CHARACTERIZATION OF DIFFERENTIALLY CULTURABLE BACTERIA IN AXENIC CULTURE AND FROM TUBERCULOSIS PATIENTS

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DECLARATION

I, Amanda McIvor, declare that this Thesis is my own unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

A. McIvor

Signature of candidate

8th day of June 2018

PRESENTATIONS ARISING FROM THIS STUDY

Amanda McIvor, Bhavna Gordhan, Neil Martison and Bavesh Kana. The use of culture filtrate enhances diagnosis of HIV-infected, sputum smear negative individuals. Poster presentation. Molecular Biosciences Research Thrust Post-graduate Research Day, 30 November 2017.

Amanda McIvor, Julian Peters, Andrea Papadopoulos, Bhavna Gordhan, Neil Martison, Matebogo Letutu and Bavesh Kana. Decline in non-culturable tubercle bacteria in HIV-positive and HIV-negative tuberculosis patients during early first-line treatment. Poster presentation. Molecular Biosciences Research Thrust Post-graduate Research Day, 8 December 2016.

Amanda McIvor was awarded second prize for this poster in the PhD/Post-Doc Poster category.

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Amanda McIvor, Julian Peters, Andrea Papadopoulos, Bhavna Gordhan, Neil Martison, Matebogo Letutu and Bavesh Kana. Decline in non-culturable tubercle bacteria in HIV-positive and HIV-negative tuberculosis patients during early first-line treatment. Abstract submitted. 10th Annual SAMRC Early Career Scientist Convention, Cape Town, South Africa, 19 – 20 October 2016.

Amanda McIvor, Julian Peters, Andrea Papadopoulos, Bhavna Gordhan, Neil Martison, Matebogo Letutu and Bavesh Kana. Decline in non-culturable tubercle bacteria in HIV-positive and HIV-negative tuberculosis patients during early first-line treatment. Poster presentation. Wits Research day, Johannesburg, South Africa, 1st September 2016.

Amanda McIvor, Detection of culturable and non-culturable bacteria in patients receiving first-line TB treatment. Oral presentation. Molecular Biosciences Research Thrust Post-graduate Research Day, University of the Witwatersrand, 3rd December 2015.

Amanda McIvor, Detection of culturable versus non-culurable bacteria in patients receiving first-line TB treatment. Poster presentation. 9th Annual SAMRC Early Career Scientist Convention, Cape Town, South Africa, 19 - 20 October 2015.

Amanda McIvor, Detection of culturable versus non-culurable bacteria in patients receiving first-line TB treatment: An endpoint analysis. Oral presentation. SoMCHAT young researchers' conference, Johannesburg, South Africa, July 2015

Amanda McIvor, Detection of differentially culturable tubercle bacilli by exogenous cyclic-AMP in drug-susceptible patients at baseline. Poster presentation. PathRed, Johannesburg, South Africa, April 2015.

PUBLICATIONS ARISING FROM THIS STUDY

Amanda McIvor, Hendrik Koornhof and Bavesh Kana. Relapse, re-infection and mixed infections in tuberculosis disease. *Pathogens and Disease*. 75(3). 3RD April 2017, ftx0202. http:// https://doi.org/10.1093/femspd/ftx020.

Manuscripts in preparation:

Amanda McIvor, Bhavna Gordhan, Neil Martinson and Bavesh Kana. Enhanced detection of *Mycobacterium tuberculosis* in smear-negative/HIV positive patients with reduced bacterial load by supplementation of the BACTEC MGIT 960 culture system with culture filtrate

Julian Peters†, **Amanda McIvor**†, Andrea Papadopolous, Tshepiso Masangana, Bhavna Gordhan, Kennedy Otwombe, Ziyaad Waja, Neil Martinson ,Matebogo Letuli , Fiona Shahim, Saloshini Ramsamy and Bavesh D. Kana. The rise and fall of differentially culturable tubercle bacilli during treatment.

†equal first author contribution

Amanda McIvor†, Julian Peters† and Bavesh Kana. BACTEC MGIT 960 correlation to most probable number assays for mycobacterial enumeration.

†equal first author contribution

ABSTRACT

During tuberculosis (TB) disease, host-derived stresses and chemotherapy are thought to drive tubercle bacilli into differential growth states. This is evidenced by the presence of differentially culturable tubercle bacilli (DCTB) in the sputum of treatment naïve TB patients. These bacteria do not form colonies on solid media but can be cultured following supplementation of liquid media with culture filtrate as a source of growth stimulatory molecules. As DCTB are non-replicating and phenotypically drug tolerant, these organisms are thought to underpin the lengthy culture diagnosis and protracted treatment period required for TB disease. The purpose of this study was to investigate the use of culture filtrate in unmasking DCTB populations to: (1) quantify these populations in treatment naïve individuals, (2) assess the response of DCTB versus conventionally culturable bacteria to first-line treatment, (3) determine the relationship between DCTB cultured in the most probable number (MPN) assay with other TB culture methods and (4) to enhance currently employed culture diagnostic methods. The results from this study confirmed that treatment naïve individuals co-infected with HIV had significantly lower quanta of DCTB in their sputum compared to their HIV-negative counterparts. These findings implicate the host immune response in influencing the prevalence of DCTB in sputum. During treatment, four patterns of decline in DCTB were described. One quarter of the patient population accumulated DCTB during the first seven days of treatment, whilst approximately the same number of individuals displayed a rapid decline in DCTB during this period. The remaining individuals either displayed static or atypical patterns of DCTB over the first 14 days of treatment. Following treatment completion, residual DCTB was cultured in approximately two thirds of the patients analysed, suggesting that bacteriological sterilization of lungs was not achieved. These observations were confirmed using a novel fluorogenic probe specific for the detection of live *Mycobacterium tuberculosis*. DCTB cultured in the MPN assay was shown to directly correlate with current TB culture methods. These findings demonstrate a potential utility for the MPN assay in early bactericidal activity studies to assess the sterilising effect of new TB drugs on DCTB populations. Furthermore, the addition of culture filtrate to the BACTEC MGIT 960 assay reduced the rates of TB detection in smear-negative, HIV-positive individuals. Collectively, these observations demonstrate that the detection of DCTB in sputum can serve as a possible biomarker for treatment response. Further long term studies are required to determine if DCTB can use used to assess the risk of relapse disease and to test the efficacy of new drugs on persistent bacterial populations.

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LIST OF SYMBOLS AND NOMENCLATURE

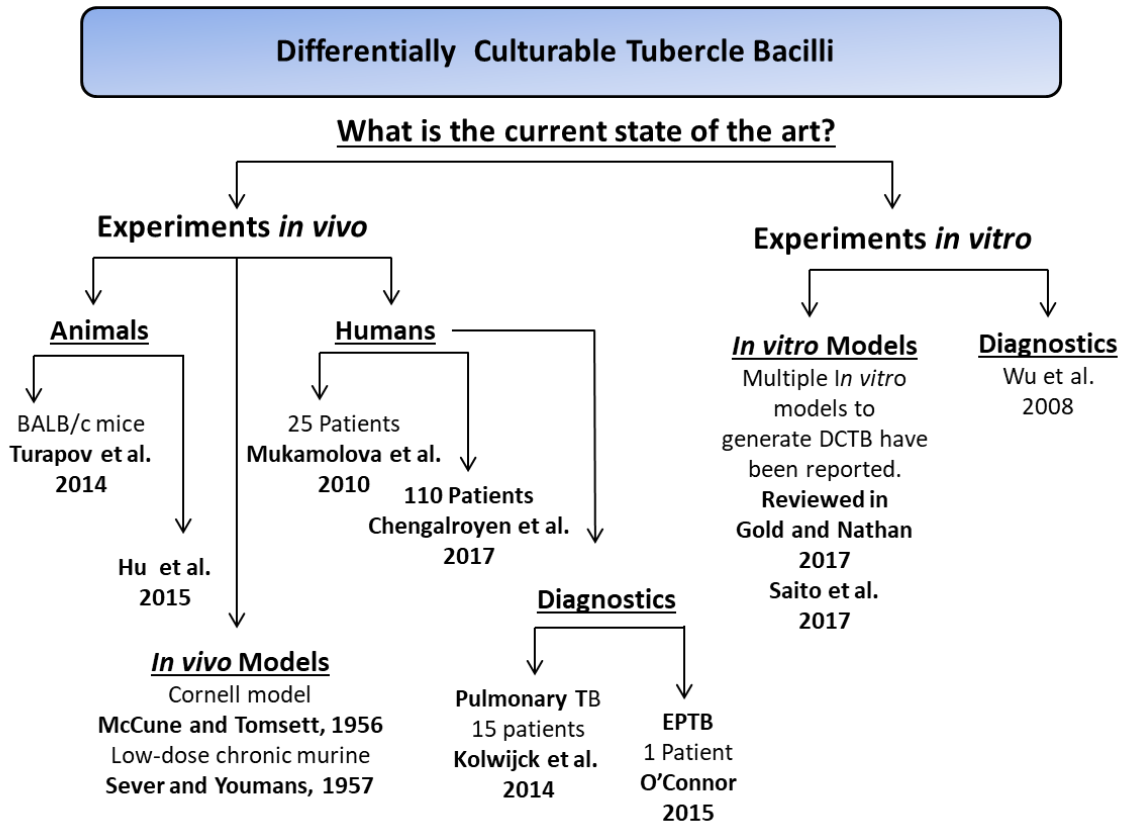
µg	Microgram
µL	Microlitre
µm	Micrometre
AFB	Acid Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
AMK	Amikacin
ART	Antiretroviral therapy
BCG	Bacillus Calmette-Guérin
BMI	Body mass index
BSC	Biological Safety Cabinet
BSL3	Biosafety Laboratory, Level 3
cAMP	Cyclic Adenosine Monophosphate
CBTBR	DST/NRF Centre of Excellence for Biomedical TB Research
CF	Culture filtrate
CFU	Colony Forming Unit
CLS	Clinical Laboratory Services
CORTIS	Correlate of Risk Targeted Intervention Study
CT	Computer Tomography
Ct	Cycle threshold
DCTB	Differentially Culturable Tubercle Bacilli
DNA	Deoxyribonucleic acid
DOTS	Directly Observed Therapy Short-Course
DR	Direct Repeat
DST	Drug Susceptibility Testing
EB	Elementary body
EBA	Early Bactericidal Activity
EDTA	Ethylenediaminetraacetic acid
EMA	European Medicines Agency
EMB	Ethambutol
EP	Extra-pulmonary
ETH	Ethionamide

