

The development and application of a high
throughput methodology to determine
MICs of Mycobacterium tuberculosis
isolates against antimicrobial agents

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Submitted in fulfillment of the requirements for the degree of Master of Medical
Science (Medical Microbiology) in the Department of Medical Microbiology and
Infection Prevention and Control, College of Health Sciences, University of
KwaZulu-Natal

As the candidate's supervisor I agree to the submission of this dissertation.

Signed: _____ Date: _____

Declaration

Plagiarism:

I, Tashmin Rampersad declare that:

(i) The work described in this dissertation has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification.

(ii) The laboratory work described in this dissertation was conducted by the candidate, where the work by others was used, it is stated and they are acknowledged and referenced.

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Presentations from this Dissertation

Rampersad T, Makume M.T and Sturm A.W. 2014. A novel high throughput methodology for *Mycobacterium tuberculosis* drug susceptibility testing, using multipoint inoculation.

- 2nd Annual College of Health Sciences Symposium, 11 – 12 September 2014, Poster presentation

Acknowledgements

My acknowledgements will be followed according to a Sanskrit phrase, “Matha Pitha Guru Deivem” which translates to Mother Father Teacher God.

I would firstly like to express my very profound gratitude to my parents (Renu and Vinesh Rampersad) for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this dissertation. This accomplishment would not have been possible without them. Thank you.

Secondly, I would like to sincerely thank my supervisor Professor A.W. Sturm for his immense patience, focused guidance, precious time and heart-warming support throughout the years of study. Dank je.

I would like to thank God for spiritual guidance and strength throughout my years of study. Om namo Narayana! Jai Matha Di!

My sincere thanks go to Dr. Mantha Makume and Dr. Sobia Parveen, for their unfailing assistance, training and support through the process of this study. Thank you very much!

My heart-felt thanks go to my only brother and sister-in-law (Nishal and Kaajal Rampersad), my dear friend (Rikayal Boodram) and a host of family and friends that are too many to mention by name but equally hold an immense importance in my life, for support and encouragement. Thank you.

The staff and students at the department of Infection, Prevention and Control – Medical Microbiology, holds my utmost gratitude for assisting and training me where it was needed. Thank you.

Lastly, I would like to thank the National Research Foundation and the College of Health Sciences for the financial support throughout my years of study.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ANTI-TB	Anti-tuberculosis
CFU	Colony Forming Units
CI	Confidence Interval
DMF	Dimethylformamide
DNA	Deoxyribonucleic Acid
DST	Drug Susceptibility Test
GEE	Generalized Estimating Equation/s
HIV	Human Immunodeficiency Virus
ICC	Intra-class correlation
IGRA	Interferon- γ Release Assay
MABA	Microplate Alamar Blue Assay
MDR	Multi Drug Resistant
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MODS	Microscopic Observation Drug Susceptibility
MRD	Multiple Replicating Device
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

NRA	Nitrate Reductase Assay
OADC	Oleic Acid-Albumin-Dextrose-Catalase
PAS	p-Aminosalicylic Acid
PCR	Polymerase Chain Reaction
QPM	Quadrant Plate Method
REMA	Resazurin Microplate Assay
SA	South Africa
SDS	Sodium Dodecyl Sulphate
TB	Tuberculosis
WHO	World Health Organization
XDR	Extensively Drug Resistant

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Ethics

This study was approved by the Biomedical Research Council of the University of KwaZulu – Natal under ethics number **BCA274/09**.

ABSTRACT

Chapter 1 of this dissertation entails the introduction, aims, objectives and the literature review. Drug susceptibility testing of *Mycobacterium tuberculosis* is time consuming and expensive. Multi-point inoculation offers the advantage of testing multiple isolates on a series of solid media with a single breakpoint concentration of a drug in each plate or a series of different drug concentrations of one drug. We aimed to determine the reproducibility of MIC determination for anti-TB drugs of *M. tuberculosis* isolates using agar dilution with multi-point inoculation and thereafter validating the results by comparing it to classic agar dilution on quadrant plates and the MTT assay.

Chapter 2 contains the manuscript that has been submitted for publication. This manuscript contains a brief introduction with the aim and objectives, and a detailed description of the methodology, results and discussion. Thirty *M. tuberculosis* isolates were grown in Middlebrook 7H9 broth with 20% Tween until mid-log phase was reached. Agar dilution MICs were determined on Middlebrook 7H10 agar for 11 anti-TB drugs at concentrations ranging from 128 to 0.125 mg/L. The agar plates were inoculated using a multi-point inoculation device with 36 points each delivering 1 μ L of a suspension of 1×10^4 cfu/ml. For the quadrant plate method and the MTT assay 100 μ l of the same suspension was used. All tests were done 3 times in triplicate. Agar dilution with multi-point inoculation was found to be reproducible within the 11 anti-TB drugs tested and correlated well with agar dilution on quadrant plates and the MTT assay for the three anti-TB drugs tested.

Chapter 3 entails a brief summary (synthesis) of the discussion found in the above-mentioned manuscript. The multi-point inoculation method has potential for wide scale application in breakpoint drug susceptibility testing as well as MIC testing of *M. tuberculosis* isolates. Lastly this dissertation contains the required references and appendices.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.0 Introduction

Mycobacterium tuberculosis is the causative agent of the highly infectious disease tuberculosis (TB), which can occur in the lungs (pulmonary TB) and other areas of the human body (extrapulmonary TB) (1). The spread of pulmonary TB usually occurs by the air-borne route through expulsion of bacteria through coughing or sneezing by individuals infected with *M. tuberculosis* (1). Immuno-compromised individuals are at a higher risk of contracting TB. Therefore, individuals infected with the human immunodeficiency virus (HIV), contract TB at higher rates than those that are HIV uninfected (1, 2).

