

**THE DEVELOPMENT AND IMPLEMENTATION  
OF A MYCOBACTERIUM TUBERCULOSIS  
RAPID DIAGNOSTIC ASSAY, USING REPORTER  
MYCOBACTERIOPHAGES**

by

**MANTHA THANDIWE MAKUME**

A thesis submitted in partial fulfilment of the requirements of the degree of

**DOCTOR OF PHILOSOPHY IN MEDICINE**

in the

**DEPARTMENT OF INFECTION PREVENTION AND CONTROL**

Nelson R. Mandela School of Medicine

College of Health Sciences

School of Laboratory Medicine

University of KwaZulu-Natal

Durban

South Africa


2013

## Declaration

Part of the experimental work described in this thesis was conducted in the department of Infection, Prevention and Control, Nelson R. Mandela School of Medicine, School of Laboratory Sciences, University of KwaZulu-Natal, Durban, under the supervision of Dr. A.W. Sturm. The other part of the experimental work described was conducted at the W.R. Jacob's Laboratory, Albert Einstein College of Medicine, Yeshiva University, Bronx, New York, USA under the supervision of Dr's William R. Jacobs and A.W Sturm.

Ethical approval for the study was obtained from the Ethics and Professional Standards sub-committee (College of Health Science), Nelson R. Mandela School of Medicine, University of KwaZulu-Natal.

This study represents the original work by the author and has not been submitted in part of whole to any other tertiary institution. Where use was made of the work of others, it has been duly acknowledged in the text

A handwritten signature in black ink, appearing to read 'Makume', is written above a horizontal line. To the left of the signature is a small, stylized symbol consisting of two vertical lines and a horizontal line forming a triangle-like shape.

M.T. Makume

## Dedication

Ke leboha Modimo, le Badimo ba Makume.

Ntai le Mantha Makume, Morontsha le Thamahane Thamahane, le qadile tsela e kgolo, la tsamaya tsela a thata, le phelela nna. Ke motho hoba ke le setloholo sa lona. Ke leboha modimo ha a mphile lona, Robalang ka Kgotso Bataung and Mapele.

Mme **Mathapi** le Ntate **Taunyane** Makume, ke mona ka tshehetso, lerato le kgothatso ya lona, ke leboha ho menahane.

**Sechaba** Makume, **Matseleng** Makume, **Frances** Degbor, **Vuyiswa** Mandita-Kuzwayo, **Siyakhona** Kuzwayo, **Setsoana** Moorosi, bana ba heso, metswalle ya sebele. Ka tshehetso le mamello ya lona, ke kgonne ho ba mona moo ke leng teng. Ke ya leboha.

Prof Sturm, a great mentor, friend and supervisor, Thank you for all your patience, for being a true and dedicated teacher

*'If you want to go quickly, go alone. If you want to go far, go together'*

## **Publications:**

Jain, P., T. E. Hartman, et al. (2012). "phi(2)GFP10, a high-intensity fluorophage, enables detection and rapid drug susceptibility testing of *Mycobacterium tuberculosis* directly from sputum samples." J Clin Microbiol **50**(4): 1362-1369.

## **Presentations:**

2009: **'Sensitivity of reporter phages to detect *Mycobacterium tuberculosis*'**

Mantha Makume, William Jacobs jr., Graham Hatfull & Willem A. Sturm.  
Federation of Infectious Diseases Societies of Southern Africa

**'Rapid detection of *Mycobacterium tuberculosis* using  $\beta$ -galactosidase reporter mycobacteriophage'**, Mantha Makume, William Jacobs jr., Graham Hatfull & Willem A. Sturm.

University of KwaZulu-Natal College of Medicine Research Symposium

2011: **'Phage based diagnostics for the detection of *Mycobacterium tuberculosis*'**

Mantha T. Makume, P. Jain, J. Kriakov, W.R. Jacobs jnr. & Willem A. Sturm  
Federation of Infectious Diseases Societies of Southern Africa

2012: **'Use of reporter mycobacteriophages in determining drug susceptibility patterns of *Mycobacterium tuberculosis* clinical isolates'** Mantha T.

Makume and Willem A. Sturm

University of KwaZulu-Natal College of Medicine Research Symposium

## **Acknowledgements**

### **FUNDING:**

Howard Hughes Medical Institute, National Research Foundation

### **STUDY SUPERVISION:**

DR's William R. Jacobs, Graham F. Hatfull, Willem A. Sturm

### **LABORATORY ASSISTANCE AND TRAINING:**

Lutchmee, Reshma & Kaloshnee, Torin and Paras

### **STATISTICAL INPUT:**

Nonhlanhla Yende

## Abbreviations

°C	Degrees Celcius
AFB	Acid Fast Bacilli
ATP	Adenine Triphosphate
BCG	Bacille Calmette Guerin
bp	base pair
BSA	Bovine Serum Albumin
CAP	Capreomycin
CFU	Colony Forming Units
CYCLO	Cycloserine
DOTS	Directly Observed Therapy Short course program
DST	Drug Susceptibility Tests
EMB	Ethambutol
ETH	Ethionamide
FAS	Ferrous Ammonium Sulphate
GFP	Green Fluorescent Protein
g	Gram
HIV	Human Immunodeficiency Virus
INH	Isoniazid

KAN	Kanamycin
kb	Kilobases
LB	Luria-Bertani
LINE	Linezolid
LJ	Löwenstein-Jensen
LRP	Luciferase Reporter Protein
M. tb	<i>Mycobacterium tuberculosis</i>
MABA	Microplate Alamar Blue Assay
MDR	Multi drug resistant tuberculosis
MIC	Minimum Inhibitory Concentration
MODS	Microscopic Observation Drug Susceptibility
MOI	Multiplicity Of Infection
MOTT	Mycobacteria Other Than Tuberculosis
MTC	Mycobacterium tuberculosis Complex
MTT	3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium
NALC	n-Acetyl Cysteine
NaOH	Sodium Hydroxide
NAP	$\rho$ -nitro- $\alpha$ -acetyl amino- $\beta$ -hydroxy propiophenone

NRA	Nitrate Reductase Assay
NTM	Non-Tuberculous Mycobacteria
OADC	Oleic acid- Albumin-Dextrose-Catalase
OFLOX	Ofloxacin
ONPG	o-nitrophenyl- $\beta$ -D-galactopyranoside
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
PhaB	Phage Amplified Biologically Assay
PZA	Pyrazinamide
REMA	Resazurin Microplate Assay
RIF	Rifampicin
RLU	Relative Light Units
STR	Streptomycin
TB	Tuberculosis
$\mu$ l	Micro litre
$\mu$ M	Micromolar
WHO	World Health Organization
X-gal	5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside
XDR-TB	Extensively drug resistant- tuberculosis

## ABSTRACT

Rapid detection of *Mycobacterium tuberculosis* is imperative in order to curb transmission. The emergence of drug resistant isolates of *M. tuberculosis* has created a need for rapid drug susceptibility testing. Apart from the test being rapid, it also needs to be inexpensive, sensitive and suitable for resource-constrained laboratories, which serve populations with a high burden of tuberculosis. We report one such rapid diagnostic test, which uses the expression of the *E. coli*-derived  $\beta$ -galactosidase enzyme reporter gene, *LacZ* in a mycobacteriophage as a detection method of tuberculosis.

The  $\beta$ -galactosidase reporter assay is a colorimetric assay, which detects single micro-colonies in the form of blue spots, within a well of a tissue culture plate, within a week. This test allows phenotypic drug susceptibility testing, where susceptibility to a drug results in absence of spots. The rapidity, ease and inexpensive way that this assay offers, satisfy the above-mentioned criteria. The sensitivity of the assay allows the detection of even pauci-bacillary disease.

Initially, the laboratory strain H37Rv was grown to log phase ( $OD_{600nm}$  0.4-0.8) in 7H9 media. The culture was centrifuged and the media decanted and the pellet washed with Tris-buffered Saline (Biochemika Fluka). The pellet was re-suspended in Tris-buffered saline and 10-fold serial dilutions were infected with *LacZ* reporter mycobacteriophage and the suspended in an X-gal (Sigma) containing agar layer (7H9 0.35% : Bacto Agar 7%; (MiddleBrook)) for up to 5 days at 37°C. For susceptibility testing, the organisms were exposed to 1<sup>ST</sup>, 2<sup>ND</sup> and 3<sup>RD</sup>-line anti TB drugs, 24 hours

prior to the assay. A total of 483 consecutive sputum specimens were collected and tested for the presence of *Mycobacterium tuberculosis* bacilli using the  $\beta$ -galactosidase assay and compared with microscopy and culture. The assay was able to correctly detect 87% of the sputum specimens, after 96 hours post incubation; bacterial growth became visible in the form of blue spots, the colour of the spots intensified up to 120 hours of incubation. Single colonies, representative of a single blue spot, were visible. The drug exposed susceptible isolates did not yield the blue colonies after 1 week of further incubation in contrast to the non-exposed control. The  $\beta$ -galactosidase assay was also found to be comparable to the recently developed and published  $\Phi^2$ GFP10 fluorescent reporter mycobacteriophage.

The  $\beta$ -galactosidase assay is an inexpensive, sensitive and rapid method to detect TB bacilli and provide a drug susceptibility profile. It detects low concentrations of bacilli, often observed among HIV co-infected patients. The assay caters especially for low resource laboratories in that it is technically non-demanding and can be performed in non-specialized laboratories, without the need for expensive machinery such as fluorescence microscopes or automated culture equipment. This assay has great potential to contribute in the control of TB pandemic.

# TABLE OF CONTENTS

<b>Declaration.....</b>	<b>ii</b>
<b>Publications.....</b>	<b>iii</b>
<b>Presentations.....</b>	<b>iii</b>
<b>Acknowledgements.....</b>	<b>iv</b>
<b>Abbreviations.....</b>	<b>v</b>
<b>Abstract.....</b>	<b>viii</b>
<b>List of Figures.....</b>	<b>xiv</b>
<b>Lists of Tables.....</b>	<b>xvi</b>

## CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

<b>1.0 INTRODUCTION.....</b>	<b>1</b>
<b>1.1 TB infections in Sub-Saharan Africa.....</b>	<b>2</b>
<b>1.2 The burden of TB in South Africa.....</b>	<b>3</b>
<b>1.3 Challenges to laboratory diagnosis of tuberculosis and drug susceptibility testing.....</b>	<b>4</b>
<b>1.4 Challenges to Current Diagnostic Methods.....</b>	<b>5</b>
<b>1.5 the development of improved TB detection methodologies.....</b>	<b>6</b>
1.5.1 Genotypic Diagnostic assays.....	6
1.5.2 Phenotypic Diagnostic assays.....	9
1.5.2.1 Microscopic Observation Drug Susceptibility (MODS) assay.....	9
1.5.2.2 Microplate Alamar Blue Assay (MABA).....	10
1.5.2.3 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.....	12
1.5.2.4 Resazurin Microplate assay (REMA).....	13
1.5.2.5 Nitrate Reductase Assay (NRA).....	14
1.5.3 Bacteriophage based assays.....	15
1.5.3.1 FastPlaque TB™ and FastPlaque-RIF™.....	15
<b>1.6 Introduction to Mycobacteriophages.....</b>	<b>16</b>
1.6.1 Genetic manipulation of <i>M. tuberculosis</i> using mycobacteriophages.....	18
1.6.2 Reporter mycobacteriophages and Reporter proteins.....	20
1.6.3 Reporter proteins: Origins, chemical reactions and applications.....	21
1.6.3.1 Green fluorescent protein (GFP).....	21
1.6.3.2 Beta-galactosidase enzyme ( <i>lacZ</i> ).....	22
1.6.3.3 Luciferase ( <i>Fflux</i> gene).....	24
1.6.3.4 Alkaline phosphatase ( <i>PhoA</i> ).....	25
<b>1.7 Reporter Proteins and their application to Mycobacteriophages.....</b>	<b>25</b>
1.7.1 The development of optimal reporter mycobacteriophages.....	26
1.7.1.1 TM4 derived reporter mycobacteriophages.....	27
1.7.1.2 L5 derived reporter mycobacteriophages.....	29
1.7.1.3 The Bronx Box and its application in detecting Luminescence.....	30

1.7.1.4	D29- derived reporter mycobacteriophages.....	31
1.7.1.5	D29 Phage- Based assays (Phage Biologically Amplified Assay).....	33
1.8.1	Further modifications to reporter mycobacteriophages.....	34
1.8.1.1	Che12-derived reporter mycobacteriophages.....	36
1.8.1.2	Second Generation reporter mycobacteriophages: Fluoromycobacteriophages.....	38

## CHAPTER 2: THE DEVELOPMENT OF THE BETA-GALACTOSIDASE REPORTER MYCOBACTERIOPHAGE AND ITS IMPLEMENTATION IN DETECTING CULTURED *MYCOBACTERIUM TUBERCULOSIS*

<b>2.0</b>	<b>INTRODUCTION.....</b>	<b>42</b>
<b>2.1</b>	<b>Aims and Objectives.....</b>	<b>45</b>
<b>2.2</b>	<b>Materials and Methods.....</b>	<b>45</b>
2.2.1	Construction of the Beta-galactosidase reporter mycobacteriophage.....	45
2.2.1.1	Plasmid (pYUBs 1228 & 1229) inoculation.....	46
2.2.1.2	Plasmid DNA preparation.....	48
2.2.1.3	Amplification of the LacZ Gene.....	48
2.2.1.4	Restriction and Purification of Plasmid and LacZ DNA.....	49
2.2.1.5	Ligation of Plasmid and DNA Molecule.....	50
2.2.1.6	Transformation of E. coli dH5 $\alpha$ .....	52
2.2.1.7	Ligation of Plasmid-DNA with Phasmid (phAE159).....	53
2.2.1.8	in vitro packaging into phage $\lambda$ heads and screening for molecule incorporation.....	53
2.2.1.9	Electroporation of M. smeg cells.....	54
2.2.1.10	High Titre lysates of incorporated DNA molecule Positive plaques.....	54
2.2.2	the Beta-galactosidase Reporter Phage Assay for the detection of Mycobacterium tuberculosis.....	55
2.2.3	Confirmation of Mycobacterium tuberculosis in culture.....	56
2.2.4	Confirmation Of $\beta$ -gal phage Host Specificity.....	56
<b>2.3</b>	<b>Results.....</b>	<b>58</b>
2.3.1	The Beta-galactosidase Reporter Phage Assay for the detection of Mycobacteria.....	58
2.3.2	Confirmation of mycobacterium tuberculosis in $\beta$ -gal assay Culture.....	61
2.3.3	Confirmation of Beta-gal Mycobacteriophage host specificity.....	62
<b>2.4</b>	<b>DISCUSSION.....</b>	<b>64</b>

## CHAPTER 3: THE IMPLEMENTATION OF THE BETA-GALACTOSIDASE ASSAY IN GENERATING DRUG SUSCEPTIBILITY PROFILES OF LABORATORY CULTURES

<b>3.0</b>	<b>INTRODUCTION.....</b>	<b>72</b>
<b>3.1</b>	<b>Aims and Objectives.....</b>	<b>77</b>
<b>3.2</b>	<b>Materials and Methods.....</b>	<b>77</b>
3.2.1	Isolate collection.....	77

3.2.2	Isolate inoculation .....	77
3.2.3	Drug exposure and Beta-gal assay.....	77
3.2.4	Statistical analysis .....	79
<b>3.3</b>	<b>Results.....</b>	<b>79</b>
3.3.1	The $\beta$ -gal assay.....	79
3.3.2	The Accuracy of the $\beta$ -gal assay using WHO drug concentrations .....	80
3.3.3	The Accuracy of the $\beta$ -gal assay with adjusted drug concentrations .....	83
<b>3.5</b>	<b>Discussion.....</b>	<b>87</b>

## CHAPTER 4: DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM SAMPLES USING A BETA-GAL REPORTER MYCOBACTERIOPHAGE

<b>4.0</b>	<b>INTRODUCTION.....</b>	<b>94</b>
<b>4.1</b>	<b>Aims and Objectives.....</b>	<b>95</b>
<b>4.2</b>	<b>Materials and methods.....</b>	<b>96</b>
4.2.1	Specimen collection .....	96
4.2.2	Decontamination of sputum specimens.....	96
4.2.3	Sputum Microscopy and culture .....	97
4.2.4	Beta-gal reporter phage assay.....	97
4.2.5	Statistical analysis .....	98
<b>4.3</b>	<b>Results.....</b>	<b>99</b>
4.3.1	Clinical data .....	99
4.3.2	Performance of the beta-gal assay .....	100
4.3.3	Microscopy and Culture Results.....	100
4.3.4	beta- gal assay compared to BACTEC MGIT 960™ .....	101
4.3.5	$\beta$ - gal assay compared to combined BACTEC MGIT 960™ and Culture on 7H11 Middlebrook agar .....	102
4.3.6	Analysis of discrepancies between BACTEC MGIT 960™, Middlebrook 7H11 culture and Smear microscopy and their effect on the accuracy of the B-gal assay .....	103
4.3.7	Contamination and growth of Mycobacteria Other Than Tuberculosis and their effect on the accuracy of the B-gal assay .....	105
4.3.8	Receiver Operating Characteristic & Area under Curve analysis of the B-gal assay.....	106
<b>4.4</b>	<b>Discussion.....</b>	<b>107</b>

CHAPTER 5: COMPARISON OF BETA-GAL AND GREEN FLUORESCENT PROTEIN EXPRESSING REPORTER PHAGES IN GENERATING DSTs OF LABORATORY CULTURES OF *MYCOBACTERIUM TUBERCULOSIS*

<b>5.0 INTRODUCTION.....</b>	<b>111</b>
<b>5.1 Aims and Objectives.....</b>	<b>112</b>
<b>5.2 Materials and Methods.....</b>	<b>113</b>
5.2.1 Inoculating the isolates .....	113
5.2.2 Drug exposure.....	113
5.2.3 Reporter mycobacteriophage assays.....	113
5.2.3.1 $\beta$ -gal reporter phage assay .....	113
5.2.3.2 Green fluorescent protein reporter phage assay .....	114
<b>5.3 Results.....</b>	<b>115</b>
<b>5.4 Discussion.....</b>	<b>123</b>

**Chapter 6: Summary**

<b>6.0 INTRODUCTION.....</b>	<b>126</b>
<b>6.1 The development of the Beta-galactosidase reporter mycobacteriophage and its implementation in detecting cultured <i>Mycobacterium tuberculosis</i>.....</b>	<b>127</b>
<b>6.2 The implementation of the <math>\beta</math>beta-galactosidase assay in generating Drug Susceptibility Profiles of laboratory cultures.....</b>	<b>128</b>
<b>6.3 Detection of <i>Mycobacterium tuberculosis</i> in sputum specimens using A Beta-galactosidase reporter mycobacteriophage.....</b>	<b>129</b>
<b>6.4 Comparison of Beta-galactosidase and Green Fluorescent Protein expressing reporter phages in generating DSTs of laboratory cultures of <i>Mycobacterium tuberculosis</i>.....</b>	<b>131</b>
<b>Conclusion.....</b>	<b>132</b>
<b>REFERENCES.....</b>	<b>133</b>

## LIST OF FIGURES

		PAGES
<b>Figure 1.1:</b>	Chemical reaction of the firefly luciferase	23
<b>Figure 1.2:</b>	Chemical reaction involving X-gal and the $\beta$ -galactosidase enzyme	24
<b>Figure 1.3:</b>	Chemical reaction involving phosphate and the alkaline phosphatase enzyme	25
<b>Figure 2.1:</b>	Enzymatic reaction of the breakdown of lactose into galactose and glucose by beta-galactosidase	44
<b>Figure 2.2:</b>	Beta-galactosidase reporter mycobacteriophage plaques in a lawn of mc <sup>2</sup> 155 <i>Mycobacterium smegmatis</i> infused with X-gal	46
<b>Figure 2.3:</b>	Figure of pYUB 1228 (5025 base pair)	47
<b>Figure 2.4:</b>	Figure of pYUB 1229 (5025bp)	47
<b>Figure 2.5:</b>	PacI and NdeI restriction map to confirm the presence of pYUBs 1228 and 1229	48
<b>Figure 2.6:</b>	The <i>LacZ</i> PCR product inserted into the reporter mycobacteriophage	49
<b>Figure 2.7:</b>	Restriction maps of pYUBs 1228 & 1229 clones	51
<b>Figure 2.8:</b>	Beta-galactosidase reporter phage assay in 24-well culture plate, detecting the presence of <i>M. tuberculosis</i> bacilli, 96 hours post infection with reporter mycobacteriophages	58
<b>Figure 2.9:</b>	Beta-galactosidase reporter phage assay in 24-well culture plate, detecting the presence of <i>M. tuberculosis</i> bacilli, 96 hours post infection with reporter mycobacteriophage	60

<b>Figure 2.10a:</b>	Bright field depiction of <i>M. tuberculosis</i> 100X Magnification	60
<b>Figure 2.10b:</b>	Fluorescence image of the figure 2.10 a, of <i>M. tuberculosis</i> infected with $\Phi^2$ GFP10 reporter phage 400X Magnification	61
<b>Figure 2.11:</b>	Solid Culture plate of <i>Mycobacterium tuberculosis</i> pure colonies	62
<b>Figure 2.12:</b>	(a) mc <sup>2</sup> 155 <i>M. smegmatis</i> control strain, with GFP and $\beta$ -gal phage plaques (b)-(d): Representative lawns of normal flora showing lack of beta-galactosidase activity and plaques	63
<b>Figure 3.1:</b>	Currently available methodologies to determine drug susceptibility profiles of <i>Mycobacterium tuberculosis</i>	74
<b>Figure 3.2:</b>	Lay-out of Isolates and drugs in a 24-well tissue culture plate	77
<b>Figure 3.3:</b>	Representative plate of the $\beta$ -gal reporter assay, detecting drugs resistance among susceptible, MDR and XDR-TB isolates	79
<b>Figure 4.1:</b>	Representative plate of the $\beta$ -gal assay depicting TB positive specimen in triplicate	98
<b>Figure 4.2:</b>	The ROC curve of the accuracy of the $\beta$ -gal phage assay	104
<b>Figure 5.1:</b>	Fluorescent microscopy pictures of <i>M. tb</i> infected with the $\Phi^2$ GFP10 reporter phage. Left: drug resistant bacilli; right: drug susceptible bacilli	113

## LIST OF TABLES

	<b>PAGES</b>
<b>Table 1.1:</b> Meta-analysis of Various Reporter Mycobacteriophages	41
<b>Table 2.1:</b> Reaction components, amounts and conditions used for amplification of target DNA	49
<b>Table 2.2:</b> Reaction components, amounts and conditions used for restriction of plasmid and target DNA	50
<b>Table 2.3:</b> Reaction components, amounts and conditions used for restriction of phAE159 and pYUB: <i>LacZ</i> clone	53
<b>Table 2.4:</b> Table of tests used to confirm the presence of <i>M. tuberculosis</i>	56
<b>Table 2.5:</b> Table of replicate experiments used to assess the assay's reproducibility	60
<b>Table 3.1:</b> Performance of currently available DST methodologies	75
<b>Table 3.2:</b> Concentrations of antimicrobial drugs initially used in the $\beta$ -galactosidase assay	77
<b>Table 3.3:</b> Overall accuracy of the Beta-galactosidase assay on clinical isolates	81
<b>Table 3.4:</b> Table of streptomycin and kanamycin results after titration of the drugs	83
<b>Table 3.5:</b> Table of capreomycin and ofloxacin results after titration of the drugs	84
<b>Table 3.6:</b> Table of reproducibility assessment of the $\beta$ -gal phage	85
<b>Table 4.1:</b> Tests and amounts of specimen used	95
<b>Table 4.2:</b> Patient demographic data	97

<b>Table 4.3:</b>	TB smears and culture results	99
<b>Table 4.4:</b>	$\beta$ -galactosidase assay compared to BACTECMGIT 960™100	
<b>Table 4.5:</b>	Performance of $\beta$ -galactosidase assay compared to culture in BACTEC MGIT™ and on 7H11 Middlebrook agar	101
<b>Table 4.6:</b>	A comparison of the Culture methodologies including smear microscopy results and their effect on the accuracy of the $\beta$ -gal assay	102
<b>Table 4.7:</b>	Summary of the performance assessment of the $\beta$ -galactosidase assay compared to culture and microscopy in various combinations	102
<b>Table 5.1:</b>	Concentrations of antimicrobial drugs initially used in the $\beta$ -galactosidase assay	112
<b>Table 5.2:</b>	Overall accuracy of $\Phi^2$ GFP10 phage assay in determining drug susceptibility	115
<b>Table 5.3:</b>	Sensitivity, specificity and overall accuracy of $\Phi^2$ GFP10 phage assay in determining drug susceptibility after titration of the STR and KAN drug concentrations	117
<b>Table 5.4:</b>	Sensitivity, specificity and overall accuracy of $\Phi^2$ GFP10 phage assay in determining drug susceptibility after titration of the CAP and OFLOX drug concentrations	118
<b>Table 5.5:</b>	A Comparison of the reporter bacteriophage tests on DST pre-determined clinical isolates	120
<b>Table 5.6:</b>	Comparison of the $\Phi^2$ GFP10 assay with the $\beta$ -gal assay	121

## APPENDIX

A: MEDIA, ANTIBIOTICS AND SUPPLEMENTS

B: PROPAGATION OF MYCOBACTERIUM

C: PROPAGATION OF MYCOBACTERIOPHAGE

D: DECONTAMINATION OF CLINICAL SPUTUM

E: PERFORMANCE OF THE BETA GALACTOSIDASE ASSAY ON CLINICAL  
SPUTUM SAMPLES

# CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

## 1.0 INTRODUCTION

Although, it is now 126 years since Koch's revolutionary study, describing *Mycobacterium tuberculosis* (*M. tb*) as the causative agent of tuberculosis (TB), the disease is still not under control. More people have died from this infection than from any other disease known to mankind (Carriere, Riska et al. 1997; Schluger 2005). TB mortality rates have dropped after the discovery of antibiotics and the improvement of living conditions in the post-industrial era. However, current trends reveal that the age-old fight against TB needs to be renewed. The emergence of transmissible drug resistant strains of tuberculosis is threatening to overwhelm many health systems, particularly in countries that are ill equipped to handle this crisis (McNerney 1999; Lawn and Nicol 2011).

The implementation of the Directly Observed Therapy Short course program (DOTS) by the World Health Organization (WHO) in 1991 has had varying success in different regions of the world (Health 2007). The aim of this program was to achieve a 70% detection rate and 85% cure rate worldwide by 2000. However, 2004 studies showed that the global case detection rate was 60% and the cure rate was 84%. These statistics, although optimistic, did not reflect the situation in sub-Saharan Africa, where it was found that the detection rate was 51% and cure rates ranged from 60 to 70% (Department of Health 2007; Health 2007; Lawn and Nicol 2011). The countries that achieved the highest success rates were developed countries, as well as some third world countries outside Africa such as Vietnam and Philippines. It should

also be noted that the detection and cure rates are based on the respective country's definitions and protocols, and these are not standardized. Hence, the caseload and cure rates should not be seen as absolute reflection of the status quo.

## 1.1 TB INFECTIONS IN SUB-SAHARAN AFRICA

Sub-Saharan Africa accounts for the heaviest burden of TB cases worldwide; which is fuelled by the Human Immunodeficiency Virus (HIV) infections, extreme poverty and failure of control mechanisms (Riska, Jacobs et al. 1997). The rise in TB prevalence has been linked to the rise in HIV infections, where it is the most frequent cause of death among immune-compromised individuals (Lawn and Nicol 2011). It is estimated that approximately 12 % of all TB deaths were HIV co-infected (Jacobs W.R. 1993; Drobniewski FA 2003; Health 2007). WHO estimated that there were 8.8 million incident cases of TB globally in 2010, 1.1 million deaths among HIV-negative cases of TB and an additional 0.35 million deaths (range, 0.32 million–0.39 million) among people who were HIV-positive. Additionally, 7.4 million cases are found in Asia and Sub-Saharan Africa (WHO 2011).

Of the 15 countries that have the highest number of TB cases in the world, 12 are in Africa. As mentioned previously, the heavy burden of TB is linked with high HIV prevalence rates where TB is reported as a cause of death in 60-70% of HIV infected patients in South Africa (Abdool Karim, Churchyard et al. 2009; Abdool Karim, Abdool Karim et al. 2010). The burden of TB is exacerbated by lack of infrastructure, poor case reporting and generally poor patient management (Health 2007). The emergence of multi-drug (MDR) and extensively drug resistant (XDR) strains of TB has put various health care systems and TB control programs at risk of collapse (Carriere, Riska et al. 1997; Riska, Jacobs et al. 1997).

## 1.2 The Burden of TB in South Africa

South Africa specifically, has the third highest TB caseload in the world. South Africa's population is only 0.7% of the world, yet accounts for 19 % of the world's adult TB cases (Department of Health 2007; Health 2007). Over a period of 5 years, TB detection rates had risen by 81% from 188,695 cases in 2001 to 342,315 cases in 2006 (Health 2007). These statistics may reflect improved case reporting, they do however also shed light on the lack of control of TB transmission. The South African department of Health has highlighted several challenges to curbing TB: poor patient management at clinic level, lack of infrastructure especially in rural areas and the emergence of drug resistant strains. WHO estimated that 8000 cases of multi-drug resistant TB (i.e.: resistance to Isoniazid (INH) and Rifampicin (RIF)) were from South Africa (Department of Health 2007).

The province of KwaZulu-Natal recorded the highest TB caseload in 2006, accounting for 31% of national TB cases. Multi-drug and extensively drug resistant cases were first recorded in this province (Department of Health 2007; Health 2007). The mortality rate due to XDR-TB (i.e.: MDR isolates with additional resistance to the fluoroquinolones and at least one of the three 2<sup>nd</sup>-line injectable drugs) exceeded 90%. This has placed the health care system under severe burden as treatment of such cases is prolonged and expensive (Gandhi, Moll et al. 2006; Department of Health 2007).

### 1.3 CHALLENGES TO LABORATORY DIAGNOSIS OF TUBERCULOSIS AND DRUG SUSCEPTIBILITY TESTING

Laboratory diagnosis, being the cornerstone of effective control of TB, has made strides in aiding to control the transmission of the disease (Hazbon, Guarin et al. 2003). However, the current methods of detection are failing to keep up with the developing crisis. The fact that Koch's microscopy and culture methods are still considered the gold standard for TB diagnosis shows the temporal difference between the evolution of *Mycobacterium tuberculosis* and improvement of TB control. Microscopic detection of acid fast bacilli (AFB) was found to be inadequate in the detection of 30-50% of cases of active tuberculosis and is unable to identify the increasing numbers of multi-drug resistant cases (Banaiee 2001). Sole reliance on the sputum smear microscopy means that it is increasingly difficult to control TB transmission (Riska, Jacobs et al. 1997; Banaiee, Bobadilla-del-Valle et al. 2003).

The emergence and increasing prevalence of both MDR and XDR-TB underscores the need for faster, cheaper, more accurate as well as safer methods to diagnose TB. Newer technologies have eased the challenge by providing a drug-resistance profile of clinical isolates within a shorter time period (Piuri, Jacobs Jr et al. 2008).

## 1.4 CHALLENGES TO CURRENT DIAGNOSTIC METHODS

Smear microscopy has been supplemented by the use of culture either on solid or in liquid media or both. The culture of specimens on solid media such as Middlebrook 7H10 and 7H11 agar plates or Löwenstein-Jensen (LJ) slants and in liquid media such as Middlebrook 7H9 broth takes between 10 and 21 days (Riska, Su et al. 1999; Drobniowski, Watterson et al. 2000; Drobniowski FA 2003). These techniques are inexpensive and relatively easy to perform. However the time it takes for a definitive result means a delay in treatment initiation, particularly in patients that are *M. tb* smear negative (Banaiee, Bobadilla-del-Valle et al. 2003; Pai, Kalantri et al. 2005).