FDA	Food and Drug Administration
FQ	Fluoroquinolone
HAART	Highly active antiretroviral therapy
HIV	Human Immunodeficiency Virus
IGRA	Inteferon Gamma Release Assay
INH	Isoniazid
IQR	Interquartile range
IRIS	Immune reconstitution inflammatory syndrome
KAN	Kanamycin
L	Litre
LAM	Lipoarabinomannan
LTBI	Latent tuberculosis infection
MCC	Medicines Control Council
MDR	Multi drug resistant
MEM	Meropenem
MFDS	Ministry of Food and Drug Safety
MGIT	Mycobacterial Growth Indicator Tube
mL	Millilitre
MPN	Most Probable Number
MRC	Medical Research Council
MTBC	*Mycobacterium tuberculosis* complex
NAAT	Nucleic Acid Amplification Test
NAG	*N*-acetyl glucosamine
NALC	*N*-acetyl-*L*-cysteine
NAM	*N*-acetyl muramic acid
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Service
NOS	Nitrogen Intermediate Species
NTM	Non-tuberculosis mycobacteria
NTP	National Tuberculosis Treatment Program
OADC	Oleic acid, Albumin, Dextrose, Catalase
OFX	Ofloxacin
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PASTA	Penicillin-Binding Protein and Serine/Threonine Kinase-Associated Domains

PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
PET	Positron Emission Tomography
PG	Peptidoglycan
PHRU	Perinatal HIV Research Unit
PPD	Purified Protein Derivative
Primer-F	Forward Primer
Primer-M	Mutant Primer
Primer-R	Reverse Primer
PZA	Pyrazinamide
RFLP	Restriction Fragment Length Polymorphism
RI	Resuscitation Index
RIF	Rifampicin
RMR	Rifampicin Mono-resistance
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
Rpf	Resuscitation-Promoting Factor
RR	Rifampicin resistant
RSA	Republic of South Africa
SDS	Sodium Dodecyl Sulphate
SOP	Standard Operating Procedure
STPRK	Serine Threonine Protein Kinase
TAE	Tris, Acetic acid, EDTA
TAG	Triacylglycerol
TB	Tuberculosis
TE	Tris-EDTA buffer
TNF-α	Tumour Necrosis Factor Alpha
Tris	Alpha, alpha, alpha-Tris-(hydroxymethyl)-methylamin
TST	Tuberculin Skin Test
TTP	Time to positivity
TU	Tuberculin Unit
USA	United States of America
VBNC	Viable But Non-Culturable
VNTR	Variable Number of Tandem Repeats

WE	Wax Ester
WHO	World Health Organization
XDR	Extremely drug-resistant
ZN	Ziehl-Neelson

OUTLINE OF PHD



What are the knowledge gaps?

- How robust are reports from current literature? Do all patients harbour DCTB pre-treatment? **Chapter 3**
- How do DCTB recovered from patient sputa respond to treatment? **Chapter 4**
- How does the most probable number assay relate to currently employed culture methods? **Chapter 5**
- Can the unmasking of DCTB be used to enhance current diagnostic methods, such as the BACTEC MGIT 960? **Chapter 6**

Research conducted in this PhD to address the above-mentioned gaps

CHAPTER 3	CHAPTER 4	CHAPTER 5	CHAPTER 6
Independent quantification and description of DCTB in pre-treatment patient cohort from Soweto and Klerksdorp, South Africa	Assess response to treatment and describe patterns of decline in DCTB in patients throughout first-line anti-TB treatment	Establish the relationship between the MPN assay and other culture-based assays, i.e. CFU and MGIT assays (performed on axenic cultures and patient samples)	Determine whether culture filtrate can enhance time to positivity by unmasking populations of DCTB in sputum samples (axenic work also performed)

BACKGROUND OVERVIEW

CHARACTERIZATION OF **DIFFERENTIALLY CULTURABLE** BACTERIA IN AXENIC CULTURE AND FROM TUBERCULOSIS PATIENTS

CHAPTER 1
Literature review of **Tuberculosis**

Topics and questions addressed in this chapter:

What is tuberculosis (TB)? Section 1.1. Introduction
What is the burden of disease? Section 1.2. Epidemiology
What causes TB? Section 1.3. Aetiology
How does TB disease develop? Section 1.4. Tuberculosis pathogenesis
What are the clinical outcomes of TB disease? Section 1.5. Clinical presentation
How is TB diagnosed? Section 1.6. Diagnosis
How is TB treated? Section 1.7. Treatment
How can TB be prevented? Section 1.8. Prevention and control in South Africa
What can be concluded? Section 1.9. Knowledge gaps related to this PhD

CHAPTER 2
Literature review of **Microbial Dormancy**

As differentially culturable organisms are closely related to dormancy and metabolic quiescence, Chapter 2 focuses on these concepts in other bacteria and more specifically in *Mycobacterium tuberculosis*

Topics and questions addressed in this chapter:

Are all bacteria in a population culturable? Section 2.1. The great plate count anomaly
What are common terms encountered in the literature? Section 2.2. Terminology and definitions
What are the stages of microbial dormancy? Section 2.3. Stages of microbial dormancy
How is dormancy related to disease? Section 2.4. Cellular quiescence and intracellular human pathogens
What about dormancy in *M. tuberculosis*?
Section 2.5. *Mycobacterium tuberculosis*: latency, active disease, the VBNC state and resuscitation
What is the scout hypothesis? Section 2.5.1. The scout hypothesis
What are the physical characteristics of non-replicating *M. tuberculosis?* Section 2.5.2. Phenotypic characteristics of non-replicating M. tuberculosis
What dormancy models are available? Section 2.5.3. *M. tuberculosis* models of non-replicating persistence
What are resuscitation promoting factors? Section 2.5.4. Resuscitation promoting factors

CHAPTER 1

TUBERCULOSIS

When it rained down sorrow
It rained all over me
When it rained down sorrow
It rained all over me

'Cause my body rattles
Like a train on that old S.P.
I've got the T.B. blues

TB blues, Jimmie Rogers, 1932

1.1 INTRODUCTION

Tuberculosis (TB) is a communicable disease in humans caused by the actinobacterium, *Mycobacterium tuberculosis*. TB is characterised pathologically by necrotising granulomatous inflammations that occur predominantly in the lung, but any organ or body tissue can be affected (Jagirdar et al., 1996, Pai et al., 2016, Dheda et al., 2016, Skoura et al., 2015). TB remains a significant global health challenge and remains a public health threat in current times, as it has been throughout history. With an estimated 1.4 million deaths per annum, TB is now the leading cause of death due to an infectious agent and ranks as one of the top ten causes of death worldwide (WHO, 2017a). Several factors continue to contribute to the public health crisis associated with TB, including a complex clinical presentation, delayed diagnosis, low rates of diagnostic pickup in low-income countries, HIV/TB co-infection and protracted treatment (Koul et al., 2011, Corbett et al., 2003, Pablos-Mendez et al., 1996, Pablos-Mendez et al., 1997, Byrd Jr et al., 2003, Pai et al., 2016, Greenaway et al., 2002). Another major barrier to the eradication of TB is the emergence of drug resistant strains of *M. tuberculosis* which has demanded the urgent development of new drugs (Koul et al., 2011, Ghandi et al., 2006, Tiberi et al., 2017, Pai et al., 2016, Laughon and Nacy, 2017, Tang et al., 2013, Dheda et al., 2017). Furthermore, the vaccine currently used for TB, Bacillus Calmette-Guérin (BCG), has demonstrated efficacy in protecting mostly against severe forms of disseminated childhood TB but fails to provide any meaningful protection against adult pulmonary TB (Colditz et al., 1994, Fine, 1995, Fine, 1988, Hart and Sutherland, 1977, Andersen and Doherty, 2005, Vaudry, 2003,

Mangtani et al., 2013). As a result, vaccination has limited impact interrupting the transmission of TB in adults (Franco-Paredes et al., 2006). Regrettably, advances in the development of a new TB vaccine have been hampered by a poor understanding of what constitutes protective immunity and how best to exploit this to create a vaccine with long lasting protection.

