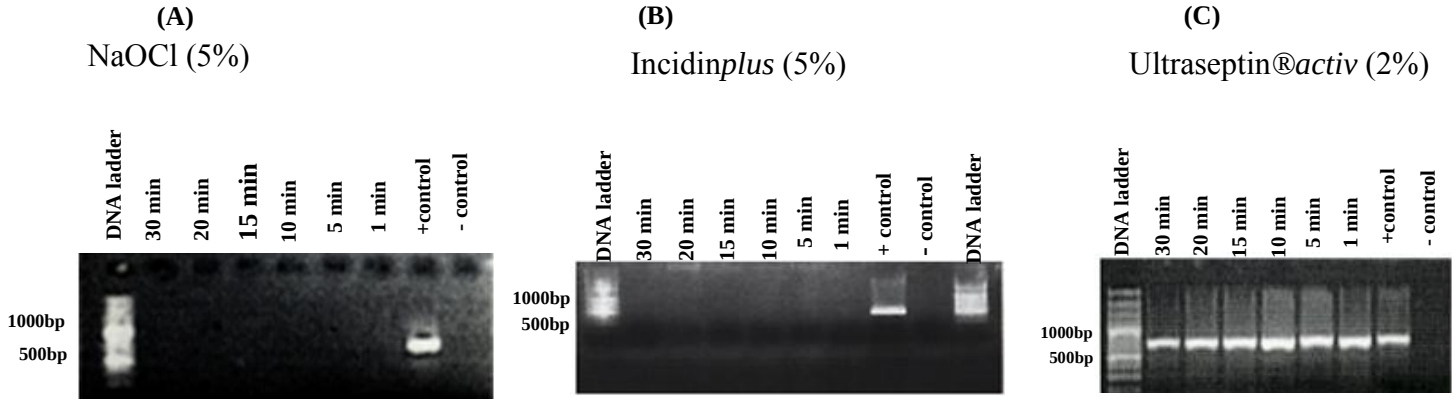


Figure 4-2 PCR amplified products of the *rpoB* gene on agarose gel after bactericide treatment

LEGEND TO FIGURE 4.2

Agarose gel of the *rpoB* gene PCR amplicons from BCG Pasteur cells exposed to (**A**) NaOCl for 30, 20, 15, 10, 5 and 1 minute(s) (**B**) incidin*plus* and (**C**) Ultraseptin®*aktiv* for the same time durations.

During the course of this study, an additional bactericide, *ortho*-phthalaldehyde (OPA) was identified in the literature as an efficient bactericide against mycobacteria. OPA was therefore also tested along with ultraseptin®*aktiv* against mycobacteria (1).

Both ultraseptin®*aktiv* (2%) and OPA (0.5%) were also bactericidal against a clinical isolate of *M. tuberculosis* which was inoculated to mid-log phase (optic density of 0.8) after 30 minutes exposure and did not induce DNA damage [data not shown].

4.1.3. Bactericidal efficacy on *M. tuberculosis* and on sputum specimens

To investigate whether ultraseptin®*aktiv* (2%) [final concentration] and OPA (0.5%) [final concentration] displayed bactericide activity against raw sputum specimens of different quality (bloody, mucoid or watery) and varying bacterial loads (scanty to 3+) were exposed for 15, 20 and 30 minutes. AFB negative sputa were included in the experiments as negative controls.

Mycobacterial growth was observed after 3 to 14 days on BACTEC MGIT 960 system in 19/34 and 15/34 liquified with NALC-NaOH or sputagest activial followed by exposure for 15 and 20 minutes to ultraseptin®*aktiv* respectively. Growth was also observed after 6 to 15 days on BACTEC MGIT 960 system in 12/30 specimens and 7/30 exposed for 15 minutes and 20 minutes respectively to OPA regardless of the liquification method (NALC-NaOH or sputagest activial). No growth was observed on either Middlebrook 7H9 liquid (inoculated for 25 days), Middlebrook 7H10 agar (inoculated for 28 days) or on BACTEC MGIT 960 system (inoculated for 42 days) after 30 minutes exposure to either sputagest activial or NALC-NaOH liquefied OPA (n= 44) or ultraseptin®*aktiv* (n=40) sterilized specimens.

Of the sputum specimens liquefied with NALC-NaOH and exposed to either OPA (n= 44) or ultraseptin®*aktiv* (n= 40) for 30 minutes, PCR amplification was inhibited in 33/40 specimens. *Figure 4.3* shows representative microscopic results after auramine-O and ZN staining of AFB positive sputum specimens unexposed as well as exposed to OPA and ultraseptin®*aktiv* for 30 minutes respectively. These results show that ZN and auramine-O staining can be obtained after exposure to both ultraseptin®*aktiv* and OPA. However, background fluorescence was observed

on sputum specimens stained by auramine-O after OPA exposure indicating that OPA may not be an appropriate candidate for microscopic identification of AFB.

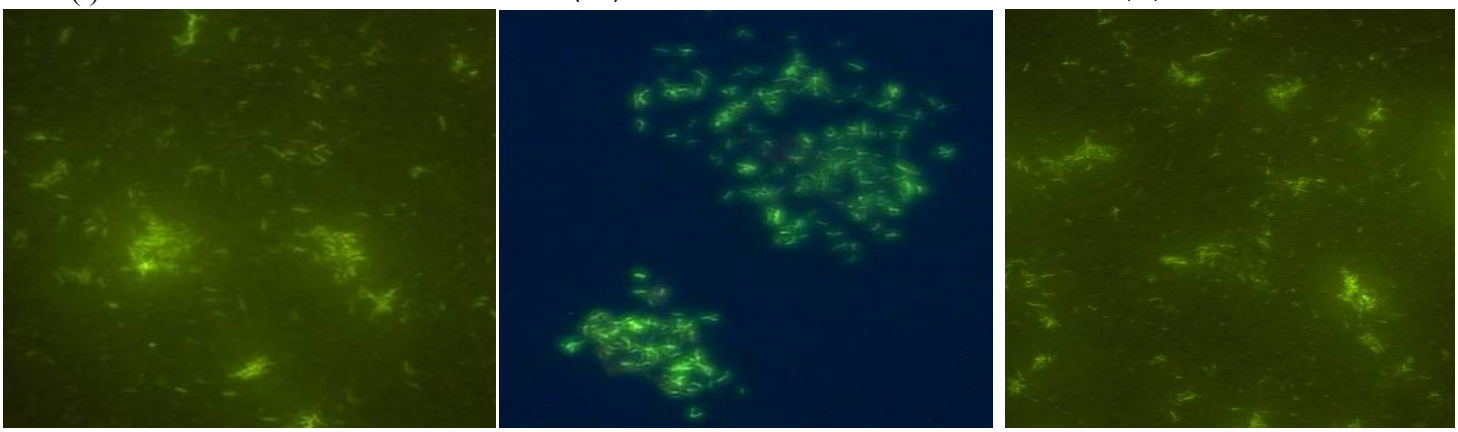
Figure 4.4A and 4.4B shows representative agarose gel electrophoresized products and MTBDR*plus* assay results after sputum specimen graded as (3+ to scanty) were liquefied with sputagest activial and exposed to OPA or ultraseptin®*aktiv* for 30 minutes. Both bactericides did not hinder PCR amplification (*figure 4.4A*) of *M. tuberculosis* DNA or the MTBDR*plus* assay on specimens liquefied with sputagest activial (*figure 4.4B*).