Aim

To determine the reproducibility of MIC determination for anti-TB drugs of *M. tuberculosis* isolates using agar dilution with multi-point inoculation and thereafter validating the results by comparing it to agar dilution on quadrant plates and the MTT assay.

Objectives

- 1) To establish MICs of 11 anti-TB drugs using 30 *M. tuberculosis* isolates with varying resistance profiles by means of agar dilution using multi-point inoculation
- 2) To validate the MICs of agar dilution using multi-point inoculation with agar dilution on quadrant plates and the MTT assay.

1.1 Tuberculosis statistics

Globally, TB is the leading cause of death by a communicable disease (1). Of new cases of TB approximately 90% occur in adults and 10% children. In 2015, the estimated number of people that were infected with *M. tuberculosis* was 10.4 million worldwide and 1.8 million of those died from the disease (1). The heaviest burden of TB is seen in parts of the world like Asia, the Indian subcontinent and Sub-

Saharan Africa, due to their population sizes, low socio-economic standing and overall poor health (1, 3, 4).

In the 17th century, TB was introduced in South Africa (SA) by European immigrants, arriving predominately from Britain and Holland (2). At that time, Europe was experiencing a TB epidemic. SA's mining industries began to develop late in the 19th century and favored the spread of infection which happened at a fast rate (2, 5). The miners were exposed to unfortunate working conditions within the mines, such as enclosed environments with silica dust exposure. Furthermore, they lived in congested hostels and suffered from malnutrition (2), both promoting the transmission of *M. tuberculosis* bacilli. The mine workers that were infected with TB were sent home, generally located in rural areas. This would ensure the distribution of TB to other members of their families (2). This cycle of TB infection still occurs presently.

Incidence rates of TB increased steadily during the 20th century, due to factors related to the apartheid policy, whereby a poorly equipped health sector was available to tackle the TB epidemic among the black population (2). Standard treatment for TB was implemented in SA by the administration of anti-TB drugs isoniazid, streptomycin and para-amino-salicylic acid, during a period of 12-18 months (2, 6). The duration of treatment was reduced to 6 months with the introduction of rifampicin and pyrazinamide, due to pyrazinamide being known to kill bacilli (containing low metabolic activity) that are not normally killed by other drugs (7), but treatment of TB with various anti-TB drugs can take up to 6 months to 2 years because it is dependent on the type of infection i.e. if susceptible or drug-resistant strains of *M. tuberculosis* are present in patients with TB (3). Incorrect use of anti-TB drugs, high prevalence of HIV/AIDS and delays in diagnosing TB gave in the last half of the 20th century rise to multi-drug resistant TB (MDR-TB) (8) and this threatened the efficacy of the TB control program (6). The situation became even worse with a further development into XDR-TB (9). This occurrence has been seen world-wide (1).

There are different levels of resistance i.e. high, intermediate and low levels of resistance, which can be seen in susceptible, MDR and XDR strains of *M. tuberculosis*. The levels of resistance affect treatment regimens of TB in that the duration of treatment, the dosage of drugs and the variety of drugs changes accordingly. These factors bring about the need for reliable and rapid drug susceptibility tests so that correct TB drug regimens can be given to the infected patients.

1.2 Diagnosis of Tuberculosis

1.2.1 Clinical Diagnosis

There are many methods of diagnosing TB. These can be separated in 2 categories depending on whether the patient has latent or active TB. Latent TB can be detected by the Mantoux test and the Interferon- γ test, whereas methods applied to diagnose active TB depend on where the site of infection is. These include physical examination, the posterior-anterior chest X-ray, computed tomography, magnetic resonance imaging, ultrasound, echocardiogram and intravenous urography (10). These techniques are outside the scope of this dissertation.

1.2.2 Laboratory Diagnosis

Laboratory detection of *M. tuberculosis* is performed by smear microscopy and culture, which forms the WHO approved gold standards (11). Smear microscopy involves staining sputum specimens with an acid-fast staining technique (12) like Ziehl-Neelsen and auramine staining. Culture methods involve inoculation of decontaminated specimens from the site of infection on culture media that promote the growth of *M. tuberculosis*. These include Middlebrook 7H11 and 7H10 agars and 7H9 broth, Lowenstein-Jenson slants (11, 12) and several others. While smear microscopy is rapid with a good specificity, its sensitivity is low. Culture based methods are highly specific and highly sensitive but have a turnaround time of 3 to 8 weeks. Because culture is more demanding and costly, low-income countries rely mainly on smear microscopy (11, 13). There are however, newer diagnostic methods that offer shorter turnaround times but are either too expensive for resource-limited countries or require expertly trained staff to conduct these (14, 15).

Other methods of laboratory detection of TB include, the MGIT 960, the BacT/ALERT 3D and ESP culture system II which are automated liquid culture systems that uses non-radiometric detection methods to measure growth by monitoring gas pressure fluctuations, i.e. consumption of oxygen or production of carbon dioxide, and these can be measured fluorimetrically or colorimetrically (16).

The Interferon- γ release assays (IGRA's) are able to detect *M. tuberculosis* infection in vitro by detecting interferon- γ that are released by T-cells upon exposure of *M. tuberculosis* antigens (17, 18). There have been claims that IGRA's can differentiate between active and latent TB, but this is not entirely true as they have shown to contain poor sensitivities in patients that are heavily burdened with *M. tuberculosis* infection or immuno-compromised, thereby making it impossible to see differences in TB infection from disease (17, 18).

1.3 Drug susceptibility tests

Drug susceptibility tests (DSTs) for *M. tuberculosis* are performed to examine drug resistance within isolates and to provide guidance in the treatment of TB (3, 19). Like for all bacteria, methods of DSTs for *M. tuberculosis* can be separated into two categories, i.e. phenotypic and genotypic DSTs. Phenotypic DSTs include the culture-based methods on solid media such as the proportion method, resistance ratio and absolute concentration method (14, 20) and methods that use liquid media with growth indicators. These include colorimetric assays such as the Microplate Alamar Blue Assay (MABA), the Resazurin Microplate Assay (REMA), the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the Nitrate Reductase Assay (NRA). Non-colorimetric assays are the Microscopic Observation Drug Susceptibility (MODS) assay, the MGIT 960 DST, the BacT/ALERT 3D DST and ESP culture system II DST (14). The genotypic DST's include the Cepheid GeneXpert system and line probe assays.