To meet the goals proposed by the END-TB strategy, multiple factors are in need of attention. These include the development of new and effective drugs, shortening of current treatment regimens, reducing the turn-around-time of diagnosis and improvements in public health related factors, such as poverty, social inequities, lack of infrastructure and access to health care facilities (Pai et al., 2016, Dheda et al., 2016). In addition, TB disease cannot be viewed in isolation and drivers of the TB epidemic, such as HIV co-infection, need to be tackled concurrently if there is any hope of completely eradicating this disease.

1.2 EPIDEMIOLOGY

1.2.1 The Global TB burden

TB is curable disease, yet recent estimates state that one quarter of the world's population is latently infected with *M. tuberculosis* (Raviglione and Sulis, 2016, Houben and Dodd, 2016). Whilst the global incidence has declined at a rate of 1.5% per year for the past 13 years, this decline has been insufficient to achieve the first target set by the WHO-endorsed END-TB strategy, which requires this decline to accelerate to 5% per annum (set for 2020, the strategy aims to achieve a 20% reduction in the TB incidence rate compared to the figures reported in 2015) (WHO, 2014a). In 2016 globally, there was an estimated 10.4 million incident cases of TB, equivalent to 142 cases per 100 000 population)(WHO, 2017a). It was estimated that approximately 65% of these cases were male and 90% of cases were adults (WHO, 2017a). TB disease is also associated with the most vulnerable populations and is disproportionately increased in HIV-infected individuals (Tornheim and Dooley, 2017). In 2016 it was estimated that 10% of all incident TB cases (74% in Africa) occurred in people living with HIV (WHO, 2017a). Globally, approximately 6.9% of new TB cases occur in children (<15 years) (WHO, 2017a), with exposed infants having the highest risk of contracting TB disease (Marais et al., 2006b).

South Africa was ranked amongst the top six countries with the highest total number of estimated incident cases in 2016 (Table 1.1), along with India, Indonesia, China, the Philippines and Pakistan, six countries which collectively accounted for 60% of the global total.

The reported incident cases per 100 000 population for these countries are depicted in Table 1.1, along with the associated HIV-positive TB incidence and mortality rates (WHO, 2017a).

Table 1.1 Burden of TB in 2016 for top six high burden countries.

Country	Population	Incident cases (2016) [a]	Incidence [b]	HIV prevalence in incident TB (%)	HIV negative TB mortality [b]	HIV positive TB mortality [b]
India	1 324 billion	2790	211	3.10	32	0.92
Indonesia	261 million	1020	391	4.40	42	5.10
China	1 404 billion	895	64	1.20	3.6	0.13
Philippines	103 million	573	554	1.10	21	0.29
Pakistan	193 million	518	268	1.30	23	1.10
South Africa	56 million	438	781	59.0	41	181

[a] Numbers in thousands

[b] Rates reported per 100 000 population

Sourced from WHO, Global Tuberculosis Report, 2017

1.2.2 HIV/TB co-infection

HIV infection is the most significant risk factor for TB disease (Havlir et al., 2008, Mahtab and Coetzee, 2017, Bruchfeld et al., 2015). Globally, an estimated 10% of TB incident cases occurred among individuals co-infected with HIV; In South Africa, this was reported as high as 59% (Table 1.1) (WHO, 2017a). Overall, 12% of all new active disease cases and 25% of all TB-related deaths occur in HIV-positive individuals (Pai et al., 2016). Furthermore, a systematic review of the literature reported that TB was the leading cause of hospitalisation and in-hospital mortality in both adults (18%) and children (10%) living with HIV worldwide (Ford et al., 2015). Africa is the epicentre for the global AIDS pandemic and consequently, the majority (approximately 75%) of HIV-associated active TB disease cases and deaths occur on this continent, mainly in sub-Saharan Africa (Pai et al., 2016).

HIV infection has an amplifying effect on the incidence of TB as it increases the risk of reactivation disease in latently infected individuals as well as facilitates the rapid progression

to clinical disease soon after an individual has been infected or re-infected with *M. tuberculosis* (Shafer et al., 1996, Daley et al., 1992, Havlir et al., 2008, Ai et al., 2016). A molecular epidemiological study employing IS*6110* restriction fragment length polymorphism (IS*6110* RFLP) genotyping illustrated a rapid spread and progression of TB disease in a residential facility for HIV-infected persons in San Francisco (Daley et al., 1992). Consistent with this, the risk of TB disease was shown to double within the first year following HIV-infection in a cohort of South African miners (Sonnenberg et al., 2005). The risk of disease progression is enhanced with advancing HIV disease (Lodi et al., 2013, Wood et al., 2000) and significantly reduced with the administration of antiretroviral therapy (ART) (Badri et al., 2002, Collaboration, 2005). An additional complication associated with HIV-TB coinfection is immune reconstitution inflammatory syndrome (IRIS). IRIS is a poorly understood immunological phenomenon whereby a paradoxical worsening of TB or unmasking of sub-clinical TB disease occurs within the first few months following ART initiation (Manabe et al., 2009, Abdool Karim et al., 2011). Management of IRIS is challenging and further underscores the need for new TB regimens that can be administered with ART.

1.2.3 Drug resistance

WHO reports routinely highlight multidrug-resistant TB (MDR-TB), wherein strains are resistant to isoniazid (INH) and rifampicin (RIF), the two most effective drugs used in the treatment of TB. Since May 2016, the WHO has issued guidance, that RIF mono-resistance is to be treated with an MDR-TB regimen (WHO, 2016). Globally in 2016, there were an estimated 600 000 cases of MDR-TB and rifampicin resistant TB (RR-TB) (4.1% occurring among new cases, and 19% amongst retreatment cases), with China, India and the Russian Federation reported as having the largest number of MDR/RR-TB cases (47% of the global total) (WHO, 2017a). Furthermore, treatment of extremely drug-resistant TB [XDR-TB; resistance to INH and RIF as well as to any fluoroquinolone and at least one of the three second line injectable drugs (i.e. amikacin, capreomycin and kanamycin)] was reported in 72 countries, with the majority of notified cases occurring in India (2464), Ukraine (1195), South Africa (967), Belarus (572) and China (525). The emergence of totally drug-resistant TB has been reported in several countries, including Italy, Iran, India and South Africa (Udwadia et al., 2012, Velayati et al., 2009, Klopper et al., 2013). These composite forms of drug-resistant TB are a major concern and continue to hamper current TB control efforts.

1.2.4 Drivers of the TB epidemic

Whilst HIV infection is a major driver of the TB epidemic, particularly in Africa, HIV-positive individuals only constitute 0.5-0.8% of the global population (Pai et al., 2016). Other contributors to the spread of TB include poverty, overcrowding, urbanization, poor housing, sanitation, stigma and other socio-economic/socio-cultural conditions which sustain transmission of both drug-susceptible and drug-resistant strains in communities. Many developing countries face particular challenges with regard to national TB control programs, several of which relating to social and public health issues, with social inequities, poor infrastructure and a lack of basic education featuring prominently (Lönnroth et al., 2009, Harling et al., 2008). Living in rural settlements that are secluded from public health facilities often results in diagnostic delays, poor healthcare delivery and decreased access to anti-TB and HIV medication. Furthermore, treatment programs for HIV and TB are often not sufficiently integrated at clinic and community level, thus complicating management of individuals with coincident infection. Further risk factors that are associated with TB disease include smoking (Bates et al., 2007, Murrison et al., 2016, Lin et al., 2007), type-II diabetes mellitus (Jeon and Murray, 2008, Lönnroth et al., 2014), alcohol abuse (Rehm et al., 2009, Imtiaz et al., 2017), malnutrition (Lönnroth et al., 2009, Cegielski and McMurray, 2004, Chandrasekaran et al., 2017) and air pollution (Sumpter and Chandramohan, 2013, Lönnroth et al., 2009, Lin et al., 2007, Lai et al., 2016) all of which highlight the need for public health interventions (Narasimhan et al., 2013, WHO, 2017a, Mathema et al., 2017). TB elimination therefore requires a combination of both therapeutic/diagnostic strategies along with prevention strategies aimed at reducing transmission and the progression to active disease taking into account the large reservoir of latent TB cases globally.