Mycobacterial DNA could still be amplified from specimens with very low bacterial load (scanty up to 3+) (*figure 4.4A*). From *figure 4.4B*, it is also evident that the bactericidal agents do not affect the efficiency of the MTBDR*plus* assay. All the specimens were known to be pan-susceptible except samples in lane 2, known to be resistant to rifampicin (weak hybridization on *rpoB*MUT2A), 8 and 12 which were known to be resistant isoniziad (INH).

From these results (*figure 4.3 and 4.4*), it can be concluded that both OPA and ultraseptin®*aktiv* when used in combination with sputagest activial do not compromise cell walls or the integrity of DNA templates for microscopy and PCR amplification respectively.

(A) Auramine staining

(i) (iii) (v)



(B) Ziehl Neelsen staining

(ii) (iv) (vi)

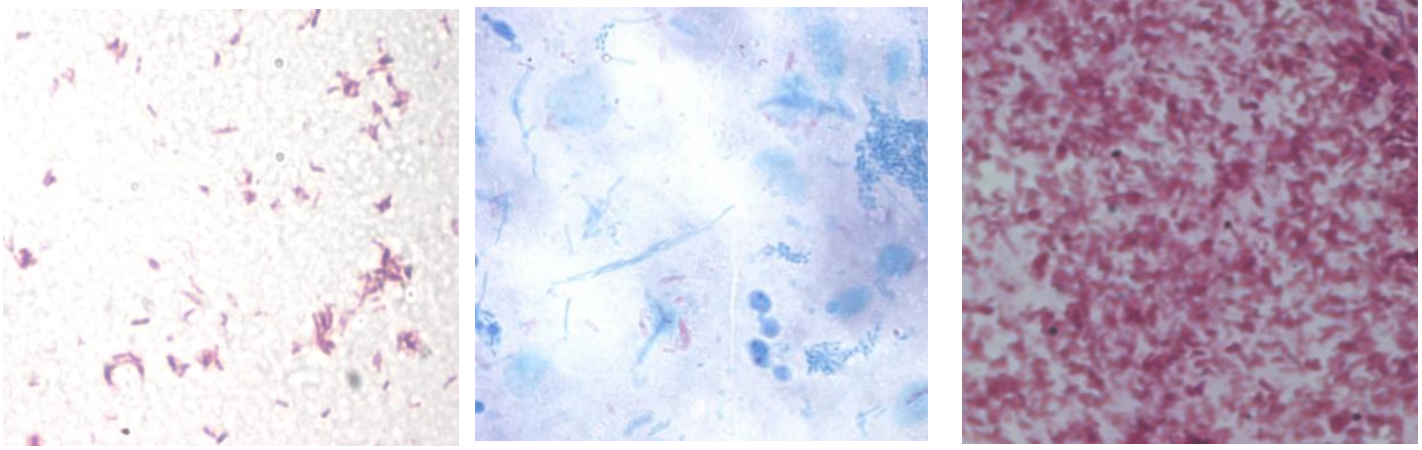


Figure 4-3 Mycobacterial stains of specimens exposed to OPA and Ultraseptin®*aktiv*

LEGEND TO FIGURE 4.3

Figure 4.3 (A and B) shows representative results of ultraseptin®*aktiv* treated auramine-O (i) and ZN (ii) stains exposed to the bactericides for 30 minutes, untreated auramine-O (iii) and ZN (iv), OPA treated auramine-O (v) and ZN (vi) stains exposed to the bactericides for 30 minutes. The specimens used for microscopy were all 2+ graded sputa.