1.3.1 Phenotypic drug susceptibility testing

The conventional culture-based methods involve a series of anti-TB drugs used against *M. tuberculosis* that are incorporated in a solid medium, usually Middlebrook agar or Lowenstein-Jenson media at the critical concentration for the drug tested (21). Media are inoculated with a standard inoculum of *M.*

tuberculosis, incubated at 35 – 37°C, thereafter being observed if growth or inhibition occur by comparing with a drug-free control (19). These methods are cheap and reliable but are also labour intensive and rely on visualizing the growth of *M. tuberculosis*, which takes up to 3-6 weeks (14, 20). The colorimetric assays mentioned above can be used for detecting *M. tuberculosis* and be used for drug susceptibility testing. The MABA utilizes an oxidation-reduction dye called alamar blue, which is an indicator of cellular viability (22). The blue dye is a non-flourescent compound in its oxidized form, that changes into a pink flourescent, reduced form, in the presence of viable cells (22). The REMA on the other hand uses the salt resazurin, which is reduced in the presence of viable *M. tuberculosis* bacilli. This results in a colour change from blue to pink (23). The MTT assay detects bacterial dehydrogenase, found in viable cells. This enzyme reduces MTT which turns from a yellow soluble substance into purple, insoluble formazan crystals. The NRA takes advantage of the ability of *M. tuberculosis* to reduce nitrate into nitrite, whereby this reduction reaction is determined by the addition of a colour-changing reagent called the Griess reagent (24). Drug susceptibility tests of the above kind are performed in liquid media in microtitre plates and colour changes are observed visually or measured by means of a spectrophotometer (22-24). Although the above-mentioned colorimetric assays yield faster and reliable results, they also carry a biohazard risk with the formation of aerosols (14).

The principle of the Microscopic Observation Drug Susceptibility assay relies on microscopic observation of the cord formation displayed by *M. tuberculosis* which can be viewed at an early stage by microscopy. Drug susceptibility testing can be done by comparing growth in medium without an anti-TB drug with media that contain drugs at the critical concentration for this method (25, 26). This assay is also rapid and cheap but needs experienced and specifically trained staff to conduct (13, 25, 26). It is also prone to contamination.

Drug susceptibility tests using the MGIT 960, the BacT/ALERT 3D and ESP culture system II can be also done, together with it being diagnostic methods. They are known to confer rapid and reliable results but require the use of expensive equipment and expertly trained staff, which makes it unfavourable for developing countries (14, 16, 27).

1.3.2 Genotypic drug susceptibility testing

The Cepheid GeneXpert System is an automated system that carries out molecular tests for tuberculosis case detection, together with its associated DST (28). The Cepheid GeneXpert System detects rifampicin resistance in *M. tuberculosis* by the use of nested real-time PCR. Rifampicin resistance is used as a marker for MDR since it is almost always combined with isoniazid resistance (29, 30). The *rpoB* gene is amplified, and probed with molecular beacons specific for mutations in the rifampicin-resistant determining region (28). The system utilizes a disposable plastic cartridge, which contains all the reagents needed for processing of the specimen and PCR. The addition of a specific bactericidal buffer in this process is the only manual step undertaken. The cartridge is then inserted into the GeneXpert device and results are retrieved within 2 hours (28).

Line probe assays e.g. INNO-LIPA Rif TB (Innogenetics, Ghent, Belgium) and Genotype MTBDR plus assay (Hain Life Sciences, Nehren, Germany) are utilized for the detection of resistance conferring mutations in a variety of genes. It is based on the principle of solid-phase hybridization, whereby PCR is used containing biotinylated primers that amplify the target DNA. The biotin is incorporated into the amplification product. These are then denatured and hybridized to capture probes attached to the solid phase. During the gel electrophoresis that follows, the amplicons migrate over the solid phase. If amplicon reaches a matching probe, it binds in that spot. Color detection is then followed by adding a conjugate which is streptavidin bound to alkaline phosphatase. This enzyme converts the chromogenic substrate into an insoluble product; this forms a colored line at the site where probe and amplicon have bound (31).

Although these genotypic methods seem rapid and efficient, there are also major drawbacks in that they are expensive, detect only identified resistance conferring mutations, require expensive equipment and expertly trained staff and detect both viable and dead *M. tuberculosis* (13, 14, 28).

1.4 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is the minimal concentration of an antimicrobial that is able to inhibit the growth of a target organism. Susceptibility testing of organisms to antimicrobials using MIC

determination is done to establish if a particular organism is either susceptible or resistant to a particular drug and to determine trends in resistance development (32). Diagnostic laboratories use MICs as a confirmation of unusual resistance in organisms and to establish a definitive answer when results from other tests are borderline (32). Minimum inhibitory concentration testing can be done to determine the efficiency of a particular drug for an entire species. If MIC tests are done for a group of isolates of one particular species then the MIC50 and MIC90 of that species can be calculated (33). MIC50 and MIC90 requires that the number of isolates to be tested results in a statistically significant outcome. The MIC50 is the concentration of drug that inhibits 50% of the isolates and MIC90 inhibits 90% of the isolates (33). The MIC obtained with a particular method is influenced by the differences in inoculum size, the composition of media (solid or liquid), pH and incubation times (7, 19, 34). It is also understood that with different methods of determining MICs, there will be a difference in the MIC values. MICs of *M. tuberculosis* isolates on solid media are, for many drugs higher than in liquid media (34). In this study the determination of MIC's of various anti-TB drugs against *M. tuberculosis* was done with three different DST's, therefore the understanding of factors that influence the outcome is of crucial importance.