1.3 AETIOLOGY

The aetiology of TB was first described by Robert Koch on the 24th of March in 1882 at the Berlin Physiological Society, a day which is now commemorated as world TB day. Koch developed a staining procedure based on Methylene blue counterstained with versuvin allowing for the discovery of the contagion:

"…it seems likely that the tubercle bacillus is surrounded with a special wall of unusual properties, and that the penetration of a dye through this wall can occur when a alkali, aniline, or similar substance is present."

(Koch, 1882)

1.3.1 Taxonomy and morphology

1.3.1.1 Taxonomy

1.3.1.1.1 The Mycobacterium tuberculosis complex

Bacteria of the family Mycobacteriaceae (of the order Actinomycetales) contain the genus of Mycobacteria, of which more than 150 different species have been identified, Figure 1.1. The vast majority of these bacteria are non-pathogenic and are classified as non-tuberculous/ environmental mycobacteria (NTM) (see section 1.3.1.1.2). There are however, several non-environmental mycobacterial species that are pathogenic. These include members of the *M. tuberculosis* complex (MTBC) as well as *Mycobacterium leprae* and *Mycobacterium ulcerans*.

The MTBC consists of eight genetically related *Mycobacterium* species and subspecies including *M. tuberculosis, Mycobacterium bovis, M. bovis* BCG, *M. bovis subsp caprae, Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium pinnipedii*, and *Mycobacterium canettii*. The majority of TB cases are caused by *M. tuberculosis* and *M. africanum*, while a small number of cases can be caused by zoonotic members of the MTBC (see Table 1.2). While members of the MTBC vary greatly in their phenotypic characteristics, mammalian host ranges, geographic distribution and pathogenicity, they represent an extreme example of genetic homogeneity, with 99.9% similarity at the nucleotide level and identical 16S rRNA sequences (Aranaz et al., 1999, Cousins et al., 1998, Brosch et al., 2002, Böddinghaus et al., 1990, Sreevatsan et al., 1997). There is also evidence to show that members of the MTBC evolved from a common ancestor, *Mycobacterium prototuberculosis* (Brosch et al., 2002, Gutierrez et al., 2005, Sreevatsan et al., 1997).

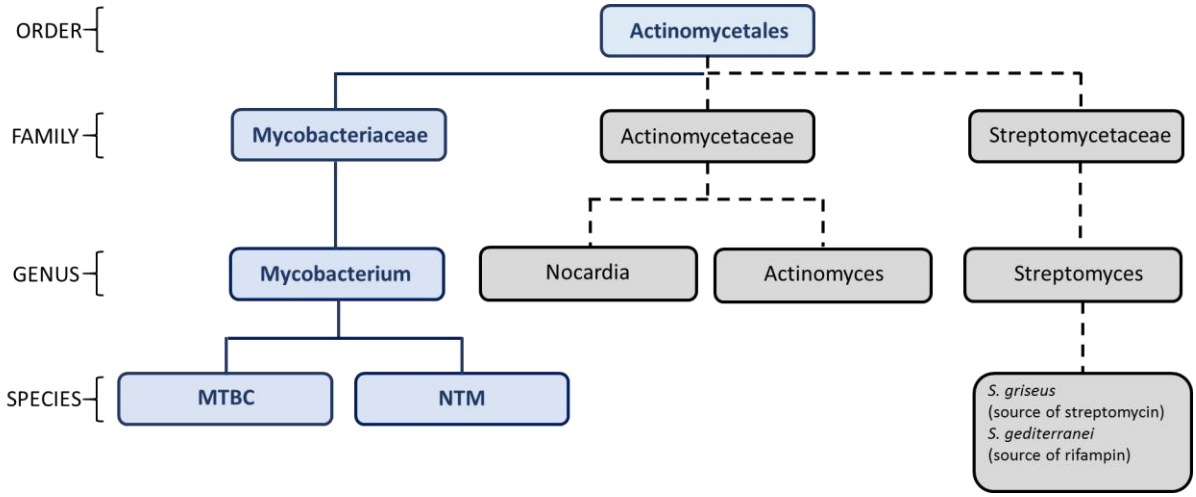


Figure 1.1 Taxonomic tree for *Mycobacterium tuberculosis* complex, non-tuberculosis mycobacteria and related species. MTBC, *Mycobacterium tuberculosis* complex; NTM, non-tuberculosis mycobacteria. Adapted from (Iseman, 2000).

Table 1.2 Primary host, mode of transmission and geographic distribution of members of the *Mycobacterium tuberculosis* complex

Organism	Primary reservoir/ Host	Transmission?	Geographic distribution
Mycobacterium tuberculosis	Human	Inhalation of droplet nuclei *	Worldwide
Mycobacterium bovis	Human and range animals (e.g. cattle)	Ingestion of contaminated milk from infected cows †; Airborne transmission ‡	Worldwide
M. bovis BCG	Human – commercial vaccine	Via immunization	Worldwide
Mycobacterium africanum	Human §	Inhalation of droplet nuclei	East and west tropical Africa. Been reported in USA
Mycobacterium caprae	Wide range of host animals (e.g. goat); human infection is rare	Inhalation of droplet nuclei	Europe
Mycobacterium microti	Vole, rodents	Inhalation of droplet nuclei	Europe, Great Britain, Netherlands
Mycobacterium mungi	Banded mongoose	ND	Africa
Mycobacterium canettii	ND human infection is rare	ND	Africa
Mycobacterium orygis	Larger mammals (e.g. oryxes, antelope, gazelle and waterbuck)	ND	Africa
Mycobacterium pinnipedii	Seal, sea-lion	ND; Transmission from sea-lion to human has been reported	Europe

* Infection can occasionally occur via the skin or gastrointestinal tract.

† The incidence has significantly decreased since the universal pasteurization of milk and milk products.

‡ Can occur human-to-human, animal-to-human and human-to-animal.

§ Infection in animals are not excluded.

Reproduced from (Tille, 2013)

1.3.1.1.2 Non-tuberculosis mycobacteria

A number of mycobacterial saprophytes are opportunistic and cause disease when the immunity of the host is compromised. For example, *Mycobacterium avium-intracelluare* of the *Mycobacterium avium-intracelluare* complex is associated with pulmonary disease and to a lesser extent, focal disease involving intestines or lymph nodes in HIV-positive patients. Disseminated disease with bacteraemia may also develop in AIDS patients with very low CD4 T-cell counts (<100/mm^3) (Gillespie and Hawkey, 2006). Examples of non-tuberculosis mycobacteria that are associated with clinical infection are given in Table 1.3.

Table 1.3 Examples of clinically relevant non-tuberculosis mycobacteria*

Disease association	Species
Pulmonary	*M. avium-intracelluare*
	M. kansasii
	M. xenopi
	M. absessus
	M. fortuitum
Lymph node	*M. avium-intracelluare*
	M. scrofulaceae
	M. malmoense
Cutaneous	*M. ulcerans*
	M. absessus
	M. fortuitum
	M. avium-intracelluare
Disseminated	*M. avium-intracelluare*
	M. kansasii
	M. fortuitum
	M. chelonae

* Reproduced from and reviewed in (Gillespie and Hawkey, 2006)

1.3.1.2 Morphology

Mycobacteria resist conventional stains, such as Gram staining, due to the high lipid content of their cell walls (approximately 60%), often appearing as 'neutral' or as unstained ghost forms. These bacteria have a unique tinctorial property whereby acid fast stains such as Ziehl-Neelsen (ZN) or auramine are used for microscopic identification. Microscopically, TB bacilli

typically appear as straight or slightly curved rods (Palomino et al., 2007). When cultivated *in vitro*, the shape and length of these bacilli can vary depending on the age of the culture and growth conditions (Shleeva et al., 2011). The different morphologies range from coccobacilli to long rods and the reported lengths and widths of these organisms range from one to 10 μm (typically 3 to 5 μm) and 0.2 to 0.6 μm, respectively (Velayati et al., 2011, Velayati and Farnia, 2012). In liquid culture, MTBC often exhibits serpentine cording allowing for the rapid and presumptive identification of TB infection using microscopy (Gonzlez et al., 1998).

1.4 TUBERCULOSIS PATHOGENESIS

The pathogenesis of TB is a complex process beginning with transmission, which can either result in the establishment of a contained infection, no infection or active disease. These and related aspects are discussed below.

1.4.1 Transmission

TB is transmitted through the production of aerosolized particles harbouring *M. tuberculosis* by infected individuals through high-velocity exhalation actions such as coughing or sneezing (Wells, 1934). These aerosols dehydrate to form droplet nucleic (1 to 5 μm) that remain suspended in the air and can subsequently be inhaled into the alveoli of a new prospective host.