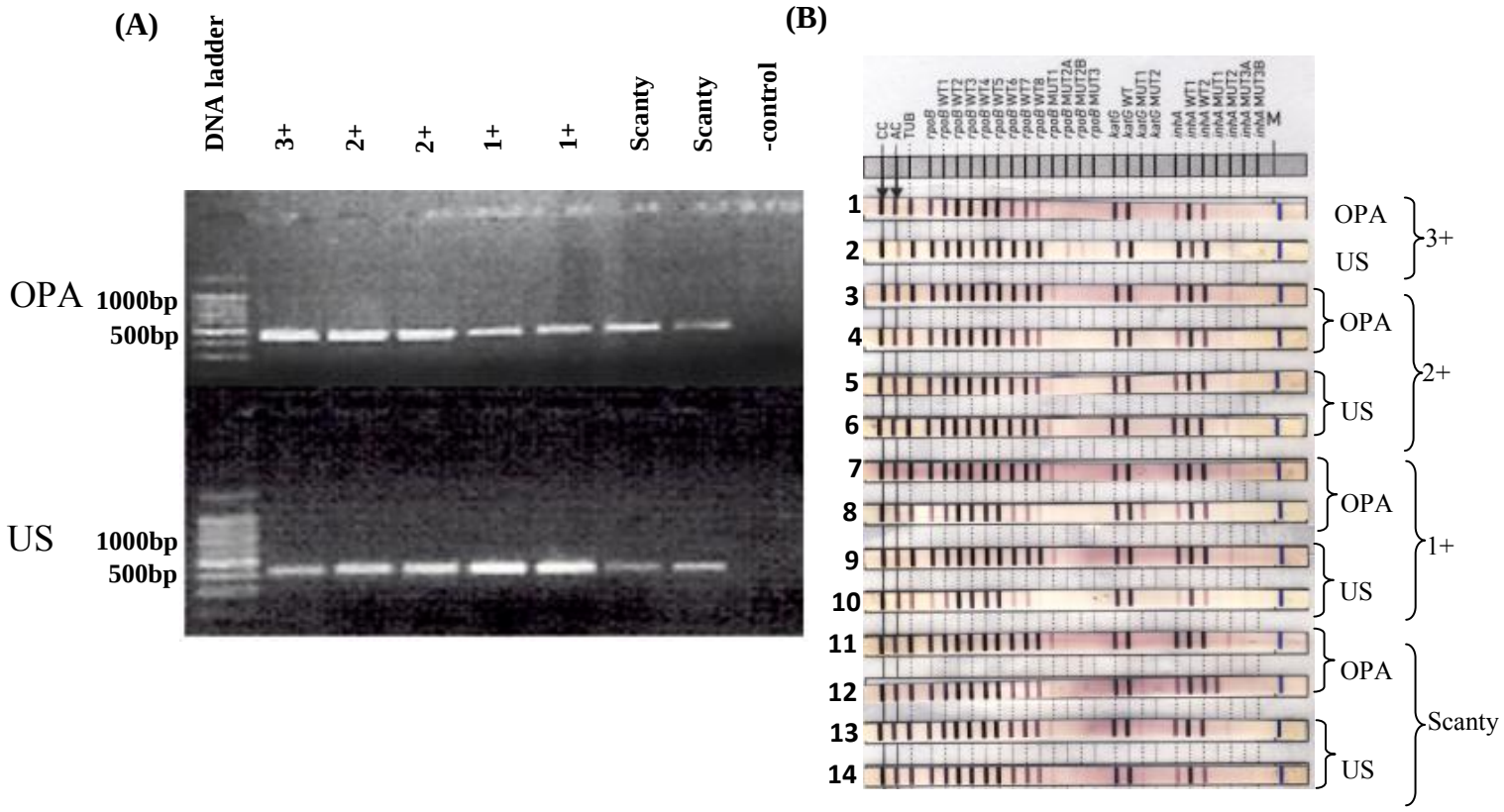


Figure 4-4 Efficiency of PCR amplification after bactericide treatment.

LEGEND TO FIGURE 4.4

Figure 4.4A*:** Agarose gel of amplicons of *RV2629* gene after sputagest activial liquefied sputum specimens 30 minutes exposure to OPA and ultraseptin®*aktiv* (US). ***Figure 4.4B: MTBDR*plus* results after sputagest activial liquefied sputum specimens 30 minutes exposure to OPA and US exposure.

4.2. Factors affecting the MTBDR*plus* assay

In an attempt to identify factors which may influence the reliability of the MTBDR*plus* assay, the following experiments were conducted:

- To determine the effect of underlying non tuberculous mycobacterium (NTM's) on the detection of drug resistant TB (Materials and Methods – project 2);
- To determine the limit of detection of drug resistant *M. tuberculosis* in hetero-resistant isolates (Materials and Methods – project 3);
- To develop a device to prevent amplicon cross contamination during the detection steps (Materials and Methods – project 4).

All the tests involving the MTBDR*plus* assay were repeated twice.

4.2.1. Non-tuberculous mycobacteria

Pure DNA from a well characterized MDR-TB clinical isolate (R439) of *M. tuberculosis* with known mutations in the *katG* 315 ACA (INH resistance) and *rpoB* gene 531 TTG (RIF resistance) was mixed with increasing concentrations of NTM DNA.

Figure 4.5 shows representative MTBDR*plus* results of different ratios made with DNA purified from the MDR-TB isolate and (**A)** *M. intracellulare* or (**B**) *M. kansasii*. From *figure 4.5A*, the MDR-TB strain could still be detected in the background of *M. intracelullare* at a ratio of 1:10. However, the results were interpreted as invalid at a ratio of 1:10 of the MDR-TB isolate in the background of *M. kansasii* as the TUB band is missing (*figure 4.5B*). During the final stages of detection with the MTBDR*plus* assay, non specific binding to probes often occurs. It is of note that there are two bands numbered 17 and 18 in *figure 4.5A* on lane 8 but not on the blank in lane 9. The bands in lane 8 do not affect interpretation of the assay since the TUB band does not come up and the drug resistance pattern is also absent.

In a typical diagnostic setting, crude boiled DNA is used for genotypic detection of *M. tuberculosis* and drug resistance testing. To investigate the reliability of the MTBDR*plus* assay on clinical isolates in the background of NTM's, crude DNA from the MDR-TB isolate was diluted with each of the 10 different NTM's in a range of dilutions varying from 1:1 to 1:200.

The results in Table 4.2 indicate that as the NTM DNA concentration increases relative to the *M. tuberculosis* DNA concentration, the TUB band which marks presence of *M. tuberculosis* disappears. In the absence of the TUB band the assay is interpreted as invalid. From these results it is evident that the MTBDR*plus* assay cannot reliably detect the presence of *M. tuberculosis* in the presence of NTM's which influence the sensitivity of this drug resistance genotyping test.

To further determine the influence of underlying bacterial or mycobacterial species on the detection of *M. tuberculosis* and the corresponding drug resistance pattern, clinical isolates previously identified by *16S rRNA* and *gyrB* sequencing that showed mixed infection with *M. tuberculosis* and NTM's were evaluated with the MTBDR*plus* test. Invalid results were obtained for 2 samples containing *M. terrae* and *M. triviale* (Table 4.3). The Genotype® Mycobacterium CM test could not identify *M. tuberculosis* in all the 5 clinical isolates [data not shown]. In addition, further investigation of the reliability of the MTBDR*plus* assay directly on sputum specimens showed that 6/19 were interpreted as invalid when NTM's and *M. tuberculosis* were present in the same sample (Table 4.4). Three specimen from 5 of the 19 specimen (961, 337(1), 848, 278 and 775) identified by the Genotype® Mycobacterium CM to be mix infected yielded invalid results on the MTBDR*plus* assay (table 4.4). The specimen 464 identified as infected with *M. tuberculosis* by *16SrRNA* sequencing and *M. kansasii* by the speciation assay could be genotyped for drug resistance whereas specimen 331 identified to contain *M. kansasii* or *gastri* by *16SrRNA* sequencing and *M. tuberculosis* by the speciation assay could not be interpreted.

In summary, these results suggest that underlying NTM's can strongly influence reliability of the MTBDR*plus* assay to detect TB and drug resistance TB.