1.5 Multi-point Inoculation

1.5.1 The origin of the Multiple Replicating Device

Microbiological procedures often require the sequential transfer of inocula of multiple isolates onto plates or into tubes (35). These procedures are generally performed manually, which can be tedious and prone to error. This brought about the development of multiple replicating devices (MRDs) that helped to circumvent these challenges and improve precision (35). The first MRD was described by Garret in 1946, where he constructed his very own 10-point inoculating device that had the ability to inoculate agar plates at 10 separate points (35). The use of this device shortened the inoculation time to 4 hours for 65 plates, giving rise to newer and more practical MRDs (35). Another device was developed and described by Tarr in 1958 which had the ability to inoculate up to 25 drops of bacteriophage onto agar plates seeded with *Staphylococcus aureus*, simultaneously (35). More mechanized features (Figure 1) of the MRD device were developed for bacteriophage typing (35).

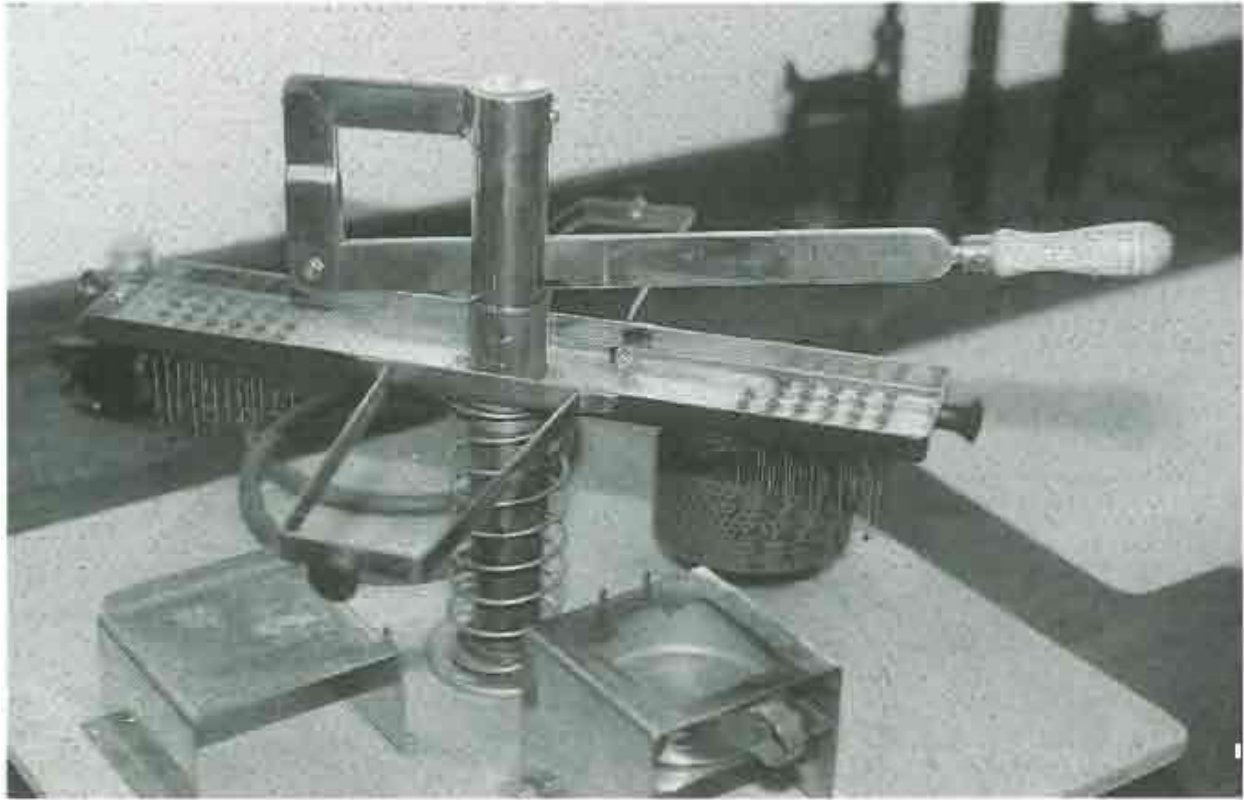


Figure 1: Image showing a multiple replicating device (MRD) modified by Lidwell (35)

Multiple replicating devices with a micro-titre format also have been developed. These contain 96 stainless steel pins. Multiple replicating devices can be used on semi-solid and solid agar plates for assays like gas detection, biochemical and motility tests of microorganisms (35). It was noticed that the pins could take up approximately 6 μ l of culture and deliver 2 μ l onto the agar plates and when this procedure was compared to conventional methods; it utilized only 5% of materials and only consumed 10% of time (35). Their major applications include antimicrobial susceptibility testing of various isolates of microorganisms and screening of compounds that can be used for antimicrobial activity (35). When testing different bacterial isolates against streptomycin, Steers et al, developed an aluminum-based 36-pin device for antimicrobial susceptibility testing (35, 36). An image of the device can be seen below (Figure 2)

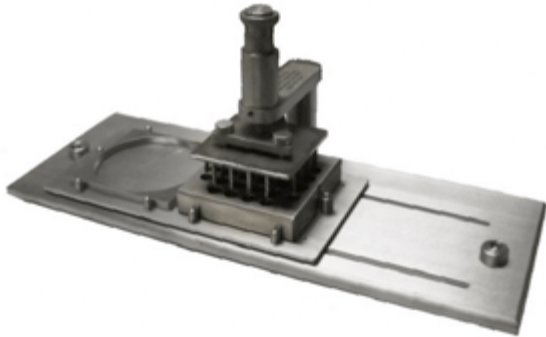


Figure 2: Image showing a multiple replicating device (MRD) modified by Steers et al.⁽³⁶⁾

1.5.2 Use of Multi-point Inoculation with various bacterial species

Multi-point inoculation techniques have been used with species of the family of *Enterobacteriaceae* for inoculation of biochemical and metabolic test media used in identification systems as well as for inoculation of drug susceptibility tests (37). When compared to conventional methods of susceptibility testing such as disc diffusion methods and test tube methods, the multi-point inoculation technique saves time, consumable costs and labor (37). The technique has been demonstrated on clinical isolates with rapid results which is good because in clinical settings, quick identification and DST of the infecting microorganism allows for the clinician to know which antibiotic regimen to administer or to adjust an empirically started regimen. It also can alert infection control teams for potential outbreaks (38).