The time-to-diagnosis factor has been overcome by the implementation of radiometric, and later, non-radiometric growth detection techniques (Riska, Jacobs et al. 1997; Riska, Su et al. 1999; Banaiee 2001; Banaiee, Bobadilla-del-Valle et al. 2003). Some of the challenges for the radiometric method were in the inherent use and discarding of radio-labelled <sup>14</sup>C-palmitate, as well as its demand on time and expertise (Riska, Su et al. 1999; Drobniowski FA 2003). This was overcome by the use of non-radiometric liquid culture reader-incubators like the mycobacterial growth indicator tube system (BACTEC 960 MGIT™), which measure the bacteria's carbon dioxide production or oxygen consumption. The liquid media is enriched with compounds that enhance the growth of *M. tb*. However, the instrument gives a positive signal only when the tubes contain 10<sup>5</sup>-10<sup>6</sup> CFU (colony forming units)/ml, thus delaying the reporting of specimen with lesser bacilli by up to 42 days. Although this phenotypic detection method was found for most specimens to be faster and more sensitive than manually read solid culture systems, it requires expensive specialised equipment that makes it inaccessible for laboratories in resource-constrained countries. Thus this technology is

usually found in developed countries and reference laboratories (Banaiee, Bobadilla-del-Valle et al. 2003; Bwanga, Hoffner et al. 2009; Ogwang, Asiimwe et al. 2009).

A methodology or assay that overcomes all of the above-mentioned factors is instrumental in the fight against TB, particularly in low resource settings. Such a test needs to be rapid, cheap, and able to provide susceptibility profiles, whilst simultaneously allowing for high throughput measures (Jacobs W.R. 1993; Hazbon, Guarin et al. 2003).

## 1.5 THE DEVELOPMENT OF IMPROVED TB DETECTION METHODOLOGIES

### 1.5.1 GENOTYPIC DIAGNOSTIC ASSAYS

Commercially available line-probe assays such as INNO-LiPA®Rif, TB kit (Innogenetics, Belgium) and GenoType® MTBDR assay (Hain Life science, Germany), focus on genetic differences between susceptible and resistant strains of *M. tb* (Brossier, Veziris et al. 2006; Pai, Kalantri et al. 2006; Bwanga, Hoffner et al. 2009). Following DNA extraction and hybridization (or lack thereof) to a set of membrane-bound probes, this indicates the presence of *M. tb* genes as well as of mutations in genes coding for the drug targets (Brossier, Veziris et al. 2006).

In a study performed by Brossier et al. 2006, it was found that the GenoType® MTBDR test was able to detect 100% of the Rifampicin resistant clinical isolates, compared to 67% of the Isoniazid resistant isolates. Their reference method was DNA sequencing from growth on LJ slants. The discrepancy among the INH resistant isolates clearly supports the contentions of

Bottger et al., 2011 and Riska et al., 2000, that not all resistance can be attributed to known single gene mutations. Thus the Genotype® MTBDR test does not adequately cover all mutations (Brossier, Veziris et al. 2006). Consequently, the Genotype® MTBDR*plus* was developed to include the *inhA* regulatory regions, in an effort to enhance detection levels among INH resistant isolates (Brossier, Veziris et al. 2009). It was found that the newer Genotype® MTBDR*plus* performed better than the original assay (86% vs. 67%). Furthermore, the Genotype® MTBDR*plus* was also able to detect low-level INH resistant isolates (Brossier, Veziris et al. 2009).

The time it takes to perform these genotypic tests and provide a resistance profile has been reported to be hours, as opposed to weeks for conventional DST (Hillemann, Rusch-Gerdes et al. 2006). The Hillemann group suggested that the Genotype® MTBDR*plus* kit was able to detect the majority of RIF resistant isolates. However, they caution against sole reliance on this kit for DST determination (Hillemann, Rusch-Gerdes et al. 2006).

Although both the Genotype® MTBDR*plus* and INNO-LiPA®Rif kits have been proven to be highly specific and sensitive when used on cultured mycobacterial isolates, they are still to be vigorously challenged in direct DST, i.e.: on sputum specimens. Furthermore, the technical expertise as well as the cost to perform such tests has proven to be prohibitive, thus limiting their use in laboratories in low-income countries. Additionally, the evolution of resistance patterns over time and the range of resistance levels, as well the lack of full knowledge of the causality of resistance, may render the kits obsolete over time (Riska, Jacobs et al. 2000; Brossier, Veziris et al. 2006; Shiferaw, Woldeamanuel et al. 2007; Bottger 2011).

The Xpert® MTB/RIF (Cepheid Inc., Sunnyvale, CA, USA) uses real time PCR to detect simultaneously *M. tb* and RIF resistance in a clinical specimen. It has been shown to have potential in replacing conventional TB diagnostic assays (Armand, Vanhuls et al. 2011), as evidenced by the South African Department of Health, who implemented its use to detect TB among the prison population in the Western Cape (2013). The fully automated, cartridge-based technology allows for the detection of TB in an unprocessed clinical specimen. The ease of use of this technology provides a basis of wider implementation at primary health care centres (Armand, Vanhuls et al. 2011; Lawn and Nicol 2011). Five different nucleic acid hybridization probes are used in the same multiplex reaction, which are complementary to different targets within the *rpoB* gene. Hybridization occurs only with wild-type RIF susceptible genes, where an occurrence of a mutation will prevent hybridization and thus fluorescence is suppressed (Lawn and Nicol 2011). The major advantage of this assay is the reduction of time to results to 2 hours with very little manual input from the user (Rachow, Zumla et al. 2011). The limits of detection of the Xpert® MTB/RIF was found to be 131 CFU/ml, making this assay approximately 100 times more sensitive than smear microscopy and approaches that of culture (Rachow, Zumla et al. 2011).

Due to the automated format of the technology, it was found to that the use of the Xpert MTB/RIF assay “poses a substantially smaller biohazard risk than direct smear microscopy and might reasonably be done without the need for special biosafety equipment, which is lacking in most resource- limited settings” (Lawn and Nicol 2011).

A South African study conducted by Theron et al., 2011 revealed that the sensitivity of this assay among smear-positive patients was 94.7% and 46.8% among smear negative patients and an overall specificity of 78.7% (Lawn and Nicol 2011).

Studies performed by Rachow et al., 2011, and Armand et al., 2011 revealed similar results. Rachow et al reported 98% sensitivity and 99% specificity for smear positives, but the sensitivity decreased to 61% among smear negative, culture positive specimens (Rachow, Zumla et al. 2011). Armand et al found 79% sensitivity, 84% specificity in smear positive and 53% sensitivity among smear negative specimens (Armand, Vanhuls et al. 2011). The shortcoming of the Rachow study, was the lack of RIF resistant specimens to assess the performance of the Xpert® MTB/RIF assay.

Although promising to aid in TB control, there have been studies that have reported false-positive results, thus decreasing the sensitivity and reducing positive predictive value to 57%. This has prompted the WHO to recommend that the results be supplemented further with culture (Lawn and Nicol 2011). Furthermore, the cost of the technology, which is substantially higher than for microscopy, could potentially be an inhibitory factor to its use (Armand, Vanhuls et al. 2011; Lawn and Nicol 2011).

## 1.5.2 PHENOTYPIC DIAGNOSTIC ASSAYS

### 1.5.2.1 *Microscopic Observation Drug Susceptibility (MODS) assay*

An assay that shows promising results is the Microscopic Observation Drug Susceptibility (MODS) assay, a relatively simple phenotypic test that utilises a readily available inverted light microscope to detect mycobacterial cording in cultures, directly from sputum specimens (Caviedes, Lee et al. 2000; Park, Bishai et al. 2002; Shiferaw, Woldeamanuel et al. 2007). The test is performed in a liquid media format in a 24-well tissue culture plate. It is known that liquid broth provides faster growth as compared to solid media. When mixed with drugs, growth will be abrogated in susceptible organisms as opposed to actively growing resistant

ones (Caviedes, Lee et al. 2000; Moore, Mendoza et al. 2004; WHO 2011). Unlike the Line probe assays, MODS has proven to be efficient in testing both smear positive and negative specimens (Shiferaw, Woldeamanuel et al. 2007). Additionally, the time to result was found to be comparable to BACTEC™ MGIT™ 960 (Becton Dickinson). The ease with which the assay is performed may alleviate the human resource and equipment costs (Park, Bishai et al. 2002; Shiferaw, Woldeamanuel et al. 2007).

Reports on the performance of the MODS assay have been encouraging, with relatively few drawbacks. This assay was able to detect 93% of RIF resistant isolates and 90% each for INH and Ethambutol (EMB), with an overall >90% specificity for the above-mentioned drugs (Park, Bishai et al. 2002; Dixit, Singh et al. 2012). The assay's turnaround time has been reported to be less than 10 days, thus vastly improving the time-delay as compared to conventional culture (Park, Bishai et al. 2002). The few concerns raised by Bwanga et al., 2009 and Dixit et al., 2012, include the cost of the inverted microscope and training of staff to read the plates accurately (Bwanga, Hoffner et al. 2009; Dixit, Singh et al. 2012). Others have drawn attention to the labour intensity and the high contamination rates (Shah, Moodley et al. 2011).

#### *1.5.2.2 Microplate Alamar Blue Assay (MABA)*

This colorimetric assay takes advantage of the oxidative ability of viable bacterial cells by yielding a colour change of the oxidised compound (Collins and Franzblau 1997; Reis, Neves et al. 2004). The blue non-oxidised compound changes to a fluorescent or pink colour, that can be read either visually or using a fluorometer. The assay has been used previously to

detect methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci (Collins and Franzblau 1997). The time to results, when used for the detection of *M. tb* was reported to be within 10 days (Franzblau, Witzig et al. 1998).

Early studies revealed that the performance of the MABA was comparable to both the agar proportion method and the BACTEC 460 methodologies (Franzblau, Witzig et al. 1998; Reis, Neves et al. 2004). A study performed by Franzblau et al., 1998 revealed a 97% agreement between the MABA and the BACTEC 460 system and the time to detection was 8 days (Franzblau, Witzig et al. 1998).

The MABA has been applied in determining drug susceptibility of clinical isolates, where the lack of colour change upon drug exposure was indicative of resistance. Reis et al., 2004, found a 98% and 100% agreement for detecting INH and RIF respectively, compared to the agar proportion method (Reis, Neves et al. 2004). This study also confirmed the results of the study of Franzblau et al., 1998 in that the time to results was 8 days. This led to the following statement: “ the cost-effectiveness, speed and quantitative MIC results, the MABA test represents an excellent alternative to antimycobacterial DST”(Chauca, Palomino et al. 2007).

Chauca et al., 2007 however state that the use of this methodology needs to be restricted to laboratories, which follow strict bio-safety standards. Thus precluding this assay to be used as a point of care diagnostic test (Chauca, Palomino et al. 2007).

### 1.5.2.3 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

The yellow dye 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced by mitochondrial dehydrogenase in living cells to produce an insoluble purple MTT formazan crystals, which can be measured either visually or spectrophotometrically (Abate, Mshana et al. 1998; Mshana, Tadesse et al. 1998). The test was initially developed to detect mammalian cell survival. Since aerobically metabolising prokaryotic cells produce a similar dehydrogenase complex, the MTT can also be used to measure proliferation as well as viability of bacteria such as *Staphylococcus aureus* and *Listeria monocytogenes* (Abate, Mshana et al. 1998). The assay was subsequently adapted to detect mycobacterial cell viability and consequently to generate drug susceptibility profiles of *Mycobacterium avium* complex isolates. The amount of formation of formazan crystallization correlated proportionally to the amount live cells (Abate, Mshana et al. 1998).

A study performed by Abate et al., 1998 revealed that the test was successful in determining drug resistance profiles of stored isolates of *M. tb*, making this assay a cost effective way of screening infected patients (Abate, Mshana et al. 1998). Additionally, a study done by Mshana et al., 1998, showed that the test can detect RIF resistant isolates within 72 hours, thus greatly reducing the turnaround time (Mshana, Tadesse et al. 1998).

The MTT assay was found to have high concordance with the BACTEC MGIT 960™ with regards to the detection of INH, RIF, EMB and STR resistance, with 100%, 99.1%, 100% and 96% sensitivity respectively (Ferrari Mde, Telles et al. 2010). This study confirmed the findings by the Montoro group (Montoro, Lemus et al. 2005).

#### 1.5.2.4 *Resazurin Microplate assay (REMA)*

The Resazurin Microplate Assay (REMA) is a rapid method that utilizes the ability of mycobacteria to reduce the sodium salt resazurin as an indicator of growth (Palomino, Martin et al. 2002). This assay is similar to the MABA in that the assay also uses an oxidation-reduction indicator to assess cell viability during drug exposure (Palomino, Martin et al. 2002). This microplate-based phenotypic method is simple to perform with turnaround time being within a week (Palomino, Martin et al. 2002; Umubyeyi, Martin et al. 2006).

A study performed by Palomino et al., 2002 found the test to yield a high sensitivity (100%) and specificity (96.2%) in detecting INH resistant isolates and 100% concordance with the agar proportion method in detecting RIF resistant isolates (Palomino, Martin et al. 2002). The Martin group went a step further in that they used the REMA to detect resistance against second-line drugs with a specificity of 100% for ethionamide, kanamycin, PAS and ofloxacin, and a sensitivity ranging from 96.8 to 100%; the sensitivity for capreomycin was found to be 84.2%. The accuracy of the REMA plate method was comparable to the agar proportion method (Martin, Camacho et al. 2003). Similar results were also reported by Umubyeyi et al., 2006, where they tested the ability of the REMA to detect ofloxacin resistance (Umubyeyi, Martin et al. 2006).

Another promising phenotypic assay is the Nitrate Reductase Assay (NRA), which uses the ability of mycobacteria to reduce nitrate to nitrite and this biochemical event can be detected by the addition of a colour-changing reagent (Griess reagent)(Angeby, Klintz et al. 2002; Bwanga, Joloba et al. 2010). LJ medium containing potassium/sodium nitrate is mixed with a drug to be tested, after which growth or lack thereof will be detected after a suitable growth period by addition of Griess reagent. Several studies revealed promising results using cultured isolates, specifically, the Angeby group who found a 94% agreement of results between the NRA assay and BACTEC 460 (Angeby, Klintz et al. 2002). When drug susceptibility results were evaluated, there was a 100% agreement in the detection of RIF resistance, a comparable agreement with INH, but as with other tests, there were several discordant results for streptomycin and ethambutol resistance (Angeby, Klintz et al. 2002). The time to results was found to be comparable to the BACTEC MGIT 960™ and 460™ technologies.

The NRA offers the advantage of not requiring anything else other than conventional laboratory consumables. A drawback to this assay was highlighted by Dixit et al, 2012, who noted that approximately 1% of *M. tb* isolates do not possess the nitrate reductase enzyme. Such isolates may be falsely labelled as susceptible (Dixit, Singh et al. 2012).

### 1.5.3 BACTERIOPHAGE BASED ASSAYS

The last two decades have seen the study and advancement of bacteriophage-based technologies in the detection of *M. tb* bacilli in clinical isolates, and their use in generating drug susceptibility profiles (Jacobs, Barletta et al. 1993; Wilson, al-Suwaidi et al. 1997; Pai, Kalantri et al. 2006; Piuri, Jacobs et al. 2009). Initially, the bacteriophage assay (PhaB assay) was based on plaque formation in a lawn of *M. smegmatis*. It relied on virucidal compounds to remove extraneous particles, thus assuming that subsequent plaques were the result of bacilli-infected viral progeny (Albert, Heydenrych et al. 2002; Albert, Trollip et al. 2002; Pai, Kalantri et al. 2006). The methodology of this assay is outlined in more detail in a later section. Subsequently, reporter bacteriophages were developed which eliminated the need for a virucidal step (Jacobs, Barletta et al. 1993).

#### 1.5.3.1 *FastPlaque TB™ and FastPlaque-RIF™*

A commercialized version of the PhaB assay, the *FastPlaque TB™*, developed in BIOTEC Laboratories Ltd, Ipswich, UK, was extensively evaluated in several studies with promising results. Briefly, the Albert group (2002) found that the assay had a 75% sensitivity and 99% specificity in their cohort of 1692 isolates, (Albert, Heydenrych et al. 2002). Furthermore, the kits could be applied directly on decontaminated specimens, further reducing the turn-around time. The drug susceptibility test version was also developed in order to detect rifampicin resistant isolates. The Albert group revealed that the *FastPlaque-RIF™* assay was able to detect 100% RIF resistance in smear positive sputum specimens, with an overall 98.8% specificity, thus the overall accuracy of the kit was 99.2%(Albert, Trollip et al. 2002).

## 1.6 INTRODUCTION TO MYCOBACTERIOPHAGES

Bacteriophages (phages) are the most numerous organisms on earth, it has been estimated that  $10^{31}$  different bacteriophages exist and all exhibit immense diversity in their genetic make-up (McNerney 1999; Pedulla, Ford et al. 2003; McNerney and Traore 2005). The significance of genomic diversity among phages is highlighted by the fact that no two genetically identical phages have been isolated (Pedulla, Ford et al. 2003). The presence and effects of phages have been observed since the earlier part of the 20<sup>th</sup> century. In 1915, Twort managed to isolate an agent that caused lysis of *Staphylococcus aureus*. He concluded the agent to be a particle (Bradley 1967). The name 'Bacteriophage' was given by d'Herelle to a particle that cleared cultures of Shigella, characterizing it as an ultramicroscopic parasite in bacteria (Bradley 1967). Due to the lytic nature of phages, they gained popularity as a potential antimicrobial agent. However, the concept was not completely developed due to the discovery and successful use of antibiotics (Bradley 1967; McNerney 1999).

Bacteriophages were found to have the ability to infect host bacteria by injecting its own DNA into the bacterial cell, using the host's machinery to generate progeny, leading eventually to lysis of the host cell (McNerney 1999). Two types of replication cycles or phases are observed among the phages namely the lytic and the temperate or lysogenic cycle. During the lytic cycle, the virus enters the host cell, replicates immediately, and lyses the host cell to release its progeny. In contrast, during the temperate cycle the phage enters the host cell, inserts its own DNA into the host's, lies dormant, replicating with the host DNA, until external conditions trigger the release of its progeny and ultimately lysis of the host cell.

Bacteriophages that infect mycobacteria, also referred to as 'mycobacteriophages', were first isolated from soil and leaf mould by Gardner and Weisner in 1947 (McNerney 1999; McNerney and Traore 2005). These particular mycobacteriophages were able to infect and lyse the fast-growing species, *Mycobacterium smegmatis* (*M. smegmatis*). Later (1954), other phages which were able to infect the slow growing *M. tb* and *M. bovis* BCG (Bacille Calmette Guerin) were discovered (McNerney and Traore 2005). To date, over 250 mycobacteriophages are known and more are being discovered and characterized (Pedulla, Ford et al. 2003).

The discovery of increasing numbers of mycobacteriophages with restricted host range, led to the consideration of using them for isolate identification and typing. The mycobacteriophage DS6A was found to be specific to bacteria of the *M. tb* complex (MTC) (McNerney 1999). DS6A and others were utilised to differentiate other strains such as *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium xenopi* and *Mycobacterium fortuitum* (McNerney and Traore 2005). The idea had several shortcomings, the main one being the large host range of several mycobacteriophages. Despite the valuable data gathered, phage typing remained a crude technique (McNerney 1999). Furthermore, the development of more discriminating molecular typing methods rendered phage typing obsolete (McNerney 1999). Thus, focus was turned to phages that could differentiate among clinically relevant and pathogenic strains of the MTC (McNerney 1999).

The idea of using the lytic ability of mycobacteriophages as a therapeutic tool against TB had been met with limited success in that phage treatment of infected animals failed to yield

positive therapeutic outcomes (McNerney 1999). Furthermore, the guinea pigs used were found to be adversely affected by phage therapy (McNerney 1999). Using mycobacteriophages to kill *M. tb* presented several challenges, namely: the transport of phages across mammalian cell membranes, where part of the bacilli reside, and decreased survival of phages in the pH reduced intra-cellular environment (McNerney and Traore 2005). Phage delivery systems were developed in this regard, where macrophage cell lines infected with *M. tb* were co-infected with *M. smegmatis* carrying the TM4 phage (McNerney and Traore 2005). The rationale was that once the *M. smegmatis* cells were lysed by the inherent TM4 phage, the surrounding *M. tb* cells would also be infected (McNerney and Traore 2005). There remained the challenge of gaining access to granulomas, which proved impenetrable and therefore prevented complete clearance of *M. tb* (McNerney and Traore 2005).

#### 1.6.1 GENETIC MANIPULATION OF *M. TUBERCULOSIS* USING MYCOBACTERIOPHAGES

Despite the advances in genetic engineering and molecular biology of *E. coli* and its infecting  $\lambda$  phages, work on mycobacteria has lagged. The factors behind the difficulty of working with mycobacteria were mainly due to the infectious nature of species of the MTC, difficulty of accessing single cells (mycobacteria grow in clumps) as well as the time it took to grow single colonies, 14-28 days (Jacobs Jr, Snapper et al. 1989; Jacobs Jr 2000).

Although the first transfection system (transfer of viral genetic material into a bacterial cell) for *M. tb* was described in 1967 and the first transduction system (the transfer of genetic material from one bacterial cell to another by a bacteriophage) reported in 1970, attempts to introduce genetic material into mycobacteria remained relatively unsuccessful (Jacobs Jr 2000). The Jacobs' group hypothesized that experiments, similar to those done on *E. coli* and

*Streptomyces lividans* could be performed on mycobacteria (Jacobs W.R. 1987; Jacobs, Kalpana et al. 1991). That is, the generation of expression vectors that would introduce recombinant DNA into mycobacteria. "By achieving this process, the study of mycobacterial genetics would be greatly enhanced" (Jacobs Jr, Snapper et al. 1989; Jacobs Jr, Kalpana et al. 1991; Jacobs Jr 2000). The ability of mycobacteriophages to infect both slow and fast growing mycobacteria made them viable candidates for the development of recombinant DNA expression vectors (Jacobs Jr, Snapper et al. 1989). Jacobs' group succeeded in generating cloning vectors (vehicles for nucleic acid transport) for mycobacteria (Jacobs W.R. 1987; Jacobs Jr, Snapper et al. 1989; Jacobs W.R., Jeffrey D. Cirillo et al, 1991; Jacobs W.R. 1993).

The analysis of a variety of mycobacteriophages revealed that the genomes of D29 and TM4 mycobacteriophages possessed cohesive ends, which, theoretically, would allow for *in vitro* packaging into  $\lambda$  phage heads (Jacobs W.R. 1993; Bardarov, Bardarov Jr et al. 2002). This was based on previous work done on non-mycobacterial phages (Jacobs Jr, Snapper et al. 1989). The methodology for introduction of DNA into a bacterial cell was based on work done with *Streptomyces* (Jacobs W.R. 1987). Furthermore, for ease of detection, Jacobs and co-workers aimed to develop a transfection system that would replicate as a plasmid in *E. coli* and a phage in mycobacteria, a phasmid. Fortuitously, they discovered that the phage TM4 possessed regions within its genome, that were not crucial to the survival of the phage, and hence could be removed or replaced with foreign DNA of choice (Jacobs W.R. 1987; Jacobs Jr, Snapper et al. 1989). The details of the processes involved are outlined below.

Briefly, the investigators chose the phage TM4 that readily infects the fast-growing *M. smegmatis* and the slow-growing *M. tb* and *M. bovis* BCG. The DNA from the phage was

restricted with a restriction enzyme compatible with the already restricted cosmid pHC79. The restricted pHC79 and the phage fragment were allowed to anneal. The ligated chimeras were then packaged into  $\lambda$  phage heads *in vitro* and transduced into *E. coli* cells. The chosen plasmid contained a selectable marker (e.g. ampicillin resistance), which was utilised to select for viable pHC79::TM4 recombinants. The viable *E. coli* recombinants were selected and then transfected onto *M. smegmatis* lawns. The surviving recombinants were analysed electrophoretically and yielded various genomic combinations of plasmid and TM4, ranging from no plasmid to large segments of plasmid within the phage. Among the recombinants with both plasmid and phage DNA, one was chosen for further analysis and thus named the first phasmid (phage and plasmid chimera) phAE1. The plasmid DNA was found to have inserted into the non-essential region of the TM4 phage genome (Jacobs W.R. 1987; Jacobs Jr, Snapper et al. 1989; Jacobs Jr 2000). The work performed by Jacobs' group revolutionised the previously impossible task of genetic manipulation of mycobacteria. This has led to the use and potential success of mycobacteriophages in either diagnosis or vaccine development.

### 1.6.2 REPORTER MYCOBACTERIOPHAGES AND REPORTER PROTEINS

Reporter proteins are routinely used as markers for biological processes and have been used in a wide variety of applications namely, cancer, plant and molecular biology studies (Gilad, P.T. et al. 2007; Kavita and Burma 2008). The most studied and extensively used reporter proteins in microbiology are luciferase, green fluorescent protein, alkaline phosphatase and  $\beta$ -galactosidase reporter markers, coded for by the *Fflux*, *gfp*, *phoA* and *lacZ* genes respectively. The advantage of using reporter proteins is in the tracking of a metabolic process of a living cell, without the need to inactivate it (Gilad, P.T. et al. 2007; Kavita and Burma 2008). The reporter gene could be incorporated into a bacterial cells and 'tracked' as

it underwent the metabolic process of concern. Thus, incorporating a reporter gene within the phage genome became a distinct possibility, where cell viability was a concern.

'Mycobacteriophages offered a promising tool for early diagnosis of drug-resistant TB in that they offer a phenotype-based result in a turnaround time similar to that of some molecular approaches at a low cost' (Hazbon, Guarin et al. 2003).

### 1.6.3 REPORTER PROTEINS: ORIGINS, CHEMICAL REACTIONS AND APPLICATIONS

#### 1.6.3.1 *Green fluorescent protein (GFP)*

The two well-studied green fluorescent proteins (GFP) are derived from the marine invertebrates, *Aequorea victoria* and *Renilla reniformis*, of which the most widely used GFP is from the *A. victoria*. The *A. victoria* was first purified and characterized by Shimomura et al., 1962, as the first light producing protein from a green bioluminescent coelenterate, aequorin. They found that the activated aequorin generated blue light as opposed to green where the green colour was generated by the excitation of an inherently fluorescent protein (Chalfie 1995). The *A. victoria* GFP is a 238 amino acid protein, with an absorbance peak of 395nm and has been used widely as a reporter of gene expression, a tracer of cell lineage and as fusion tag to monitor protein localization within living cells (Cubitt, Heim et al. 1995). The initial demonstration that this protein could be utilised in gene expression was performed in *E. coli* and the nematode *C. elegans*. In *C. elegans*, the protein was attached to a promoter utilised only in a few nerve cells and results showed marked fluorescence throughout the cytoplasm of these nerve cells. This initial experiments showed that GFP could be used as a reporter protein to track processes in live cells, without the need for cell permeabilization or

toxic detection methods (Chalfie 1995). The fusion tagging was successfully demonstrated by adding the GFP to the N- or C-terminus of the *exuperantia* (EXU) gene of the *Drosophila* oocyte. The tagging showed no deleterious effects on the function of the EXU gene (Chalfie 1995). The wild-type GFP has since been modified to increase fluorescence intensity, altered excitation and emission spectra. The major advantage of utilizing this protein is ability to be tracked in living cells, its specificity and lack of cross-reactivity.

#### 1.6.3.2 *Beta-galactosidase enzyme (lacZ)*

The *E. coli*  $\beta$ -galactosidase enzyme is a product of the *LacZ* operon, which was central in the development of the Jacob and Manod's operon model (Cohn 1957; Matthews 2005). The 464kDa protein, was found to have 2 catalytic activities, namely hydrolysing the disaccharide lactose into the monosaccharide's galactose and glucose as well as converting lactose to allolactose, which in itself, acts as a natural inducer for the lac operon(Matthews 2005). The enzyme is one of the most widely used reporters of gene expression in molecular biology (Griffith and Wolf Jr 2002). Although the enzyme has strict specificity for sugars in the galactosyl position, it was found to be adept at hydrolysing  $\beta$ -D-galactopyranosides, lending this promiscuity to exploiting a wide variety of chromogenic substrates, such as 5-bromo-4-chloro-3-indoyl- $\beta$ -D-galactopyranoside (X-gal) and o-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG)(Griffith and Wolf Jr 2002; Anson and Limberis 2004; Matthews 2005).

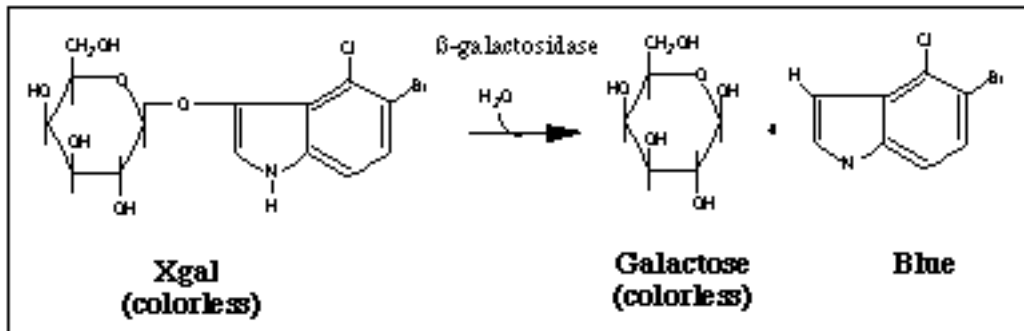


Figure 1.1: Chemical reaction involving x-gal and the beta-galactosidase enzyme <http://www.sci.sdsu.edu>

The  $\beta$ -galactosidase enzyme was proven to have a wide range of applications, and has been used in studies with both prokaryotic and eukaryotic cells. The soluble indigo-blue reaction product released by the enzyme from X-gal, has been found to be stable and readily detectable, thus allowing for assays to be re-examined over long periods of time (Anson and Limberis 2004).

1.6.3.3 Luciferase (*Fflux gene*)

The luciferase reporter protein, derived from the firefly, yields quantifiable photons by catalysing the interaction of luciferin substrate and adenosine triphosphate (Jacobs, Barletta et al. 1993).