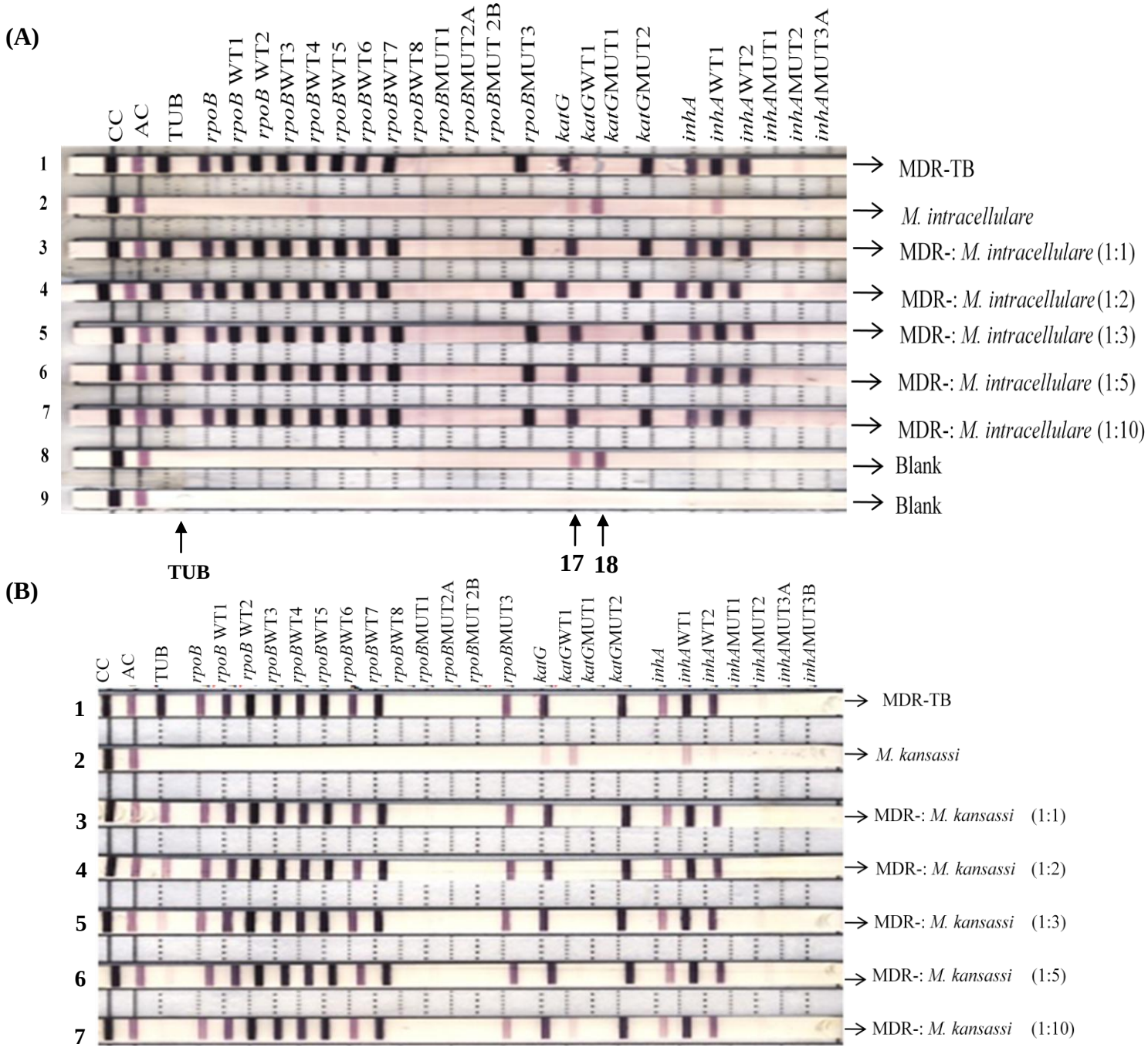


Figure 4-5 MTBDR*plus* assay results

LEGEND TO FIGURE 4.5

Figure 4.5A: Lane 1, MDR-TB DNA isolate (R439), lane 2 ***M. intracellulare***, lane 3-7 Mix DNA of the MDR-TB DNA and M. ***intracellulare*** from 1:1, 1:2, 1:3, 1:5 and 1:10 dilution respectively, lane 8-9 are blanks (negative control). *Figure 4.5B*: Lane 1, MDR-TB DNA isolate (R439), lane 2 ***M. kansasii***, lane 3-7 Mix DNA of the MDR-TB and ***M. kansasii*** from 1:1, 1:2, 1:3, 1:5 and 1:10 respectively.

Table 4-1 MTBDR*plus* results for crude DNA mixtures of NTM's and the MDR-TB isolate.

NTM	Ratio	TUB	INH					RIF		MTBDR*plus* results interpretation
			katG			*inhA*				
			Wt	*kat*GMUT		*Wt*	Mut	*Wt*	*rpo*BMUT3	
				1	2					
MDR-TB	1	✔	✘	✘	✔	✔	✘	✘	✔	MDR-TB
M. peregrinum	1:100	✔	✔	✘	✔	✔	✘	-	✔	Drug resistant to RIF and both resistant and susceptible INH
M. bovis	1:50	✔	✔	✘	✔	✔	✘	-	✔	Drug resistant and susceptible to INH and RIF resistant
	1:150	✘	-	✘	-	-	-	-	-	Invalid
M. abscessus	1:150	✔	✘	✘	✔	✘	✘	-	✘	INH monoresistant
M. kansasii	1:100	✔	✔	✘	✔	✔	✘	-	✔	Drug resistant to RIF and both resistant and susceptible INH
	1:200	✘	-	✘	-	-	-	-	-	Invalid
M. fortuitum	1:50	✔	✔	✘	✔	✔	✘	-	✔	Drug resistant and susceptible to both RIF and INH
M. chelonae	1:55	✘	-	✘	-	-	-	-	-	Invalid
M. intracellulare	1:50	✘	-	✘	-	-	-	-	-	Invalid
M. avium	1:100	✘	-	✘	-	-	-	-	-	Invalid
M. xenopi	1:50	✔	✔	✔	✔	✔	✘	-	✔	Drug resistant to RIF, both susceptible and resistant to INH
M. terrae	1:100	✘	-	✘	-	-	-	-	-	Invalid

LEGEND TO TABLE 4.1

TUB= presence of *M. tuberculosis,* X= no hybridization on probe

Clinical isolate	*16SrRNA* sequencing	*gyrB* sequencing	MTBDR*plus* results interpretation
6865	*M. kumamotonense*	*M. tuberculosis*	*M. tuberculosis* (MDR-TB)
7426	*M. triviale*	*M. tuberculosis*	No TB, *katG* MUT1and *katG* MUT2 detected
7618	*M. terrae*	*M. tuberculosis*	No TB, susceptible pattern
7690	*M. acapulcensis*	*M. tuberculosis*	*M. tuberculosis* susceptible pattern

Table 4-2 Mixed NTM and *M. tuberculosis* in clinical isolates.

LEGEND TO TABLE 4.2

Shows the corresponding 16*SrRNA*, *gyrB* gene sequencing and MTBDR*plus* results of the 4 mixed isolates.

4.2.1.1. MTBDR*plus* on sputa

Table 4-3 Speciation and drug resistance genotyping of isolates directly from sputum specimens without culture.