Agar dilution with multi-point inoculation is a phenotypic culture-based method for determination of MICs that is able to test a large number of isolates at a time, using less materials and cheap equipment (35).

Chapter 2: Submitted Manuscript

Manuscript Reference number: JCM01176-17

The development and application of a high throughput methodology to determine MICs of
Mycobacterium tuberculosis isolates against antimicrobial agents

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ABSTRACT

Drug susceptibility testing (DST) of *Mycobacterium tuberculosis* is time consuming and expensive. With multi-point inoculation, multiple isolates can be tested on solid media with a single breakpoint concentration or a series of different drug concentrations to establish the MIC. This reduces consumables and labour costs. Multi-point inoculation has not been used with *M. tuberculosis*. Since its characteristic clumping could influence reproducibility of DST using multi-point inoculation, we performed MICs for anti-TB drugs of *M. tuberculosis* isolates using this inoculation technique. The results were compared with classic agar dilution on quadrant plates and with the MTT assay.

Thirty *M. tuberculosis* isolates were grown in Middlebrook 7H9 broth with 20% Tween until mid-log phase. Agar dilution MICs were determined on Middlebrook 7H10 agar for 11 anti-TB drugs at concentrations ranging from 128 to 0.125 mg/L. The agar plates were inoculated using a multi-point inoculation device with 36 points each delivering 1 µL of a suspension of 1×10^4 cfu/ml. For the quadrant plate method and the MTT assay 100 µl of the same suspension was used. All tests were done three times in triplicate.

Agar dilution with multi-point inoculation was found to be reproducible with the 11 anti-TB drugs and correlated well with agar dilution on quadrant plates and the MTT assay for the three anti-TB drugs tested.

The multi-point inoculation method has potential for wide scale application in breakpoint DST as well as MIC testing of *M. tuberculosis* isolates.

INTRODUCTION

Tuberculosis (TB) is the leading cause of death by communicable diseases (1). In 2015, the estimated number of people that were infected with *Mycobacterium tuberculosis* was 10.4 million worldwide and 1.8 million of those died from the disease (1). Developing countries bear the heaviest burden with Asia and Africa accounting for 61% and 26% respectively of the number of new cases (2). TB is also the leading killer of people infected with HIV, with 35% of HIV related deaths attributed to this infection (2).

The emergence of multidrug-resistant (MDR) strains threatens the efficacy of TB control programs worldwide (3). Factors associated with MDR-TB are HIV infection, control programs not being followed appropriately and the delay or absence of laboratory diagnosis. In many under resourced countries the diagnosis of TB relies solely on microscopy where drug-susceptibility testing (DST) is not done. With classic DST the results become available with considerable delay. To control drug resistant TB, faster and cheaper methods are needed (4).

There are many DST methods available. The gold-standard include culture-based methods such as the proportion method, resistance ratio and absolute concentration method (4,5). These methods are labour intensive and rely on visualizing growth of *M. tuberculosis* which takes up to 3 – 6 weeks (5). There are a number of colorimetric assays that use reagents which change colour, indicative of growth. These tests include the Microplate Alamar Blue Assay (MABA), the Resazurin Microplate Assay (REMA), the 3(4,5-

dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the Nitrate Reductase Assay (6). The Microscopic Observation Drug Susceptibility (MODS) assay is a rapid and cheap technique however it needs experienced and trained staff to conduct (7-9). All of these are rapid assays but they pose a biohazard risk with the formation of aerosols (4). The MGIT 960, the BacT/ALERT 3D and ESP culture system II are automated liquid culture systems that use expensive equipment, which is not suitable for under-resourced countries (4,10).

Genotypic DSTs such as the Cepheid GeneXpert system and line probe assays have many drawbacks in that the reagents as well as the required equipment are expensive, detect only identified resistance conferring mutations, require expertly trained staff and detect both viable and dead *M. tuberculosis* (4,11,12).

The above mentioned drawbacks of DSTs, bring about the need for cheaper and less labor intensive procedures. Agar dilution with multi-point inoculation is a phenotypic culture-based method that is able to test a large number of isolates at a time, using less materials and cheap equipment (13). We report on the reproducibility of agar dilution with multi-point inoculation and compare this method with agar dilution on quadrant plates and MTT.

METHODOLOGY

Inoculum preparation

Thirty *Mycobacterium tuberculosis* strains with known resistance profiles were used in this study. The resistance conferring genes of these isolates have been sequenced previously (14). Two strains were fully susceptible, thirteen were MDR and fourteen were XDR. H37Rv was included as a susceptible control. Strains were inoculated in 10 ml Middlebrook 7H9 broth containing 10% oleic acid-albumin-dextrose-catalase (OADC) (Difco Laboratories, Detroit, Mich.), 0.2% glycerol and 0.05% Tween 80 (Appendix A).

The broths were incubated at 37°C in a shaking incubator until an OD_{600nm} reading of 0.7 – 1 was reached. Each strain was sonicated at 5Amps for 20 seconds (Misonix Sonicators) and left undisturbed on the bench at room temperature for 15 to 20 minutes to allow remaining clumps to settle. The top-layer was then siphoned off and diluted till an OD_{600nm} reading of approximately 0.7. Ethics approval for the study was granted by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (BCA274/09).