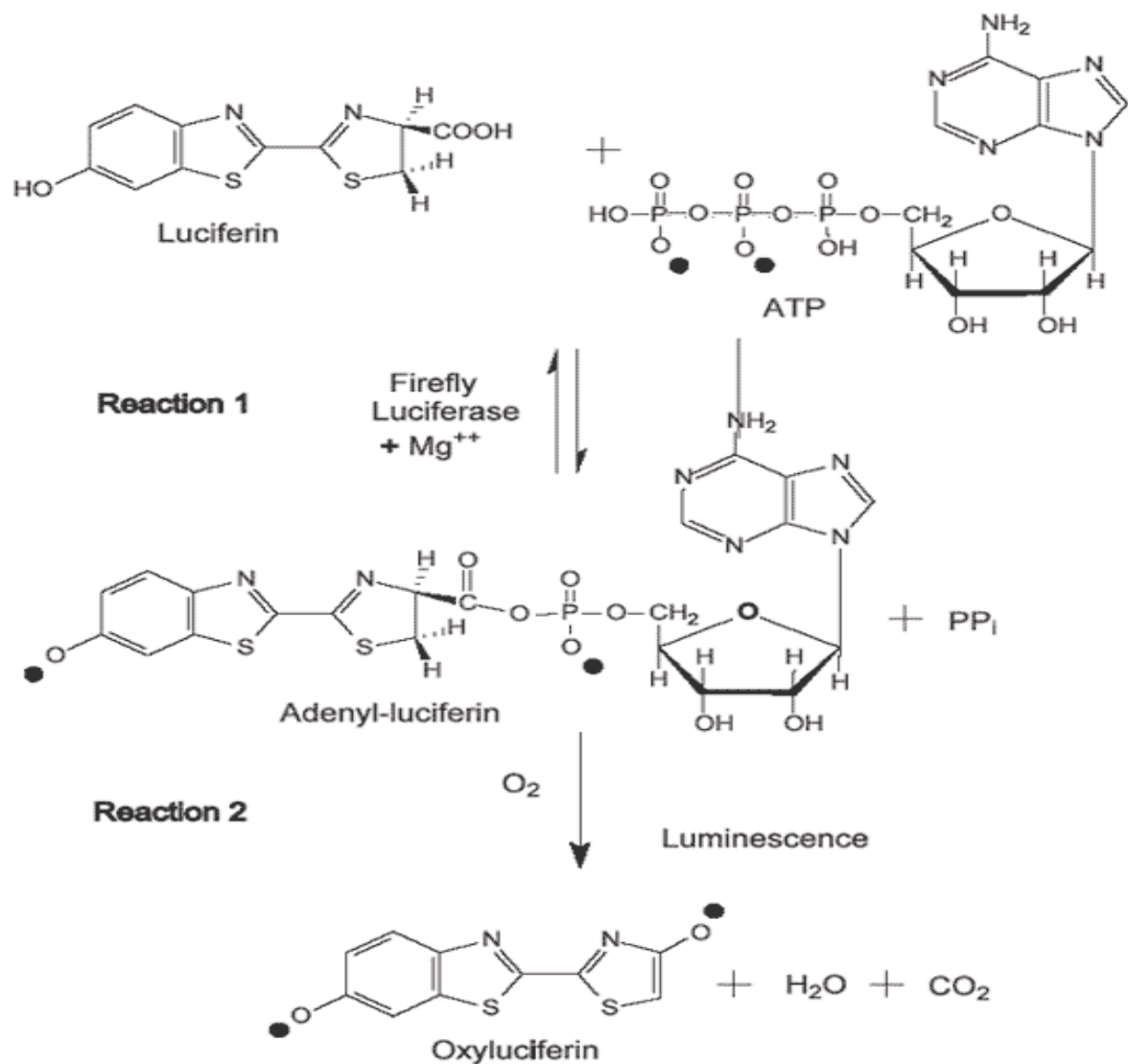


Figure 1.2: Chemical reaction of the firefly luciferase <http://www.sigmaaldrich.com>

1.6.3.4 Alkaline phosphatase (PhoA)

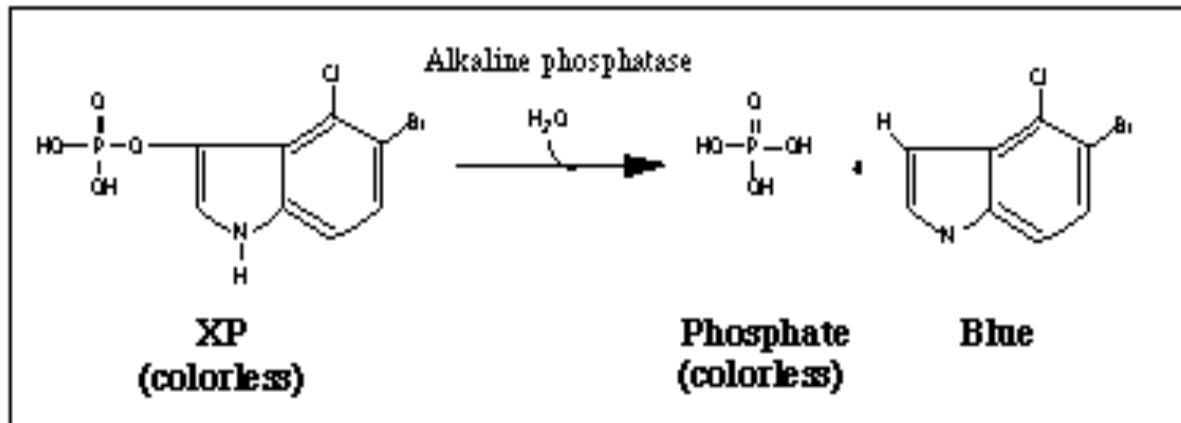


Figure 1.3: Chemical reaction involving phosphate and the alkaline phosphatase enzyme <http://www.sci.sdsu.edu>

Alkaline phosphatase is another reporter protein with enzymatic activity. However it is not popular in comparison to the beta-galactosidase or the firefly luciferase.

## 1.7 REPORTER PROTEINS AND THEIR APPLICATION TO MYCOBACTERIOPHAGES

The earlier success of introducing foreign DNA into mycobacteria led to the consideration of using reporter gene-phage complexes to detect mycobacteria. The biological characteristics of mycobacteriophage (infects and lyse viable host cells only) were ideal for developing a series of reporter mycobacteriophages (Jacobs, Kalpana et al. 1991). While the original phasmid included the cosmid and phage DNA, for the generation of a reporter phage, the cosmid DNA was excised and replaced with a reporter gene (1993), leading to the construction of phAE39 and phAE40 which contained the *Flux* gene (Jacobs W.R. 1993).

Hence, the first reporter phage containing the *Eflux* gene was developed expressing the gene product (luciferase) that would interact with luciferin (substrate) and the host ATP to produce light (0.85 photons/ molecule of substrate), indicating host cell viability (Jacobs W.R. 1993; Banaiee 2001). Furthermore, the premise that reporter phages only infect viable mycobacteria led to the use of reporter mycobacteriophage to establish drug susceptibility. This was achieved by adding anti-tuberculosis drugs to the medium and measuring light production indicating the presence of viable cells and thus resistance to the drug used (Jacobs W.R. 1993).

### 1.7.1 THE DEVELOPMENT OF OPTIMAL REPORTER MYCOBACTERIOPHAGES

Previously, the molecular and genetic mechanisms behind the ability of phages to accept foreign DNA and infect host bacteria were unknown or rather, not studied. The manipulation of TM4, L5 and D29 phages occurred (1987) before their genomes were sequenced in 1998. It became apparent that knowledge of the genome and molecular mechanisms surrounding phage-host interaction would elucidate previously unknown features of mycobacteria (Ford, Stenstrom et al. 1998). Thus, the Hatfull group undertook the task of sequencing the available and widely used mycobacteriophages (Pearson, Jurgensen et al. 1996; Bardarov, Kriakov et al. 1997; Ford, Sarkis et al. 1998; Ford, Stenstrom et al. 1998; Peña, Stoner et al. 1998; Pedulla, Ford et al. 2003; van Kessel and Hatfull 2007).

Four mycobacteriophages, TM4, L5, D29 and recently Che12, have been studied and utilised extensively as tools to detect *M. tb*, as well as to determine drug susceptibility profiles of mycobacterial isolates.

#### 1.7.1.1 *TM4 derived reporter mycobacteriophages*

The mycobacteriophage TM4 was first isolated in 1984, by Timme and Brennan (Ford, Stenstrom et al. 1998). This bacteriophage genome is similar in size (approximately 50kb) to most of the mycobacteriophages isolated and studied to date and possesses cohesive termini, which lends the ability for foreign gene insertion (Ford, Stenstrom et al. 1998). It is able to infect a wide range of hosts, such as the slow growing *M. tb*, *M. avium* and *M. paratuberculosis* and the fast growing non-pathogenic *M. smegmatis*. It does however, infect *M.bovis* BCG at a reduced efficiency (Ford, Stenstrom et al. 1998). Analysis of the plaque morphology does not immediately reveal the phage to be either lytic or temperate because it forms hazy or turbid plaques on *M. smegmatis* lawns (Ford, Stenstrom et al. 1998), contrary to the clearly temperate L5 phage. An extensive review of the biology of TM4 phage is provided elsewhere (Ford, Stenstrom et al. 1998). This thesis will focus on the subsequent use of the TM4 phage to detect mycobacterium bacilli. Of note, is the fact that this phage was sequenced 11 years (Ford, Stenstrom et al. 1998) after its prodigious use for the detection of *M. tb* (Jacobs W.R. 1993).

The TM4 mycobacteriophage was amongst the first mycobacteriophages to be used for recombineering experiments. As mentioned above, the Jacobs group elected to insert the reporter gene *Fflux* into it's genome (Jacobs W.R. 1993). Briefly, in order to ensure that the luciferase gene would be expressed within mycobacteria, the *Fflux* gene was cloned into the extra-chromosomal plasmid pYUB180 and the integration plasmid pGS16. The recombinant plasmids were then electroporated into *M. smegmatis* mc<sup>2</sup> 155 host cells. The host cells were

serially diluted to test for the sensitivity of detection, where it was found that expression of luciferase could be detected in  $5 \times 10^1$  cells/ ml.

The luciferase reporter phasmid (chimera of plasmid and phage) was constructed by inserting the *E. coli* cosmid pYUB216 into the TM4 non-essential region. The resultant phAE39 was found to infect *M. tb* readily; however, it did not as efficiently infect *M. bovis* BCG host cells. Subsequent host range mutants of phAE39 were isolated, where one (phAE40) was chosen for further analysis. The phAE40 was transfected into *M. smegmatis* and evaluated for light production. Luciferase activity was measurable for more than 3 hours post infection, for both *M. bovis* BCG and *M. smegmatis* cells (Jacobs W.R. 1993). The success of the phAE40 phasmid, led to its use in exploring its ability to distinguish between drug-susceptible and drug-resistant mycobacteria. Culture isolates of resistant and susceptible (*M. bovis* BCG and *M. smegmatis*) mutant cells, with or without antibiotics, were infected with phAE40 phage and luciferase activity measured. Light emission was observed and thus the phAE40 was able to distinguish between drug resistant and drug susceptible isolates (Jacobs W.R. 1993).

Although the TM4 was found to be capable of detecting *M. tb* and determining susceptibility of isolates, it was found to be somewhat limited in its ability to generate prolonged light output. The reason for the reduced light production was presumably due to premature lysis of host cell (Sarkis, Jacobs et al. 1995). Therefore a phage with an ability to form lysogens, and delay host cell lysis, needed to be found to improve detection. These concepts lead to the development of other, more sensitive, lysogenic and specific phasmids.

#### 1.7.1.2 L5 derived Reporter Mycobacteriophages

The decline of light production by the phage phAE40 due to lysis of the infected bacteria, led the Jacobs' group to investigate a temperate phage, which has the ability to form lysogens, thus prolonging the production of light (Sarkis, Jacobs et al. 1995). Thus, the L5 mycobacteriophages was chosen. It was among the first mycobacteriophages to be completely sequenced by the Hatfull group (Sarkis, Jacobs et al. 1995).

The preferred host for L5 is *M. smegmatis*. However, unlike the TM4, the L5 does not form plaques efficiently in *M. bovis* BCG and *M. tb* lawns (Sarkis, Jacobs et al. 1995). Together with genome characterization, Sarkis, Jacobs jr. and Hatfull constructed a phasmid, using the L5 phage. Genes 6-12 of the L5 phage and the *Fflux* gene were inserted into the *E. coli* shuttle vector (pMD31) to yield a phasmid phGS24. This phasmid was passaged several times in *M. smegmatis* where two resultant phasmids (phGS15 and phGS18) were analysed further. Restriction analysis of the two phasmids revealed that there were deletions of several L5 genes. The conclusion was that the L5 genome does have non-essential regions, which are not crucial to the survival of the phage.

The plaque morphology of both phasmids (phGS15 and phGS18) was found to be somewhat similar to the wild type L5 phage. The measurement of luciferase activity revealed a short 20-minute lag period, followed by a rapid increase in light production. It was also revealed that the light output from phGS18 was five-fold higher than the reference phAE40. Surprisingly, light production remained for longer periods than with phAE40. Several theories were formulated to explain this phenomenon, the most plausible one being the L5's

ability to form stable lysogens caused the prolonged and increased light output (Sarkis, Jacobs et al. 1995). The efficiency of the L5-based phage as reporter phage was proven, however, there was a major shortcoming. As previously mentioned, the L5 phage did not form plaques efficiently on either *M. tb* or *M. bovis* BCG, making it useless in detecting clinically relevant, mycobacterial strains (Pearson, Jurgensen et al. 1996). Regardless of this limitation, important findings were alluded to. Gene 71 of the L5 phage, may have a more significant role to play in increasing the sensitivity of the luciferase reporter phage (LRP) assay and the ability of a phage to form lysogens which may influence the expression of the *Fflux* gene.

#### 1.7.1.3 *The Bronx Box and its application in detecting Luminescence*

The development and application of the Luciferase reporter mycobacteriophage, offered a viable option in the diagnosis of TB, however, the use of expensive machinery (luminometer) threatened to inhibit the assay's use in developing countries. An alternate approach was developed, whereby a Polaroid film in a device called the 'Bronx Box' was developed as a 'low cost' alternative to the expensive luminometer (Hazbon, Guarin et al. 2003). It was found that detection of *M. tb* as well as DST assay results were in almost 100% agreement with the reference methods. Additionally, the Bronx box detected more than 80% of INH and RIF resistant isolates, in a shorter time period than the proportion method assay (Hazbon, Guarin et al. 2003). However, the Bronx Box also had the disadvantage of only being operational in the dark, which would invariably pose health risks especially when working with highly infectious material such as *Mycobacterium tuberculosis*.

#### 1.7.1.4 D29- derived reporter mycobacteriophages

The phage TM4 was proven to be able to accept foreign DNA in the form of the luciferase encoding gene *Fflux* (Jacobs W.R. 1987; Jacobs W.R. 1993). The phage was able to infect both slow- and fast-growing mycobacteria (Jacobs W.R. 1993). Additionally, the phasmid (phAE40) was able to differentiate between drug susceptible and drug resistant strains of mycobacteria. However, the challenge remained in the phasmid's inability to detect small numbers of bacilli, thus leading to the development of the L5 phage based reporter (phGS18) with increased sensitivity and prolonged light output. As mentioned before, the L5 phage does not infect the clinically relevant *M. tb*. Hence, the Jacobs and Hatfull groups investigated the possibility of converting the closely related D29 (i.e. to L5) phage into a reporter phage, in a similar manner as the TM4 and L5 phages.

The choice of the D29 phage was based on its sequence homology to the L5 temperate phage, as well as the D29 phage's ability to infect a wider range of mycobacterial hosts than L5 (Pearson, Jurgensen et al. 1996; Ford, Sarkis et al. 1998). Froman et al. discovered the D29 mycobacteriophage in 1954. It infects a large variety of mycobacterial species such as slow and fast growing mycobacteria, *M. bovis* BCG and even *M. leprae*, making it a good candidate for further development as a reporter phage (Pearson, Jurgensen et al. 1996; Ford, Sarkis et al. 1998; Peña, Stoner et al. 1998). Additionally, it is a lytic phage, which exhibits highly efficient infectivity as seen in *M. smegmatis* infection studies, where pro-phage was released within 90 minutes of infection (Pearson, Jurgensen et al. 1996). This phage has similar genome size (48kb) as the previous two phages (TM4 and L5), and it has cohesive ends.

Pearson et al., 1996 found that the expression of the luciferase cassette in the D29 phage was similar to the reference phAE40 (TM4-derived phage). The significant difference between the D29-based phBD8 and TM4-based phAE40 was the sharp decline of the phBD8's light output during infection (Pearson, Jurgensen et al. 1996). This sharp decline was seen as a reflection of the phasmid's infective efficiency and its lytic nature (Jacobs W.R. 1993; Pearson, Jurgensen et al. 1996).

Although the phBD8 was relatively more sensitive than the phAE40, it required further modifications in order to delay host cell decline. It is worth noting that investigations into the conversion of mycobacteriophages into reporter phages elucidated the biology of the phages themselves. For instance, host immunity to super-infection by D29 was found to increase the light output of the D29 phasmids, in that, by infecting *M. smegmatis* L5 lysogens with phBD2 and phBD8, production of light increased significantly. Another phenomenon was observed with regards to the orientation of the luciferase cassette within the genome. Two types of D29-based reporter phages were observed, the one type had its transcription direction away from the cohesive terminus and the other type was placed in the opposite direction. The orientation of the cassette clearly influenced the light production, where the first type of orientation produced light and the other did not (Pearson, Jurgensen et al. 1996). This prompted further investigation into the biology of the phages.

The effect in homo- and hetero immunity to infection and its applications in the sensitivity of phages were studied and it was found that the gene product (gp71) of the L5 phage plays a significant role in host cell immunity to infection. Furthermore, the directionality of the luciferase cassette and its influence on light production needed further study as well as the

ability of the mycobacteriophage head to package DNA. It was found that the D29 phasmids has the ability to package a larger genome than the wild-type (D29); pBD2 is 6% larger than the original genome (Pearson, Jurgensen et al. 1996).

#### 1.7.1.5 *D29 Phage- Based assays (Phage Biologically Amplified Assay)*

As early as 1964 Tokunaga and Sellers investigated the mechanism of D29 bacteriophage infection of *M. smegmatis* (Tokunaga and Sellers 1964). Since then, numerous studies have been reported ranging from mycobacterial genome manipulation and the application of the phage for detection of *M. tb* as well as for drug susceptibility testing (Jacobs, Barletta et al. 1993). McNerney and Wilson, 1979 followed by David et al., 1980, hypothesised that the D29 phage could ‘in-theory’ be used to determine drug susceptibility of *M. tb*, by exposing the bacilli to drugs, and allowing for phage infection thereafter. They showed that *M. smegmatis* ‘indicator’ cells could be used to determine the presence of viable D29 infected *M. tb* bacilli. Thus the Phage Biologically Amplified Assay (PhaB) was developed and extensively promoted as an inexpensive technology (Wilson, al-Suwaidi et al. 1997; McNerney, Wilson et al. 1998; McNerney 1999; Traore, Ogwang et al. 2007; Zhu, Cui et al. 2011).

Briefly, *M. tb* bacteria are mixed with phages, which are then allowed to adsorb and infect the cells. Exogenous phages are consequently destroyed and the bacteria are plated onto a lawn of ‘phage-sensitive’ cells (*M. smegmatis*). A phage-infected bacterium would eventually lyse, and the released phage progeny form a visible plaque in the lawn of indicator cells (Jacobs, Barletta et al. 1993; McNerney, Wilson et al. 1998). The simplicity of the assay as well as its fast turn-around time of 2 to 4 days made it a viable option for use in low to middle-income countries (Wilson, al-Suwaidi et al. 1997; Eltringham, Wilson et al. 1999; Park, Drobniewski et al. 2003).

### 1.8.1 FURTHER MODIFICATIONS TO REPORTER MYCOBACTERIOPHAGES

The period between 1987 (the development of a phasmid), 1993 (the expression of Luciferase gene), 1995/6 (the development of L5-based phasmid) and 1996 (the development of the D29-based phasmid), served to increase the knowledge base regarding mycobacteriophages and their accompanying host mycobacteria. The phAE40 was among the first reporter phages to be developed and was thus maintained as a reference phage for further improvements. Since the phAE40 phage, subsequent reporter phages had not improved the assay significantly, thus researchers went back to the phAE40 system in order to improve it.

The Jacobs group concentrated on the lytic ability of the phAE40 phage, with the focus of attempting to delay the process, thus enhancing its detection sensitivity (Carriere, Riska et al. 1997). The concept of gene cassette location and transcriptional direction within the phage genome was explored for potential increased light output. Thus after a round of plasmid vector insertions, three phages, which had different locations and different transcriptional locations within the genome were isolated (phAE80, 81 and 82). From this step, the luciferase gene was inserted to yield phages (phAE83-86). Light kinetic studies revealed that the phage phAE85 produced 3-10 times more light than the reference phAE40, thus it was chosen for further study and possible manipulation. It was worth noting that the transcriptional direction and location of the *Fflux* gene did not affect gene expression, because phAE83 and 84 (opposite orientation) did not differ in their light output (Carriere, Riska et al. 1997).

Upon further evaluation of the phAE85, it was found that it did not form plaques on *M. bovis* BCG, however the light production was significantly higher than phAE40. Hence, a temperature sensitive mutation was introduced, where the phage would be able to propagate on *M. smegmatis* at 30°C but fail to form plaques at 37°C, the procedural temperature of the luciferase assay. The rationale behind this, was to obtain a phage that could deliver the luciferase gene into the host without lysing the cell, thereby imitating the temperate L5 phage. The mutation inhibits the phage's ability to form plaques at 37°C, inherently extending the light production after host infection (Carriere, Riska et al. 1997). Light kinetic studies showed success in this regard, however, cell lysis still occurred. Therefore, another round of mutants were developed from the original TM4 phage as opposed to the existing phasmids.

A temperature-sensitive mutation was introduced before the insertion of the *Fflux* gene, where the phAE88 phasmid showed increased sensitivity and prolonged light output (Carriere, Riska et al. 1997). Furthermore, the phAE88 produced 9 times more light than the reference phAE40, peaking at 21 hours and was able to detect as few as 120 cells after 12 hours, versus 10<sup>4</sup> cells and 3.2 hours for the phAE40. It was also found that the phAE88 has, like the pBD2 phage, the ability to package a genome larger than its wild type (Carriere, Riska et al. 1997).

Its use in antibiotic susceptibility tests revealed the phAE88 to be more sensitive and produced results more rapidly than the phAE40: less than 30 hours as opposed to 40 hours for the phAE40. The increased and prolonged signal outputs allowed for the development of light capture methods (1.7.1.3), that would eventually eliminate the use of the expensive luminometer, making this assay suitable for resource-constrained laboratories.

Although the overall sensitivity for detection as well as for drug susceptibility testing of the phAE40-phAE88 phages remained favourable in comparison to traditional methods, there still remained the challenge that, they infected several species of mycobacteria (Riska, Jacobs et al. 1997). Therefore, a luciferase-luciferin- ATP reaction after exposure to this phage does not necessarily mean that the reacting organism is *M. tb*. Hence, a compound  $\rho$ -nitro- $\alpha$ -acetyl amino- $\beta$ -hydroxy propiophenone (NAP) that has selective inhibitory activity against *M. tb* complex (MTC) was used in conjunction with the LRP assay. NAP served to differentiate between mycobacteria other than tuberculosis (MOTT) and MTC by inhibiting peptide chain elongation of bacteria belonging to the MTC, ultimately abrogating light production by these species (Riska, Jacobs et al. 1997).

The study performed by the Jacobs' group revealed an increase in specificity exceeding 97% through the addition of NAP. The study also provided valuable information regarding the optimal concentration (10  $\mu$ g/ml) and optimal incubation period (24 hours) of NAP as well as the minimal bacilli concentration of  $10^6$  CFU/ml (Riska, Jacobs et al. 1997).

#### 1.8.1.1 *Che12- derived reporter mycobacteriophages*

Based on previously encountered problems with other reporter phages, the Rama group (2008) sought to investigate an alternative phage, bypassing the lytic activity and gradual light output decline of the TM4, the non-specificity of L5 and the temperature-sensitive mutation requirements of the D29 phage (Kumar, Loganathan et al. 2008). Soil samples around the TB clinic of Chennai, India, yielded a temperate phage that was labelled Che12 after the place of discovery. The host range of this phage was tested and it was found to have

a wide host range (i.e. infects more than 12 mycobacterial species) including *M. tb*, *M. smegmatis* and *M. bovis* BCG. This made Che12 an ideal candidate for further study (Kumar, Loganathan et al. 2008).

Subsequent genetic manipulations, similar to the previous phages, yielded a reporter phasmid phAETRC16, which was found to have increased light output as compared to the TM4-based phAE129 phage. The phAETRC16-infected bacteria observed a steady increase in light output peaking at 72 hours post-infection versus 4 hours for the phAE129 phage. Light kinetic experiments on the *M. tb*, clinical isolates revealed a 4-log increase in relative light units (RLU) after 7 days as opposed to 8 hours with the reference phages. The light output was similar in all strains of *M. tb* (Kumar, Loganathan et al. 2008). The use of Che12 and its ability to sustain light production up to 7 days provided various laboratories the flexibility to design suitable test formats.

The Che12 was revealed to be an ideal phage for the detection of mycobacteria, however, further studies are required particularly for its suitability in drug susceptibility testing. One could argue that based on previous work, Che12 would presumably be equally apt at providing susceptibility profiles of clinical isolates. Furthermore, the wide host range of Che12, including non-tuberculous species may require further modifications such as the addition of NAP (Riska, Jacobs et al. 1997). Additionally, unlike previous workers who were focused on the sensitivity of the test phage, the Che12 investigators failed to highlight the amount of bacilli cells required for an effective result. Hence, regardless of Che12's ability to detect *M. tb*, an extensive study on other aspects such as rapidity, specificity and sensitivity needs to be conducted.

#### 1.8.1.2 *Second Generation reporter mycobacteriophages: Fluoromycobacteriophages*

The development of the second generation of reporter mycobacteriophages by the Hatfull group (Piuri, Jacobs Jr et al. 2008), was prompted by the need to circumvent the expensive luminometer as well as the ability of a rapid test to discern mycobacterial bacilli from other accompanying sputum pathogens. These fluoromycobacteriophages (fluorophages) make use of equipment such as a fluorescence microscope or a flow cytometer. Such equipment is readily available: most TB laboratories use the fluorescence microscope to read auramine stained smears while many laboratories use a flow cytometer for CD4 counts (Piuri, Jacobs Jr et al. 2008).

Although the fluorophages are in their initial stages of development, potential major advantages over the previous reporter mycobacteriophages is in the ease of use and time required to perform the test. The specificity of infection of the fluorophages means that, mycobacterial bacilli can be detected, using a microscope, within a heterogeneous population of bacteria (Piuri, Jacobs Jr et al. 2008).

The construction and evaluation of the fluorophages was based on the already established TM4-temperature sensitive mutant (phAE87). The rationale behind the choice of this mycobacteriophage is mentioned above. Briefly, the TM4 temperature sensitive phage provided a sensitive assay, where it replicates at 30°C and expresses its reporter protein at 37°C thus allowing for detection at the optimal growth temperature of pathogenic mycobacteria. (Carriere, Riska et al. 1997; Piuri, Jacobs Jr et al. 2008). Initially, the reporter genes for green fluorescent protein (*Gfp*) and ZsYellow (*ZsYellow*) were each fused to the

plasmid (pYUB854) and transferred to the phAE87, generating a phAE87::*hsp60-EGFP* and phAE87::*hsp60-ZsYellow* respectively (Piuri, Jacobs Jr et al. 2008).

The fluoromycobacteriophages were evaluated for their fluorescing ability by infecting *M. smegmatis* cells and were found to produce fluorescence only in infected cells. Furthermore, upon mixing with *DsRED* expressing *E. coli* cells, green fluorescing *M. smegmatis* cells were discernable from the red *E. coli* cells with no overlap (Piuri, Jacobs Jr et al. 2008). The sensitivity of the phAE87::*hsp60-EGFP* was evaluated and found to be able to detect as few as 100 cells at 400-fold magnification (Piuri, Jacobs Jr et al. 2008).

Infection of *M. tb* yielded similar results as with *M. smegmatis* after 16 hours of phage infection; however, only 37-67% of cells fluoresced as opposed to 70% of *M. smegmatis* cells. The reason for this was not clearly determined (Piuri, Jacobs Jr et al. 2008).

An additional feature of fluoromycobacteriophages is their ability to maintain fluorescence after para-formaldehyde fixation (effectively killing mycobacteria). This feature makes the fluoromycobacteriophages a better option for reporter assays, as it reduces the hazard of working with infective specimens. Fixation experiments reveal that fluorescence is maintained up to 14 days post infection allowing for possible off-site evaluation and flow cytometry (Piuri, Jacobs Jr et al. 2008). Fluoromycobacteriophages were found to be specific in detecting resistant strains, especially resistance to rifampicin and streptomycin.

A distinct potential drawback of fluoromycobacteriophages is in their inability to discriminate between MTC and NTM (non-tuberculous mycobacteria) species, due to the non-specificity of its TM4 parent phage. Although the fluoromycobacteriophages may eliminate the use of the expensive luminometer, a flow cytometer or fluorescence microscope is still required, which ultimately may prove a stumbling block for use in resource-constrained laboratories. Piuri states that high HIV prevalence would require laboratories to purchase flow cytometers (Piuri, Jacobs et al. 2009). Since high HIV prevalence is invariably accompanied by a high TB incidence, incorporating fluoromycobacteriophages for TB detection would increase the cost effectiveness of that equipment.

**Table 1.1: Summary of Various Reporter Mycobacteriophages**

Study Year	Type and number of specimen	Phasmid used	Reference methodology	Time to results	Sensitivity	Specificity	Overall performance	Reference
1999	Reference strain collection & clinical strains	phAE85 (LRP)	BACTEC 460	72 hrs. (post MGIT culture)	50% EMB 89% STR 100% INH 100% RIF	97% EMB 67% STR 100% INH 100% RIF	Favourable	(Riska, Su et al. 1999)
2001	Sputum samples (n=523)	phAE142 (LRP)	MGIT-960 system Acid fast staining BACTEC-460 LJ method	2-4 days, 94% of DST results obtained within 48 hrs.	94% MTC 100% RIF 100% STR 100% EMB 85.7% INH	100% MTC 100% RIF 100% STR 100% EMB 95.3% INH	Favourable	(Banaiee 2001)
2003	MGIT clinical cultures (n=72)	phAE142 (LRP)	MGIT-960 system Acid fast staining BACTEC-460 NAP differentiation test	72 hrs. (post MGIT culture <sup>a</sup> ) 9 days (post BACTEC 460 culture <sup>b</sup> )	97.2% for MTC 100% for INH 100% for STR 100% for RIF 100% for EMB	100% MTC 100% INH 100% STR 100% RIF 94.4% EMB	Three time faster than BACTEC-460, Nearly 3 X faster than MGIT	(Banaiee, Bobadilla-del-Valle et al. 2003)
2003	Clinical isolates (n=51)	phAE85 (LRP)	Culture (7H9) Proportion method	54-94 hours (post culture)	100% for RIF 78.9% for STR 40% for EMB 100% for INH	96% for RIF 93.1% for STR 95.3% for EMB 96% for INH	Favourable	(Hazbon, Guarin et al. 2003)
2008	MGIT clinical isolates (n=191)	phAE142 (LRP)	BACTEC 460 system Agar proportion method MGIT 960 system	48 hrs. (post culture)	98.4% overall agreement with ref. method 100% for RIF 96.9 % for INH	100% for RIF 97.7 for INH	Favourable	(Banaiee, January et al. 2007 )

# CHAPTER 2: THE DEVELOPMENT OF THE BETA-GALACTOSIDASE REPORTER MYCOBACTERIOPHAGE AND ITS IMPLEMENTATION IN DETECTING CULTURED *MYCOBACTERIUM TUBERCULOSIS*

## 2.0 INTRODUCTION

The classic approach of diagnosing tuberculosis through laboratory tests has, for decades been smear microscopy following acid fast staining and culture on Lowenstein-Jensen slants and Middlebrook agar plates (1.4). However, these methods are failing to keep up with the developing crisis among third world countries. Since smear microscopy does not provide resistance profiles, use of this approach only for diagnosis of tuberculosis needs to be abandoned with the emergence of drug resistant transmissible strains. The presence of these strains has increased the urgency of development of tests that can rapidly detect the presence of *M. tb* in a clinical specimen and, with equal rapidity provide a resistance profile of the organism present. Such tests need to be affordable in resource constrained settings and safe to be performed in peripheral laboratories (1.4) (Piuri, Jacobs Jr et al. 2008).

With this in mind, several tests have been developed in recent years. These can be broadly divided in three categories. Firstly, improved culture methodologies mainly by introducing indicators of growth rather than observing the bacterial growth itself. These methodologies

have been discussed in 1.4 and 1.5.2. Secondly, molecular biology based tests are rapid but expensive and can only indicate resistance through the detection of mutations that have formerly been linked to a resistant phenotype. These genotypic tests have been described in 1.5.1. Thirdly, the last category of tests uses mycobacteriophages to detect viable cells of *M. tb*. The principles of this approach have been discussed in 1.7.

Given the shortcomings of the established technologies, it is imperative that a methodology or assay be developed, that can be performed in peripheral laboratories and in resource constrained settings and produce results, including a resistance profile within a week. It should be possible to perform such a test without expensive, high-maintenance equipment and it should be cheap (Hazbon, Guarin et al. 2003).

An assay that uses reporter mycobacteriophages could potentially meet all those criteria. The development of methods to do this (Jacobs, Barletta et al. 1993) has been described in 1.6 and 1.7. Since only viable bacteria will be able to express the introduced reporter gene, the use of such bacteriophages to diagnose infection while providing a resistance profile has become a distinct possibility.

In 1993, the Jacobs' lab constructed the phAE39 and phAE40 reporter phages (Jacobs W.R. 1993), which contained the *Flux* gene. These phages were able to identify the presence of *M. tb* in culture and identified resistance (Jacobs W.R. 1993; Banaiee 2001).