Sample	*16S rRNA* sequencing	*gyrB* sequencing	Genotype Mycobacterium®CM results interpretation	MTBDR*plus* results interpretation
919	no amplification	no amplification	*M. tuberculosis* complex	*M. tuberculosis* (susceptible)
645	Uncultured *Corynebacterium sp.**	no amplification	*M. tuberculosis* complex	*M. tuberculosis* (susceptible)
937	*M. tuberculosis* complex	*M. tuberculosis*	*M. tuberculosis* complex	Invalid
961	*M. tuberculosis* complex	no amplification	*M. avium*, *M. tuberculosis* complex, *M. kansasii*	Invalid
337 (1)	*M. tuberculosis* complex	*M. tuberculosis*	*M. avium*, *M. tuberculosis* complex,	Invalid
339	*M. tuberculosis* complex	*M. tuberculosis*	no amplification	*M. tuberculosis* (susceptible)
848	*M. tuberculosis* complex	no amplification	*M. avium*, *M. tuberculosis* complex	*M. tuberculosis* (INH Monoresistant)
472	*M. tuberculosis* complex	no amplification	*M. tuberculosis* complex	*M. tuberculosis* (susceptible)
773	*M. tuberculosis* complex	no amplification	*M. tuberculosis* complex	*M. tuberculosis* MDR-TB
516	*M. tuberculosis* complex	*M. tuberculosis*	*M. tuberculosis* complex	Invalid
278	*M. avium*	no amplification	*M. avium*, *M. tuberculosis* complex, *M. kansasii*	*M. tuberculosis* (pattern absent)
775	bad sequencing	*M. tuberculosis*	*M. tuberculosis* complex, *M. fortuitum*	Invalid
842	*M. tuberculosis* complex	*M. tuberculosis*	no amplification	*M. tuberculosis* (MDR-TB)
877	*M. tuberculosis* complex	*M. tuberculosis*	no amplification	*M. tuberculosis* (susceptible)
821	*M. tuberculosis* complex	*M. tuberculosis*	*M. tuberculosis* complex	*M. tuberculosis* (MDR-TB)
203	*M. tuberculosis* complex	*M. tuberculosis*	*M. tuberculosis* complex	Invalid
464	*M. tuberculosis* complex	*M. tuberculosis*	*M. kansasii*	*M. tuberculosis* (susceptible)
331	*M. kansasii* or *M. gastric*	no amplification	*M. tuberculosis* complex	Invalid
913	*M. tuberculosis* complex	*M. tuberculosis*	*M. tuberculosis* complex	*M. tuberculosis* (susceptible)

4.2.2. Hetero-resistance

Reviewing the genotypic data of *M. tuberculosis* culture from a longitudinal reference bank at Stellenbosch University, Tygerberg, Cape Town, South Africa, showed that both wild type and mutant cells may be present in the same sputum specimen reflecting hetero-resistance (unpublished data: Dr E. Streicher, 2010). To determine the detection limit of the MTBDRp*lus* assay for identifying drug resistance in hetero-resistant isolates, purified DNA from a pan-

susceptible and MDR-TB were mixed at different ratio's with the amount of DNA of the drug resistant isolate kept constant.

Figure 4.6 shows that the MDR-TB RIF and INH resistance markers (*rpoB*MUT3 and *katG*MUT1) become weaker as a function of increasing wild type *M. tuberculosis* DNA. According to the manufacturer's scoring instructions, the MDR-TB strain would be missed when the ratio of mutant to wild type exceeds 1:50.

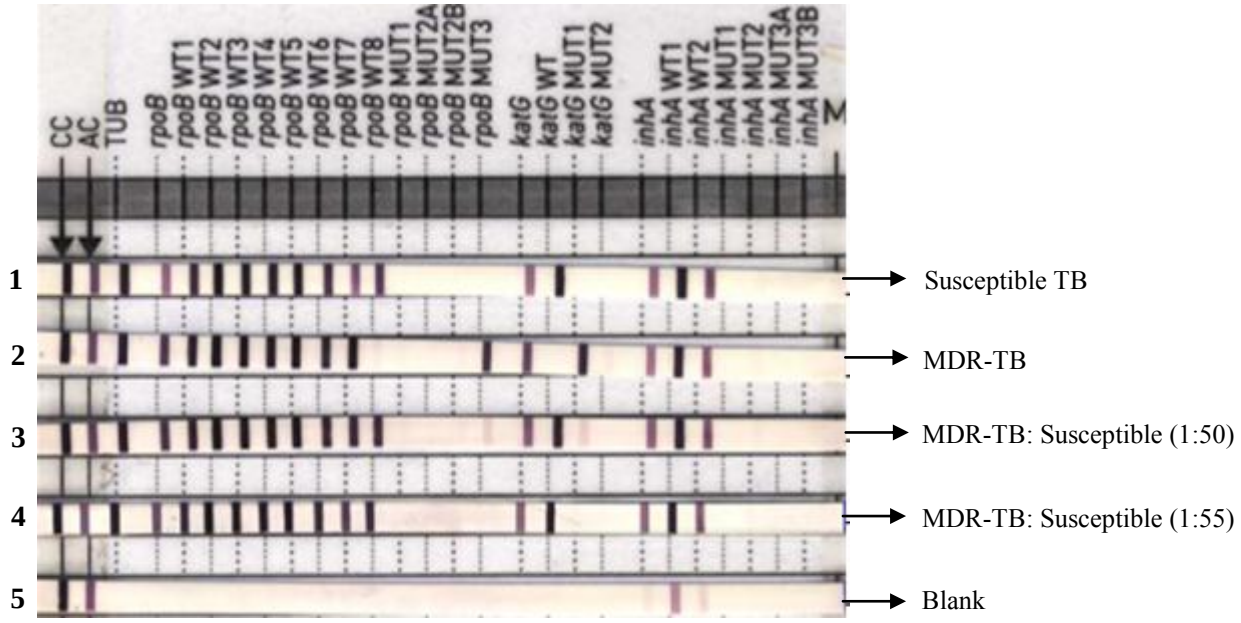


Figure 4-6 MTBDR*plus* assay results for the hetero-resistant ratio mixtures

LEGEND TO FIGURE 1.6

Lane 1, Pan-susceptible-TB isolate (1260), lane 2 MDR-TB isolate (2126), lane 3-4 Mix DNA of the pan-susceptible TB and MDR-TB DNA 1:50 and 1:55 dilution respectively, lane 5, blanks (negative control).

4.2.3. Improving the MTBDRplus assay

PCR-based assays including the MTBDR*plus* assay are prone to amplicon cross contamination. This study aimed to develop a single closed line probe device which will prevent amplicon cross contamination during the final steps involved in the detection of genotypes.