> *"…most droplets atomized into air evaporate almost instantly, leaving disease germs drifting like cigarette smoke in the droplet nuclei."*
>
> (Riley and O'Grady, 1961)

In March 2016, a workshop was held at the National Institute of Health to identify potential research gaps and find possible solutions to halt TB transmission with the ultimate goal of reducing new infections to zero. The three major themes that were addressed included: (1) infectiousness and susceptibility (i.e. which individuals are infectious and who is prone to becoming infected?), (2) drivers of the TB epidemic and (3) interventions to halt transmission (Shah et al., 2017b, Churchyard et al., 2017, Turner et al., 2017, Mathema et al., 2017, Dowdy et al., 2017, Auld et al., 2017). Transmission of *M. tuberculosis* involves both host and

bacterial-related factors. For example, factors associated with infectiousness include the immune status of the index case as well as lung cavitation, extent of disease and bacillary load (Dheda et al., 2010, Turner et al., 2017, Churchyard et al., 2017). In terms of susceptibility and exposure, closeness of contact, exposure duration, room ventilation, barrier host defences as well as the new host's immune status (including macrophage function and mucosal immunity) are important in the establishment of a new infection (Turner et al., 2017). Furthermore, certain strains, including drug-resistant strains, have also been associated with increased virulence and transmission. Indeed, primary resistance (transmission of resistant TB strains) has been reported globally (Ghandi et al., 2006, Wells et al., 2007, Moro et al., 1998, Ritacco et al., 1997, Shah et al., 2017a). An in-depth understanding of factors that drive transmission are also required, as well as tools to monitor ongoing transmission within a community (Mathema et al., 2017). Until the advent of molecular methods for the typing and characterization of *M. tuberculosis* strains, primary TB disease, usually in children, was generally thought to occur as a consequence of infection with a single *M. tuberculosis* strain which becomes dormant, resulting in latent TB infection (LTBI). Active TB disease in adults was considered to be predominantly the result of endogenous reactivation of the dormant strain that caused the original infection or failure to contain the initial infecting episode in the form of LTBI (Stead, 1967)**.** With the advent of molecular-based genotyping, various studies have subsequently demonstrated that many incident TB cases occur as a result of recent transmission (associated with a short latency/incipient period) as opposed to reactivation of a historic infection, thus challenging past 'misconceptions' regarding the transmission of TB (Alland et al., 1994, Small et al., 1994). Studies indicating high rates of re-infection versus relapse disease, recent transmission and mixed infections have also challenged previous paradigms regarding TB transmission (McIvor et al., 2017).

TB transmission is a process that is complex. An improved understanding of this and ongoing research is required to improve knowledge and understanding in this regard. In addition, the implementation of multiple strategies that include both established and new interventions (at biomedical and social levels) are required to halt transmission.

1.4.2 Outcomes of infection

Following primary infection, multiple scenarios can take place. The first involves the clearance and complete eradication of organisms by the host immune system. However, in most cases primary infection may lead to the establishment of LTBI, whereby the disease is contained and in this case, the physiological state of the bacilli remains enigmatic. In few cases (roughly 5-10%), the inhaled bacilli may begin to multiply resulting in the rapid progression to active disease, Figure 1.2 (Koul et al., 2011).

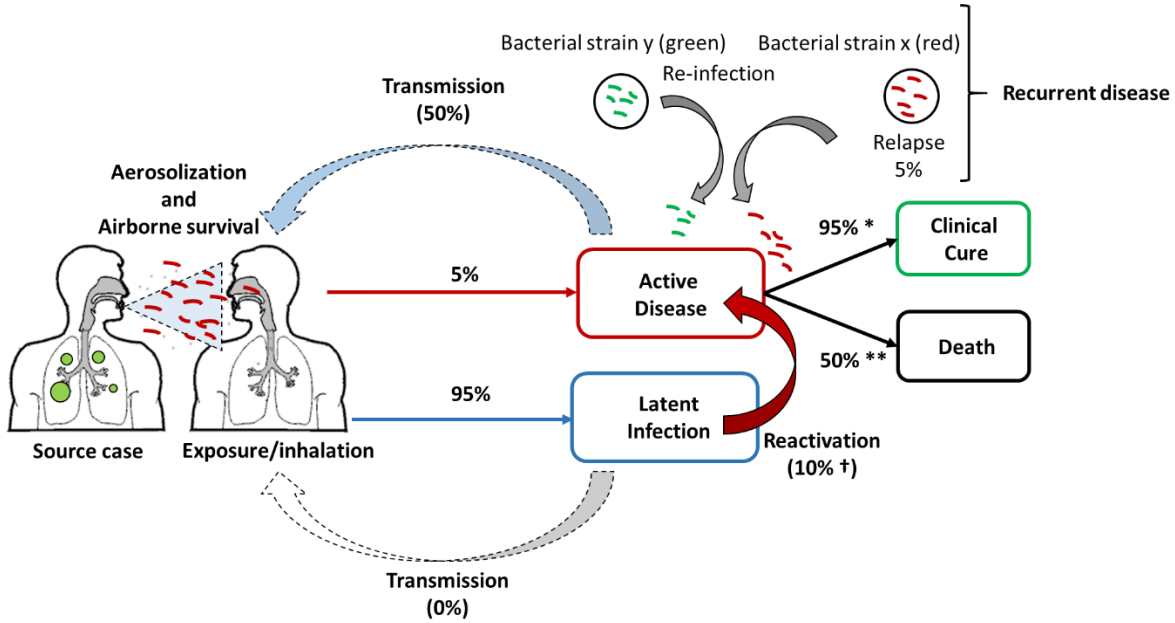


Figure 1.2 Stages of *M. tuberculosis* infection. Infection with *M. tuberculosis* can occur via the inhalation of viable organisms (bacterial strain x, red), where close enough contact to the diseased host seems necessary. Following a transmission event, in the majority of cases, latent tuberculosis infection (LTBI) is established (blue box), but a small number of individuals will rapidly progress to active TB disease (red box). Globally, there is a large reservoir of latent TB cases. These individuals, if immune-competent, carry a 10% life time risk of developing active disease (†). Disease reactivation is commonly associated with immune suppression and this event can occur years after the initial infection. Immune-compromised individuals have an increased risk of developing active disease at a rate of seven to 10% per annum. In drug-susceptible TB disease (denoted by *), approximately 70 to 95% of patients will recover following treatment completion (Chaulk and Kazandjian, 1998), whereas 5% will relapse (bacterial strain x, red) (Koul et al., 2011). The treatment success rates in drug-resistant TB disease are much poorer (ranging from 40 to 70% in multi-drug resistant cases, to 20 to 60% in patients with extremely drug-resistant TB) (Dheda et al., 2014). Furthermore, patients with previous TB infection are at increased risk of being re-infected with a different strain (bacterial strain y, green). Patients who are untreated (denoted by **) have high mortality rates. Adapted from Koul et al. 2011. Drawn by Amanda McIvor.

1.4.3 Immune pathogenesis

Following inhalation, the infecting bacilli are engulfed by resident alveolar macrophages (Schlesinger et al., 1990, Tailleux et al., 2005, Hirsch et al., 1994, Kang et al., 2005), epithelial cells, dendritic cells (Tailleux et al., 2003, Geijtenbeek et al., 2003) and neutrophils that are directed at killing bacteria. However, *M. tuberculosis* is able to resist eradication and uses macrophages as a niche for survival and replication. Through the process of phagocytosis, tubercle bacteria gain access into the macrophage through the recognition of cell wall glycolipids and the formation of toll-like receptor heterodimers (Aderem and Ulevitch, 2000, Flynn and Ernst, 2000). Following entry, *M. tuberculosis* is able to prevent phagosome maturation by preventing fusion of the phagosome and lysosome (Russell, 1998, Russell, 2011). The infected macrophages migrate and transport *M. tuberculosis* via the lymphatic system to the hilar lymph nodes where a primary lesion known as the 'Ghon focus' is formed (Frieden et al., 2003), and T-cells are primed, followed by clonal expansion (Dheda et al., 2010).