We describe the development of a *lacZ* containing reporter mycobacteriophage ( $\beta$ -gal phage), which codes for and expresses the  $\beta$ -galactosidase enzyme upon infection of a mycobacterial bacillus (1.6.3.2). This reporter phage was used to develop an assay with a shorter turn-around time and does not require specialised equipment.

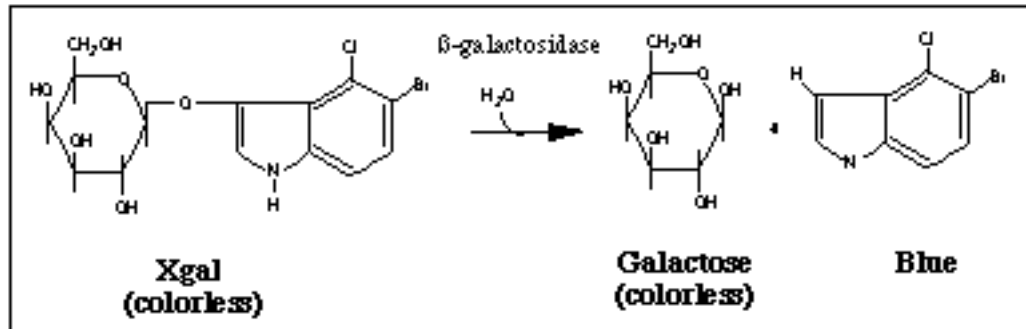


Figure 2.1: Enzymatic reaction of the breakdown of lactose into galactose and glucose by beta-galactosidase

<http://www.sci.sdsu.edu>

Similar to the luciferase (*Flux*) (Jacobs, Barletta et al. 1993) and the Green Fluorescence Protein (*gfp*) (Piuri, Jacobs et al. 2009) reporter phages, the  $\beta$ -gal reporter phage ( $\beta$ -gal phage) is a TM4-based phage with a *lacZ* gene insert. The signal generated by the  $\beta$ -gal reporter phage differs from the others in that it does not need specific and expensive equipment to be visualised. The colour generated by the  $\beta$ -gal phage can be seen by the naked eye. Therefore the use of this reporter phage is not only cheaper but can be done in rural or peripheral laboratories without complicated equipment.

## 2.1 AIMS AND OBJECTIVES

Objective: To develop an affordable and rapid diagnostic test for tuberculosis that can be performed without the use of expensive and complicated equipment.

Aim 1: To develop a  $\beta$ -galactosidase reporter mycobacteriophage that has the ability to detect *M. tb* bacilli in a timely manner, with a sensitivity and specificity comparable to currently available technologies. This part of the project was performed in collaborations with William R. Jacobs jr. of the Einstein College of Medicine, Bronx, NY, USA.

Aim 2: To develop a test format for easy detection using *M. tb* in culture

Aim 3: To determine the assay's sensitivity by assessing the lowest detectable bacillary concentration

Aim 4: To determine the assay's specificity by testing common TB culture contaminants.

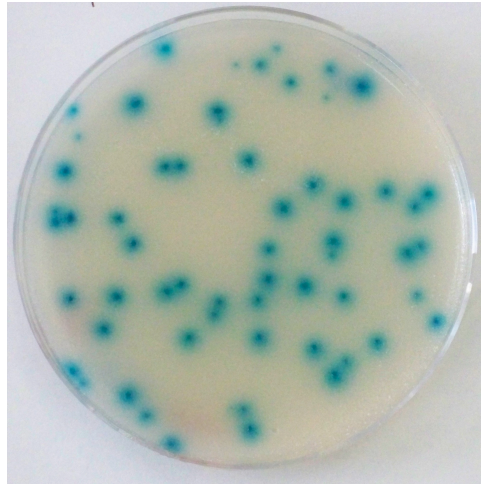
## 2.2 MATERIALS AND METHODS

### 2.2.1 CONSTRUCTION OF THE BETA-GALACTOSIDASE REPORTER MYCOBACTERIOPHAGE

The  $\beta$ -gal phage was first constructed *in silico* using the clone manager software program (SciEd, Cary NC, USA)

The TM4 mycobacteriophage formed the basis of the *in vitro* construction (Jacobs W.R. 1993). The host range (the range of bacterial species that can be infected with the phage) of TM4 includes the species within the MTC complex as well as *M. smeg.* Whereas Piuri et al., 2009 utilised the phAE87 as their conditionally replicating derivative (Piuri, Jacobs et al. 2009), we

chose to use the phAE159. The *lacZ* gene was fused to the *Mycobacterium bovis* BCG Hsp60 promoter to drive expression in pYUB1228 and 1229 plasmid derivatives. The construct was transferred to the phAE159 shuttle phasmid to generate a phAE159::*hsp60-LacZ*. This was packaged into  $\lambda$ -phage heads and propagated in fast growing *M. smeg* strain mc<sup>2</sup> 155 (Fig. 2.2). High titre stocks of 10<sup>10</sup> PFU/ml were prepared and stored for use in subsequent assays.



**Figure 2.2:**  $\beta$ -gal reporter mycobacteriophage plaques in a lawn of mc<sup>2</sup> 155 *M. smeg* infused with X-gal

#### 2.2.1.1 Plasmid (pYUBs 1228 & 1229) inoculation

The plasmids pYUBs 1228 and 1229 (Figure 2.3 & 2.4) found in *E. coli*, were used as vectors. These are available in high copy number and possess a gene coding for ampicillin resistance as well as unique restriction sites for the insertion of the reporter gene. The fast growth rate of *E. coli* makes it an ideal host in cloning experiments.

*E. coli* DH5  $\alpha$  was inoculated in 5ml Luria-Bertani (LB) broth containing carbenicillin at a final concentration of 150  $\mu$ g/ml which was incubated at 37°C overnight.

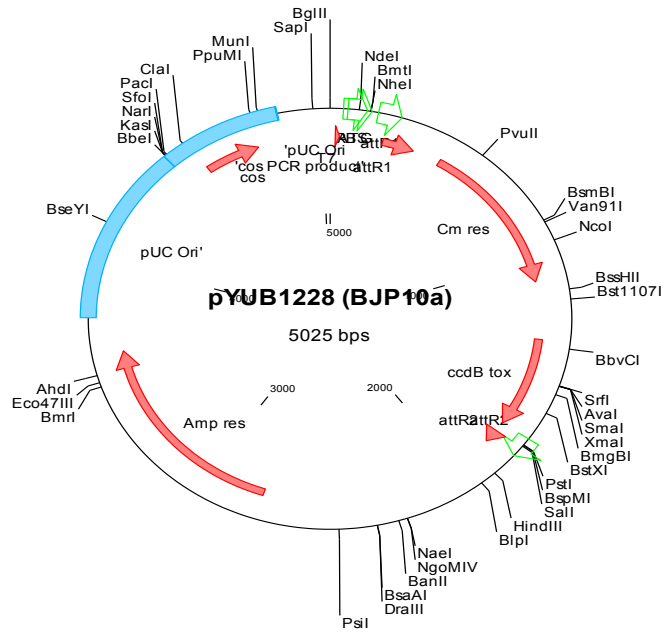


Figure 2.3: pYUB 1228 (5025 base pair)

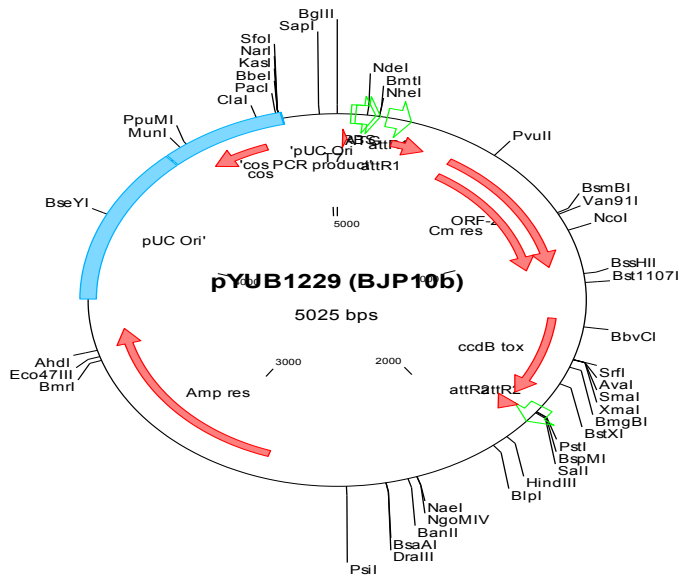


Figure 2.4: pYUB 1229 (5025bp)

### 2.2.1.2 Plasmid DNA preparation

Following overnight incubation of the plasmid containing *E. coli* culture, the plasmid DNA was extracted using the Qiaprep kit (QIAGEN™) according to the manufacturer's instructions. Verification of the DNA products was done by electrophoresis in 1% agarose, showing 5025 bp products (Figures 2.3 and 2.4). Additionally, the DNA products were restricted using NdeI and PacI restriction enzymes to confirm the presence of the specific plasmids (Figure 2.5).



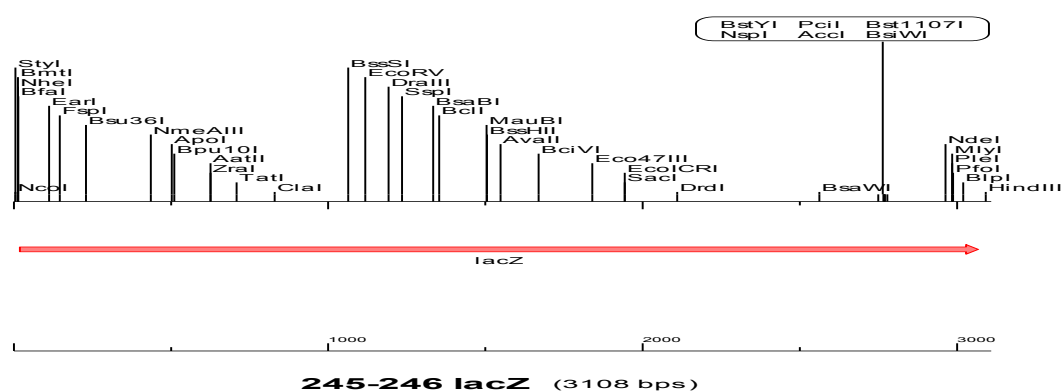
Figure 2.5: PacI and NdeI restriction map to confirm the presence of pYUBs 1228 and 1229; lanes 1 & 12: Molecular weight markers, lanes 2 & 6: unrestrictied plasmids, lanes 3-5 and 7-9, PacI and NdeI restriction products

### 2.2.1.3 Amplification of the LacZ Gene

The *LacZ* gene (Fig 2.6), to be inserted into the TM4 phage was prepared by PCR amplification, using the following primer pair: Forward primer- 5' TTCACACAGGATCCAGCATG 3' and Reverse primer- 5' GCGCTGTGTGCCTCGATTGT. The PCR conditions are shown in Table 2.1.

**Table 2.1: Reaction components, amounts and conditions used for amplification of target DNA**

	Amounts	Reaction conditions
Total reaction amount	50 µl	35 cycles of
Buffer	5 µl (10x)	95°C for 1 min
Enzyme (Taq polymerase)	1 µl	95°C for 30 sec
dNTPs	1 µl	58°C 30 Sec
Primers (F & R)	1 µl	72°C for 3 min
Template DNA	1 µl (10µg/ml)	72°C for 1 min
Water	40 µl	Hold at 4°C



**Figure 2.6: The *LacZ* PCR product inserted into the reporter mycobacteriophage**

After electrophoresis in 1% agarose, the expected 3108 bp amplification product was identified

#### 2.2.1.4 Restriction and Purification of Plasmid and *LacZ* DNA

The plasmids pYUB 1228 and 1229 were cut with restriction enzymes HindIII and NdeI to accommodate the insertion of the target (*lacZ*) DNA molecule. Simultaneously, the *lacZ* amplicons were restricted with the same restriction enzymes. Details of the reactions conditions are given in Table 2.2. The restrictions produced ‘sticky ends’ in preparation for ligation between the plasmid and *LacZ* DNA molecule.

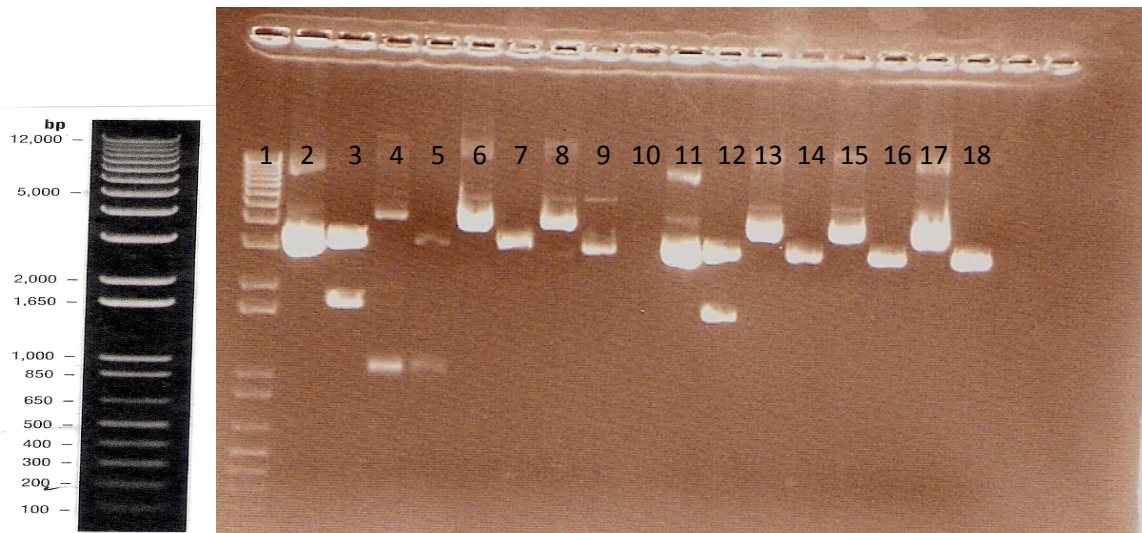
**Table 2.2: Reaction components, amounts and conditions used for restriction of plasmid and target DNA**

	<b>Plasmids restriction</b>	<b>LacZ DNA Molecule</b>
<b>Total reaction amount</b>	20 µl	20 µl
<b>Template DNA</b>	10 µl	16 µl
<b>BSA (Bovine serum albumin)</b>	0.5 µl	0.5 µl
<b>Buffer</b>	2 µl	2 µl
<b>Enzyme</b>	1,1 µl (HindIII, NdeI)	1,1 µl
<b>Water</b>	5.5 µl	-
<b>Incubation conditions</b>	37°C for 90 -120 minutes	37°C for 90 -120 minutes

The restriction products were verified by means of 1% agarose gel electrophoresis. Cutting the plasmids with HindIII and NdeI yielded two fragments of 1816 and 3209 bp respectively. The 3209 bp fragment was excised from the gel and purified, using the QIAquick PCR Purification Kit (QIAGEN™), according to the manufacturer's instructions. The *LacZ* 3108 bp molecule was also restricted to yield three fragments of 2964, 127 and 17 bp, of which the larger fragment (2964 pb) was excised and purified (not shown).

#### 2.2.1.5 *Ligation of Plasmid and DNA Molecule*

The plasmid DNA and the *LacZ* DNA were ligated using the Quick Ligation™ Kit (NEB) sealing the sticky ends of the plasmid and target DNA, briefly; 10µl of the DNA molecule was combined the 10µl plasmid and 10µl Quick Ligase Buffer and 1µl Ligase Enzyme and allowed to stand at room temperature for 30 minutes. A verification gel was run to show that the products of 2964 bp (target DNA) and 3209 bp (plasmid DNA) respectively, did circularise to form a 6173 bp fragment (figure 2.7).



**Figure 2.7: Restriction map of pYUBs 1228 & 1229 clones; Lane 1: Molecular Weight Marker (1Kb) Plus DNA Ladder, Lane 2: pYUB 1228 unrestricted plasmid control, Lane 3: pYUB 1228 restricted plasmid control, Lane 4: pYUB1228-*lacZ* clone 1, unrestricted, Lane 5: pYUB1228-*lacZ* clone 1 restricted, Lane 6: pYUB1228-*lacZ* clone 2 unrestricted, Lane 7: pYUB1228-*lacZ* clone 2 restricted, Lane 8: pYUB1228-*lacZ* clone 3 unrestricted, Lane 9: pYUB1228-*lacZ* clone 3 restricted, Lane 10: empty Lane 11: pYUB 1229 unrestricted plasmid control, Lane 12: pYUB 1229 restricted plasmid control, Lane 13: pYUB1229-*lacZ* clone 1 unrestricted, Lane 14: pYUB1229-*lacZ* clone 1 restricted, Lane 15: pYUB1229-*lacZ* clone 2 unrestricted, Lane 16: pYUB1229-*lacZ* clone 2 restricted, Lane 17: pYUB1229-*lacZ* clone 3 unrestricted, Lane 18: pYUB1228-*lacZ* clone 3 unrestricted**

#### 2.2.1.6 Transformation of *E. coli* dH5 $\alpha$

The transformable strain of *E. coli* dH5 $\alpha$  was made competent by washing twice with CaCl<sub>2</sub> (100 mM) and MgCl<sub>2</sub> (100 mM) salts. The culture was used immediately or stored in 10-20% glycerol at -70°C. The plasmid::lacZ ligate was added to the competent *E. coli* for transformation.

The following procedure was followed:

- 100ng DNA /10  $\mu$ l of the ligation product is added to 50 $\mu$ l competent *E. coli* dH5 $\alpha$  cells
- Incubated on ice for 30 min
- Incubated at 42°C for 1 min
- Incubated on ice for 1 min
- 250  $\mu$ l SOC - LB medium supplemented with 0.2g/L KCl, 2g/L MgCl<sub>2</sub>, 2g/L MgSO<sub>4</sub> and 4g/L glucose (Sigma Aldrich®) was added and incubated at 37°C for 30 min

Bacteria were inoculated on agar plates containing 50  $\mu$ l Carbenicillin [50  $\mu$ g/ml] and 20  $\mu$ l 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-gal) final concentration [40  $\mu$ g/ml] and incubated at 37°C overnight

After incubation, the blue 'transformed' colonies and the white 'untransformed' colonies were picked separately, inoculated in LB broth and incubated overnight. DNA from the propagated blue colonies was extracted according to the Plasmid DNA preparation protocol (2.2.1.2) and restricted using PaeI. The process was verified by agarose gel electrophoresis.

Phasmid (pHAE 159) DNA, a derivative of TM4 was also restricted using PaeI to create sticky ends. Details of both reactions are summarised in table 2.3.

**Table 2.3: Reaction components, amounts and conditions used for restriction of phAE159 and pYUB:*LacZ* clone**

	Phasmid (phAE159) restriction	pYUB: <i>LacZ</i> clone DNA Molecule
<b>Total reaction amount</b>	20 $\mu$ l	20 $\mu$ l
<b>Template DNA</b>	10 $\mu$ l	8 $\mu$ l
<b>BSA (Bovine serum albumin)</b>	0.5 $\mu$ l	0.5 $\mu$ l
<b>Buffer</b>	2 $\mu$ l	2 $\mu$ l
<b>Enzyme PaeI</b>	1 $\mu$ l	1 $\mu$ l
<b>Water</b>	6.5 $\mu$ l	8.5 $\mu$ l
<b>Incubation conditions</b>	37°C for 90 -120 minutes	37°C for 90 -120 minutes

2.2.1.7 *Ligation of Plasmid-DNA with Phasmid (phAE159)*

The PaeI restricted plasmid::*LacZ* DNA clone was ligated to the PaeI restricted phAE159 by adding 5  $\mu$ l Plasmid::*LacZ* DNA and 5  $\mu$ l Phasmid (100ng/ml) to 10  $\mu$ l ligation buffer and 1  $\mu$ l ligase enzyme reaction mixture. This was incubated at room temperature for 30 min. Ligation products were subjected to 1% agarose gel electrophoresis for verification.

2.2.1.8 *in vitro packaging into phage  $\lambda$  heads and screening for molecule incorporation*

The ligation products (shuttle phasmid) were packaged into  $\lambda$  phage heads (MaxPlax™ Lambda Packaging Extracts), according to the manufacturer's (Epicenter® Biotechnologies) instructions. Briefly: 10 $\mu$ l ligation mix was added to 10  $\mu$ l packaging mix and this was incubated at room temperature for 3 hours. Following this, 400  $\mu$ l *E. Coli* HB101 cell suspension were added and incubated at 37 °C for 1 hour. The cells were centrifuged for 1 min at 3000 g and the pellet re-suspended in 400  $\mu$ l SOC / LB medium. The cells were plated onto X-gal (20  $\mu$ g/ml) and carbenicillin (100  $\mu$ g/ml) -containing plates and incubated at 37°C overnight.

The colonies that appeared blue and carbenicillin resistant were picked and grown in LB broth, in a shaking incubator at 37°C overnight. Following incubation, DNA was extracted, using the Qiagen mini-prep kit and the process verified by electrophoresis after *PacI* restriction.

#### 2.2.1.9 *Electroporation of M. smeg cells*

The shuttle phasmid DNA was prepared and electroporated into competent *M. smeg* mc<sup>2</sup> 155 cells, according to Sambrook and Russel's Molecular cloning Handbook (2001). Briefly, a 10- $\mu$ l volume of phasmid DNA was mixed with 400  $\mu$ l *M. smeg* cells and added to chilled electroporation cuvettes. The mixture was stored on ice for 20 minutes. This was followed by electroporation (pulse settings: 2.5 kv, 100 ohm and 25  $\mu$ F) for 3 seconds. The electroporated *M. smeg* was mixed with 1 ml Middlebrook 7H9 broth and incubated for 1 hour at 37°C. The cells were then plated onto Middlebrook 7H10 plates containing 50  $\mu$ l X-gal (50 $\mu$ g/ml) and incubated at 30°C for 48 hours (Figure 2.2).

#### 2.2.1.10 *High Titre lysates of incorporated DNA molecule Positive plaques*

Mycobacteriophages were harvested from the blue plaques (Figure 2.2) and amplified using *M. smeg* incubated at 37°C for 48 hours. Titres of 10<sup>10-13</sup> PFU/ml were harvested, stored in Mycobacteriophage buffer pH 8.6 (MP) (1M Tris-HCl, 1M MgSO<sub>4</sub>, 5M NaCl, and 2mM CaCl) at 4°C until use.

### 2.2.2 THE BETA-GALACTOSIDASE REPORTER PHAGE ASSAY FOR THE DETECTION OF MYCOBACTERIUM TUBERCULOSIS

The assay was performed using 24-well tissue culture plates. The mycobacterial cells were grown in Middlebrook 7H9 broth supplemented with 10% oleic acid- albumin-dextrose-catalase (OADC; Difco Laboratories, Detroit, Mich.), 0.2% glycerol and 0.05% Tween 80 to mid-log phase or an  $OD_{600nm}$  0.7-1 was reached. The cultures were aliquoted in volumes of 1.5 ml into micro centrifuge tubes and centrifuged at 3 000 g for 3 minutes. The cells were washed twice with MP buffer. This served to remove Tween 80, which is known to impede phage infection as well as removing the 7H9, which also has the potential to interfere with colour development during the assay. The pellet was re-suspended in 750  $\mu$ l Tris buffered (pH 7.6) saline and six 100-fold serial dilutions (10  $\mu$ l pellet in 990  $\mu$ l saline) were made in order to assess the minimum detection level.

100 $\mu$ l from each dilution was added to the wells of a tissue culture plate and 200  $\mu$ l of bacteriophage suspension of  $1 \times 10^4$  to  $1 \times 10^9$  PFU/ml was added to each of the bacilli containing wells. Wells without either the phage lysate or bacilli served as negative controls.

To allow adhesion to and infection of the bacteria by the phages, the plates were put on a horizontal shaker for 90 minutes at 37°C. Following this, 1 ml of Top agar (7% Agarose, 3.5% Middlebrook and 150  $\mu$ l/ 100 ml Top agar of a X-gal solution (50 mg/L)) was added to the bacilli: phage mixture. The plate was incubated at 37°C for 72 hours. Photographs were taken at every 24 hours to monitor colour change (Figure 2.8).

### 2.2.3 CONFIRMATION OF MYCOBACTERIUM TUBERCULOSIS IN CULTURE

In order to confirm the presence of *M. tb* in the  $\beta$ -galactosidase detection system, several confirmatory tests were employed on the same culture used for the  $\beta$ -gal phage assay. Briefly

**Table 2.4: Table of tests used to confirm the presence of *M. tb***

Test	Amount of Original culture used	Experiment conditions	Refer to figure
BACTEC MGIT 960™	500 $\mu$ l	37°C in MGIT machine for 7 days/ until positive	Not shown
GFP Fluorescence	300 $\mu$ l	37°C for 24 hours	2.11
7H10 Solid culture	50 $\mu$ l	37°C for 21 days	2.12

### 2.2.4 CONFIRMATION OF B-GAL PHAGE HOST SPECIFICITY

Diagnostic tests for pulmonary tuberculosis are performed on sputum specimens that are, by nature contaminated with oropharyngeal flora, needed to be established that the host specificity of the  $\beta$ -gal phage was restricted to its mycobacterial hosts. In order to assess this, a selection of non-acid fast bacteria grown from the sputum received in the laboratory to diagnose tuberculosis were chosen to determine their reaction in the  $\beta$ -gal phage assay.

Some of the sputum samples that showed contamination despite NALC-NaOH decontamination (Kent and Kubica 1985) were plated onto Middlebrook 7H11 agar plates and grown for 7 days at 37°C. Ten colonies with different morphology were chosen and inoculated onto blood agar

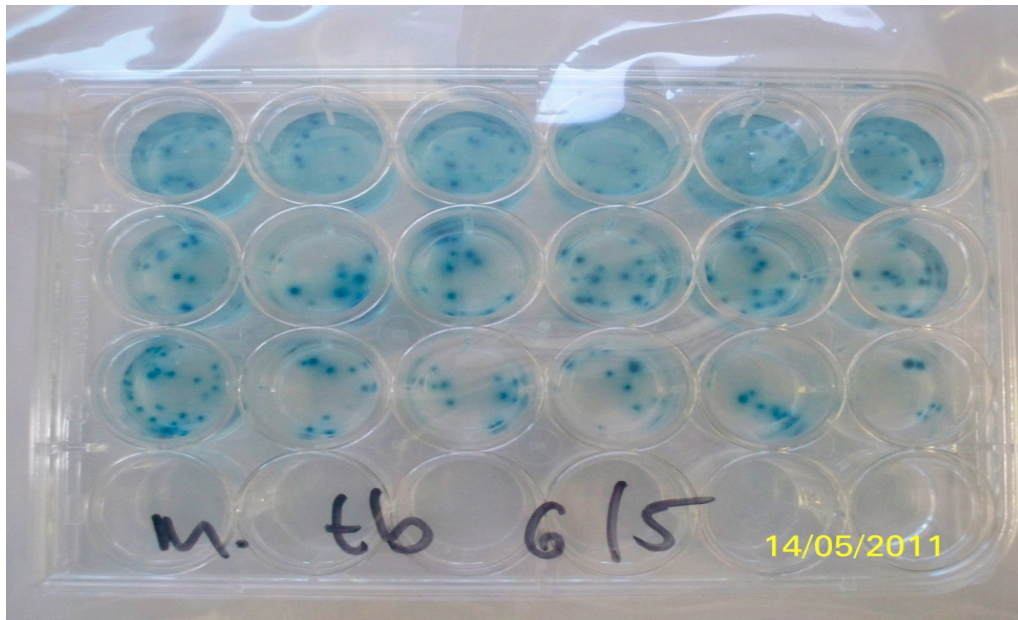
plates, to allow for single colony selection (Not Shown). Single colonies of which were assumed to be different species, were selected and grown in Middlebrook 7H9 broth at 37°C for 48 hours.

Middlebrook 7H9 broth supplemented with 50 mg/L of X-gal was cooled to 45°C. A volume of 500µl broth culture of each species was added to 3 ml of X-gal infused Top agar. After mixing, the bacteria was poured over a Middlebrook 7H11 agar plate and allowed to solidify. 10 µl of the β-gal phage ( $2.0 \times 10^9$ pfu/ml) was spot-inoculated on the surface. A similarly prepared plate containing *M. smeg* mc<sup>2</sup> 155 served as control (Figure 2.12 a-d). The plates were incubated at 37°C for 48 hours and subsequently assessed for 'blue spot' plaques, indicative of phage infection and reactivity.

## 2.3 RESULTS

### 2.3.1 THE BETA-GALACTOSIDASE REPORTER PHAGE ASSAY FOR THE DETECTION OF MYCOBACTERIA

Figure 2.8 shows the application of  $\beta$ -gal reporter phage assay on the 10-fold serial dilutions of *M. tb* (H37Rv) (across) exposed to different concentrations of bacteriophages (down). The wells containing  $10^8$  cfu/ml per well show evenly coloured agar. In these wells, the amount of  $\beta$ -galactosidase produced is so high that it has diffused throughout the agar layer.



**Figure 2.8:  $\beta$ -gal reporter phage assay in 24-well culture plate, detecting the presence of *M. tb* bacilli, 96 hours post infection with reporter mycobacteriophages**

The infection of mycobacterial cultures by the  $\beta$ -gal phage was held in a semi-solid matrix (Top-Agar), and thus allowed for the expression of the  $\beta$ -galactosidase enzyme and colour change, which could be visualised without the need for equipment. Each blue 'spot' within the well

represented a colony-forming unit, thus making this assay optimal for visualising isolates with low bacillary count. The specificity of the assay was also proven by the lack of reactivity in the bottom row (negative control) of the tissue-culture plate.

From figure 2.9, it was found that the bacillary count as low as  $10^0$  cells could be detected by a lysate count as low as  $10^5$  PFU/ml, the concentration of the lysate could however be lowered as long as the bacillary count is high. The  $10^0$  and 0 CFU/ml columns showed some reactivity in at the  $10^9$  PFU/ml concentration, which prompted optimisation by decreasing the MOI or lysate concentration to a low enough PFU/ml to avoid false sensitive detection. It was found that at 'too high' concentrations, the inherent phages tended to react with compounds in the Middlebrook (7H9) media. For the purposes of sensitivity and reproducibility, the lysate concentration of  $10^6$  PFU/ml was chosen as the constant. From the figure above, it is also evident that the higher the bacillary count, the more uniform the well colour, whereas individual spots became more pronounced as the bacillary load decreased. As with the previous figure 2.8, the sensitivity of the assay was further proven by lack of reactivity in the last column of the plate, where there were no bacilli present.

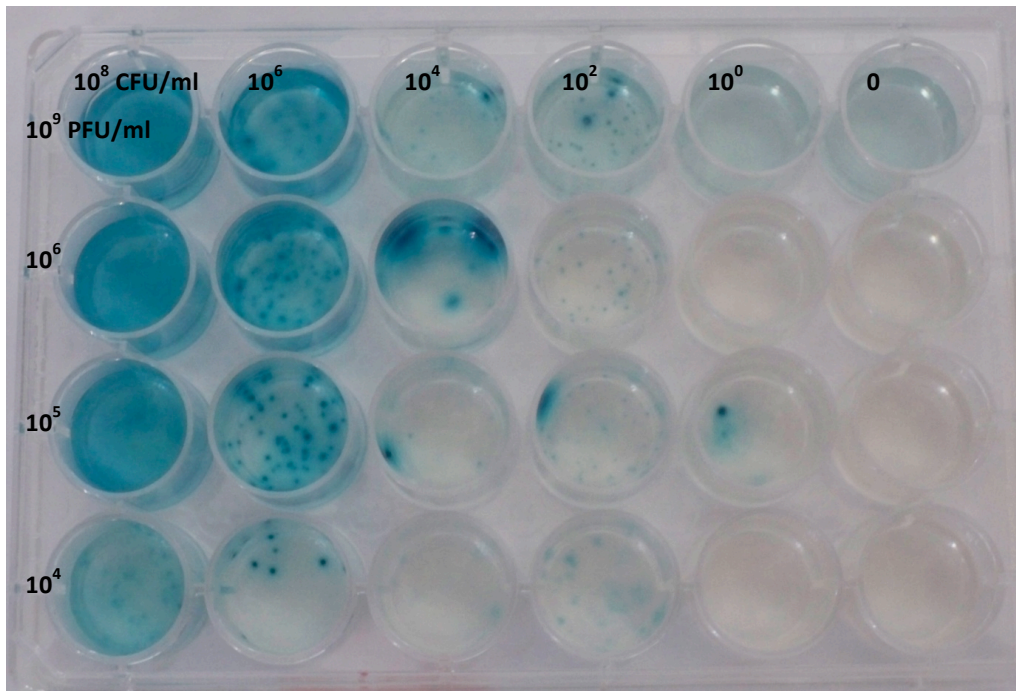


Figure 2.9:  $\beta$ -gal reporter phage assay in 24-well culture plate, detecting the presence of *M. tb* bacilli, 96 hours post infection with reporter mycobacteriophage

The sensitivity of the assay was further assessed by sequentially decreasing the concentration of both the bacillary count as well as the viral count of the phage lysate. The assay was repeated several times to assess reproducibility, see table 2.5.