Agar-dilution MICs with multi-point inoculation

Agar dilution with multi-point inoculation was used to determine the MIC of *M. tuberculosis* isolates for isoniazid, rifampicin, ethambutol, ethionamide, streptomycin, kanamycin, amikacin, capreomycin, ciprofloxacin, ofloxacin, and clofazimine. Double dilutions of the drugs were made in sterile OADC (10%) enriched Middlebrook 7H10 agar supplemented with casitone (5.3%) and glycerol (0.2%) (Appendix A). The concentrations ranged from 128 mg/L to 0.125 mg/L for all 11 drugs. The agar containing the drug dilutions was poured into 90 mm Petri dishes and allowed to solidify.

The strains were inoculated onto the plates using a Steers replicator, delivering approximately 10 cfu per spot. The plates were sealed in gas-permeable plastic bags and incubated in a regular incubator at 37°C for 21 days.

Confirmation tests

To validate the results obtained with multi-point inoculation, two established methods for MIC determination were performed with a selection of the strains using three of the antimicrobial drugs: isoniazid, streptomycin and kanamycin. These methods were agar dilution on quadrant plates (QPM: quadrant plate method) and micro-broth dilution with MTT growth detection.

The QPM differed from the multi-point inoculation method in that the surfaces of the agar quadrants were flooded with 100µL of the bacterial suspension (approximately 1×10^3 cfu/quadrant). The plates were sealed in gas-permeable plastic bags and incubated in a regular incubator at 37°C for 21 days.

For the MTT, a micro-broth dilution test was performed using the same drug concentrations as for the agar dilution methods in 100µL volumes using Difco™ Middlebrook 7H9 broth containing 10% OADC (Difco Laboratories, Detroit, Mich.), 0.2% glycerol and 0.05% Tween 80. Hundred microliter of bacterial suspension was added to the wells containing the drug dilutions (approximately 1×10^3 cfu/100µL), followed by incubation at 37°C for 7 days. All tests were performed in flat-bottom 96 well plates. After this incubation period, 20µl of the MTT solution (5µl/mg) (Appendix B) was added to each well followed by incubation for a further 24 hours at 37°C. Purple precipitates were then observed to indicate growth. A 1:1 solution containing 20% sodium dodecyl sulphate (SDS) and 50% dimethyl formamide (DMF) (Appendix B) was added to the wells to dissolve the precipitate to facilitate reading (15).

Statistical analysis was done using an ordinal regression model (GEE). An intra-class correlation (ICC) of 1 indicates the replicates are identical. An ICC greater than 0.8 indicates good intra-class correlation.

RESULTS

Table 1 shows the results of MIC determination of 30 *M. tuberculosis* isolates against 11 anti-TB drugs using agar dilution with multi-point inoculation. The test was performed three times in triplicate in order to determine reproducibility. Identical results were found with streptomycin, kanamycin and amikacin (ICC=1; $p < 0.001$). Poor reproducibility was found with ethambutol (ICC=0.4; $p=0.7$). The ICC values for the seven other drugs showed good correlation with ICC values varying between 0.95 and 0.8.

To validate the results, 11 of the isolates in triplicate were tested against isoniazid, streptomycin and kanamycin, comparing the agar dilution with multi-point inoculation with classic agar dilution in

quadrant plates and with the MTT test. The results are shown in table 2. The multi-point inoculation technique showed similar results as those presented in Table 1. ICC values for isoniazid obtained with the quadrant plate technique were within the satisfactory range ($p=0.03$) and were good for the other two drugs. However, MICs obtained were 2 to 4 dilutions higher than with the multi-point inoculation. The MTT test results were highly reproducible for all 3 drugs. The MICs obtained with this method were lower than with the quadrant plate method but higher than with multi-point inoculation.

DISCUSSION

Multi-point inoculation using a Steers replicator is a well-established method to inoculate agar plates with multiple bacterial isolates (16). It is mostly used in determining resistance profiles of fastidious organisms that need prolonged incubation. Incubation beyond 16 to 24 hours diminishes the reliability of disc diffusion susceptibility testing. Since *M. tuberculosis* is a slow growing organism, incorporation of the test drugs in agar or broth is applied for its susceptibility testing. If agar is used, the plates are inoculated with a suspension of bacteria which are evenly distributed over the surface. Multi-point inoculation allows for testing of multiple strains on one agar plate. The technique is used for the determination of MICs of fastidious organisms like *Neisseria gonorrhoeae* and strict anaerobes. We applied this inoculation method with *M. tuberculosis* and showed good reproducibility with most of the drugs tested. Only ethambutol showed poor correlation between tests. This is not surprising because ethambutol is known to give inconsistent results in most tests (4, 17).

We then compared the MICs of three drugs obtained with multi-point inoculation with the results of two other methods. The multi-point inoculation technique results shown in table 2 were similar to the previous results (table 1), which supported the conclusion that the test is reproducible. It is well known that different methods result in differences in MIC values therefore; the differences between the 3 tests can be explained by the differences in the inoculum sizes. In both the agar dilution on quadrant plates and the

MTT assay, the inoculum size was approximately 1×10^4 cfu/100 μ L, whereas in the multi-point inoculation technique the inoculum size was approximately 10 cfu per spot. The Steers replicator makes use of pins that deliver approximately 1 μ L of the inoculum onto the agar plates. Therefore the inoculum sizes in both the agar dilution on quadrant plates and the MTT assay is approximately 100 times more than with the multi-point inoculation technique. Inoculum sizes are known to affect the outcome of drug susceptibility tests (18-20).

Another point to note about the differences in the MICs between the 3 tests is that the two agar dilution methods use solid media, as opposed to the MTT assay that uses liquid media. The critical concentrations of the anti-TB drugs differ in liquid and solid media (18,21). MICs on solid media, for many drugs are higher than in liquid media (18). The points above could be the reason that the MTT assay MIC results were lower than with the quadrant plate method but higher than the multi-point inoculation MICs.