4.2.3.1. Development of a closed line probe assay device

The device

The device that was developed consists of wire clumps, rubber tubes, a plastic 4-way connector and modified 7ml plastic tubes (*figure 4.7*). The rubber tubes are divided into 7 compartments by wire clumps (*figure 4.7*). Each compartment contains the reagents used in the different steps followed during the final detection steps of the MTBDR*plus* assay (*figure 4.7*). These compartments respectively contain the hybridization buffer, stringent wash solution, conjugant, 2 sections for the rinse solution, ddH_2O and finally for detection the substrate. A single strip of the MTBDR*plus* membrane is inserted inside the device and closed by the wire clumps. The denaturing reagent (20µl) is first mixed with the PCR amplicons (20µl) into a 1ml tube before introduction of the two into the 1st chamber. After the hybridization buffer is allowed to mix with the membrane, the denatured amplicons (40µl) are injected with a 1ml syringe into the rubber tube on the compartment with the membrane. The hybridization buffer and stringent wash solution compartments also act as reservoirs. The different reagents are pushed through the wire clumps to the compartment containing the membrane and once the respective buffer or wash solution is finished, it pushed onto the one of the reservoirs. The single tube device is then placed onto a shaking oven at 45ºC during the hybridization and stringent wash steps. The rest of the assay is performed at room temperature.

The single tube MTBDR*plus* device was evaluated on 2 samples, one of which was a pan-susceptible and the other one was an MDR-TB isolate (*figure 4.8*). A blank was also included as a negative control (*figure 4.8*). The results indicate that the device could successfully identify both the pan-susceptible and MDR-TB pattern correctly and that the blank was also completely clean (*figure 4.8*).

This is a completely sealed device and no amplicons will leak out of the device to cause cross contamination with other samples. This device is a prototype for proof of concept and in the long term may offer prospects for automation and for patenting.

A single tube line-probe prototype that is user friendly is proposed (*figure 4.9*). It differs from the proof of concept device in that these improvements could be made; (i) linking of the

compartments into a linear structure, (ii) use of tap-like clamps instead of wire clamps, (iii) incorporation of a reservoir underneath the different compartments with a tap-like clamp within the compartment containing the strip membrane, (iv) a plastic sealed well which contains the denaturing buffer onto which the PCR product can be injected into with a syringe onto a rubber septum.

It is proposed that the device (*figure 4.9*) can be further improved by (i) creation of a platform which links several of these devices in 1 unit (*figure 4.10*), and by using (ii) a machine which opens and closes the taps automatically.

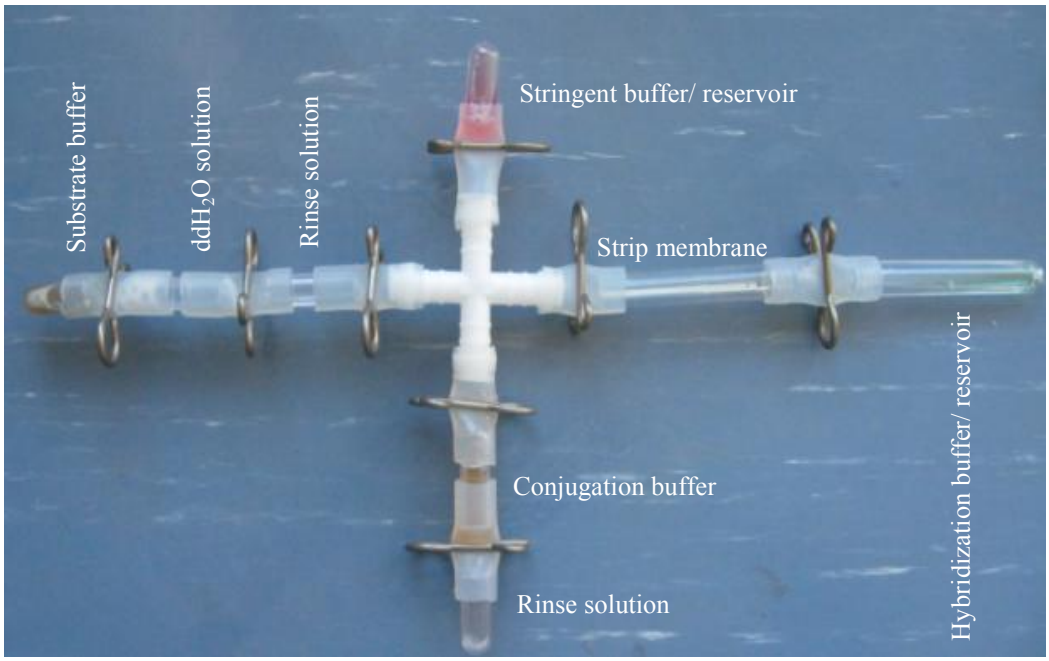


Figure 4-7 MTBDR*plus* prototype of a single tube device.

Figure 4-8 Single tube MTBDR*plus* assay results

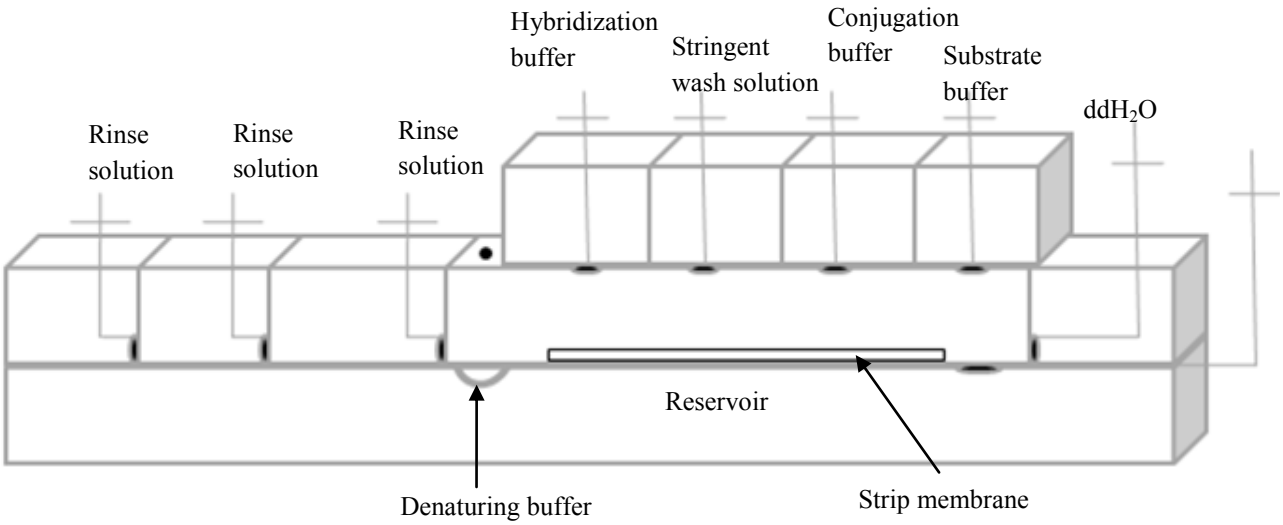


Figure 4-9 MTBDR*plus* prototype of a single tube device.