Table 2.5: Table of replicate experiments used to assess the assay's reproducibility

Experiment #	Lowest bacillary count detection (CFU/ml)	Lowest phage particle used (PFU/ml)	Background reactivity
1	$10^2$	$10^6 \pm 10^2$	no
2	$10^1$	$10^9 \pm 10^3$	yes
3	$10^1$	$10^6 \pm 10^2$	no
4	$10^1$	$10^7 \pm 10^1$	yes

### 2.3.2 CONFIRMATION OF MYCOBACTERIUM TUBERCULOSIS IN B-GAL ASSAY CULTURE

As mentioned in 2.2.3, several other tests were used to confirm the presence of *M. tb* in the cultures used for the  $\beta$ -gal assay. Figures 2.10a & b and 2.11 show the infection of the *M. tb* bacilli by the  $\Phi^2$ GFP10 ((Jain, Hartman et al. 2012), when viewed under bright-field microscopy at 400X magnification and its corresponding fluorescence image as well as a 7H10 plate showing colonies of *M. tb* (2.11). Both tests, together with the BACTEC MGIT960™ confirmed the presence of *M. tb* (H37Rv) pure culture.

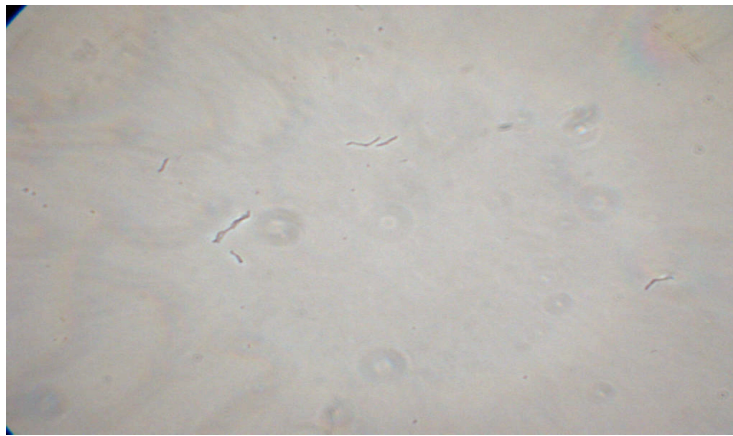


Figure 2.10a: Bright field depiction of *M. tb* 400X Magnification

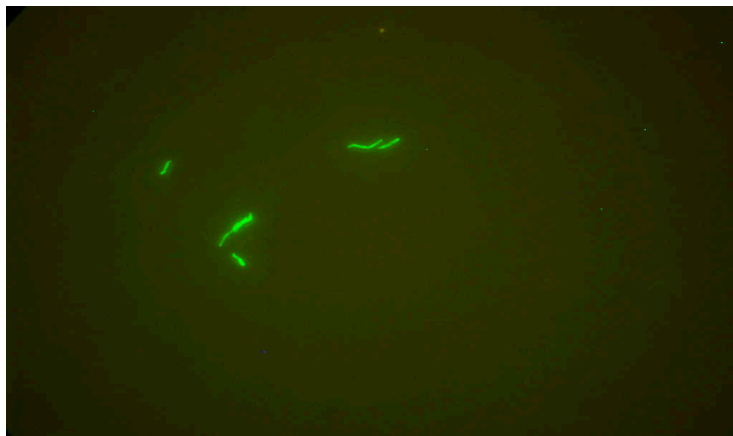
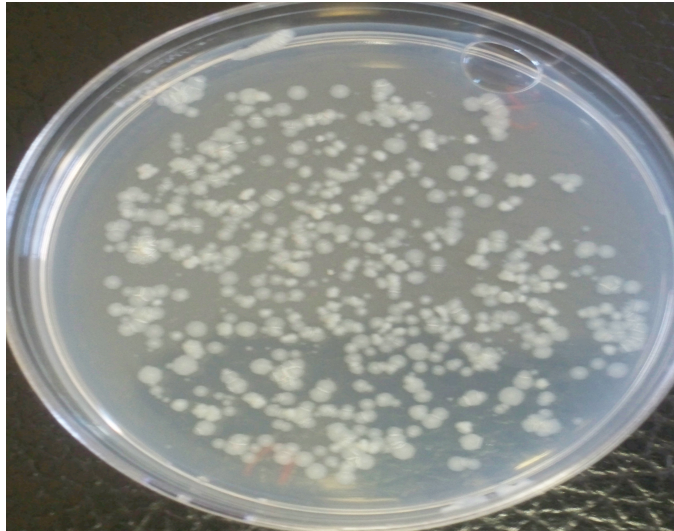


Figure 2.10b: Fluorescence image of the figure 2.11a, of *M. tb* infected with  $\Phi^2$ GFP10 reporter phage 400X Magnification



**Figure 2.11: Solid Culture plate of *M. tb* pure colonies**

### 2.3.3 CONFIRMATION OF BETA-GAL MYCOBACTERIOPHAGE HOST SPECIFICITY

Figure 2.12 shows the host specificity of the  $\beta$ -gal and  $\phi^2$ GFP10 reporter phage. The non-mycobacterial isolates obtained from sputum specimens of patients with tuberculosis did not show any  $\beta$ -galactosidase activity. In contrast, the *M. smeg* laboratory strain mc<sup>2</sup> 155 (fig. 2.12 a) did show blue spots at the site where the phage had been dropped onto the lawn of growth indicative of phage activity.

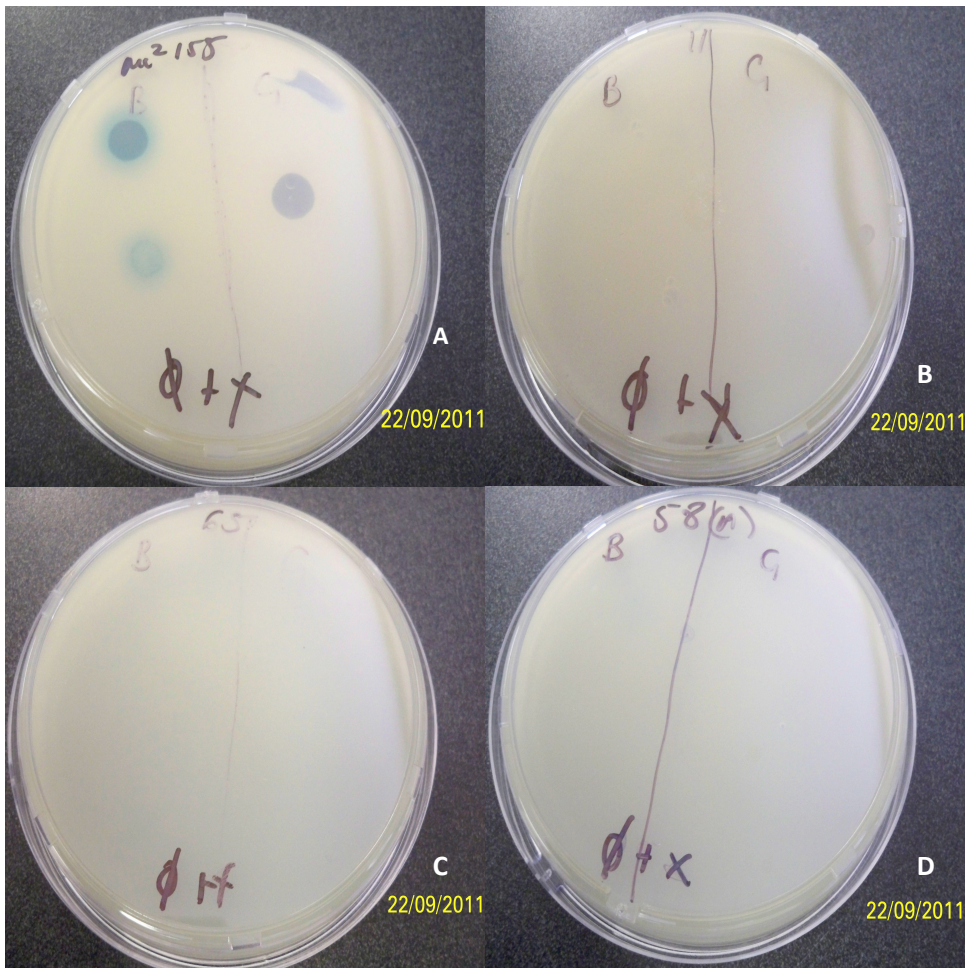


Figure 2.12: (a): mc<sup>2</sup>155 *M. smeg* control strain, with GFP and  $\beta$ -gal phage plaques (b)-(d): Representative lawns of normal flora showing lack of beta-galactosidase activity and plaques

## 2.4 DISCUSSION

Our study revealed that genetically manipulated mycobacteriophages were able to sensitively and specifically detect *M. tb* in culture. The substrate X-gal was broken down into a soluble 5-bromo-4-chloro-3-indoyl by the  $\beta$ -galactosidase enzyme produced by bacteria infected with the reporter gene. This was clearly visible without the use of other machinery.

The significance of the results is in the ease with which the assay can be performed. Whereas acid fast staining is relatively rapid compared to other mycobacterial detection techniques, the use of  $\beta$ -gal reporter phages surpasses the use of staining in that larger sample amounts can be used. Furthermore, the sensitivity of the reporter phages to infect mycobacterial bacilli, even at low counts makes the use of this assay an added advantage over staining.

The BACTEC MGIT 960™ or radiometric technologies may have greatly improved the detection rate of *M. tb*; however, they require machinery, which may not always be affordable in low-resource settings. In addition, equipment requires expert maintenance that may not be available in such settings. Thus the  $\beta$ -gal phage assay can be used as a reliable substitute because it requires readily available 37°C incubator, disposable culture plates and culture medium. The propagation of the  $\beta$ -gal reporter phage can be done, without the need for purchase, using the same materials that are available for the assay itself (section 2.2.1.12).

The study also reiterates the specificity of the reporter phage (sections 2.2.4 & 2.3.3), where oropharyngeal flora grown from a representative sputum specimen did not show any reaction after exposure to the phage. This was to ascertain that false positives would not

occur, should the sputum specimen not be sufficiently decontaminated. Whereas the BACTEC MGIT 960™ can result in false positivity due to contamination, the  $\beta$ -gal phage test faced no such challenge.

In comparison to the BACTEC MGIT 960™ time to detection of 10-21 days, the  $\beta$ -gal assay's time to yield results was between 72 to 96 hours, making this a clear advantage particularly when 'time to detection' is considered a vital part of transmission control. This assay has potential clinical significance particularly in resource-limited settings, which invariably are the same settings that have the highest TB burden. The ease of performance, the sensitivity and specificity, time to detection as well as the low cost of the assay has major advantages over the currently available laboratory diagnostic methodologies.

To date, several mycobacteriophage technologies that serve to detect *M. tb* have been developed, ranging from the *FASTplaqueTB*™ and *FastPlaque-Response*™ (Cosmos Biomedical) and the McNerney group's PhaB Assay utilizing the D29 mycobacteriophage (McNerney, Wilson et al. 1998; McNerney, Kambashi et al. 2004) to Jacobs' (Jacobs, Barletta et al. 1993; Pearson, Jurgensen et al. 1996; Bardarov, Dou et al. 2003; Jain, Hartman et al. 2012) and Hatfull (Piuri, Jacobs et al. 2009; Jain, Hartman et al. 2012) groups' reporter protein infused mycobacteriophages. Our reporter mycobacteriophage is more aligned with, as well as an improvement of the original Jacobs' reporter mycobacteriophages.

In comparison to the *FASTplaqueTB*™ and McNerney's assays, our study compares favorably in that, the former assays require the inactivation of the extraneous phages using the virucidal agent ferrous ammonium sulphate (FAS), additionally their phages are not reporter

protein-infused. Our phages do not require inactivation and are genetically manipulated to include and express reporter protein (Biotec 2004; McNerney, Kambashi et al. 2004). The need for a virucidal compound for the D29 and *FASTplaqueTB™* assay was deemed necessary due to the inclusion or use of sensor cells, *M. smeg mc<sup>2</sup> 155* in the case of McNerney's assay (McNerney, Kambashi et al. 2004). FAS inactivates extraneous phages, which may interfere with plate readings, thus decreasing the risk of false positivity. Our assay does not require the use of sensor cells; thus invalidating the need for FAS inactivation because of our reliance of expression of the reporter protein, which can only occur upon infection of viable bacilli (Jacobs, Barletta et al. 1993).

Both the *FASTplaqueTB™* and the McNerney D29 assays reported sensitivities of 75% and 60% respectively and specificities above 90% of both assays (Biotec 2004; McNerney, Kambashi et al. 2004). The sensitivity of all three phage-based (ours included) assays can detect as little as 10 cells per ml of culture, which is a definite improvement on the microscopy, which requires 10<sup>4</sup> cells/ml. This has very important clinical implications where it has been shown that HIV positive or immune-compromised individuals often fail to produce sputum of high bacillary count.

As with the *FastPlaque TB™* assay, our results compare favorably in that results are available within a week, although the *FASTplaqueTB™* reported a turn around time of 2 days. An important factor to consider in comparing our assay to McNerney's D29 assay, is that although both tests are performed at 37°C, the major difference is that our reporter phages have a temperature (*ts*) mutation that promotes propagation and cell lysis at 30°C and reporter protein expression without lysis at 37°C. This serves to allow cell lysis during phage

propagation and inhibition of cell lysis during the assay at 37°C.

Our assay is more aligned with work previously done by both the Jacobs' and Hatfull groups, where the luciferase reporter phage was developed by the Jacobs group (Jacobs, Barletta et al. 1993) and the fluorescent reporter mycobacteriophages were developed by the Hatfull group (Piuri, Jacobs et al. 2009) and more recently the generation of more efficient fluorescent mycobacteriophages by the Jacobs group (Jain, Hartman et al. 2012). However, the cost required to perform diagnostic tests using the above-mentioned reporter phages:

Luminometer and luciferase substrate in the case of the luciferase reporter phage and a flow cytometer and fluorescent microscope for the fluorophages prompted us to consider cheaper ways to use the same format, without the need of expensive machinery or specialized technical training. This led to the development of an assay where the results could be visualized and retain similar specificity and sensitivity as the older generation reporter phages.

The initial reporter mycobacteriophage (*Fflux*), developed in 1999 was reported to detect as few as 500 cells per ml (Jacobs, Barletta et al. 1993). Further improvements were made with regards to the strength of expression (Sarkis, Jacobs et al. 1995), the length of signal retention (Sarkis, Jacobs et al. 1995; Carriere, Riska et al. 1997) even the mode of signal detection (Riska, Su et al. 1999; Hazbon, Guarin et al. 2003), which aided in the detection of even fewer cells in any given sample, as well as the time it took to yield a results (less than 48 hours).

Our study reporter phage built on the backbone of those already efficient reporter phages as mentioned in section 2.2.1, by replacing the *Fflux* or *gfp* genes with the *lacZ* gene.

Newer reporter phages developed by the Hatfull group (Piuri, Jacobs et al. 2009) eliminated the need for the use of an expensive luminometer by inserting commonly used green and yellow fluorescent reporter proteins, thus employing the use of a more readily available fluorescent microscope. The 2009 study, was performed similar to the current study by using laboratory culture isolates, and their study revealed similarities in sensitivity and specificity of the reporter phages.

We added a step to verify specificity of the reporter phage by attempting to infect other bacterium, similar to the Rybniker and Kumar studies (Rybniker, Kramme et al. 2006; Kumar, Loganathan et al. 2008) both of which revealed that TM4 mycobacteriophage (which all the above-mentioned reporter phages are based on), have narrow host range preference, and none outside the Mycobacterium complex family. Our confirmatory study (section 2.2.4) concurs with the above-mentioned revelations.

In the face of current TB incidence and infection rates, is it imperative that laboratory diagnosis or detection of bacilli is rapid and reliable. Coupled with high infection rates, the realities facing the most heavily burdened countries are that they often lack the resources or the manpower to conduct diagnostics using the currently available technologies. Our assay aims to circumvent the prohibitive costs, without compromising on the quality or reliability of the results.

As mentioned in Chapter 1, in order for any TB control measures to be effective, rapid, reliable and efficient diagnostic technologies are required, thus our assay aims to fulfill that

role by decreasing the turnaround time to results. The current Gold standard for TB diagnosis (smear microscopy and culture), rely on the amount and growth of bacilli respectively, factors which often either decrease sensitivity or compromise on the time to diagnosis (section 1.1.3).

In as much as the  $\beta$ -gal phage assay has major clinical implications, it is still to be tested in its ability to generate a drug susceptibility pattern in both cultured specimen and sputum samples in a larger clinical trial setting. Furthermore, our study did not take into account the potential reactivity with mycobacteria other than tuberculosis (MOTTs). The reason behind this lies in the fact that in the current infection climate, Sub-Saharan Africa has more TB infections than MOTTs or patients are more likely to be infected with TB than MOTTs. This however should does not discount the fact that MOTTs are found among South African miners (personal communication).

When laboratory diagnosis is coupled with clinical diagnosis, MOTTs can be successfully excluded thus decreasing the need to account for MOTTs exclusion in our test, or rather depending on the cohort of the patients, the prevalence of MOTTs need to be assessed before large-scale exclusion. However, should the incidence of MOTTs be found to be significant, Riska et al, 1997 conducted a study for excluding MOTTs whilst using the phage assay (Riska, Jacobs et al. 1997), with reasonable success. "The compound (*p*-nitro- $\alpha$ -acetylamino-  $\beta$ -hydroxy propiophenone (NAP), which has selective inhibitory activity against members of the *M. tb* complex, can be used in conjunction with the luciferase reporter mycobacteriophage (LRP) assay to differentiate photon production by the tubercle bacillus from that by mycobacteria other than tuberculosis (MOTT)." - (Riska, Jacobs et al. 1997). NAP can also be

applied to the  $\beta$ -gal assay.

Although we have established that the  $\beta$ -gal reporter mycobacteriophage can 'specifically' and sensitively detect *M. tb*, further studies are needed, particularly in the clinical setting, where a sputum sample from TB suspected patients could be used to detect the presence of TB bacilli. Other studies evaluated the efficiency of their reporter phages on MGIT cultures, (Banaiee, Bobadilla-Del-Valle et al. 2001; Banaiee, Bobadilla-del-Valle et al. 2003; Bardarov, Dou et al. 2003), the aim is to ultimately apply this technology directly onto sputum samples, which would serve to decrease the time to diagnosis. However, several factors have to be taken into account when applying the phage assay to sputum samples, namely the effect of current decontamination procedures and chemicals may have on the integrity of the reporter phage.

Based on the premise of 'infection of viable bacilli', it stands to reason that drug susceptibility patterns could in theory be generated by the  $\beta$ -gal phage, following drug exposure. This aspect of susceptibility testing has been performed by others with reasonable success (Jacobs, Barletta et al. 1993; Banaiee, Bobadilla-del-Valle et al. 2003; Bardarov, Dou et al. 2003; Banaiee, January et al. 2008; Piuri, Jacobs et al. 2009), prompting us to investigate further the ability of our reporter phage to successfully generate a drug susceptibility pattern. The recommendation is first to investigate its viability using stored laboratory cultures then apply then technology in a clinical setting on sputum samples.

The  $\beta$ -gal reporter mycobacteriophage assay is a novel technique that can be successfully applied to detecting *M. tb* in culture. This methodology offers a viable alternative to the

currently available detection methodologies, with major advantages, in that the cost of performing the test is comparable to the cheapest test. The time to detection is lowered by the fact that our assay does not rely on the growth to colony formation of the bacilli, but merely on their metabolic activity. The specificity of the assay is accounted for by use of the parent TM4 reporter mycobacteriophage, which infects mycobacteria only. Others previously assessed this as well as in our confirmatory tests.

The sensitivity of the assay has been proven to be high, especially in the phages' ability to yield a positive reaction of individual bacilli. The ease of performing the assay nullifies the need for further intensive technical training. The use of tissue culture plates and an incubator for the assay, allows for easier access to the technology because no extraneous machinery, outside the conventional laboratory is needed, making it a promising technology to implement.

In conclusion, this assay, subject to further testing may be a clear alternative to other currently available technologies, particularly in resource-constrained settings.

# CHAPTER 3: THE IMPLEMENTATION OF THE BETA-GALACTOSIDASE ASSAY IN GENERATING DRUG SUSCEPTIBILITY PROFILES OF LABORATORY CULTURES

## 3.0 INTRODUCTION

A majority of those infected with tuberculosis reside in low-income or developing countries, which can ill-afford the burden placed on them by this disease (McNerney, Wilson et al. 1998; Eltringham, Wilson et al. 1999; McNerney 1999). The situation is further exacerbated by the emergence of drug resistant strains of TB. Originally, it was thought that this was 'acquired largely as a result of incomplete treatment regimens but also as a result of spread from index cases of resistant TB' (Jacobs, Barletta et al. 1993; Eltringham, Wilson et al. 1999) but it has become evident that it is mainly a problem of direct spread (Moodley, Shah et al. 2011). This situation threatens to overwhelm the existing control efforts (Bwanga, Hoffner et al. 2009).

The accepted 'Gold standard' for generating drug susceptibility test (DST) results is the 1 % proportional method on drug-embedded, Löwenstein-Jensen (LJ) slants, or Middlebrook 7H10 agar. On both media, it usually takes several weeks to yield results (Jacobs, Barletta et al. 1993; McNerney, Wilson et al. 1998; Mengatto, Chiani et al. 2006; Yzquierdo, Lemus et al. 2006; Traore, Ogwang et al. 2007; Bwanga, Hoffner et al. 2009). In the current state of drug resistance, these methods fall short of aiding in the control of transmission of resistant organisms since adjustment of the treatment regimens occurs too late. The BACTEC-MGIT™

960 (BD, Sparks, MD, USA technologies) has been developed, to curb the challenge of time to detection. This automated broth culture system is based on quenching of fluorescence by bacterial metabolic products, allowing growing organisms to be detected. If drugs are added to the broth, growth will indicate resistance (Park, Bishai et al. 2002). This process takes approximately 10 days. The costs of this system has largely impeded their use in low-resource settings (McNerney, Wilson et al. 1998; Banaiee, Bobadilla-Del-Valle et al. 2001; Park, Bishai et al. 2002; Banaiee, Bobadilla-del-Valle et al. 2003; Piuri, Jacobs et al. 2009; Dixit, Singh et al. 2012).

As mentioned earlier, research into the development of rapid drug susceptibility tests (DSTs) has yielded a variety of tests, which could be roughly categorised into tests based on phenotype and genotype of the isolates (1.5). The reporter mycobacteriophage based tests belong to the former category and the development of these is the primary focus of this study.

Initially, most of the DST technologies were focussed on detecting RIF and INH resistance as primary markers for MDR-TB. The advent of XDR-TB prompts for a wider target selection by including 2<sup>nd</sup> or even 3<sup>rd</sup> line anti-TB drugs. An incorporation of the 2<sup>nd</sup> and 3<sup>rd</sup> line drugs in DST assays, will provide a clearer path to treat infected patients, by providing treatment according to the infecting isolate.

Figure 3.1 gives an overview of the currently available assays that can be used to determine drug susceptibility profiles of either laboratory cultures or directly from patient specimens. Their exact modes of action have been discussed in more detail in Chapter 1 section 1.5.2. This section will focus mainly on their performance by outlining their mode of action,

sensitivities and specificities given in table 3.1. The table is based on the latest representative studies performed to assess the performance of each assay.

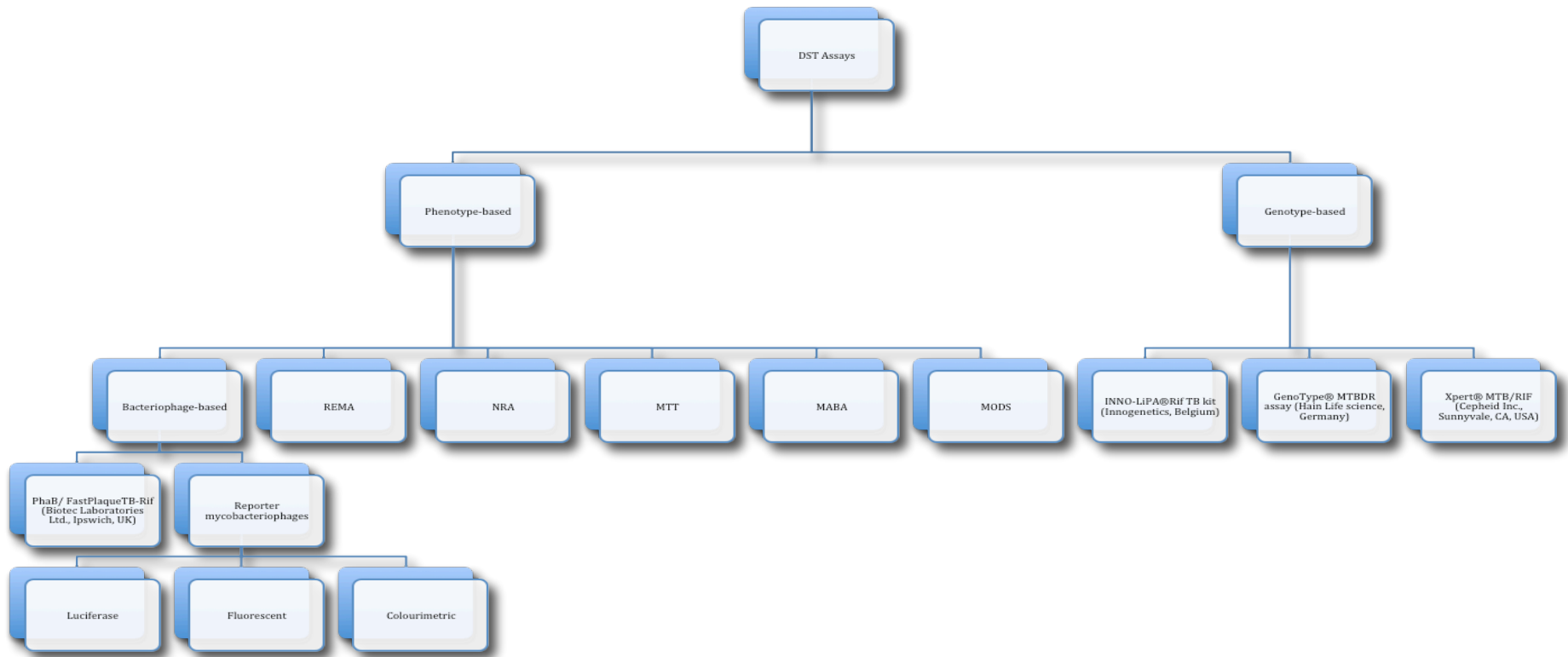


Figure 3.1: Currently available methodologies to determine drug susceptibility profiles of *Mycobacterium tuberculosis*

**Table 3.1: Performance of currently available DST methodologies**

Assay	Reference method	Time to results <sup>a</sup>	Sensitivity	Specificity	Accuracy	Reference
<b>MODS</b>	1% agar proportion method	±18 days	91%	96%	93.3%	(Dixit, Singh et al. 2012)
<b>NRA</b>	1% Agar proportion method	± 14 days; 7 days	98%; 96-100% for RIF & INH	100%; 96-100% for RIF & INH	99%; 100% for RIF & INH	(Dixit, Singh et al. 2012); (Bwanga, Joloba et al. 2010)
<b>MABA</b>	BACTEC MGIT 960™	± 8 days	93% for RIF 100% for INH	100% for RIF 64% for INH	100% for RIF 66% for INH	(Bwanga, Joloba et al. 2010)
<b>MTT</b>	BACTEC MGIT 960™	± 8 days	71% for RIF 77% for INH	94% for RIF 86% for INH	67% for RIF 61% for INH	(Bwanga, Joloba et al. 2010)
<b>REMA</b>	1% Agar Proportion method; BACTEC MGIT 960™	8 days	93%; 71% for RIF 88% INH	98%; 94% for RIF 57% of INH	95%; 67% for RIF 47% for INH	(Dixit, Singh et al. 2012) (Bwanga, Joloba et al. 2010)
<b>PhaB assay</b>	LJ Slants	48 hours	79%	89%	86%	(Zhu, Cui et al. 2011)
<b>FastplaqueRIF</b>	BACTEC 460 system	48 hours	100% for RIF	99%	99	(Albert, Trollip et al. 2002)
<b>Luciferase reporter phage</b>	BACTEC MGIT960™ and 1% agar proportion method	48 hours	100% for RIF 100%; 86% for INH 100% for EMB 94% for STR	100% RIF 97% for INH	100% for RIF 97% for INH	(Banaiee, January et al. 2008) (Bardarov, Dou et al. 2003)
<b>Fluorescent reporter phage<sup>b</sup></b>	1% Agar Method:	48 hours;	100% for KAN 100% for RIF;	100% for KAN 100% for RIF	100% for both RIF and KAN	(Jain, Hartman et al. 2012)

a = earliest time when all the isolates results yielded results; b = performed on pure culture only

### 3.1 AIMS AND OBJECTIVES

Objective: The newly developed  $\beta$ -galactosidase assay (Chapter 2) will be used to generate drug susceptibility profiles of clinical isolates of *M. tb*. The isolates had already been assessed for their drug susceptibility using the 1% agar proportion method but results were only available once the  $\beta$ -galactosidase assay had been completed. This was therefore a blinded study.

## 3.2 MATERIALS AND METHODS

### 3.2.1 ISOLATE COLLECTION

Sixty-nine (69) well characterised isolates from the Department of Infection Prevention and Control (UKZN) were chosen. Their drug susceptibility profiles ranging from fully susceptible to extensively drug resistant were determined using the 1% proportion method. The isolates had been previously stored at -70°C.

Ethics approval number: BCA 27409

### 3.2.2 ISOLATE INOCULATION

1 ml of the each bacterial suspension was thawed and inoculated in 10 ml Middlebrook 7H9 broth supplemented with 10% oleic acid- albumin-dextrose-catalase (OADC; Difco Laboratories, Detroit, Mich.), 0.2% glycerol and 0.05% Tween 80. The tubes were incubated at 37°C until an OD<sub>600nm</sub> 0.7-1, was reached, representing mid-log growth phase.

### 3.2.3 DRUG EXPOSURE AND BETA-GAL ASSAY

One ml of broth culture of each isolate was added to a well according to plate layout (Figure 3.2). The drugs were added to a final concentrations outlined in Table 3.2 and the plates were incubated for 24 hours at 37°C. Following drug exposure, 200 µl of the β-gal reporter phage (10<sup>6</sup> pfu/ml) (MOI: 10-20) was added to each well, without the removal of Tween 80.

**Table 3.2 Concentrations of antimicrobial drugs initially used in the  $\beta$ -galactosidase assay**

	Test concentration (mg/L)
isoniazid	1
rifampicin	1
ethambutol	1
streptomycin	2
ethionamide	1
kanamycin	2
ofloxacin	10
capreomycin	5

The plates were incubated at 37°C for 2 hours to allow for adsorption of the phage and infection of the bacterial cells. One ml of 3.5% agarose (final concentration) in Middlebrook 7H9 broth with 20mM X-gal was added and allowed to solidify. The plate was sealed in an O<sub>2</sub> permeable bag, incubated at 37°C for 5 days and assessed for colour change every 24 hours. The experiments were performed in triplicate to ensure reproducibility.