Bacteria are able to move hematogenously to various sites in the body and through the draining lymph system. The presence of *M. tuberculosis* in the pulmonary interstitium leads to a local inflammatory response attracting dendritic cells, monocytes, macrophages and T-cells to the infected area where the primary lesion matures into a granuloma – the hallmark of TB. Early stages of granuloma development are dependent on the production of tumour necrosis alpha (TNF-α) by infected macrophages and T-cells (Guirado and Schlesinger, 2013, Algood et al., 2005, Russell et al., 2009). TNF-α along with other inflammatory cytokines [e.g. IL-1β, IL-2, IL-6, IL-12, IL-15, IL-17, IL-18, IFN-γ (pro-inflammatory Th1 profile)], anti-inflammatory cytokines [IL-4, IL-10, TGF-β (Th2 profile)] and chemokines (e.g. IL-8) attract natural killer T cells, neutrophils, CD4 and CD8 to the area of infection (Ramakrishnan, 2012, Pagan and Ramakrishnan, 2017, Russell, 2007). Sustained signalling of TNF-α is required to maintain these chemokine levels resulting in cellular recruitment and retention (Russell et al., 2009, Algood et al., 2005, Kindler et al., 1989, Roach et al., 2002, Saunders and Britton, 2007). Interestingly, a granulatomous response has been shown to occur in TNF-deficient mice; However, this response is delayed in onset and the structures remain poorly organized and become necrotic (Gil et al., 2006). Maintenance of the granuloma and outcome of infection is determined by the interplay between pro-inflammatory and anti-inflammatory cytokines. While TNF-α is one of the predominant cytokines in granuloma formation, an excess may lead to severe inflammation resulting in necrosis, liquefaction and overt tissue destruction. An

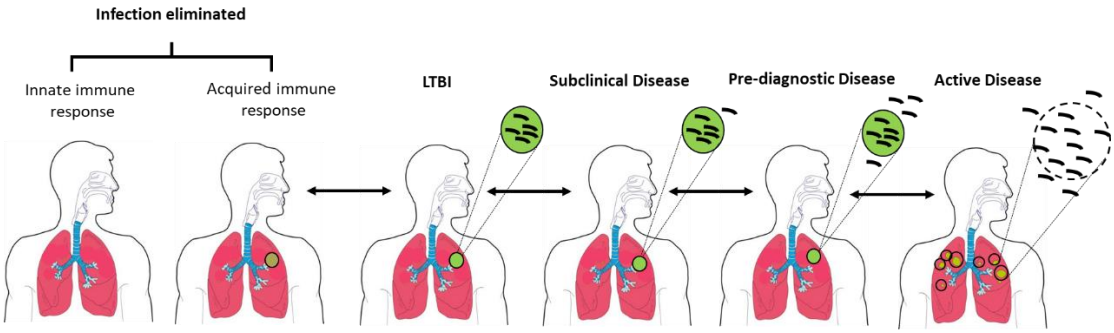
example of this is IRIS in HIV/TB co-infected patients with low CD4 T-cell counts on highly active anti-retroviral therapy (HAART). The resulting expansion of CD4 cells in the presence of a high antigen load or bacterial burden results in an aggressive granulomatous response manifesting in new, worsening or recurrent signs and symptoms (Lipman and Breen, 2006, Namale et al., 2015).

The granuloma is often described as a 'double edged sword', preventing dissemination of disease but also functioning as a site for bacterial replication (Gideon et al., 2015, Ehlers and Schaible, 2012, Ramakrishnan, 2012). Originally viewed as protective physical barrier preventing dissemination of the invading pathogen (Saunders and Cooper, 2000), studies conducted in infected zebra fish embryos (*M. marinum*) have suggested that the granuloma may function as a protective niche for the pathogen allowing for replication and facilitation of transmission (Ramakrishnan, 2012, Davies et al., 2014).

PET-CT scans have illustrated the dynamic nature of granulomas in patients with active TB not responding to therapy (Coleman et al., 2014). In human infection, three types of granulomas have been described, namely solid, necrotic and caseous granulomas (Gengenbacher and Kaufmann, 2012).

1.4.4 Progression from latent infection to active disease

In most cases of LTBI, the infection is contained by the host's immune system involving a balance of macrophages, dendritic cells and T-cells, resulting in asymptomatic infection. However, in a subset of these individuals (approximately 5-10%) reactivation to active disease will occur (Selwyn et al., 1989, Selwyn et al., 1992). Reactivation disease can take place after a few months or decades following the initial infection and sub-clinical persistence. Risk factors that are associated with reactivation disease include HIV-infection, poverty, malnutrition, alcoholism, diabetes, tobacco smoking, silicosis, chronic renal failure needling dialysis, immune suppressive therapy as well as individuals older than the age of 65 (Ai et al., 2016, Lönnroth et al., 2009).



TST	Negative	Positive	Positive	Positive	Positive	Usually Positive
IGRA	Negative	Positive	Positive	Positive	Positive	Usually Positive
Smear	Negative	Negative	Negative	Negative	Negative/Positive	Negative/Positive
Culture	Negative	Negative	Negative	Intermittently Positive	Positive	Positive
Infectious	No	No	No	Sporadically	Yes	Yes
Symptoms	None	None	None	Mild or None	Mild	Mild to Severe
Treatment	None	None	Preventative Therapy	Multidrug Tx	Multidrug Tx	Multidrug Tx

Figure 1.3 The spectrum of tuberculosis disease – from *M. tuberculosis* infection to active pulmonary disease. Historically, tuberculosis (TB) disease was viewed in a binary fashion comprising either latent TB infection (LTBI) or active disease. More recently, TB disease is considered as a spectrum, extending from sterilizing immunity to active infection and clinical disease (Barry et al., 2009). Furthermore, the extent of infection/disease can advance or reverse depending on changes in host immunity and disease comorbidities. In one scenario, complete eradication of *M. tuberculosis* may occur following exposure by the host's innate or acquired immune response. When *M. tuberculosis* is not eliminated by the immune system, bacilli can exist in a non-replicating state (associated with LTBI). LTBI can be detected by a positive tuberculin skin test (TST) and interferon gamma release assay (IGRA) and the absence of clinical symptoms. The clinical spectrum of TB disease is also inclusive of subclinical TB (mild or no clinical symptoms but positive culture results), pre-diagnostic TB (symptoms are present, but not severe enough to seek clinical attention) and clinical disease (clinical seeking of care, positive diagnosis) (Mathema et al., 2017). Treatment of TB disease (subclinical to active disease) requires a multidrug treatment (Tx) regimen. Adapted from Pai et al. 2016. Drawn by Amanda McIvor.

1.5 CLINICAL PRESENTATION OF TB DISEASE

The various outcomes of infection include LTBI, primary disease, secondary disease (also referred to as post-primary/progressive or reactivation disease) and extra-pulmonary TB disease. These stages of TB disease have different clinical presentations and further detailed below:

1.5.1 Latent infection

Persons with LTBI are infected with *M. tuberculosis* as evident by means of a positive immunological test result, i.e. tuberculin skin test (TST) or IFN-γ release assays (IGRA), but have no clinical symptoms of disease. In LTBI, bacilli can persist in the host for many years or

a life time without causing active disease. Indeed, evidence for extremely long latency periods in untreated humans was illustrated in a molecular-based study conducted in Denmark. While typing historical freeze dried *M. tuberculosis* strains, the investigators came across a 13-band DNA pattern collected in 1961 that matched the fingerprint of a specimen obtained in 1994. The isolates were from a father (1961) and son (1994) (Lillebaek et al., 2002).

LTBI in clinical medicine was previously viewed as a homogenous entity; however, current data suggests that LTBI can exist as a spectrum of infectious states ranging from sterilizing immunity where *M. tuberculosis* is eradicated by the host immune response, or incipient TB (characterized by a biomarker signature of progression), or subclinical disease, where the probability of reactivation is high (Barry et al., 2009, Esmail et al., 2016, Shnappinger and Ehrt, 2016, Fiore-Gartland et al., 2017). Indeed, [8F]-fluoro-2-deoxy-D-glucose (FDG) positron emission tomography (PET) in combination with computed tomography (PET/CT) imaging identified heterogeneous lesions in patients with LTBI including infiltrates, fibrotic scars and active nodules which were indicative of active disease (Esmail et al., 2016). A subset of individuals with PET/CT scans indicative of subclinical disease developed TB symptoms during a 6 month follow-up period illustrating an increased risk of reactivation disease. These findings further complicate diagnosis and treatment and stress the need for the identification of biomarkers that discriminate patients at risk for reactivation disease who can be prioritised for preventative therapy. The use of a highly specific transcriptomic biomarker (Zak et al., 2016) is being considered for a risk-targeted, test-and-treat strategy with the aim of identifying individuals who have a high risk of progressing to active TB disease (Fiore-Gartland et al., 2017). The performance of this biomarker is currently being assessed in a South African study designated 'CORTIS' (Correlate of Risk Targeted Intervention Study) involving a large cohort of both high-risk and low-risk individuals (Fiore-Gartland et al., 2017).

1.5.2 Pulmonary TB Disease

Traditionally, pulmonary TB has been characterised as either primary or secondary based on the time between the initial infection and the development of clinical disease. Primary disease has been defined to occur within 'five years of the initial infection' (Vynnycky and Fine, 1997, Holm, 1969) and in molecular epidemiology terms is said to occur as a result of recent transmission events. Primary and secondary TB disease have different clinical and radiographic presentations which are discussed below.

1.5.2.1 Primary TB disease

Primary disease occurs in a host that is previously unexposed and therefore unsensitised, and is characterised by the formation of the Ghon complex (ATS, 2000, Small and Fujiwara, 2001, Ober, 1983). This pathognomic macroscopical lesion consists of a granuloma, normally in the middle or lower zones of the lung (referred to as the Ghon focus) in conjunction with hilar and/or paratracheal lymphadenopathy (Heemskerk et al., 2016). Primary disease is often asymptomatic and the only evidence of disease is a positive immunological diagnostic test (Palomino et al., 2007). In some cases, symptoms may include low-grade fever, malaise and erythema nodosum. Generally primary TB disease has a favourable outcome, with healing resulting in fibrosis and/or calcification in the majority of individuals cases resulting in a Ranke complex which is visible on a chest X-ray (Heemskerk et al., 2016). In adult patients, primary TB disease is paucibacillary, non-contagious, difficult to diagnose and varies in severity (Palomino et al., 2007).