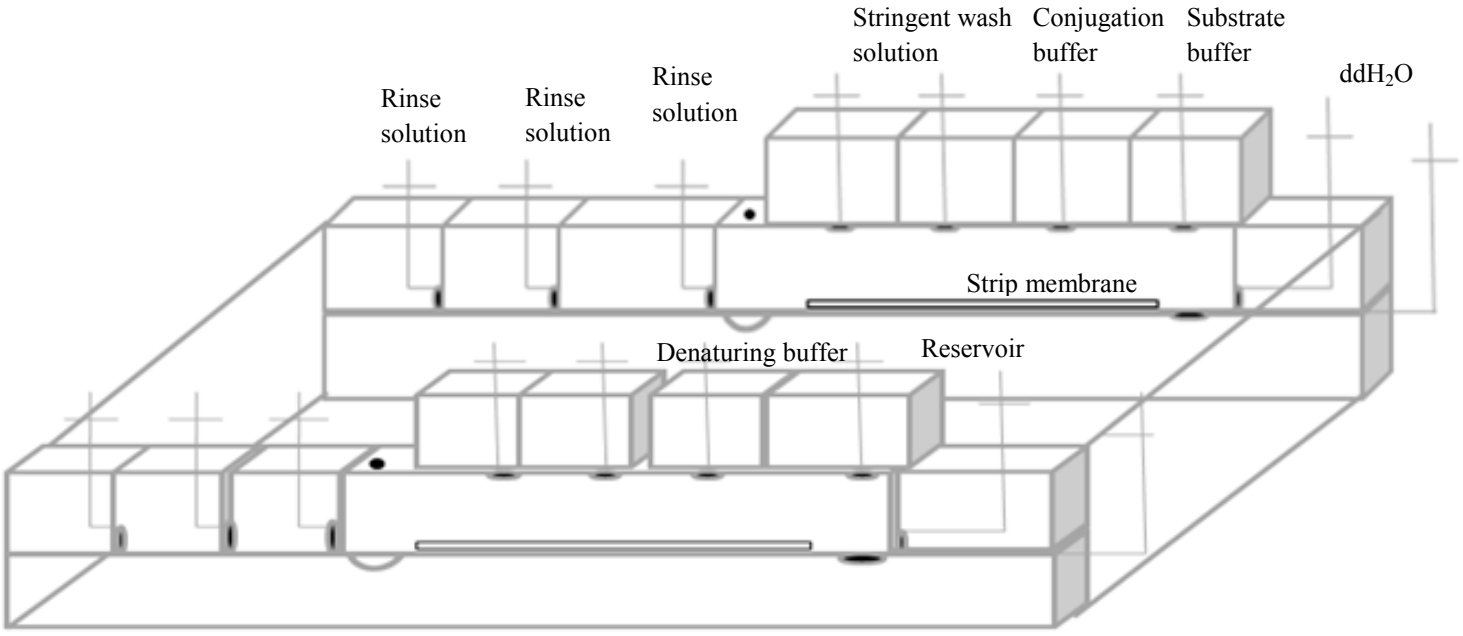


Figure 4-10 Proposed patentable prototype MTBDR*plus* device

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CHAPTER 5

Discussion and Conclusion

This study covers two main aspects; it demonstrates a methodology for liquification and sterilization of viable mycobacteria in sputum specimens prior to genotypic analysis for rapid drug resistance testing outside the confinement of a biosafety level 3 facility as well as highlights factor(s) which may influence reliability of the MTBDR*plus* assay.

Four commercial bactericides (sodium hypochlorite (NaOCl), incidin*plus*, *ortho*-phthalaldehyde (OPA) and ultraseptin®*aktiv*) which were reported previously to efficiently sterilize *M. tuberculosis* were identified. They were selected based on their proposed mechanism of action according to the chemical structures of their active moieties, lack or low toxicity, cost effectiveness and ease of access. The bactericides were evaluated for their bactericidal effect in BCG Pasteur cells, a clinical *M. tuberculosis* isolate (R439) and directly on acid fast bacilli (AFB) positive sputum specimens with different quality and bacterial loads. Eight AFB negative sputum specimens were included as controls.

The study confirms that sodium hypochlorite (5%), incidin*plus* (5%), ultraseptin®*aktiv* (2%), and *ortho*-pthalaldehyde (OPA) (0.5%) are bactericidal against actively growing mycobacteria and that their activity is not hindered by the presence of organic matter (in sputum) or bacterial load after 30 minute exposure. Our findings are in accordance with previous studies which reported cellular degradation and mycobacterial death after exposure to sodium hypochlorite, incidin*plus*, OPA and ultraseptin®*aktiv* (1,6,7,9,10,14,17). In this study however, bactericidal agent(s) which can effectively sterilize mycobacteria without compromising mycobacterial stainability or inhibit PCR amplification are required for tuberculosis (TB) diagnosis and rapid drug resistance genotyping by PCR-based methods. Although incidin*plus* and sodium hypochlorite are bactericidal, they hindered PCR amplification. OPA was shown to influence mycobacterial fluorescence staining as the agent also stains proteins present in sputa resulting in background fluorescence noise. Ultraseptin®*aktiv* was therefore identified as the most promising

bactericide which does not negatively impact on staining of mycobacterial cells and also provides a template for efficient PCR amplification.

Due to the properties of ultraseptin®*aktiv*, an algorithm which can be used at primary health care clinics with minimal infrastructure was proposed (*figure 5.1)*. A sputum bottle containing ultraseptin®*aktiv* can be used to collect the sputum and microscopy with an LED microscope can be done on site while TB suspects or retreatment patients wait at the clinic for the results *(figure 5.1*). If AFB positive, treatment can be initiated immediately. Positive specimens can then be batched for safe transport and drug resistance genotyping at a central facility. It has previously been shown that the turnaround time for routine genotypic diagnosis of TB varies between 2-20 days (5). The approach outlined in this study has several advantages, i) the protocol can be conducted in any primary health care clinic by a person with minimal training, ii) TB diagnosis and initiation of treatment can be obtained on the same day while the patient waits at the clinic, iii) rapid diagnosis will decrease the percentage of defaulters, iv) safe transport of batched samples, v) PCR based drug resistance genotyping can be done directly on sputum specimens, vi) rapid drug resistance genotyping will enable rapid proper therapy to patients thereby decreasing the rate of transmission to contacts. Liquification of sputum specimens prior to bactericidal sterilization is an essential step which may be problematic. This study does not provide a defined protocol for initial liquification and subsequent sterilization by ultraseptin®*aktiv*. This problem may be circumvented by using a sputum bottle with a rubber lead instead of the conventional plastic lead which would enable injection of ultraseptin®*aktiv* onto the liquified sputum. The sputum bottle presented to the TB suspect would already contain the liquification reagent (sputagest activial). Upon subsequent sputum liquification, ultraseptin®*aktiv* would then be injected onto the liquified sputum.