Drug free control	RIF	INH	EMB	ETH	STR	KAN	CAP	OFLO
Isolate 1								
Isolate 2								
Isolate 3								
Isolate 4								

**Figure 3.2: Lay-out of Isolates and drugs in a 24-well tissue culture plate RIF = Rifampicin; INH= Isoniazid; EMB= Ethambutol; ETH= Ethionamide; STR= Streptomycin; Kan= Kanamycin; CAP= Capreomycin; OFLO= Ofloxacin**

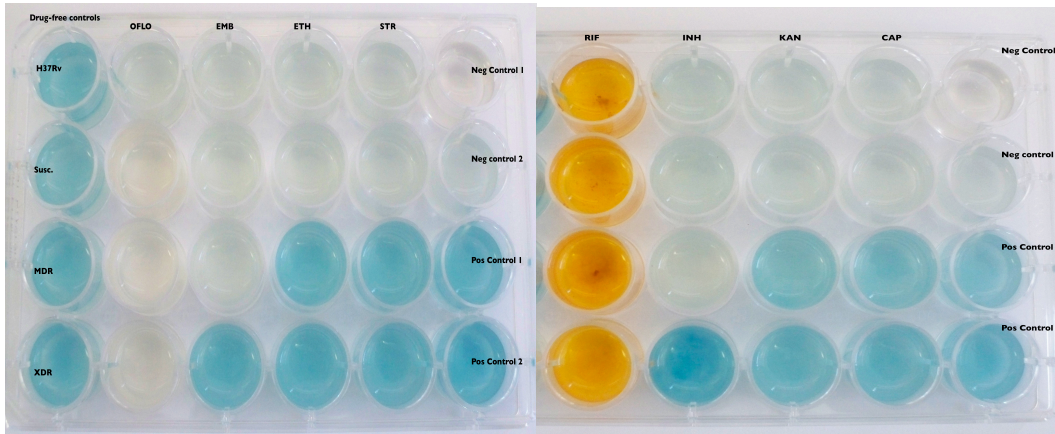
### 3.2.4 STATISTICAL ANALYSIS

To determine the validity of  $\beta$ -galactosidase assay against the 1% proportion method, sensitivity, specificity, positive predictive and negative predictive values were calculated. The McNemar test the difference between paired proportions and Cochran-Armitage test for trend was used to test for possible trends across different drug concentrations.

## 3.3 RESULTS

### 3.3.1 THE B-GAL ASSAY

Figure 3.3 is a depiction of the assay, showing the test wells and the internal controls. The first 2 rows are the H37Rv control strain and the susceptible strain, which did not yield the colour blue after exposure to the drugs. The MDR and XDR-TB strains show a colour reaction on most of the wells, indicative of resistance to the drugs. The rifampicin wells were found to be a challenge due to the potential colour interference with the X-gal. The orange colour often made it difficult to discern the blue reaction of the X-gal.



**Figure 3.3: Representative plates of the  $\beta$ -gal reporter assay, detecting drugs resistance among susceptible, MDR and XDR-TB isolates. Negative control 1: X-gal +  $\beta$ -gal phage only; Negative control 2: X-gal + H37Rv cells only; Positive control 1 ( $mc^2$  155) + X-gal +  $\beta$ -gal phage; Positive control 2 (H37Rv)+ X-gal +  $\beta$ -gal phage**

### 3.3.2 THE ACCURACY OF THE B-GAL ASSAY USING WHO DRUG CONCENTRATIONS

The time to results was determined to be approximately 96 hours with half the isolates yielding results at 72 hours. Table 3.3 below shows the accuracy of the  $\beta$ -gal assay in correctly determining drug resistance. Raw data shown in Appendix E.

The  $\beta$ -galactosidase reporter phage assay was found to have a wide range of concordance with the 1% Agar proportion reference method (45.0%- OFLO to 93.8%- INH). When assessing the 1st line drugs (RIF, INH & STR) it was found that the accuracy of the  $\beta$ -galactosidase assay was higher than of the 2nd line drugs (KAN, CAP & OFLO). Additionally, the McNemar test for agreement showed that initially, there was a statistical overall agreement between the  $\beta$ -gal assay and the 1% agar proportion methods for all the drugs with the exception of rifampicin and ethambutol. However, this did not affect the sensitivity or accuracy of the  $\beta$ -gal assay in determining RIF and EMB resistance among the isolates.

In order to fully assess the impact of drug concentration on the accuracy of the assay, further modifications of the assay i.e.: titrations of the drug concentrations were performed on the drugs that revealed unsatisfactory accuracy. The drug concentrations of these drugs were diluted 2-fold from 2 mg/L to 1 mg/L and 0.5 mg/L for kanamycin; 5 mg/L to 2.5 and 1.25 mg/L for capreomycin and 10 mg/L to 5 and 2.5 mg/L for ofloxacin.

Table 3.3: Overall accuracy of the Beta-galactosidase assay on clinical isolates

Drug	β-gal assay	Agar Proportion method		Sensitivity (%)	Specificity (%)	PPV <sup>a</sup> (%)	NPV <sup>b</sup> (%)	Accuracy <sup>c</sup> (%)	McNemar Test (p)
		Resistant (n)	Susceptible (n)						
RIF	Resistant (n)	45	0	81.8	100	100	47.4	84.4	0.0020
	Susceptible (n)	10	9						
INH	Resistant (n)	53	2	96.3	77.8	96.4	77.8	93.8	1.00
	Susceptible (n)	2	7						
EMB	Resistant (n)	43	10	95.6	47.4	81.1	81.8	81.3	0.04
	Susceptible (n)	2	9						
ETH	Resistant (n)	39	8	86.7	57.9	83.0	64.7	78.1	0.79
	Susceptible (n)	6	11						
STR	Resistant (n)	42	4	82.4	62.9	91.3	50	79.7	0.27
	Susceptible (n)	9	9						
KAN	Resistant (n)	24	6	68.6	79.3	80	67.6	73.4	0.33
	Susceptible (n)	11	23						
CAP	Resistant (n)	20	9	57.1	69	69	57	62.5	0.31
	Susceptible (n)	15	20						
OFLOX	Resistant (n)	0	35	0	100	0	45	45.0	N/A
	Susceptible (n)	0	29						

a= Positive predictive value; b= negative predictive value; c= the number of correct results over the total number of results expressed as a percentage;

### 3.3.3 THE ACCURACY OF THE B-GAL ASSAY WITH ADJUSTED DRUG CONCENTRATIONS

A decrease in the concentration of streptomycin from 2mg/L to 0.5mg/L improved the accuracy of the assay by 2% (table 3.4); this is confirmed by the Cochran-Armitage trend test ( $p=0.02$ ), which revealed a significant trend in accuracy when the drug concentration was decreased.

Similar to streptomycin, the effect of decreasing the drug concentration of kanamycin served to improve the accuracy of the assay by 14% when using half the original drug concentration, however, this percentage falls slightly to 12% when diluting the drug further to 0.5mg/L (table 3.4). The Cochran-Armitage value of  $p=1.00$  further confirms that diluting the drug by 4-fold, does not necessarily increase the accuracy of our assay.

A 2-fold dilution of capreomycin yielded a significant increase (27%) in the accuracy of the assay (table 3.5), with a Cochran-Armitage value of  $p=0.01$ .

Ofloxacin drug concentration of 10mg/L was the highest concentration used amongst all the above-mentioned drugs, and also proved to have the lowest accuracy (table 3.3). When the concentration was decreased 4-fold (2.5mg/L), the accuracy of the assay revealed the highest increase of 36% compared to all other adjusted drugs (table 3.5). The sensitivity of the assay also showed a significant increase from 5.1 to 77% (table 3.5).

**Table 3.4: Table of streptomycin and kanamycin results after titration of the drugs**

	Drug Conc	β-gal assay	Agar proportion		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	McNemar Test (p)	Cochran –Armitage test (p)
			Resistant	Susceptible							
STR	2mg/L	Resistant	42	4	82.4	62.9	91.3	50	<b>79.7</b>	0.27	
		Susceptible	9	9							
	1mg/L	Resistant	44	4	86.3	69.2	91.7	56.3	<b>82.5</b>	0.55	
		Susceptible	7	9							
	0.5 mg/L	Resistant	45	5	88.2	61.5	90	57.1	<b>82.5</b>	1.00	
		Susceptible	6	8							
KAN	2mg/L	Resistant	24	6	68.6	79.3	80	67.6	<b>73.4</b>	0.04	
		Susceptible	1	23							
	1mg/L	Resistant	32	5	91.4	82.8	86.5	88.9	<b>87.5</b>	0.73	
		Susceptible	3	24							
	0.5 mg/L	Resistant	35	9	100	69.0	79.5	100	<b>85.9</b>	0.004	
		Susceptible	0	20							

**Table 3.5: Table of capreomycin and ofloxacin results after titration of the drugs**

	β-gal assay		Agar proportion		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	McNemar Test (p)	Cochran Armitage (p)
	Drug Conc		Resistant	Susceptible							
CAP	5mg/L	Resistant	20	9	57.1	69	69	57	<b>62.5</b>	0.31	
		Susceptible	15	20							
	2.5 mg/L	Resistant	33	7	94.3	75.9	82.5	91.7	<b>85.9</b>	0.18	
		Susceptible	2	22							
	1.25mg/L	Resistant	35	7	100	75.9	83.3	100	<b>89.1</b>	0.02	
		Susceptible	0	22							
OFLOX	10 mg/L	Resistant	2	0	5.1	100	100	46.8	<b>46.8</b>	<0.001	
		Susceptible	33	29							
	5mg/L	Resistant	18	3	51.4	89.7	85.7	60.5	<b>68.8</b>	0.03	
		Susceptible	17	26							
	2.5mg/L	Resistant	27	3	77.1	89.7	90.0	76.5	<b>82.8</b>	0.23	
		Susceptible	8	26							

The reproducibility of the assay was assessed using the same clinical isolates. As mentioned previously, the assay was performed in triplicate at the original concentration and was found to be 98% reproducible (data not shown). The reproducibility aspect was repeated with drug titration, using between 51 and 69 isolates (table 3.6). The assay was found to be reproducible (average 96%).

**Table 3.6: Table of reproducibility assessment of the  $\beta$ -gal phage**

	Concordance	Discordance	Excluded <sup>a</sup>	Total Included	Percent agreement (%)
<b>STR</b>	46	5	18	51	90,2
<b>KAN</b>	51	1	17	52	98
<b>CAP</b>	59	0	10	59	100
<b>OFLO</b>	67	2	0	69	97.1

a: Excluded due to contamination of one or more of the replicates

### 3.5 DISCUSSION

The  $\beta$ -gal reporter phage assay has proven to be adequate in determining the drug susceptibility profiles of clinical isolates. The accuracy of the test, subsequent to titrations improved significantly, particularly with second-line anti TB drugs (tables 3.4 &-3.5). The high accuracy ( $85\% \pm 5\%$ ) of the assay for both first and second-line drugs proves that this assay can indeed form part of a panel of tests that could be used to determine drug susceptibility. The turnaround time for results was found to be within 5 days, a significant factor in TB control and individual patient treatment outcomes.

Originally, the initial concentrations of the drugs used, were determined as per the 'gold standard' 1% agar proportion method, however there was a high level of discrepancy, where some of the drugs (kanamycin, capreomycin and ofloxacin) skewed in the direction of too many isolates being read as susceptible, thus giving false results and lowering the accuracy of the assay (table 3.3). Therefore an adjustment of the test concentration was needed. The optimal concentration was established by titrating the drugs by 2-fold serial dilutions. The high initial drug concentration turned out to be responsible for lowered accuracy of the assay (Tables 3.4-3.5).

As mentioned previously, the original concentration used for determining drug susceptibility patterns were based on the breakpoints advised for the 1% proportion method in agar, which is a solid-medium based method. As the beta-galactosidase assay is a liquid-medium based methodology, the drug concentrations used, as in solid culture may yield incorrect results.

This is further confirmed by the WHO guidelines, which recommend lowered drug concentrations when working with liquid as opposed to solid culture methods (WHO 2009).

Our original concentrations (STR; 2mg/L), (KAN; 2mg/L), (CAP; 5mg/L) and (OFLO; 10mg/L) were applicable to either Middlebrook 7H10 or 7H11 solid culture plates, thus the subsequent reduction in concentration was found to be more in line with the WHO recommended concentrations for liquid methodologies. The breakpoint concentration for kanamycin was not reported. However, judging by the lower concentration for other drugs as well as the increased accuracy of our assay when decreasing the concentration, it would be reasonable to assume that a concentration of 0.5 to 1mg/L would be optimal.

The microplate formats of the MABA, MTT, NRA and REMA assays are similar to our  $\beta$ -galactosidase assay, in that we utilised 24-well tissue culture plates. Additionally, the above mentioned assays have been developed to be read visually as well as spectrophotometrically, whereas, our assay has been adapted to be read visually, thereby removing the need for expensive equipment thus serving resource poor settings, which may not have the equipment.

A study performed by Chauca et al., 2007, evaluated MABA's performance on TB clinical isolates, revealed that MABA's ability to correctly predict a RIF resistant isolates was 80.3% and a negative predictive value of 99.7% (Chauca, Palomino et al. 2007). Our study differs in this regard, in that our assay's positive predictive value was calculated to be 100% and the negative predictive value was 47% (table 3.3). The INH positive and negative predictive values for both our study and Chauca et al., 2007 were comparable at 96%, 77% and 99%, 76% respectively. The differences for RIF could be due to the lack of drug titration to

determine the optimal concentrations of RIF. These values may improve if the drugs were titrated.

In our assay we used closed and bagged plates. This resulted in a reduced biosafety hazard and less contamination as compared to the luciferase assay or the spectrophotometric readable assays, which require removing of the lid during reading (Chauca, Palomino et al. 2007). Furthermore, time to results of the MABA is approximately 8 days in contrast to the 4 or 5 days for our assay.

Another test that has similarities to our plate format is the MTT assay. This relies on colourimetry to report resistance or susceptibility of *M. tb* isolates. A study performed by Ferrari et al., 2010 revealed a 100% concordant results in detecting INH and ETH resistance among a group of isolates taken from LJ slants and 99.5% and 95.8% agreement in detecting RIF and STR susceptibility respectively (Ferrari Mde, Telles et al. 2010). Our study's percentages of agreement are slightly lower than the reported MTT study (table 3.1), however, the difference in drug concentrations may have bearing on the slightly lowered percentages. The concentrations used by Ferrari et al. 2010, (0.25 INH, 0.5 RIF, 4 ETH and 1mg/L STR) were often much lower than the concentrations in our study (table 3.2). This difference in drug concentrations highlights the need for standardization when newer phenotypic tests are implemented, as well as determining the optimal drug concentration for any given geographical area.

A study by Dixit et al., (2012) evaluating the NRA assay on clinical isolates found very high predictive values and accuracy levels in detecting RIF, INH and STR resistance. Similar to the Ferrari et al., (2010) the concentrations of the drugs also differed significantly with our study, possibly explaining our slightly lower predictive values and accuracy levels. However, the drawback of the NRA assay lies in the possibility of absence of a nitrate reductase system among *M. tb* isolates, whereas our  $\beta$ -gal reporter phage is specifically designed to detect mycobacterium bacilli and not rely on their enzymatic properties.

Using the REMA assay, Umubyeyi et al. (2006) reported 100% accuracy for ofloxacin at 2mg/L. We found this to be 83% at 2.5mg/L in our assay.

Liquid-based microplate assays such as REMA, MTT and NRA have the potential for aerosol generation when the plates are opened. This critical factor is catered for in our assay by 'fixing' the bacilli in place using X-gal infused 'top' agar. The top agar serves a two-fold purpose in that, it 'holds' the bacilli in one place for visualisation of beta-galactosidase expression, as well as limiting aerosol production. The cost of performing the  $\beta$ -galactosidase assay is comparable to the microplate phenotypic assays in that it requires very little reagents, equipment and expertise.

When taking into consideration the phage-based assays, the luciferase assay performed by Banaiee et al., revealed an overall 98% (100% RIF and 96% INH) agreement with the BACTEC method (Banaiee, January et al. 2008), whereas our study showed a 89% (84% RIF and 94% INH) agreement. Similar results were observed by Bardarov et al., (2003), who reported DST profiles on sputum samples using the luciferase reporter phage. They revealed that their assay had an overall 95% agreement (table 3.1). Our assay's accuracy was 8%

higher than their 86%, which could be attributed to the RIF drug concentration (2,10 and 40 mg/L) they used. The lower agreement of the RIF resistance detection may among other things be due to the colorimetric nature of the assay. The orange RIF powder may have interfered with the colour development in the  $\beta$ -galactosidase assay, causing misreading of the positive samples (figure 3.3). In this regard, the RIF testing may require more optimisation such as 'washing off' of the drug after the 24-hour drug exposure, prior to the addition of the phage. However, this may decrease the sensitivity of the assay as the washing may inherently cause bacilli loss. The time to results for the luciferase assay was 24 hours unlike the  $\beta$ -galactosidase assay that needs 96 hours. However, this is offset by the fact that the luciferase assay requires expensive machinery (luminometer) and reagents (Luciferin) while our assay's only 'costly' factor is the X-gal powder. Thus the cost consideration may be the distinguishing factor when choosing either phage-based method.

Both methods are phenotype based assays that require little training to perform, an added advantage to implementing them at peripheral clinical laboratories (Banaiee, January et al. 2008). In an effort to reduce the need for an expensive luminometer, The Bronx box is a cheaper alternative for the luminometer (section 1.7.1.2) (Riska, Su et al. 1999). The luciferase assay's performance in generating DST using 2<sup>nd</sup> line anti-TB drugs has yet to be determined.

The *FastPlaque RIF-TB<sup>TM</sup>* assay was found to have a good correlation (99.2%) with the BACTEC method in determining RIF resistance, with a turnaround time of 2 days (Albert, Trollip et al. 2002) compared with our assay's 84% accuracy. Similar results were reported by Traore et al., 2007 who used an in-house phage amplified biologically (PhaB) assay (Traore, Ogwang et al. 2007).

The difference in the assays' accuracy could be attributed to their different modes of reading. The *FastPlaque RIF-TB™* method relies on the formation of plaques whereas our assay is colorimetric based which was mentioned above. As with the luciferase assay, the *FastPlaque RIF-TB™* assay is relatively easy to perform, with a faster time to results compared to the BACTEC MGIT™, however it is only geared to detecting RIF resistance in contrast to our assay, which was used to detect resistance to 8 drugs.

In the context of increasing drug resistance among infected individuals, prompt detection and determination of drug susceptibility of *M. tb* is imperative. As previously mentioned, the current methodologies used to generate DSTs are either prohibitively expensive or require technical expertise that is not readily available in developing countries. Thus our  $\beta$ -galactosidase reporter phage assay has the potential to alleviate the laboratory constraints of generating DSTs in a prompt and accurate manner.

The preliminary assessment of cost per isolate (drugs included) was found to be less than US \$3 compared to \$39 for the BACTEC MGIT test. Additionally, the reporter phage can be propagated in-house for use without additional cost. The most expensive component of the assay is the X-gal powder, which is still cheaper than the cost of the luciferin for the luciferase assay. The drugs used for our assay's DST determination are routinely used in other reference methodologies.

Another factor in diagnosis of drug resistant TB is the turnaround time. The current gold standard, agar proportion method takes 3 weeks to yield results, during which time the

infected patient may be left untreated or treated with less efficacious medications. This often leads to poor treatment outcomes and increased risk of transmission. Our assay reports results within one week of performing the test.

Although other tests such as the MTT, MODS, and MABA have been recommended for use in referral laboratories (Chauca, Palomino et al. 2007), our test has the potential to be used at primary health care laboratories. The simplicity of the assay requires equipment (37°C incubator, a refrigerator for storage and a class II hood) that already forms part of a basic laboratory.

Currently, the  $\beta$ -gal assay cannot be used as a sole source of DST information, however it offers the potential to be a rapid preliminary determination. As the assay was used on cultures with predetermined susceptibilities, it still needs to be thoroughly tested on isolates from primary sputum specimens. As mentioned in Chapter 2, the potential for detecting MOTTS is high as the TM4 phage (which forms the backbone of the  $\beta$ -gal reporter phage) also infects MOTTS, however with the inclusion of NAP that inhibits MTC, this issue can be resolved.

In order to ensure that the assay eventually reaches the 'Gold standard' status, further optimisation has to be performed, particularly the drug concentrations used. The test has the potential to be used on primary sputum cultures and eventually other tissues.

# CHAPTER 4: DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM SAMPLES USING A BETA-GAL REPORTER MYCOBACTERIOPHAGE

## 4.0 INTRODUCTION

The use of smear microscopy to detect acid-fast bacilli (AFBs) is still a hallmark technology for the diagnosis of tuberculosis in many developing countries (Caviedes, Lee et al. 2000; Albert, Heydenrych et al. 2002). This method relies on a relatively high bacillary count, however as previously noted, most cases of HIV co-infected TB patients are seen as smear negative because of a low bacillary count. As stated by Albert et al., 2002 “significant morbidity and mortality are associated with TB patients with negative sputum smears” (Albert, Heydenrych et al. 2002). It is also possible that the smear negative patients may later become smear positive if left untreated, further contributing to the transmission of the disease (Albert, Heydenrych et al. 2002).

Smear microscopy does not provide a drug susceptibility profile of the bacteria that infect the patient (Caviedes, Lee et al. 2000). Therefore, sole reliance on AFB results is insufficient to curb this disease. Several technologies have been developed to detect mycobacterial bacilli, ranging from phenotypic-based to genotypic based (figure 3.1). The fastest assays, which are based on detection of mutations, are prohibitively expensive and unsuitable in low-resource settings. The classic tests on LJ slants or Middlebrook agar plates as well as the automated culture based techniques (BACTEC MGIT 960™) are able to diagnose paucibacillary disease

and can provide drug susceptibility profiles but these methods are slow.

The application of reporter bacteriophages promises cheaper, simpler, rapid tests that show potential both in their ability to detect low bacillary counts as well as simultaneous drug susceptibility testing (DST). Since this test uses a phenotypic approach, it should potentially, be able to provide full susceptibility profiles. This chapter reports on the application of the  $\beta$ -gal expressing reporter mycobacteriophage as a diagnostic test for pulmonary tuberculosis (PTB).

#### 4.1 AIMS AND OBJECTIVES

Objective: To assess the performance of the  $\beta$ -gal assay, when applied to clinical sputum samples.

Aim 1: To determine the ability of the  $\beta$ -gal reporter mycobacteriophage to detect *M. tb* in sputum specimens obtained from patients suspected of having PTB.

Aim 2: To compare the sensitivity, specificity and accuracy of the  $\beta$ -gal assay with classic smear microscopy and culture.

## 4.2 MATERIALS AND METHODS

### 4.2.1 SPECIMEN COLLECTION

Sputum specimens were collected from consecutive patients with symptoms and signs of PTB attending the R.K. Khan's chest clinic. Retrospective chart reviews were done to verify the clinical diagnosis, gender and ethnicity. Since further clinical details were irrelevant for analysis of the laboratory data, these were not collected. (BREC approval number BE064/08)

### 4.2.2 DECONTAMINATION OF SPUTUM SPECIMENS

The sputum specimens were digested and decontaminated using the NALC-NaOH method. Briefly, NALC-NaOH working solution (4g N-acetyl-L-cysteine, 2.94% Sodium Citrate and 2% Sodium Hydroxide) was added to the specimen in equal volume and mixed thoroughly for 15 seconds or until the specimen was no longer mucoid. The specimen was incubated at room temperature for 15 minutes. Phosphate buffer (pH7.6) was added to a final volume of 50 ml in order to neutralize the effect of NaOH. The specimen was centrifuged at 3000 g for 20 minutes at 4°C and the supernatant discarded. The resultant pellet was re-suspended in 3 ml broth buffer (4 ml Middlebrook 7H9 broth and 36 ml PBS) and mixed thoroughly. The sample was then incubated in 7H9-OADC-40% Glycerol broth at 37°C for 72 hours to allow for bacilli recovery, following harsh treatment of the decontamination procedures.

### 4.2.3 SPUTUM MICROSCOPY AND CULTURE

Sputum microscopy was performed using Auramine and Ziehl-Nielsen acid-fast staining techniques. Culture was performed in MGIT broth, incubated in the automated BACTEC-960 incubator and on Middlebrook 7H11 plates incubated in sealed gas-permeable bags in a CO<sub>2</sub> incubator. Volumes of decontaminated specimen used for each test are shown in table 4.1.

**Table 4.1: Tests and amounts of specimen used**

<b>TEST</b>	<b>VOLUME (µl)</b>
Smear microscopy	
Ziehl-Nielsen	15
Auramine	15
Culture	
MGIT broth	500
Middlebrook 7H11 plate	50

Direct DST testing was performed once a positive BACTEC MGIT960™ was confirmed, however the scope of this chapter did not include the DST testing.

### 4.2.4 BETA-GAL REPORTER PHAGE ASSAY

The assay was performed as described in the former chapter, paragraph 3.2.2, with minor modifications; briefly, 2 ml of the NALC-NaOH treated specimen was washed with phage buffer (50 mM Tris pH7.6, 150 mM NaCl, 10 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>) by adding 5 ml buffer, vortexing for approximately 30 seconds and centrifuging at 3000 g for 5 minutes. This was done twice. The pellet was re-suspended in 1.5 ml phage buffer resulting in an end volume of 2 ml. Three aliquots of 500 µl were added to separate wells of a 24 well tissue culture plate.

This equals the amount of specimen used in the MGIT broth (Table 4.1). To each of the wells, 200  $\mu$ l  $\beta$ -gal phage ( $10^6$  PFU/ml) was added and the mixture was incubated for 90 minutes at 37°C. Following this, 1 ml of X-gal infused Top Agar (final agar concentration 3.5%) was added. The tissue culture plate was sealed in a gas permeable bag and incubated for 21 days at 37°C. The six wells in the bottom row contained the various controls (Figure 4.1). The first positive control contained 10  $\mu$ l *M. smeg* mc<sup>2</sup> 155 with the phage and X-gal reagent, the second *M. tb* H37Rv with the phage and X-gal reagent and the third 10  $\mu$ l of *M. smeg* mc<sup>2</sup> 155 without the phage and X-gal reagent. The two negative controls contained *M. smeg* mc<sup>2</sup> 155 and *M. tb* H37Rv respectively with X-gal reagent but without the phage.

#### 4.2.5 STATISTICAL ANALYSIS

To determine the validity of  $\beta$ -gal assay against the MGIT culture, sensitivity, specificity, positive predictive and negative predictive values were calculated. The McNemar test was used to establish the difference between paired proportions.

## 4.3 RESULTS

### 4.3.1 CLINICAL DATA

All patients had symptoms and signs of pulmonary tuberculosis. Table 4.2 shows the retrospectively collected basic demographic data. The cohort included individuals as young as 12 and as old as 89 years. The average age was 42 years. The male to female ratio was 1:1. The majority of the study cohort (46%) was of black African ethnicity.

**Table 4.2: Patient demographic data**

Average (range) age		42.2 (12 - 89)
Gender	no. (%) male	178 (37)
	no. (%) female	180 (37)
	no. (%) unknown	125 (26)
Ethnicity	no. (%) black	222 (46)
	no. (%) indian	128 (27)
	no. (%) white	4 (0.8)
	no. (%) coloured	4 (0.8)
	no. (%) unknown	125 (26)

### 4.3.2 PERFORMANCE OF THE BETA-GAL ASSAY

Figure 4.1 shows the assay format. The blue spots represent  $\beta$ -gal activity of one or a clump of mycobacterial cells present in the sputum. The diffuse blue colour in the wells containing specimen no. 76 is the result of the presence of large numbers of bacilli resulting in confluent “spots” (diffuse colour).

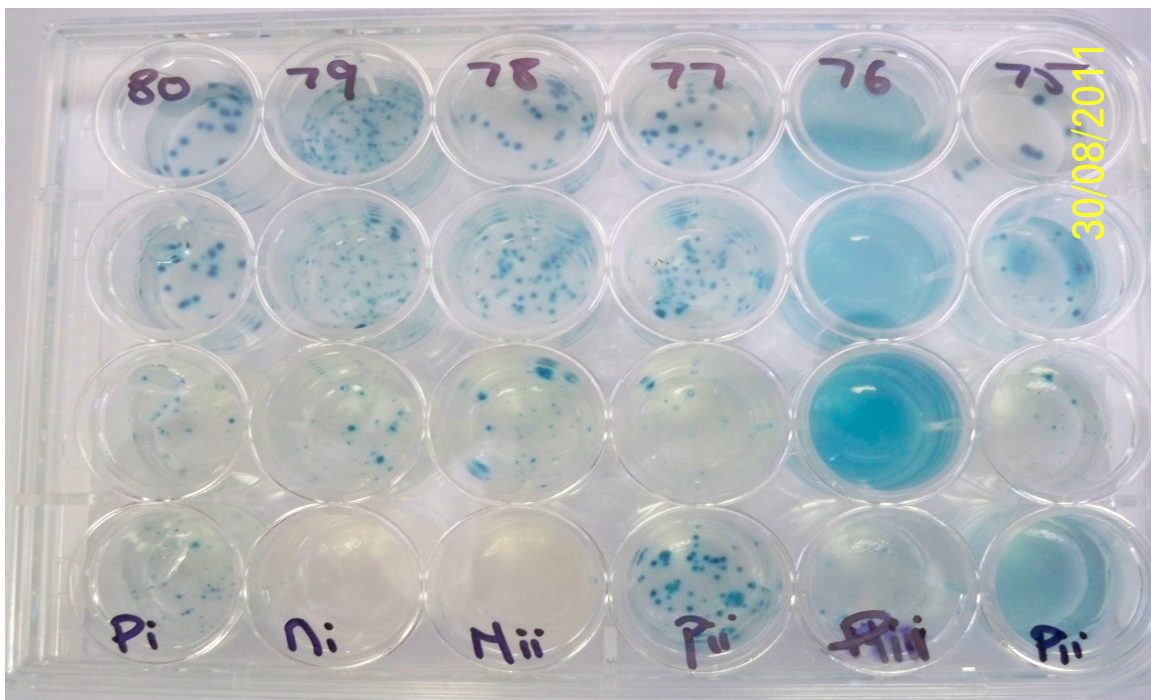


Figure 4.1: Representative plate of the  $\beta$ -gal assay depicting specimen in triplicate. Pi = Positive control ( $mc^2$  155 + X-gal +  $\beta$ -gal phage); Ni = negative control ( $mc^2$  155 + X-gal -  $\beta$ -gal phage); Mii = negative control (H37Rv + X-gal -  $\beta$ -gal phage); Pii = Positive control (H37Rv + X-gal +  $\beta$ -gal phage); Piii = Positive control ( $mc^2$  155-10  $\mu$ l + X-gal +  $\beta$ -gal phage)

### 4.3.3 MICROSCOPY AND CULTURE RESULTS

Table 4.3 describes the microscopy and culture results within the patient cohort. The yields of both liquid and solid culture were found to be identical at 20% each. As expected, AFB microscopy was significantly less sensitive (8.4 % overall and 41 % of the culture positives). The finding of a 20% prevalence rate in the RK Khan Hospital Chest Clinic cohort is in

accordance with expected TB statistics in KwaZulu-Natal South Africa. The low number of TB positive specimens detected by smear microscopy highlights its lack of sensitivity in the HIV era.

**Table 4.3: TB smears and culture results**

	<b>BACTEC MGIT</b>	<b>Solid Culture (7H11)</b>	<b>Smear microscopy (combined)</b>
<b>No. (%) positive</b>	101 (20.2%)	102 (20.4%)	42 (8.4%)
<b>No. (%) negative</b>	383 (76.8%)	396 (79.4%)	457 (91.6%)
<b>No. (%) contaminated</b>	15 (3%)	1 (0.2%)	0
<b>Total</b>	499 (100%)	499 (100%)	499 (100%)

#### 4.3.4 BETA- GAL ASSAY COMPARED TO BACTEC MGIT 960™

The  $\beta$ -gal assay results were in concordance with the BACTEC MGIT 960™ liquid-based diagnostic test. Four hundred and twenty one specimens out of a total of 484 (table 4.4) were found to be in agreement with the BACTEC MGIT 960™, reflecting an accuracy of 87 %. The sensitivity of the test was 84.2 %, the specificity 87.7%, the positive predictive value (PPV) 64.4 % and the negative predictive value (NPV) 95.5 %. The McNemar test revealed a value of  $p < 0.001$ , meaning that there was a statistically significant difference in proportions of positive and negative results for the two assays.