1.5.2.2 Post-primary TB disease

Post primary TB is defined as TB disease that arises in a previously sensitized host, i.e. infection can progress despite an adequate specific immune response (Palomino et al., 2007, ATS, 2000, Small and Fujiwara, 2001). Post-primary TB disease often occurs many years after the initial infection as a result of 'endogenous' reactivation of the primary focus. However, post-primary disease can also occur by the inhalation of new bacilli, referred to as 'exogenous' re-infection. Often, reactivation disease (defined as the re-emergence of a historic infection that was contained by the immune system) and re-infection (defined as exogenous reinfection with a distinct *M. tuberculosis* strain to that from the original infection) are clinically indistinguishable and molecular fingerprinting methods such as IS*6110*, spacer oligonucleotide genotyping or whole genome sequencing are required for differentiation (Heemskerk et al., 2016).

Post-primary TB most commonly manifests as pulmonary disease, although lymphatic dissemination can occur and almost any organ of the body can be involved (see section, 1.5.3). In pulmonary disease, radiographic manifestations typically include upper lobe lesions, cavitation and fibrosis (Lee et al., 1993, Lee et al., 2000, Jeong and Lee, 2008). However, molecular epidemiology studies employing IS*6110* RFLP genotyping to differentiate primary

disease (by recent infection) from reactivation disease have shown that the radiographic features are often similar between the two (Jones et al., 1997, Geng et al., 2005).

The signs and symptoms during the initial disease phase are often not severe, non-specific and gradual in onset. With disease progression, the most frequently reported symptom is a persistent and productive cough with purulent or mucous expectoration, as well as the typical triad of fever, weight loss/wasting and night sweats reported in 95, 75, 55 and 45% of patients, respectively (Davies et al., 2014). For further information, the reader is referred to an excellent review regarding the pathogenesis of post-primary TB (Hunter et al., 2014).

1.5.3 Extra-pulmonary TB disease

Prior to the HIV epidemic, the majority of reported TB cases were pulmonary (about 85%) with the remaining 15% being extra-pulmonary or involving both pulmonary and extra-pulmonary sites (Farer et al., 1979). In 2006, analysis of 13,779 TB cases in the United States reported that 21% of these cases displayed extra-pulmonary (EP) TB, 6.9% were concurrent EP-pulmonary TB and approximately 1.8% were disseminated (milliary TB) (CDC, 2007). Various studies have shown that whilst there has been a decrease in the total number of TB cases, there has been a proportionate increase in the number of EPTB cases (Peto et al., 2009, Farer et al., 1979, Rieder et al., 1990, García-Rodríguez et al., 2011). Factors that contribute to the risk of acquiring EPTB include HIV status, gender, ethnicity, age and BCG vaccination status (Yang et al., 2004, Rodrigues et al., 1993, Peto et al., 2009, Cailhol et al., 2005, Antony et al., 1995, Yang and Kong, 2015). In HIV-infected patients, the risk of developing EPTB is 5-fold that in HIV-negative patients (Yang et al., 2004, Yang and Kong, 2015) which is most likely associated with a deficiency in CD4 T-cells (Jones et al., 1993). In terms of the ethnicity, African-Americans and individuals born in South Asian countries (living in the United States) have higher incidence rates of EPTB compared to Caucasians (Farer et al., 1979, Yang et al., 2004, Asghar et al., 2008, Fiske et al., 2010). The observed increase in risk may be due to host-related genetic factors, social factors or vitamin D deficiency (Sita-Lumsden et al., 2007, Yang and Kong, 2015). Female gender and young age (less than 25 years) have also been described as risk factors for EPTB (Yang et al., 2004, Rieder et al., 1990). It is not clear why women are more likely to have EPTB compared to men; however, hormones, smoking status and TB exposure have been put forward as possible reasons for the increase in risk (Hudelson, 1996, Holmes et al., 1998, Yang and Kong, 2015). In addition, virulence of different *M. tuberculosis*

strains, mode of transmission and the innate immunity of the host have also been considered (Farer et al., 1979, Yang and Kong, 2015). The most common forms of EPTB are pleural TB, lymph node TB and disseminated TB (Palomino et al., 2007, Pai et al., 2016). Diagnosis of EPTB is challenging as it can present with varied clinical manifestations that mimic other diseases and sampling of sites can prove challenging (Yoon et al., 2004). For these reasons, the WHO now recommends the GeneXpert MTB/RIF assay to diagnose TB lymphadenitis and meningitis (Steingart et al., 2014, Pai et al., 2016, Boehme et al., 2010).

1.6 DIAGNOSIS

1.6.1 Latent infection

Diagnosis of LTBI can be made after active TB disease has been ruled out as current immunological tests have limited capacity to distinguish between active disease and latent infection. For adults, the exclusion of active TB disease is based on the absence of clinical symptoms, including a normal chest radiograph, as well as a negative bacteriological result [i.e. sputum smear microscopy, culture or nucleic acid amplification tests (NAAT)]. For children, the absence of clinical symptoms is usually sufficient to exclude active disease. The commercially available tests to diagnose LTBI include the TST or 'Mantoux test' as well as whole blood IGRA tests.

1.6.1.1. Tuberculin skin testing

The TST is a method of assessing cell-mediated immunity to purified protein derivative (PPD) tuberculin, and has been widely used as a screening tool for LTBI since the 1930s (Daniel, 2006). The test is performed by administering an intradermal injection of 5 tuberculin units (TU) of PPD S or 2 TU of PPD RT23 (WHO, 2014b). The reaction is measured 48 to 72 hours following administration by measuring the diameter of the induration on the forearm in mm (WHO, 2014b, Nayak and Acharjya, 2012, Menzies et al., 2008). The sensitivity of this test is compromised in patients with poor immunity (i.e. HIV-infection, malnutrition, corticosteroid therapy or children following measles infection) or in cases of overwhelming TB disease (for e.g. miliary TB disease in young children) (Nayak and Acharjya, 2012) (www.cdc.gov/tb/publications/factsheets/testing/skintesting.pdf). False positive reactions can also occur due to cross reactivity with the BCG vaccine, although this is unlikely after the first two years following vaccination at birth, or exposure to NTM in the environment (Nayak and

Acharjya, 2012, Farhat et al., 2006). The interpretation a positive result varies according to the population or individual tested, i.e. The cut-off for a negative skin test is lowered from 10 mm to 5 mm in patients who are immune compromised (Pai et al., 2014, Dheda et al., 2016). The TST is the most widely used test for the screening of LTBI due to its low cost and ease of use and is the preferred method in certain groups such as young children and routine screening of laboratory personnel and health care workers (Enarson, 2004, Pai and Banaei, 2013). A skin test, utilising more specific *M. tuberculosis* antigens (RD1) is currently under development to replace PPD (Pai and Sotgiu, 2016).

1.6.1.2 Interferon gamma release assays

The two currently used whole blood IGRA tests that are available on the market are the QuantiFERON®-TB Gold In-Tube tests (Cellestis/Qiagen, Carnegie, Australia) and the T-SPOT.TB assay (Oxford Immunotec, Abingdon, United Kingdom) (Pai et al., 2014). These *in vitro* diagnostic tests measure the amount of IFN-γ produced by T-cells in whole blood on exposure to RD-1 encoded *M. tuberculosis* antigens (namely a 6kDa early secretory antigenic target and culture filtrate protein 10) (Sørensen et al., 1995, Pai et al., 2014, Mahairas et al., 1996). The concentration of IFN-γ present is determined using an ELISA test. The advantages of IGRA tests is that they are more specific than TST as the antigens included in the assay are highly specific to *M. tuberculosis* and are not encoded in the BCG genome and most NTMs (Pai et al., 2016, Andersen et al., 2000). Therefore, infection with NTMs is unlikely to yield a false positive result. In addition, previous vaccination with BCG will not affect the results. The drawbacks of these assays are that they are expensive and require more expertise to perform than the TST, making it difficult to implement this technology for routine use in developing nations with a high burden of TB (WHO, 2015b).

Whilst research has shown that the TST and IGRA are acceptable methods for the diagnosis of LTBI, both tests have a low predictive value and cannot differentiate between active disease and LTBI (Pai et al., 2014, Sester et al., 2011, Pai et al., 2016, Pai and Sotgiu, 2016). The development of high predictive tests that are able to identify individuals who are at risk of reactivation disease and would benefit from preventative therapy would serve as an invaluable tool and strategic method of treatment to reduce the numbers of individuals with active disease and thus reduce transmission of *M. tuberculosis* within the community.

1.6.2 Active disease

Screening for active TB disease can be performed using imaging technologies such as chest X-rays and PET/CT scans; however, diagnosis of the disease primarily relies on microbiological methods including smear microscopy, culture-based methods and molecular methods, each of which is detailed below.