There are many advantages of agar dilution with multi-point inoculation as compared to the conventional agar dilution on quadrant plates, such as the Steers replicator used in the multi-point inoculation technique contains 36 pins and wells, which in turn can test 36 different isolates against 1 drug concentration at a time. The time taken to conduct an MIC with 12 dilutions using the multi-point inoculation technique on 30 isolates against 1 drug in triplicate was approximately 1 hour, as compared to the conventional agar dilution on quadrant plates in triplicate, took approximately 9 hours. The preparation of media for the two methodologies was significantly different as well, where it was a much longer and tedious process to make media for the quadrant plate method as compared to the multi-point inoculation method. These advantages of the multi-point inoculation method ultimately indicate that it is a highly efficient and reproducible drug susceptibility test for *M. tuberculosis* isolates.

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Table 1: MIC of 30 *M. tuberculosis* isolates against 11 anti-TB drugs using agar dilution with multi-point inoculation

	No. of isolates	Median	Range	ICC	95% CI	P - Value
Isoniazid	27	16	8 - 32	0.8	0.7 to 0.9	<0.001
Streptomycin	30	16	0.5 - 128	1	0.9 to 0.9	< 0.001
Rifampicin	29	128	2 - 128	0.99	0.9 to 0.9	<0.001
Ethambutol	29	32	4 - 32	0.4	0.06 to 0.7	0.731
Ethionamide	30	64	2 - 128	0.9	0.8 to 0.9	< 0.001
Kanamycin	30	4	1 - 128	1	0.9 to 0.9	<0.001
Amikacin	30	4	0.5 - 128	1	0.9 to 0.9	< 0.001
Capreomycin	30	4	0.5 - 32	0.95	0.9 to 0.9	< 0.001
Ciprofloxacin	30	2	0.125 - 4	0.9	0.8 to 0.9	< 0.001
Ofloxacin	30	1	0.5 - 4	0.89	0.8 to 0.9	< 0.001
Clofazimine	27	1	0.125 - 16	0.8	0.6 to 0.9	< 0.001

Difference in no. of isolates for isoniazid, rifampicin, ethambutol and clofazimine is due to a few isolates being contaminated

Table 2: Comparison of MICs of 11 *M. tuberculosis* isolates determined by three methods.

		Isoniazid	Streptomycin	Kanamycin
Multi-point Inoculation	Median (Range)	32 (16-32)	4 (2-128)	4 (4-128)
	ICC (95% CI)	0.89 (0.7-0.9)	0.99 (0.9-0.9)	0.99 (0.99-0.99)
	P - value	<0.001	<0.001	<0.001
Agar dilution on quadrant plates	Median (Range)	128 (128-128)	128 (8-128)	16 (8-128)
	ICC (95% CI)	0.75 (0.48-0.92)	0.99 (0.99-0.99)	0.99 (0.98-0.99)
	P - value	0.03	<0.001	<0.001
MTT Assay	Median (Range)	128 (32-128)	8 (2-128)	8 (4-128)
	ICC (95% CI)	0.97 (0.9-0.99)	1.0 (0.99-0.99)	0.99 (0.97-0.99)
	P - value	<0.001	<0.001	<0.001

Chapter 3: Synthesis

Multi-point inoculation using a Steers replicator is a well-established method to inoculate agar plates with multiple bacterial isolates (36). We applied this inoculation method with *M. tuberculosis* and showed good reproducibility with most of the drugs tested. Only ethambutol showed poor correlation between tests. This is not surprising because ethambutol is known to give inconsistent results in most tests (14, 39).

We then compared the MICs of three drugs obtained with multi-point inoculation with the results of two other methods. The multi-point inoculation technique results shown in table 2 of chapter 2 were similar to the previous results (Table 1), which supported the conclusion that the test is reproducible. It is well known that different methods result in differences in MIC values therefore; the differences between the three methods can be explained by the differences in inoculum size. In both the agar dilution on quadrant plates and the MTT assay, the inoculum size was approximately 1×10^3 cfu/100 μ L, whereas in the multi-point inoculation technique the inoculum size was approximately 10 cfu per spot. The Steers replicator makes use of pins that deliver approximately 1 μ L of the inoculum onto the agar plates. The volume of inoculum used in both the agar dilution on quadrant plates and the MTT assay is 100 μ L. Therefore, the inoculum size in both these methods is approximately 100 times higher than with the multi-point inoculation technique. Inoculum sizes are known to affect the outcome of drug susceptibility tests (7, 19, 34).

Another point to note about the differences in the MICs between the three tests is that the two agar dilution methods use solid media, as opposed to the MTT assay that uses liquid media. The critical concentrations of the anti-TB drugs differ in liquid and solid media (39, 40). MICs on solid media are for many drugs higher than in liquid media (39). The points above could be the reason that the MTT assay MIC results were lower than with the quadrant plate method but higher than the multi-point inoculation MICs. The overall limitations of the study can be seen by the fact that the number of isolates that were tested was less if compared to the number of isolates that the DST methods can accommodate. Also, the *M. tuberculosis* isolates used was stored isolates and not direct clinical isolates. An extension of this study is possible where routine DSTs for MIC testing can be used, e.g. the currently established MGIT 960 DST, together with the multi-point inoculation method can be compared.

The advantages of agar dilution with multi-point inoculation compared to agar dilution on quadrant plates is the ability to test 36 different isolates against 1 drug at a time resulting in a shorter time period to conduct the tests. Therefore, multi-point inoculation is less labor intensive. The multi-point inoculation method proved to be a highly efficient and reproducible drug susceptibility test method for *M. tuberculosis* isolates.