**Table 4.4  $\beta$ -gal assay compared to BACTEC MGIT 960™**

		BACTEC MGIT 960™		Total
		Positive (n)	Negative (n)	
$\beta$ -gal assay	No. Positive (%)	85 (85%)	47 (12.3%)	<b>132</b>
	No. Negative (%)	16 (15.8)	336 (87.7%)	<b>352</b>
	<b>Total</b>	<b>101 (100%)</b>	<b>383 (100%)</b>	<b>484</b>

The time to results of the  $\beta$ -gal assay was 4 days (96 hours) compared to the BACTEC MGIT 960™'s 14 to 21 days on the same series of specimens. Fifteen specimens were excluded from analysis either due to contamination of the BACTEC MGIT 960™ or the presence of MOTTs.

#### 4.3.5 BETA- GAL ASSAY COMPARED TO COMBINED BACTEC MGIT 960™ AND CULTURE ON 7H11 MIDDLEBROOK AGAR

Since routine culture in the Department of Infection Prevention and Control consists of the combination of BACTEC MGIT 960™ and 7H11 Middlebrook agar, this combination was also used to assess the accuracy of the  $\beta$ -gal assay. Table 4.5 shows that 425 out of 486 specimen were found to be in agreement with the combination of solid culture and BACTEC MGIT 960™ at the much shorter time period of 4 days. With this combination as gold standard, the overall accuracy was 87.4%, the sensitivity 82.7% and the specificity 88.8%. The PPV and NPV were 68.4% and 75.2% respectively. Thirteen (2.6%) specimens were reported to be either MOTTs or contaminated, and were thus excluded from the analysis.

Table 4.5: Performance of  $\beta$ -gal assay compared to culture in BACTEC MGIT™ and on 7H11 Middlebrook agar

		<b>BACTEC MGIT &amp; Culture (7H11)</b>		
		<b>Positive</b>	<b>Negative</b>	<b>Total (n)</b>
<b>Beta-gal test</b>	<b>No. Positive (%)</b>	91 (82.7)	42 (11.1)	<b>133</b>
	<b>No. Negative (%)</b>	19 (17.3)	334 (88.9)	<b>353</b>
	<b>Total (n)</b>	<b>110 (100%)</b>	<b>376 (100%)</b>	<b>486</b>

#### 4.3.6 ANALYSIS OF DISCREPANCIES BETWEEN BACTEC MGIT 960™, MIDDLEBROOK 7H11 CULTURE AND SMEAR MICROSCOPY AND THEIR EFFECT ON THE ACCURACY OF THE $\beta$ -GAL ASSAY

Fifteen specimens were found to be smear positive but negative with both BACTEC MGIT 960™ and Middlebrook 7H11 agar culture. It was found that 12 of the 15 microscopy positive, culture negative specimens were positive for *M. tb* in the  $\beta$ -gal assay. Thus an analysis was performed assessing the performance of the  $\beta$ -gal assay as a gold standard that considered a positive BACTEC MGIT 960™ culture, a positive Middlebrook 7H11 culture and or smear microscopy positive results as true positives. The results are shown in table 4.6.

**Table 4.6: A comparison of the Culture methodologies including smear microscopy results and their effect on the accuracy of the  $\beta$ -gal assay**

	No. (%) BACTEC MGIT 960™; solid Culture and smear microscopy		
	Positive	Negative	Total (n)
<b>No. (%) beta-gal assay</b>			
<b>Positive</b>	99 (20)	42 (8.5)	141 (28.5)
<b>Negative</b>	19 (3.8)	334 (67.6)	353 (71.5)
<b>Total (n)</b>	<b>118 (23.9)</b>	<b>376 (76.1)</b>	<b>494 (100)</b>

Table 4.7 shows the performance assessment of the  $\beta$ -gal assay when 3 different gold standards are used (tables 4.5-4.6). The overall accuracy tends to increase with the number of tests included in the gold standard. However, this does not reach statistical significance ( $p < 0.001$ ).

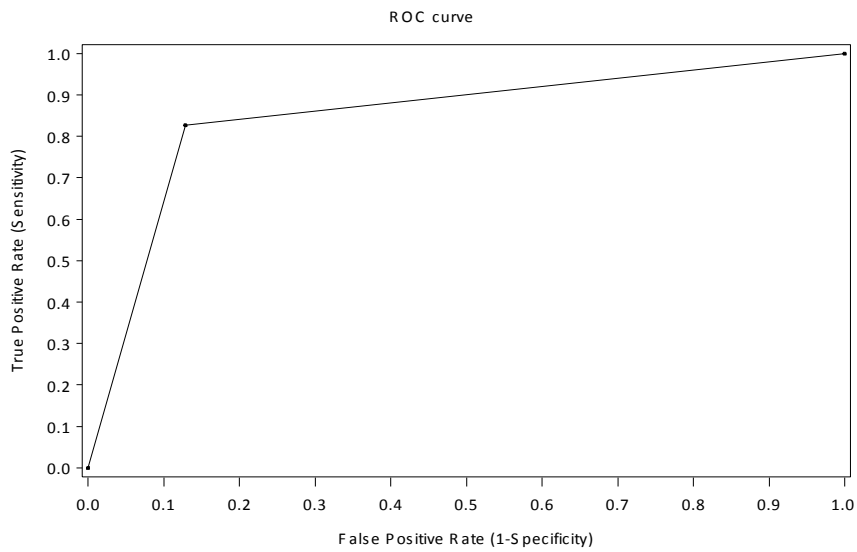
**Table 4.7: Summary of the performance assessment of the  $\beta$ -gal assay compared to culture and microscopy in various combinations**

	$\beta$ -gal reporter phage assay	BACTEC MGIT 960™ and Middlebrook 7H11	BACTEC MGIT 960™ and Middlebrook 7H11 and microscopy
Sensitivity (%)	84.2	82.7	83.9
Specificity (%)	87.7	88.8	88.8
PPV (%)	64.4	68.4	70.2
NPV (%)	76.9	75.2	73.2
Accuracy (%)	87.0	87.4	87.7

#### 4.3.7 CONTAMINATION AND GROWTH OF MYCOBACTERIA OTHER THAN TUBERCULOSIS AND THEIR EFFECT ON THE ACCURACY OF THE $\beta$ -GAL ASSAY

Of the 14 specimens that produced a positive reading in the BACTEC MGIT 960™ system but did not grow *M. tb*, 4 (0.8%) contained a MOTT and 10 (2%) were contaminated with a bacterial species not belonging to the genus *Mycobacterium*. All four MOTT containing specimens were smear negative and all were isolated on Middlebrook 7H11 agar. Of the 10 contaminated specimens, three were smear positive and two of these had a positive culture on Middlebrook 7H11 agar. Four of the contaminated specimens were found to be negative for all the tests, including the  $\beta$ -gal assay. One specimen that was contaminated was found to be  $\beta$ -gal assay positive and 4 contaminated MGIT cultures were positive either by solid culture, or smear and were positive by the  $\beta$ -gal assay. Only one contaminated specimen found to be culture positive was not confirmed by the  $\beta$ -gal assay.

#### 4.3.8 RECEIVER OPERATING CHARACTERISTIC & AREA UNDER CURVE ANALYSIS OF THE B-GAL ASSAY



**Figure 4.2: The ROC curve of the accuracy of the  $\beta$ -gal phage assay**

A combination of the cultures (solid and MGIT) were compared with the  $\beta$ -gal assay results to determine the ability of the  $\beta$ -gal assay to discriminate true positives from the cohort, using the ROC and AUC analysis. The data was plotted in the above graph and the AUC was found to be 0.85.

## 4.4 DISCUSSION

The  $\beta$ -gal assay has the ability to diagnose PTB in sputum specimens in a rapid manner. The assay revealed a 87% accuracy in determining TB positivity when compared to gold standards. It provides a significant improvement compared to smear microscopy and other phenotypic rapid tests (table 3.1). The ease of performing the assay is comparable to the non-automated solid culture methods; however, the time to results was found to be significantly shorter than the culture methods.

The high accuracy of the  $\beta$ -gal assay, the ease of performance, its low cost and the fact that there is no need for expensive equipment shows the potential of this assay to be implemented in resource-constrained settings. As mentioned previously, most countries with a high burden of TB fall in this category. The short time to results of the assay provides an important added advantage. There are currently no other methods that satisfy the above-mentioned criteria. The presence of MOTTs among the clinical specimen highlights the need of including NAP treatment to exclude MTCs from the MOTT containing specimens. It is envisaged that the addition of NAP treatment to the cost of performing the assay will not affect the time it takes to yield definitive results. From figure 4.2, and the AUC value of 0.85, the  $\beta$ -gal assay was revealed to be able to efficiently discriminate the true positive TB specimen among the cohort, which invariable increases its value as a diagnostic tool.

The closely related assays, PhaB or *FastPlaque-TB™* formed the basis for the design of this study. Both methods utilise the D29 mycobacteriophage, which infects and lyses mycobacteria. However, as mentioned previously, both methods rely on the ability of the

phage to be inactivated and the use of *M. smegmatis* 'indicator cells' for the assay to be read. On the contrary, our assay utilises the TM4 genetically manipulated mycobacteriophage, which expresses the  $\beta$ -gal protein upon infection of viable *M. tb* cells, and the signal is reported by change in colour of the X-gal from colourless to blue. Thus our assay eliminates the need for indicator cells, which may be prone to yield false positive results due to inadequate inactivation of extraneous phages.

The *FastPlaque-TB™* and the PhaB assay, similar to our assay, can be applied to detect *M. tb* directly in sputum. Several studies have been performed to assess the accuracy of these assays, and both have been found to be comparable in their sensitivity and specificity. In a study performed by Albert et al., 2002, in a South African setting, using the *FastPlaque-TB™* assay, it was revealed that the assay had 72.5% sensitivity, 99% specificity, 91%PPV and 96% NPV, whereas our assays revealed a higher sensitivity of 84%, but a lower specificity of 87.7% (section 4.3.4). The PPV and NPV for our assay were slightly lower than the *FastPlaque-TB™*. Overall, our assay was found to be comparable to the *FastPlaque-TB™* (Albert, Heydenrych et al. 2002).

Similarly, a more recent study done by Zhu *et al.*, 2011 assessing the PhaB assay found lower sensitivity and higher specificity for their assay of 54% sensitivity, 99% specificity and accuracy of 63% compared to our assay's 87% accuracy. The above-mentioned studies both used sputum specimens for direct clinical testing, and the reference method was culture on L-J slants.

Several (15) specimens were found to be smear positive but culture negative, of which 12 were found positive by our assay, revealing 80% sensitivity among this group. The Rachow et al. (2011) group found a similar occurrence among their Tanzanian cohort when evaluating the efficacy of the Gene Xpert (Rachow, Zumla et al. 2011). However, they found 61% sensitivity with their Gene Xpert, which is significantly lower than our assay.

Several explanations have been postulated to explain smear positive, culture negative specimens: firstly, dead bacteria seen in the smears of sputum from patients on treatment, secondly, non-cultivable MOTTs and thirdly, death of bacteria in transport either due to environmental conditions or by drug levels in the expectorated sputum. Of these possibilities, only non-cultivable MOTTs would turn the  $\beta$ -gal assay positive if these MOTTs were susceptible to infection by the phage and non-viable bacteria wouldn't yield a positive result. Another possibility is that stainable but not cultivable *M. tb* remains viable which could be  $\beta$ -gal positive. Two such patients have been seen in King Edward VIII Hospital in Durban: one with a liver abscess that contained large amounts of acid fast bacilli on smear but remained repeatedly culture negative. The patient was not on treatment. The IS6110-based PCR was positive and the patient responded to first line treatment. This makes it highly likely to be active disease with *M. tb* as the cause. The second case was similar but the site of infection was a lymph node package in the neck (A.W. Sturm, personal communication). These observations support the possibility that the smear positive, culture negative cases that were positive in the  $\beta$ -gal assay were in fact true positives.

Some species of mycobacteria that belong to the MOTT group are known to be susceptible to T4 phage infection. Those specimens that were found to be MOTT and were  $\beta$ -gal assay positive could be false positives. This problem can be avoided by including NAP treatment of

the specimen. However, a mixture of MOTT with *M. tb* cannot be excluded. Without further identification of the MOTTs, their clinical importance cannot be assessed.

The latest but expensive rapid test for diagnosis of tuberculosis, the Xpert test (Cepheid Sunnyvale, California, USA), has sensitivity higher than smear (61%) but lower than culture (Rachow, Zumla et al. 2011). That is also the case for our test where it was able to detect 82.7% of the isolates, which were confirmed by both liquid and solid culture (table 4.7). The differences in sensitivity may be explained by the fact that most studies that evaluate novel assays included samples that were under more stringent selective conditions whereas our study, similar to the Rachow, used sequential suspected TB patients without any selection criteria (Rachow, Zumla et al. 2011). In another study performed by Scott et al, 2011 in similar South African settings, they found that the Gene Xpert's ability to detect MTB in sputum specimen was comparable to our result (86%) (Scott, McCarthy et al. 2011).

In conclusion, this work shows that the  $\beta$ -gal assay is a promising assay in detecting TB from the sputum of patients with PTB. However, further studies are needed, particularly larger scale studies in order to determine the applicability of the assay in clinical laboratories. In order to ascertain the specificity and sensitivity of all clinical samples, in future, it would be advantageous to perform confirmatory tests on 'contaminated' MGIT specimen, using the  $\beta$ -gal assay. The 87% sensitivity of the assay among sputum specimens proves that the assay shows promise in the arena of rapid TB diagnostics.

# CHAPTER 5: COMPARISON OF BETA-GAL AND GREEN FLUORESCENT PROTEIN EXPRESSING REPORTER PHAGES IN GENERATING DSTs OF LABORATORY CULTURES OF *MYCOBACTERIUM TUBERCULOSIS*

## 5.0 INTRODUCTION

The Jacobs and Hatfull laboratories have developed a number of different reporter mycobacteriophages. The first one to be reported was the luciferase reporter phage which was successfully used for rapid identification of *M. tb* as well as for DST from culture (Jacobs, Barletta et al. 1993; Banaiee, Bobadilla-Del-Valle et al. 2001; Banaiee, Bobadilla-del-Valle et al. 2003; Bardarov, Dou et al. 2003). Recently, they reported on the development of a green fluorescent protein expressing mycobacteriophages; *gfp* and *ZsYellow* (see section 1.8.1.2) (Piuri, Jacobs et al. 2009), however, the existing reporter phages were found lacking in their ability to identify mycobacteria directly from clinical specimens.

The primary factors limiting the use of previous fluorophages have been the poorly fluorescent reporter signals, which required prolonged exposure time to distinguish fluorescent mycobacteria from background auto-fluorescence as well as “the inability of fluorophages to

produce detectable signal in clinical sputum samples” (Jain, Hartman et al. 2012). The above-mentioned challenges were overcome by engineering a fluorophage with a more powerful promoter, an improved vector backbone, and a more intense fluorescence reporter gene. Thus the construction of the  $\Phi^2$ GFP10 fluorophage that allowed direct comparison of cultured *M. tb* and yielded 100-fold more per-cell signal than the previous fluorophages.

We compared the performance of the  $\beta$ -gal and the ‘newly engineered’ green fluorescent protein expressing mycobacteriophage ( $\Phi^2$ GFP10) in determining drug susceptibility profiles of stored clinical isolates.

## 5.1 AIMS AND OBJECTIVES

Aim 1: To determine the accuracy of the  $\Phi^2$  GFP10 phage in detecting drug susceptibility patterns of a set of clinical isolates with predetermined drug susceptibility profiles.

Aim 2: To compare the sensitivity, specificity and accuracy of both the  $\beta$ -gal and  $\Phi^2$  GFP10 phages in detecting drug susceptibility.

## 5.2 MATERIALS AND METHODS

### 5.2.1 INOCULATING THE ISOLATES

Sixty-nine stored clinical isolates with varying resistance patterns (3.3.2) were inoculated in 10 ml OADC and glycerol enriched Middlebrook 7H9 liquid media. The cultures were incubated at 37°C till mid-log phase of bacterial growth ( $OD_{600nm}$  0.7-1.0) was reached.

### 5.2.2 DRUG EXPOSURE

One ml of broth culture of each isolate (5.2.1) was added to the wells of a 24-well culture plate. Anti-tuberculosis drugs were added to a final concentration as outlined in table 5.1. The plates were incubated for 24 hours at 37°C following which, 500  $\mu$ l of each culture was removed and aliquoted into 2 ml microfuge tubes to be used for the  $\Phi^2$  GFP10 phage assay. The rest of the drug-exposed culture was used for the  $\beta$ -gal assay. Drug free controls of each isolate were also included in the assay.

### 5.2.3 REPORTER MYCOBACTERIOPHAGE ASSAYS

#### 5.2.3.1 *$\beta$ -gal reporter phage assay*

The  $\beta$ -gal reporter phage (200  $\mu$ l;  $10^6$  pfu/ml) (MOI: 10-20) was added to each isolate containing well, followed by incubation at 37°C for 2 hours to allow for adsorption and infection. One ml of 3.5% Agarose in Middlebrook 7H9 (Top Agar) infused with 20mM X-gal was added to each well

and allowed to solidify. The plate was sealed in an O<sub>2</sub> gas-permeable bag, incubated at 37°C for 5 days and assessed for colour change every 24 hours.

#### 5.2.3.2 *Green fluorescent protein reporter phage assay*

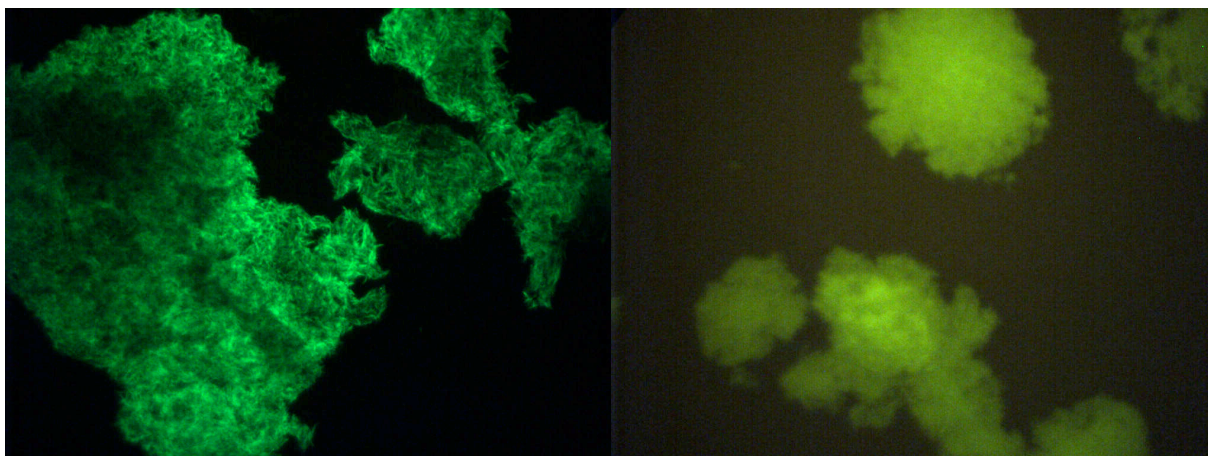
Three hundred µl of the Φ<sup>2</sup>GFP10 reporter phage (10<sup>9</sup> pfu/ml) or an MOI of 100 was added to each isolate-containing microfuge tube and allowed to stand at 37°C overnight to allow for adsorption, infection and expression of the green fluorescent protein. Following this, the microfuge tube was centrifuged at 3000 g for 5 minutes, the supernatant was removed and 10 µl of the pellet was spotted onto a microscope slide and read using a fluorescent microscope (Leica™)

**Table 5.1: Concentrations of antimicrobial drugs initially used in the Φ<sup>2</sup> GFP10 assay**

	Test concentration (mg/L)
isoniazid	1
rifampicin	1
ethambutol	1
streptomycin	2
ethionamide	1
kanamycin	2
ofloxacin	10
capreomycin	5

### 5.3 RESULTS

Fig 5.1 shows the results of the  $\Phi^2$ GFP10 reporter phage infected tuberculosis bacilli. The assay is based on the premise that viable drug resistant bacilli will express the green fluorescent protein and thus yield positive fluorescence, as seen in figure 5.1 left. Drug susceptible isolates, subsequent to drug exposure, resulted in non-viable (bactericidal) or metabolically inactive (bacteriostasis) bacilli that were unable to produce the fluorescent protein and were therefore not fluorescent as seen below in figure 5.1 right.



**Figure 5.1: Fluorescent microscopy pictures of *M. tb* infected with the  $\Phi^2$ GFP10 reporter phage. Left: drug resistant bacilli; right: drug susceptible bacilli**

Four isolates were excluded from the analysis, as their positive controls did not yield any fluorescence, despite repeat inoculation rounds. Thus only 65 isolates were included in the analysis.

Table 5.2 shows the initial results of the  $\Phi^2$ GFP10 phage assay, where the assay showed medium to high accuracy; 77.4% (65.1 -90.5) for the first line anti TB drugs. The accuracy of the test for

the second-line drugs however showed markedly lower values (52.4% OFLO and 46% CAP), prompting a repeat of the experiments using lower drug concentrations as described in chapter 3. The McNemar test revealed that the GFP assay was in agreement with the DST assay for rifampicin streptomycin and kanamycin, although the sensitivity and accuracy of the latter 2 drugs were found to be low and thus repeated with lowered drug concentrations.

Table 5.2: Overall accuracy of  $\Phi^2$ GFP10 phage assay in determining drug susceptibility

Drug	$\beta$ -gal assay	$\Phi^2$ GFP10 phage assay		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	McNemar Test (p)
		Resistant (n)	Susceptible (n)						
RIF	Resistant (n)	53	5	98.1	44.4	91.4	80.0	90.5	0.22
	Susceptible (n)	1	4						
INH	Resistant (n)	53	8	98.1	11.1	86.9	50	85.7	0.04
	Susceptible (n)	1	1						
EMB	Resistant (n)	41	10	93.2	0	68.3	0	65.1	<0.001
	Susceptible (n)	3	0						
ETH	Resistant (n)	43	19	97.7	0	69.3	0	68.3	<0.001
	Susceptible (n)	1	0						
STR	Resistant (n)	27	4	47.1	62.2	85.7	25.0	57.1	0.24
	Susceptible (n)	27	9						
KAN	Resistant (n)	21	4	60.0	86.2	84.0	64.1	73.0	0.50
	Susceptible (n)	14	25						
CAP	Resistant (n)	3	3	8.8	89.7	50	45.6	46.0	<0.001
	Susceptible (n)	31	26						
OFLOX	Resistant (n)	4	0	11.4	100	100	48.3	52.4	<0.001
	Susceptible (n)	31	29						

a= Positive predictive value; b= negative predictive value; c= the number of correct results over the total number of results

Table 5.3 shows significant increases in the assay's accuracy at decreased concentrations for all the drugs that were optimised. The increase in sensitivity of the assay was more pronounced among the capreomycin and ofloxacin exposure experiments, with increases in accuracy of almost 50% for both drugs.

Table 5.3: Sensitivity, specificity and overall accuracy of  $\Phi^2$ GFP10 phage assay in determining drug susceptibility after titration of the STR and KAN drug concentrations

	Concentration	$\Phi^2$ GFP10	Agar proportion		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	McNemar Test (p)	Cochran – Armitage test (p)
			Resistant	Susceptible							
STR	2mg/L	Resistant	24	4	47.1	69.2	85.7	25.0	51,6	1.00	
		Susceptible	27	9							
	1mg/L	Resistant	34	4	66.3	69.2	89.5	34.6	67,2	0.55	
		Susceptible	17	9							
	0.5 mg/L	Resistant	44	4	86.3	69.2	91.7	56.3	82,8	0.55	
		Susceptible	7	9							
KAN	2mg/L	Resistant	21	4	60.0	86.2	84.0	64.1	71,9	0.03	
		Susceptible	14	25							
	1mg/L	Resistant	27	3	77.1	89.7	90.0	76.5	82,8	0.23	
		Susceptible	8	26							
	0.5 mg/L	Resistant	31	2	88.6	93.1	93.9	87.0	90,6	0.68	
		Susceptible	4	27							

Table 5.4: Sensitivity, specificity and overall accuracy of  $\Phi^2$ GFP10 phage assay in determining drug susceptibility after titration of the CAP and OFLOX drug concentrations

	Concentration	$\Phi^2$ GFP10	Agar proportion		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	McNemar Test (p)	Cochran – Armitage test (p)
			Resistant	Susceptible							
CAP	5mg/L	Resistant	3	3	8.8	89.7	50	45.6	45,3	<0.001	
		Susceptible	31	26							
	2.5 mg/L	Resistant	20	3	58.8	89.7	87.0	65.0	73.0	0.01	<0.001
		Susceptible	14	26							
	1.25mg/L	Resistant	33	3	97.1	89.7	91.7	96.3	93.7	0.02	
		Susceptible	1	26							
OFLOX	10 mg/L	Resistant	4	0	11.4	100	100	48.3	51,6	<0.001	
		Susceptible	31	29							
	5mg/L	Resistant	19	0	51.3	100	100	64.4	75.0	<0.001	<0.001
		Susceptible	16	29							
	2.5mg/L	Resistant	28	0	80.0	100	100	80.6	89.1	0.23	
		Susceptible	7	29							

The  $\Phi^2$ GFP10 phage assay was compared to the  $\beta$ -gal assay. The results are shown in table 5.5.

The accuracy of both assays was comparable except for Ethambutol where the accuracy of the  $\beta$ -gal assay was significantly higher than the accuracy of the  $\Phi^2$ GFP10 phage assay. Table 5.6 shows the level of agreement between the two assays.

Table 5.5: A Comparison of the reporter bacteriophage tests on DST pre-determined clinical isolates

Drug	DST		$\beta$ -gal assay					$\Phi^2$ GFP10				P	
	Resistant (n)	Susceptible (n)	Resistant (n)	Susceptible (n)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Resistant (n)	Susceptible (n)	Sensitivity (%)	Specificity (%)		
RIF	55	9	Resistant (n)	45	0	81.8	100	84.4	Resistant (n)	53	5	98.1	44.4
			Susceptible (n)	10	9				Susceptible (n)	1	4		
INH	55	9	Resistant (n)	53	2	96.3	77.8	93.8	Resistant (n)	53	8	98.1	11.1
			Susceptible (n)	2	7				Susceptible (n)	1	1		
EMB	45	19	Resistant (n)	43	10	95.6	47.4	81.3	Resistant (n)	41	10	93.2	0
			Susceptible (n)	2	9				Susceptible (n)	3	0		
ETH	45	19	Resistant (n)	39	8	86.7	57.9	78.1	Resistant (n)	43	19	97.7	0
			Susceptible (n)	6	11				Susceptible (n)	1	0		
STR	51	13	Resistant (n)	45	5	88.2	61.5	82,8	Resistant (n)	44	4	86.3	69.2
			Susceptible (n)	6	8				Susceptible (n)	7	9		
KAN	35	29	Resistant (n)	32	5	91.4	82.8	87,5	Resistant (n)	31	2	88.6	93.1
			Susceptible (n)	3	24				Susceptible (n)	4	27		
CAP	35	29	Resistant (n)	35	7	100	75.9	89,1	Resistant (n)	33	3	97.1	89.7
			Susceptible (n)	0	22				Susceptible (n)	1	26		
OFLOX	35	39	Resistant (n)	27	3	77.1	89.7	82,8	Resistant (n)	28	0	80.0	100
			Susceptible (n)	8	26				Susceptible (n)	7	29		

Table 5.6: Comparison of the  $\Phi^2$  GFP10 assay with the  $\beta$ -gal assay

Drugs	Total Isolates	Concordant	Discordant	% Agreement
RIF	65	61	4	93,8
INH	65	57	6	87,7
EMB	65	51	14	78,5
ETH	65	54	11	83,1
STR	66	60	6	90,9
KAN	66	61	5	92,4
CAP	65	61	4	93,8
OFLO	66	64	2	98,5
PZA	65	65	0	100
CYCLO	65	65	0	100
LINE	65	64	1	98,5

## 5.4 Discussion

The  $\Phi^2$ GFP10 bacteriophage was able to generate DSTs of stored clinical isolates with reasonable accuracy. Similar to the  $\beta$ -gal phage assay, the drug concentrations used with the assay played a pivotal role in the accuracy of the assay. The performance of both the  $\Phi^2$ GFP10 and the  $\beta$ -gal assay were comparable, with the  $\Phi^2$ GFP10 time to results being shorter than the  $\beta$ -gal phage assay by a few days (3 days).

The  $\Phi^2$ GFP10 assay revealed an accuracy ranging from 94% in detecting CAP resistant isolates to 68% in detecting ETH resistant ones. The differences could be attributed to the mode of action of ETH as opposed to CAP. The statistical test McNemar test revealed a positive trend in detecting RIF, STR and KAN isolates. Tables 5.3 and 5.4 showed significant

increases in the assay's accuracy at decreased concentrations for all the drugs that were optimised. Table 5.5 showed notable differences in accuracy when considering EMB and ETH between the  $\Phi^{2GFP10}$  and the  $\beta$ -gal assays, which could be attributed to the mode of the assays themselves, with the latter assay providing more drug exposure over the course of days as opposed to the overnight incubation of the  $\Phi^{2GFP10}$  assay.

The current study aimed to compare the performance of the  $\beta$ -gal phage with the  $\Phi^{2GFP10}$  phage. The comparison was made using stored clinical samples of predetermined DST profiles. The study by Jain et al., 2012 showed that the  $\Phi^{2GFP10}$  fluorophage was able to detect 100% of *M. tb* in spiked sputum samples, as well as in 7 clinical samples from the Durban chest clinic (Jain, Hartman et al. 2012). The Jain study's sample numbers was significantly less than the current study, where only 7 samples were tested compared to our 65 isolates. The increase in sample size may well provide a more accurate measure of the  $\Phi^{2GFP10}$  assay's performance than Jain et al's study. Additionally, the study placed focus on H37Rv-spiked sputum specimen, whereas our study was on pure culture.

The performance of the  $\Phi^{2GFP10}$  assay, as confirmed by the Jain et al, 2012 study gives further evidence in the promise of the test to deliver an accurate DST profile of TB isolates. The dichotomous nature of the assay also provides a platform where the assay could be performed with minimal training of staff. Compared to the first generation luciferase assay, the  $\Phi^{2GFP10}$  assay is less complicated and more cost effective in that it requires a fluorescent microscope as opposed to a luminometer and the luciferin substrate.

This study was performed using stored clinical isolates, which were then inoculated and grown under laboratory conditions. Further study of the assay's performance under clinical conditions is needed in order to confirm this assay for use in detecting TB and generating DST's. The Jain study attempted to duplicate clinical settings by 'spiking' sputum specimen with *M. tb* culture and our test went a step further in generating DSTs of known clinical specimen. Further study, as with Chapter 4, where clinical specimens are used to generate DST using the  $\Phi^2$ GFP10 phage would be beneficial.

The accuracy of the  $\Phi^2$ GFP10 assay is comparable to the  $\beta$ -gal assay. Both bacteriophage assays were able to detect drug resistances among the clinical isolates with relative ease of performance, cost and time to results. The assays have the potential to be implemented in routine laboratory settings, supplementing the current insensitive, expensive, and laborious and time consuming techniques.

# Chapter 6: Summary

## 6.0 INTRODUCTION

The high TB incidence and prevalence rates dictate that more stringent TB control measures be undertaken. Currently available methodologies for the diagnosis of TB are either too slow in reporting results, or too expensive to implement in limited-resource settings. This negatively impacts on TB control. The development and implementation of a mycobacteriophage-based assay, the  $\beta$ -galactosidase reporter phage assay, aims to circumvent the current challenges facing TB detection. We successfully developed a novel reporter bacteriophage as well as a cheaper, more convenient assay format, using a tissue-culture plate and a semi-solid agar based matrix (top agar) infused with the metachromatic substrate X-gal.