The importance of implementation of a new generation of rapid drug resistance genotyping methods has been highlighted by the WHO's endorsement of the MTBDR*plus* assay in 2008 (16). In a recent study in Tanzania, weak hybridization on the Mycobacterium® genotype CM (line probe assay also from Hain-life science) for *M. tuberculosis* lead to subsequent treatment of a patient for TB, however on follow-up after treatment failure presence of a non-tuberculous mycobacterial infection caused by *M. sherrisii* was detected(3). Drug resistance to 1st line drugs

of NTM's and misclassification of NTM infection as MDR-TB has also been documented (12,13). Several studies have also demonstrated and reported the phenomenon of hetero-resistance (4,8,11,15). The detection limit has thus far only been reported in QT-PCR based assays (18). Quantitative RT-PCR based assays have been reported to be highly sensitive at detecting hetero-resistance at low DNA concentrations of either allele irrespective of probe system used (Taqman MGB, molecular beacon, FRET MGB probes (2,18). Detection was shown to be determined primarily based on the amount of each allele and secondly by their respective ratios (18).

In this study 3 factors which may negatively impact on the results of this assay were investigated: (i) presence of NTM's, (ii) hetero-resistance and (iii) cross contamination. The results showed that underlying NTM's found in local clinical isolates adversely affects the MTBDR*plus* assay in pure or crude DNA and sputum samples. These results suggest that the MTBDR*plus* test cannot be relied upon to test for TB and drug resistance TB in samples with underlying NTM's. In high TB burden countries where the dual HIV-TB epidemic and consequently NTM-TB co-infection are high, the MTBDR*plus* can therefore not be relied upon. Re-evaluation of the reliability of the MTBDR*plus* assay in settings with high TB and/ NTM-HIV co-infections is thus required and alternative genotypic tests are therefore needed to validate or compliment the MTBDR*plus* assay. We also report that the detection limit of the MTBDR*plus* assay in hetero-resistance isolates is 1:55. The limitation of this study may include the use of pure and crude DNA concentrations as they are not indicative of the number of cells present. Consequently, a study using known mixtures of mycobacterial colony forming units instead of purified DNA concentrations may be more informative.

Implementation of the MTBDR*plus* assay may present more problems than anticipated, given not only the rudimentary cost and complexity of the tests but also the battle with cross contamination during the multiple steps required. Currently only highly specialized laboratories are able to perform this test. To combat amplicon cross contamination, a patentable closed tube line probe device which enables detection of *M. tuberculosis* and drug resistance genotyping for multiple specimens is proposed. This device is completely closed, thereby preventing any

external amplicons from contaminating samples during the final steps of detection of drug resistance with the MTBDR*plus* assay.

In summary, this study identifies a sterilizing agent which can be used at primary health care clinics for diagnosis of TB and subsequent drug resistance genotyping by a PCR-based method. It also highlights problems with the WHO endorsed MTBDR*plus* assay and a prototype device to overcome amplicon cross contamination during the final detection steps is proposed.

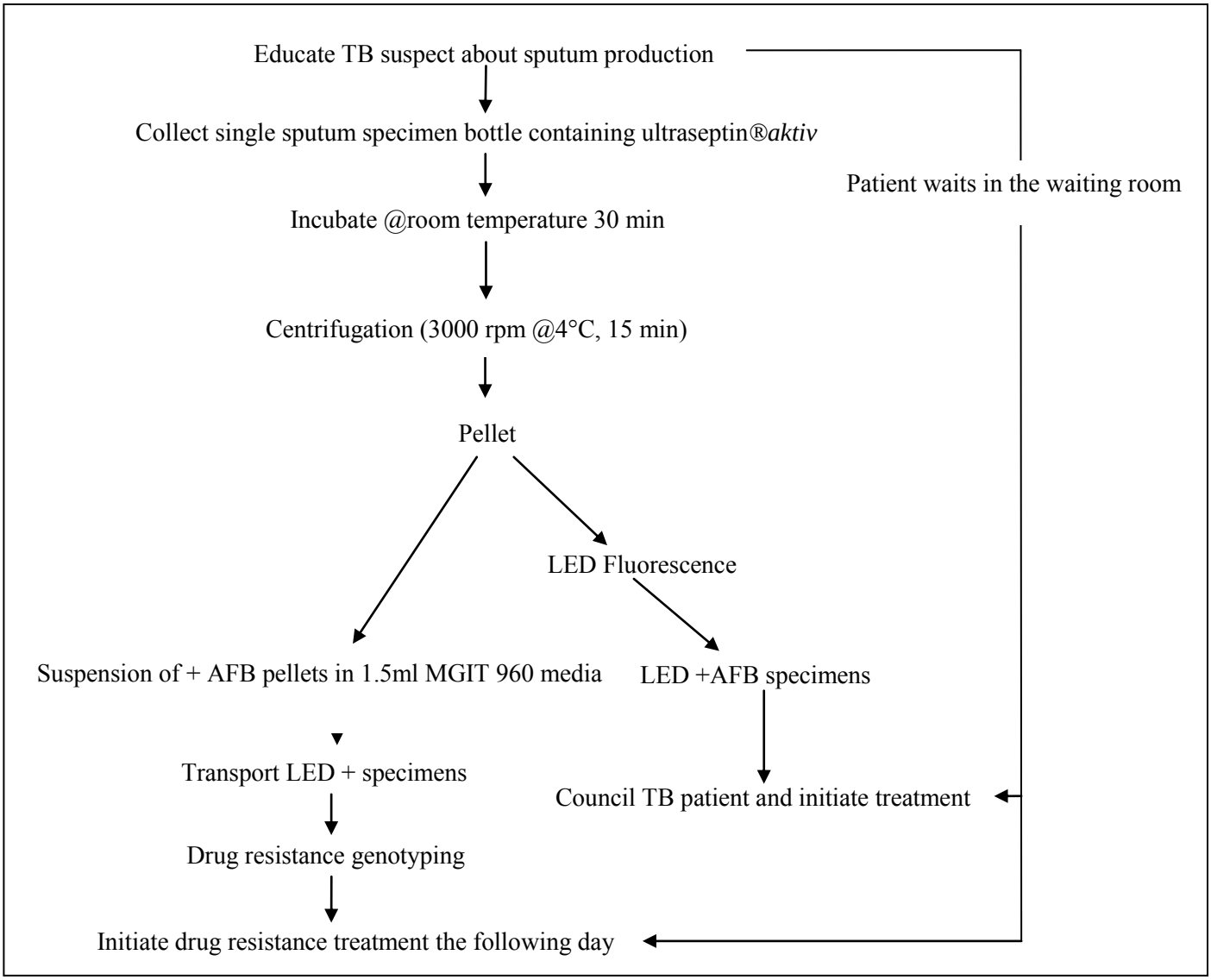

Figure 5-1 Flow diagram of proposed on site same day sputum smear diagnosis protocol.

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