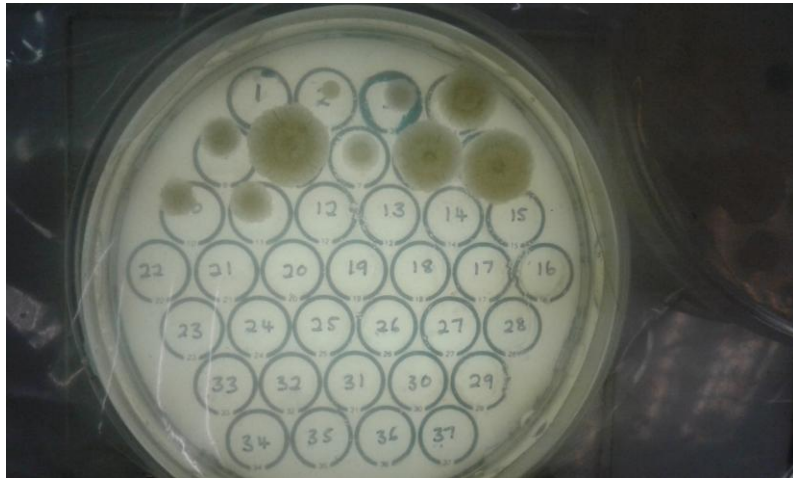


Figure 3: Image showing a multi-point inoculation plate for 11 *M. tuberculosis* isolates on Middlebrook 7H10 agar (control plate i.e. contains no drug)

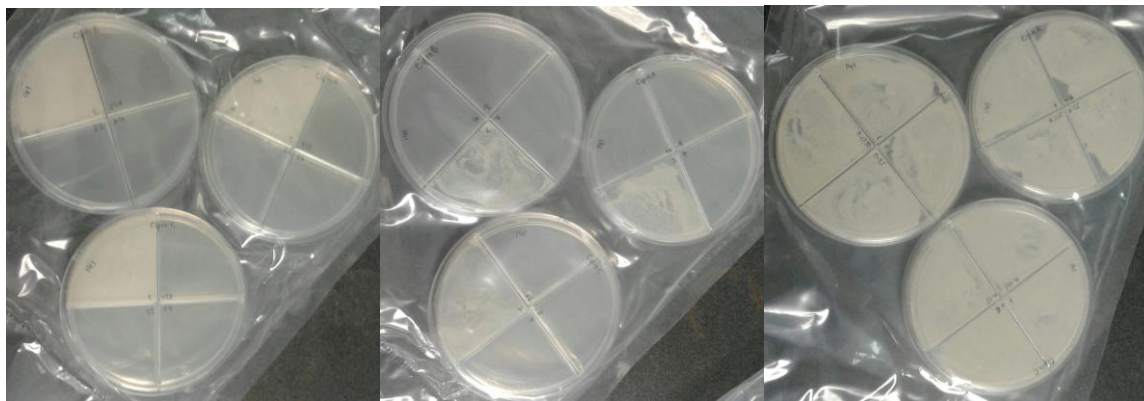


Figure 4: Images showing the quadrant plate method for one *M. tuberculosis* isolate on Middlebrook 7H10 agar with one drug.

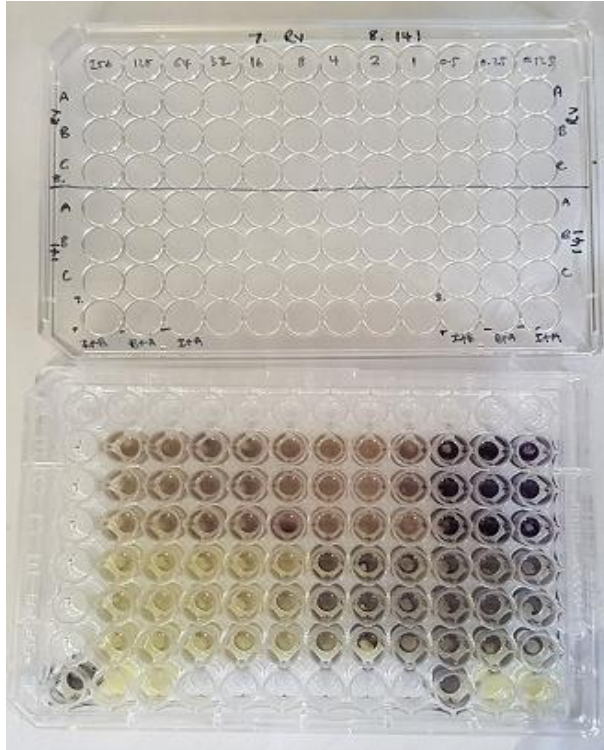


Figure 5: Image showing a MTT assay plate containing 2 *M. tuberculosis* isolates for one drug

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APPENDIX A

A.1.1 Middlebrook 7H9 Broth

4.7g Middlebrook 7H9 powder

2ml glycerol

0.5ml Tween 80

900ml distilled water

The above materials were mixed and autoclaved at 121°C for 15 minutes

100ml OADC was added once the media was cool.

A.1.2 Middlebrook 7H10 Agar

19g Middlebrook 7H10 powder

1g casitone

5ml glycerol

900ml distilled water

The above materials were mixed and autoclaved at 121°C for 15 minutes

100ml OADC was added once the media was cool.

A.1.3 Formula for drug potency for highest concentration of drug

Drug Potency (mg/L) = Highest concentration required (mg/L) × Volume (mL) / Potency

A.1.4 Amount of powder (drug) required

Mass of drug (g) = Drug Potency (mg/L) × amount of media (mL) × dilution factor × amount of stock solution required (mL)

APPENDIX B

B.1: MTT Assay reagents

B.1.1: 20% Sodium Dodecyl Sulphate

4g Sodium Dodecyl Sulphate was dissolved in distilled water to a volume of 20ml.

B.1.2: 50% Dimethylformamide

10ml Dimethylformamide

10ml distilled water

The two reagents were mixed.

B.1.3: MTT Solution

0.075g MTT powder was dissolved in Phosphate Buffer Saline to a volume of 15ml.