The newly developed  $\beta$ -galactosidase reporter phage contains a *lacZ* gene, which expresses the enzyme beta-galactosidase upon infection of a mycobacterial cell. Furthermore, expression of the enzyme is dependent on the viability of the bacilli, which creates the possibility of using the assay to generate drug susceptibility profiles by incorporating anti-TB drugs into the assay. The reporter phage is then used to establish whether the metabolism of the mycobacteria has been interrupted, indicating susceptibility.

## 6.1 THE DEVELOPMENT OF THE BETA-GALACTOSIDASE REPORTER MYCOBACTERIOPHAGE AND ITS IMPLEMENTATION IN DETECTING CULTURED *MYCOBACTERIUM TUBERCULOSIS*

The mycobacteriophage TM4, together with the phasmid phAE159 formed the backbone of the  $\beta$ -galactosidase bacteriophage, which has a temperature sensitive mutation. The gene *LacZ*, responsible for expressing the beta- galactosidase enzyme, was ligated to the phasmid phAE159 that formed the  $\beta$ -gal phage. The genetically enhanced mycobacteriophage was able to sensitively and specifically identify *Mycobacterium tuberculosis* in culture.

The techniques used in implementing the assay, was to infect the bacilli with a  $10^6$  PFU/ml reporter phage lysate at an MOI of 100, and incubate for 90 minutes at 37°C to allow for adsorption of the phage into the bacilli and the ultimate expression of the beta galactosidase enzyme. The expressed enzyme was able to successfully breakdown the X-gal substrate into a soluble 5-bromo-4-chloro-3-indoyl. This was clearly observable as blue spots with the 'naked' eye.

The format of the  $\beta$ -gal assay allows for readily available laboratory material to be used to perform the test, lowering the cost of TB detection. Furthermore, the  $\beta$ -gal reporter phage can be propagated in-house, further lowering the cost of the assay.

The specificity of the assay was clearly demonstrated in sections 2.2.4 and 2.3.3 where oropharyngeal flora did not elicit a reaction from the assay. This was seen as a major improvement from the BACTEC MGIT 960™, where there is a potential for false positives

should a specimen be insufficiently decontaminated. Additionally, the time to positive results was found to be significantly lower (72-96 hours) than conventional as well as automated culture.

The  $\beta$ -gal assay was found to be highly sensitive (section 2.3.1), in that a bacillary count of  $10^0$  was detectable, using this reporter phage. This was comparable to other bacteriophage-based assays, such as the *FASTplaqueTB™* and the McNerney D29 (PhaB) assays. This provides confidence in the assay's ability to detect the often-low bacillary counts amongst immune-compromised HIV infected patients.

The ease of performing the assay nullifies the need for further intensive technical training and this, together with its low costs and speedy 'time to detection' makes this assay a valuable tool to control TB transmission.

## 6.2 THE IMPLEMENTATION OF THE BETA-GALACTOSIDASE ASSAY IN GENERATING DRUG SUSCEPTIBILITY PROFILES OF LABORATORY CULTURES

Sixty-nine (69) stored clinical isolates from the laboratory of the Department of Medical Microbiology and Infection Prevention were used to generate drug susceptibility profiles, using the  $\beta$ -gal assay. The DST's of the isolates had previously been determined using the 1% agar proportion method and the investigator was blinded to the results till the analysis phase was reached.

The cultures were initially grown to mid-log phase, and then exposed to anti-tuberculosis drugs at the breakpoint concentrations as recommended by WHO. Following drug exposure,

the bacilli were exposed to the  $\beta$ -gal phage and allowed to express the reporter enzyme in X-gal infused Top-agar.

The  $\beta$ -gal assay was able to successfully generate drug susceptibility profiles of the stored clinical isolates. Initially, with drug concentrations as advised for testing in solid media, the accuracy of the  $\beta$ -galactosidase reporter phage assay was found to be lower than expected with values for ofloxacin 45%, capreomycin 62.5%, kanamycin 73.4% and streptomycin 79.7%(section 3.3.2). When the drug concentrations were decreased, approaching the advised concentrations for liquid media, the accuracy increased: ofloxacin 82.8%, capreomycin 89.2%, kanamycin 85.9% and streptomycin 82.5%. This highlights the need to pay particular attention to the type of media used in an assay when drug concentrations are determined. Figure 3.3 clearly showed that the  $\beta$ -gal phage was able to discern between susceptible, MDR and XDR-TB, in a tissue-culture plate format. Furthermore, unlike other plate format techniques and indeed other bacteriophage methodologies, our study included anti-tuberculosis drugs used to treat MDR and XDR infected patients, which yielded promising results in terms of accuracy. The time to results of this assay in generating accurate DST was less than one week. However, there is a need for further optimisation.

### **6.3 DETECTION OF MYCOBACTERIUM TUBERCULOSIS IN SPUTUM SPECIMENS USING A BETA-GALACTOSIDASE REPORTER MYCOBACTERIOPHAGE**

Four hundred and eighty three (483) consecutive sputum specimens were collected from the R.K.Khan's hospital chest clinic from patients presenting with symptoms and signs

compatible with TB. The  $\beta$ -gal assay results were compared with the results of conventional diagnostic tests (smear microscopy and culture).

The  $\beta$ -gal assay was able to accurately diagnose TB in 87.4% of the patients, with a sensitivity of 82.7% and a specificity of 88.8% (section 4.3.5). The phage assay also showed a high concordance with the BACTEC MGIT 960™ results, correctly reporting 425 out of 486 results. This shows promise in utilising the phage assay as a supplementary diagnostic test in conjunction with the conventional assay. It is worth noting that the  $\beta$ -gal assay's time to results was significantly shorter than average time to detection with the BACTEC MGIT 960™, 96 hours vs. 14 days. When comparing conventional culture (solid and liquid) with the  $\beta$ -galactosidase reporter assay (Table 4.7), the latter assay compared favourably with conventional tests.

Some specimens that contained MOTTs reacted when subjected to the  $\beta$ -galactosidase assay; however, the number was insignificant (0.8%) to affect the accuracy of the  $\beta$ -gal assay. As mentioned in sections, 1.8.1, 2.4, 3.5, and 4.4, selective inhibition of species of the *M. tuberculosis* complex can be discerned using NAP, allowing differentiation between MOTTs and *M. tb*.

Further studies are needed, including the application of the  $\beta$ -gal assay, on specimens from patients with extra-pulmonary TB.

## 6.4 COMPARISON OF BETA-GALACTOSIDASE AND GREEN FLUORESCENT PROTEIN EXPRESSING REPORTER PHAGES IN GENERATING DSTs OF LABORATORY CULTURES OF MYCOBACTERIUM TUBERCULOSIS

Sixty-nine (69) stored clinical TB isolates were used to determine the ability of the  $\Phi^2$  GFP10 phage to generate DSTs and to compare the results with the performance of the  $\beta$ -gal phage on the same isolates. The isolates were grown to mid-log phase and exposed to the drugs as mentioned in sections 3.2.3 and 5.2.2. Aliquots of the drug-exposed cultures were infected with  $\Phi^2$  GFP10 and the  $\beta$ -galactosidase reporter phages respectively. Incubation with the  $\Phi^2$  GFP10 phage was overnight and the culture was then spotted onto a microscope slide for viewing. The results of the  $\Phi^2$  GFP10 assay were dichotomous in that, fluorescing samples were deemed resistant to a drug, and the non-fluorescent samples susceptible. The turn-around time was 10-16 hours in contrast to the  $\beta$ -galactosidase assay, which took 96 hours to yield a definitive result.

The accuracy of the  $\Phi^2$  GFP10 was comparable to the  $\beta$ -galactosidase assay, for 1<sup>st</sup> and 2<sup>nd</sup>-line anti-TB drugs. It was noted that WHO recommendations for drug concentrations were too high for the liquid/semi-liquid-based methodologies, thus the drugs were titrated to lower concentrations, which yielded higher accuracy for both the assays. The sensitivity of the  $\Phi^2$  GFP10 assay was found to be in the upper 80% to mid 90%, comparable to the  $\beta$ -galactosidase assay (tables 5.3- 5.5). Furthermore, there was a high concordance between the tests (table 5.6).

## Conclusion

We have developed a  $\beta$ -galactosidase reporter phage assay for the detection of *Mycobacterium tuberculosis* directly in sputum specimens within 96 hours. Due to its simple format, the test is cheap and does not require specialised training or equipment. The test shows promising results for use as drug susceptibility test. The phenotypic nature of the assay allows, in principle no restriction with respect to the type of drug tested. As mentioned previously, drug susceptibility testing still requires further development and optimisation.

# APPENDIX A

## Media, Antibiotics, and Supplements

### **LB BROTH, MILLER (LURIA-BERTANI MEDIUM)**

1000 ml dH<sub>2</sub>O

25 g LB powder (Difco catalog no. 244610)

Dissolve LB powder in the dH<sub>2</sub>O Sterilize by autoclaving for 20 minutes

### **MIDDLEBROOK 7H9 LIQUID MEDIA**

1000 ml

- 4.7 g Middlebrook 7H9 powder (Difco, catalog no. 0713-01-7)
- 100 ml Albumin-Dextrose-Saline (ADS) or Oleic Acid-Dextrose-Catalase (OADC)  
(recipe below or BD Microbiology Systems, catalog no. 212351)
- 10 ml 50% glycerol
- 2.5 ml 20% Tween-80
- Dissolve Middlebrook 7H9 powder in 900 ml dH<sub>2</sub>O, then add ADS (or OADC), glycerol, and Tween-80
- Sterilize by filtration through 0.22-micron pore membrane.

### **LIQUID (0.05%) TWEEN 80 MEDIA FOR STOCK MYCOBACTERIA**

1000 ml

- 4.7 g Middlebrook 7H9 powder
- 12.5 ml 40% Glycerol\*
- 2.5 ml 20% Tween-80\*
- 900 ml d.H<sub>2</sub>O (Distilled water)
- Autoclave and stand at RT (Room Temp) until 55°C

- 100 ml ADC\*
- 1 ml CB (Carbenicillin) 50 µg/ml\*
- 1 ml CHX (Cyclohexamide) 10 µg/ml\*
- Aliquot 25 ml into 100 ml Schott Bottles
- Label and store at 4°C

\*: reagents already made

### **SOLID MEDIA FOR GROWTH AND PROPAGATION OF MYCOBACTERIUM**

1000 ml

- 19 g Middlebrook 7H10 powder
- 12.5 ml 40% glycerol
- 890 ml d.H<sub>2</sub>O (Distilled water)
- Autoclave and stand at RT (Room Temp) until 55°C
- 10 ml CaCl<sub>2</sub> 0.1 M
- 100 ml ADC
- 1 ml CB (Carbenicillin) 50 µg/ml
- 1 ml CHX (Cyclohexamide) 10 µg/ml
- Pour 25 ml of warm Media into 90 mm petri dishes, or 100 ml into 150 mm plates.
- Allow media to solidify and label plates
- Store at 4°C

### **SOLID MEDIA WITH X-GAL FOR GROWTH AND PROPAGATION OF B-PHAGE**

1000 ml

- 19 g Middlebrook 7H10 powder
- 12.5 ml 40% glycerol
- 890 ml d.H<sub>2</sub>O (Distilled water)
- Autoclave and stand at RT (Room Temp) until 55°C

- 10 ml CaCl<sub>2</sub> 0.1 M
- 100 ml ADC
- 500 µl X-gal (50 mg/ml)
- 1 ml CB (Carbenicillin) 50 µg/ml
- 1 ml CHX (Cyclohexamide) 10 µg/ml
- Pour 25 ml of warm Media into 90 mm petri dishes.
- Allow media to solidify and label plates
- Store at 4°C

### **LIQUID MEDIA FOR MYCOBACTERIAL GROWTH AND PHAGE INFECTION**

1000 ml

- 4.7 g Middlebrook 7H9 powder
- 12.5 ml 40% Glycerol
- 900 ml d.H<sub>2</sub>O (Distilled water)
- Autoclave and leave standing at RT
- 100 ml ADC
- 1 ml CB (Carbenicillin) 50 µg/ml
- 1 ml CHX (Cyclohexamide) 10 µg/ml
- Aliquot (**50 ml**) into 100 ml Schott Bottles
- Label and store at 4°C

### **SOLID COMPONENT OF TOP AGAR FOR PHAGE INFECTIONS**

1000 ml

- 4.7 g Middlebrook 7H9 powder
- 7 g Agar/ Agarose
- 900 ml d.H<sub>2</sub>O (Distilled water)
- Aliquot (**50 ml**) into 100 ml Schott Bottles

- Label, autoclave and store at 4°C

### **ALBUMIN-DEXTROSE-SALINE (ADS)**

950 ml dH<sub>2</sub>O

- 8.1 g NaCl
- 50 g Bovine Serum Albumin Fraction V (BMB catalog no.100-021)
- 20 g D-dextrose
- Dissolve NaCl and BSA in dH<sub>2</sub>O; add D-dextrose
- Split into four 250 ml aliquots in centrifuge bottles and spin (8500rpm) at 4°C for 30 minutes to precipitate insoluble material
- Sterilize clarified solution by filtration through a 0.22 micron pore membrane
- Incubate bottles at 37°C overnight to detect possible contamination
- Store at 4°C

### **OLEIC ACID-DEXTROSE-CATALASE (OADC)**

1000 ml

- 950 ml dH<sub>2</sub>O
- 8.5 g NaCl
- 50 g Bovine Serum Albumin Fraction V (BMB, cat.100-021)
- 20 g D-dextrose
- 40 mg catalase
- 0.5 g Oleic acid
- Dissolve NaCl and BSA in dH<sub>2</sub>O; add D-dextrose, catalase, and oleic acid.
- Split into four 250 ml aliquots in centrifuge bottles and spin at 4°C for 30min to precipitate insoluble material
- Sterilize clarified solution by filtration through a 0.22 micron pore membrane
- Incubate bottles at 37°C overnight to detect possible contamination
- Store at 4°C.

### **20% TWEEN-80**

100 ml

- 20 ml Tween-80 (Fisher, cat. T164-500)
- 80 ml dH<sub>2</sub>O
- Heat to 56°C to facilitate solubilization of Tween
- Sterilize by filtration through a 0.22 micron pore membrane
- Store at 4°C wrapped in foil.

### **50% GLYCEROL** (Fisher Brand catalog no.G33-1)

100 ml

- 50 ml Glycerol
- 50 ml dH<sub>2</sub>O
- Heat to 56°C to facilitate solubilisation
- Sterilize by filtration through a 0.22 micron pore membrane
- Store at room temperature

### **CARBENICILLIN**

- Filter sterilize a 100mg/ml stock solution of carbenicillin disodium salt (Sigma catalog no. C-1389) made in dH<sub>2</sub>O
- Use a final concentration of 50-100mg/l for *Escherichia coli*.

### **KANAMYCIN**

- Filter sterilize a 50mg/ml stock solution of kanamycin monosulfate (Lab Scientific catalog no. 5409) made in distilled water
- Use a final concentration of 10-20mg/l for mycobacteria and 35-50mg/l for *Escherichia coli*.

## **HYGROMYCIN B**

50mg/ml in PBS sterile liquid stock (Roche Catalog no. 843 555)

Use a final concentration 50mg/liter for mycobacteria and 100mg/liter for *Escherichia coli*

## **MP BUFFER**

1000 ml

- 50 mM Tris pH7.6
- 150 mM NaCl
- 10 mM MgSO<sub>4</sub>
- 2 mM CaCl<sub>2</sub>
- 30ml 5M NaCl; 50 ml 1M Tris-HCl, pH7.4; 10 ml 1M MgSO<sub>4</sub>
- Make up to 1 liter with dH<sub>2</sub>O
- Filter sterilize through 0.2 µm filter.

## **X-GAL SOLUTION (5-bromo-4-chloro-indolyl-β-D-galactopyranoside)**

10 ml of 50mM

- **204.32 mg X-gal power (Sigma B4252)**
- 10 ml Dimethyl Sulphoxide (Sigma Catalogue no. 472301)
- Aliquot 500 µl into microcentrifuge tubes and store at -20°C until use

## **DRUG REAGENTS FOR DRUG SUSCEPTIBILITY TESTING**

- Make up 10 ml Stock Solution at 128 mg/L with filter sterilized d.H<sub>2</sub>O or DMSO
- Aliquot 1 ml into microcentrifuge tubes
- Store at -20°C until use, thaw one tube at a time

- Add 1 ml to each tube to make up 64 mg/L Working solution
- Store at -20°C until use

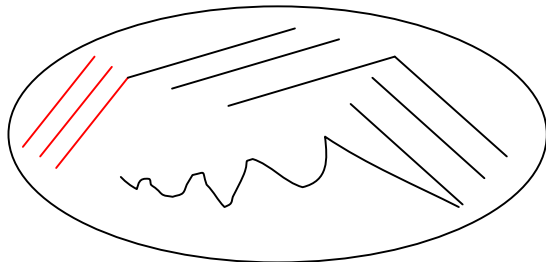
<b>DRUG</b>	<b>STOCK SOLUTION (MG/L)</b>	<b>SOLVENT 10 ML</b>	<b>POWDER MASS (G)</b>
<b>RIFAMPICIN (RIF)</b> (Sigma Catalogue no. R3501)	128	d.H <sub>2</sub> O	
<b>ISONIAZID (INH)</b> (Sigma Catalogue no. I3377)	128	d.H <sub>2</sub> O	0.176
<b>ETHAMBUTOL (EMB)</b> (Sigma Catalogue no. E4630)	128	d.H <sub>2</sub> O	0.355
<b>ETHIONAMIDE (ETH)</b> (Sigma Catalogue no. E6005)	512	d.H <sub>2</sub> O: DMSO (1:1)	0.851
<b>STREPTOMYCIN (STR)</b> (Sigma Catalogue no. S6501)	128	d.H <sub>2</sub> O	0.933
<b>CAPREOMYCIN (CAP)</b> (Sigma Catalogue no. C4142)	128	d.H <sub>2</sub> O	0.965
<b>OFLOXACIN (OFLO)</b> (Sigma Catalogue no. O8757)	128	d.H <sub>2</sub> O	0.463
<b>PYRAZINAMIDE (PZA)</b> (Sigma Catalogue no. P7136)	128	d.H <sub>2</sub> O	0.158
<b>CYCLOSERINE (CYCLO)</b> (Sigma Catalogue no. C6880)	128	d.H <sub>2</sub> O	0.131
<b>LINEZOLID (LINE)</b> (Sigma Catalogue no. PZ0014)	512	DMSO	1.727

# APPENDIX B

## Propagation of Mycobacterium

### CULTURING *MYCOBACTERIUM SMEGMATIS* ON SOLID MEDIA

1. Streak loopful of stock *M. smeg* using a sterile blue loop



2. Invert plate and store at 37°C for 48-72 hrs
3. Choose well separated colony from plate and pick in the middle of a small colony
4. Transfer to liquid Tween 80 media for propagation.

### CULTURING *MYCOBACTERIUM SMEGMATIS* IN LIQUID MEDIA

1. Aliquot 25 ml liquid media **with** Tween-80 into 100 ml Schott bottle
2. Immerse picked colony into liquid media
3. Incubate whilst shaking at 37°C for 24-48 hrs
4. Ensure mycobacteria are not 'clumpy'
5. Grow till OD (optical density) of 1.0

### PROPAGATION OF *MYCOBACTERIUM SMEGMATIS* IN LIQUID MEDIA

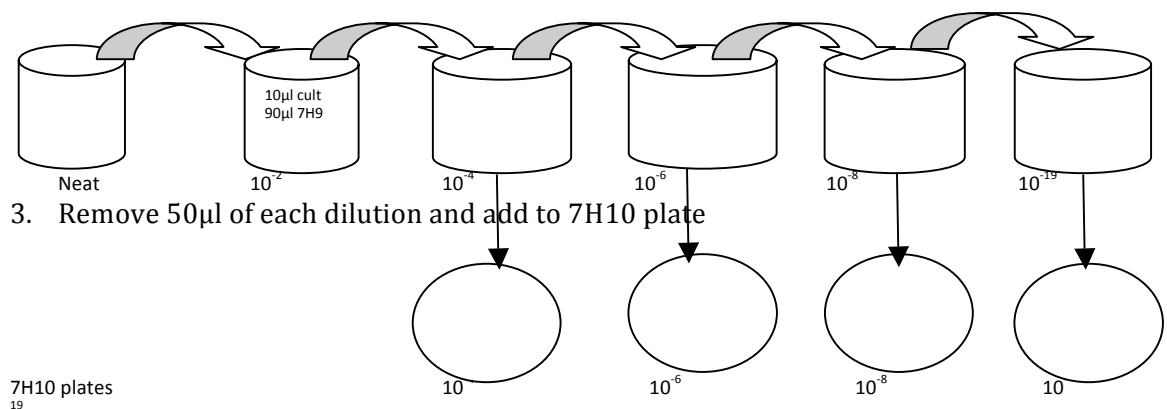
1. Add 500µl *M. smeg* from tween-80 liquid media to 25 ml liquid media **without** Tween-80
2. Incubate whilst shaking at 37°C for 24-48 hrs
3. Ensure mycobacteria are not 'clumpy'
4. Grow till OD (optical density) of  $\leq 1.0$

### DETERMINATION OF BACILLI CONCENTRATION USING A SPECTROPHOTOMETER

1. Take 3 ml of culture and add to cuvette
2. Add 3 ml of control culture (media without *M. smeg*) add to cuvette
3. Take readings of culture concentration using a spectrophotometer

### DETERMINATION OF BACILLI CONCENTRATION USING SOLID MEDIA

1. Remove 10 $\mu$ l culture from cuvette and add to 90 $\mu$ l liquid media
2. Make 100-fold serial dilution, up to 10<sup>-10</sup>



4. Disperse culture evenly across plate using sterile plastic loop, **avoid the edges of plate**
5. Wait for culture to dry on plate
6. Invert plate, label with inoculum name, your name, date and dilution of inoculum
7. Incubate plate at 37°C for 48 hours

### COLONY COUNTING

1. 24- 48 hours post inoculation, look for colony formation on solid media
2. Observe plate with between 20 and 200 colonies, count colonies individually.
3. Calculate the concentration/ml of colonies:

- Convert volume used from  $\mu\text{l}$  to ml
- Divide number of colonies by the above volume
- Multiply the answer by the dilution factor

Eg.: 20  $\mu\text{l}$  of  $10^{-8}$  was plated out and yielded 50 colonies,

$$20 \mu\text{l} = 0.02 \text{ ml}$$

$$(50 \text{ colonies} / 0.02 \text{ ml}) \times 10^8 = \text{CFU/ml}$$

### **CORRELATING SPECTROPHOTOMETER READINGS AND COLONY COUNT**

1. Remove 5 ml *M. smeg* culture
2. Take spec. reading of neat sample
3. Dilute neat culture in 10-fold dilutions with liquid growth media
4. Remove 30  $\mu\text{l}$  from each cuvette and plate out in duplicate
5. Incubate at 37°C for 24-48 hours
6. Do colony count and add to data collection

# APPENDIX C

## Propagation of Mycobacteriophage

### Important to remember:

- Phages require  $\text{CaCl}_2$  for optimal infection of host cell
- Tween-80 inhibits phage infection of host, Never infect phage into Tween-80 grown host
- Be aware that phage propagation is always optimal at  $30^\circ\text{C}$  **NOT**  $37^\circ\text{C}$

### SPOT TEST OF MYCOBACTERIOPHAGE

1. Grow a 50 ml culture of *M. Smeg* to late-log or stationary phase in 7H9 liquid media
2. Add 1 ml  $\text{CaCl}_2$  to 50 ml 7H9
3. Warm the solid TOP agar in microwave oven
4. Add liquid media (7H9) to TOP agar to make MiddleBrook Top Agar
5. Label date of addition of 7H9 (can only last for 3 days!)
6. Aliquot 300  $\mu\text{l}$  – 500  $\mu\text{l}$  of *M. Smeg* into disposable tube
  - a. Prepare 10-fold serial dilutions of original stock into phage buffer ( $10^{-1}$  -  $10^{-10}$ )
  - b. Prepare 10 (1 ml eppies)
  - c. Aliquot 90  $\mu\text{l}$  phage buffer into above tubes
  - d. Add 10  $\mu\text{l}$  stock phage into first tube, remove 10  $\mu\text{l}$  from same tube and add to the next e.t.c.
7. Draw grids on Petri dish, labelling each grid with the corresponding concentration
8. Carefully spot 5  $\mu\text{l}$  from the eppies onto corresponding grid, include original stock
9. Allow spots to dry ( $\pm 20$  min)

**10. Only invert plate when spot is completely dry**

11. Incubate the plates at 30°C or 37°C for 24-48 hrs

**12. Don't forget to invert the petri dish to avoid condensation from lid!**

13. Observe for spot formation

This is rough estimate for: 1.) presence or absence of phage 2.) concentration (titre) of phage.

**DETERMINATION OF MYCOBACTERIOPHAGE TITRE**

1. Grow host mycobacteria (*M. smeg*) till  $OD_{600\text{ nm}}$  1.0
2. Prepare 12 plates of solid media i.e. label with [ ], date and phage name
3. Make a 10-fold serial dilution of stock phage ( $10^{-1}$  to  $10^{-10}$ )
4. Aliquot 300- 500  $\mu\text{l}$  host cells into 10 (corresponding to dilutions) sterile 10 ml tubes + Control tube
5. Add 30-50  $\mu\text{l}$  of phage dilution to host tube
6. Allow for phage infection of host ( $\pm 20$  min)
7. Add 1 ml  $\text{CaCl}_2$  to 50 ml 7H9
8. Warm the solid TOP agar in microwave oven
9. Add liquid media (7H9) to TOP agar to make MBTA
10. Label date of addition of 7H9 (can only last for 3 days!)
11. Add 3 ml warm MBTA to phage: host tube, mix by flicking and add contents to plate
12. Swirl plate and allow to solidify ( $\pm 20$  min)
13. Control plates: 1.) host **without phage** and 2.) MBTA **without phage and host**
14. Incubate plates (inverted) at 30°C or 37°C for 24-48 hrs

**PLAQUE COUNTING**

1. 24- 48 hours post inoculation, look for plaque formation on solid media

2. Observe plate with between 20 and 200 plaques, count plaques individually.
3. Calculate the concentration/ml of phage particles:
  - a. Convert volume used from  $\mu\text{l}$  to ml
  - b. Divide number of plaques by the above volume
  - c. Multiply the answer by the dilution factor

Eg.: 20  $\mu\text{l}$  of  $10^{-8}$  was plated out and yielded 50 plaques,

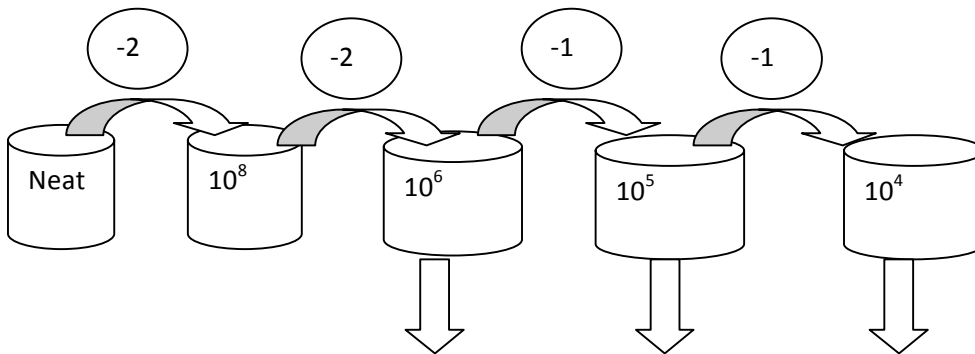
20  $\mu\text{l}$  = 0.02 ml

(50 colonies / 0.02 ml) x  $10^8$  = PFU/ml

## PHAGE PROPAGATION AND HIGH TITRE CREATION

### PREPARATION OF BIG PLATE INFECTION

1. Work out the titre which will give you a web pattern on the big plate ( $10^5$  PFU/ml)
  - a. If you have  $10^{10}$  PFU/ml:
  - b. Do serial dilution to get to  $10^5$  PFU/ml



2. Add 100  $\mu\text{l}$  of last 3 dilutions to 1 ml host cells
3. Incubate to 15 minutes
4. Add phage:host cells to 10 ml MBTA
5. Plate out
6. Incubate for 48 hrs and look out for web pattern

**After 48 hrs:**

From the absolute titre determination plate, identify plate with web-formation of plaques

1. Remove plates from fridge and warm in HOT room (plates must be warm, to prevent clumping of MBTA)
2. Take OD reading of host cells (ideally used fresh inoculate)
  - Should be around 1.0 OD<sub>600nm</sub>
3. Add 18 ml host cells to 50 ml conical tube
4. Prepare dilution microfuge tubes (label)
5. Make 10-or 100 fold serial dilution to desired dilution for web formation
6. Add 2 ml phage of appropriate dilution to host cells and incubate for 15 min
7. Prepare and label plates including 2 negative controls
  - 1 = host without phage
  - 2 = top agar alone (make this a smaller on (90 mm)
8. Prepare top agar (make 180 ml)
9. Add Phage: host (20 ml) to 180 ml Top agar
  - Swirl and add quickly to plate
  - Swirl plate to ensure adequate coverage
10. Incubate plate at required temp until near clearance is observed (24-48 hours)
11. Add 10 ml phage buffer to each plate and incubate at 4°C O/N
12. Siphon-off buffer with syringe
13. Filter through 0.2 µm filter and determine the titre of the lysate

# APPENDIX D

## DECONTAMINATION OF CLINICAL SPUTUM

The method used to decontaminate and digest sputum samples is the Kent and Kubica 1985 method using N-acetyl cysteine- Sodium Hydroxide method

- The sputum specimen was transferred from the specimen jar to a 50ml conical tube
- An equal volume of NALC working solution (4g N-acetyl-L-cysteine, 2.94% Sodium Citrate and 0.5% (NaOH) Sodium Hydroxide) to specimen was added to the clinical specimen and mixed thoroughly for 15 seconds or until the specimen was not mucoid
- The specimen was incubated at room temperature for 15 minutes.
- Phosphate buffer (pH7.6) was added to fill the 50 ml conical tube in order to neutralize the effect of NaOH
- The specimen was centrifuged at 3000 g for 20 minutes at 4°C and the supernatant discarded
- The resultant pellet was re-suspended in 3 ml Broth buffer (4 ml 7H9 and 36 ml PBS) and mixed thoroughly

# APPENDIX E

## Performance of the Beta Galactosidase assay on clinical sputum samples

		BACTEC MGIT		
		Positive	Negative	Total (n)
<b>Beta-gal</b>	<b>Positive</b>	85	47	132
	<b>Negative</b>	16	336	352
<b>Total (n)</b>		<b>101</b>	<b>383</b>	<b>484</b>
Sensitivity (%)		84.2		
Specificity (%)		87.7		
Positive predictive value (%)		64.4		
Negative predictive value (%)		76.9		
Accuracy (%)		87.0		

		BACTEC MGIT & Culture (7H11)		
		Positive	Negative	Total (n)
<b>Beta-gal</b>	<b>Positive</b>	91	42	133
	<b>Negative</b>	19	334	353
<b>Total (n)</b>		<b>110</b>	<b>376</b>	<b>486</b>
Sensitivity (%)		82.7		
Specificity (%)		88.8		
Positive predictive value (%)		68.4		
Negative predictive value (%)		75.2		
Accuracy (%)		87.4		

		<b>BACTEC MGIT; Culture and Smear microscopy</b>		
		<b>Positive</b>	<b>Negative</b>	<b>Total (n)</b>
	<b>Beta-gal</b>			
	<b>Positive</b>	99	42	141
	<b>Negative</b>	19	334	353
<b>Total (n)</b>		<b>118</b>	<b>376</b>	<b>494</b>
Sensitivity (%)		83.9		
Specificity (%)		88.8		
Positive predictive value (%)		70.2		
Negative predictive value (%)		73.2		
Accuracy (%)		87.7		