1.6.2.1 Smear microscopy

Sputum smear microscopy is currently widely used in low- to middle-income countries to diagnose active TB disease. It has several advantages including low cost, it is relatively simple to perform, it has a fast turn-around-time and it can be performed in very basic laboratory set-ups (Ryu, 2015). Furthermore, it has a high specificity in areas where TB is prevalent. For a smear to be positive, microscopy requires at least 5000 to 10 000 acid fast bacilli (AFB) per mL of sputum (Getahun et al., 2007), thus enabling it to identify the most infectious individuals (Luelmo, 2004, Perkins, 2000). Whilst some studies have reported a sensitivity of more than 80% in diagnosing pulmonary TB disease (Grzybowski et al., 1974, Behr et al., 1999), others have reported low and variable results (range 20 to 60%) (Aber et al., 1980, Urbanczik, 1985). Whilst smears are convincingly positive in individuals with cavitary pulmonary disease, they are often negative in individuals with extensive, non-cavitary disease (Canetti, 1955, Gomes et al., 2003, Palaci et al., 2007). In HIV-positive individuals, smear microscopy has a reduced sensitivity detecting only 22 to 43% of active cases (Getahun et al., 2007, Tattersfield, 2005). In addition, the diagnosis of paediatric TB remains a challenge due to difficulty in collecting respiratory specimens and the presentation of TB disease in children (Marais et al., 2006b, Zar et al., 2005, Marais et al., 2006a, Chiang et al., 2015). Smear-negative TB is associated with unfavourable treatment outcomes, highlighting the need for more sensitive and rapid tests to detect smear-negative cases (Hargreaves et al., 2001, Harries et al., 1999). For these reasons, the WHO now conditionally recommends the GeneXpert MTB/RIF as the initial line of diagnosis in adults and children who are suspected of having active TB disease (WHO, 2013b)

The two most commonly used microscopy techniques to diagnose active TB disease are conventional microscopy [carbol-fuschin methods, i.e. ZN or Kinyoun acid-fast stains] and fluorescent microscopy (auramine-O or auramine-rhodamine). Conventional staining can be viewed using artificial light or reflected sunlight (Foulds and O'brien, 1998), whereas fluorescent microscopy utilises an acid-fast fluorochrome dye and bacilli are viewed under an

intense light, i.e. a halogen lamp or high-pressure mercury vapour lamp (Steingart et al., 2006). A systematic review analysing 45 studies comparing conventional microscopy with fluorescent microscopy concluded that fluorescent microscopy was more sensitive than conventional microscopy (Steingart et al., 2006). It was suggested that the successful widespread implementation of fluorescence microscopy in TB endemic regions may improve TB case-finding via the increased sensitivity of the method as well as the decreased time spent on microscopic examination, whereby fluorescent microscopy uses a 25X objective lens compared to conventional microscopy that requires a 100X objective lens (Steingart et al., 2006). The limitations of fluorescent microscopy are that it is more expensive and it requires additional maintenance and operational requirements. These factors hamper its application as a point-of-care diagnostic in poorer regions. Efforts to improve the sensitivity of microscopy have been assessed and these include prior decontamination of the specimen with NaOH or bleach (Cattamanchi et al., 2010, Srikanth et al., 2009) as well as concentration through centrifugation and the use of magnetic beads (Albert et al., 2011).

1.6.2.2 Culture-based methods

Sputum culture remains the gold standard method for the diagnosis of active TB disease and is required for downstream line drug-susceptibility testing (DST), mycobacterial speciation and strain typing (Chegou et al., 2011). In resource poor settings, where this technology cannot be afforded for every specimen, the WHO recommends that culture is performed on patients with suspected pulmonary TB who are repeatedly smear-negative. In addition, it is also recommended for drug-susceptibility surveillance as well as to confirm cases of treatment failure and relapse disease (WHO, 2017b). Culture is more sensitive than microscopy and requires between ten and 100 viable organisms per mL of sputum (Colebunders and Bastian, 2000, van Zyl-Smit et al., 2011). The improved sensitivity of culture in HIV-positive individuals was demonstrated in a review of a consecutive sample of 109 patients from Khayalitsha, South Africa, where 49% of patients had negative direct microscopy smears but positive *M. tuberculosis* cultures (Coetzee et al., 2004). The main limitation with respect to culture is that *M. tuberculosis* is a slow growing pathogen, requiring up to eight weeks to yield a positive result (Ryu, 2015, Reinisch and Kaufmann, 1961). This delay is further exacerbated in regions where HIV is prevalent since the incubation time is linked to the bacillary load which is generally reduced in HIV-positive individuals (Johnson et al., 1998, Brindle et al., 1993). Conventional culture involves the growth of organisms on solid media made from egg

[Löwenstein–Jensen (LJ)] or specialised agar (e.g. Middlebrook 7H10/11). More recently, automated liquid culture systems such as the MB/BacT (Biomerieux), BACTEC 9000 (Becton Dickinson Diagnostic Systems, Sparks, MD) and BACTEC MGIT 960 assay (Becton Dickinson Diagnostic Systems, Sparks, MD), have become more widely available (Pfyffer et al., 1997b, Pfyffer et al., 1997a, Chew et al., 1998). These systems detect growth by measuring changes in gas pressure, the production of carbon dioxide or the consumption of oxygen fluorometrically or colourimetrically. Liquid culture systems have a faster turn-around-time and are more sensitive than solid culture; however, a drawback of these methods are that they are more prone to contamination (Hanna et al., 1999, Cruciani et al., 2004).

1.6.2.2.1 Culture-based drug-susceptibility testing

Traditionally, the procedure to determine phenotypic drug resistance was carried out using the proportion method with solid agar. An *M. tuberculosis* isolate was considered to be resistant if more than 1% of colonies grew on media containing a critical concentration of a particular drug (Canetti et al., 1969). This method is being replaced by a standardised protocol adapted for the BACTEC MGIT 960 (Dheda et al., 2016).

1.6.2.3 Molecular diagnostics

In the past, identification of *M. tuberculosis* from culture was made on the basis of colony morphology, growth characteristics and select biochemical reactions in the laboratory (Mabilat et al., 1994). Recently developed molecular methods have allowed for the definitive identification of *M. tuberculosis* DNA, metabolites and cell wall products from culture samples. These methods include nucleic acid hybridization probes, line probe hybridization assays, DNA sequencing and matrix-assisted laser desorption/ionisation time of flight mass spectrometry (Anochie et al., 2012, Ceyssens et al., 2017). Concerns regarding the severity of TB disease, nosocomial transmission and patient management have led to the further development of molecular assays that can be performed directly on patient specimens so as to eliminate the culture step and subsequently reduce the turn-around-time (Moore et al., 2005). In addition, these assays allow for the identification of common genetic mutations that lead to phenotypic resistance with certain first-line antibiotics. These methods include the commercial, WHO approved Hain MTBDR*plus* line probe assay (Hain Lifescience, Nehren, Germany) and

nucleic acid amplification tests (NAATS), namely the GeneXpert MTB/RIF test (Cepheid Sunnyvale, CA) as well as the amplified *M. tuberculosis* direct test (MTD; Hologic Gen-Probe), which have both received FDA approval and clearance.

The GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA), developed in 2004, is a PCR-based test that integrates DNA extraction, genomic amplification as well as the semi-quantitative detection of MTBC and resistance to RIF (WHO, 2014d). Resistance to RIF is determined using a hemi-nested PCR to amplify the resistance determining region of the *rpoB* gene (Helb et al., 2010). The test is performed in a single, commercially available cartridge that is simple to perform and greatly reduces the risk of contamination due to its closed amplification system. Initially, designed as a point of care tool, the GeneXpert MTB/RIF assay has a fast turn-around-time, providing results within two hours. In addition, the test is simple to perform and requires minimal training, therefore making it an ideal diagnostic for peripheral health care facilities.

Since 2010, the WHO has recommended the use of this test for individuals with suspected pulmonary disease in developing nations with a high prevalence of TB (WHO, 2014d). The assay has a limit of detection of 131 organisms per mL (Helb et al., 2010, Theron et al., 2011) and a sensitivity of 89% and 67% in smear-positive and –negative individuals with pulmonary TB disease, respectively (Steingart et al., 2014). Since the GeneXpert MTB/RIF assay is able to detect *M. tuberculosis* in approximately 70% of smear-negative pulmonary TB cases, it is an attractive diagnostic tool in the HIV-positive population (Boehme et al., 2011), particularly as this demographic are still capable of transmitting TB (Behr et al., 1999). However, in two studies the sensitivities were reported to be as low as 43 and 28% in smear-negative TB patients (Lawn et al., 2011, Sohn et al., 2014). The assay is also beneficial in identifying certain forms of EPTB disease, i.e. TB meningitis in HIV-infected individuals (Patel et al., 2013). Between 2010 and 2016, the assay has been implemented in 130 countries with more that 23 million test cartridges purchased (Albert et al., 2016). This test has had a drastic impact on TB diagnosis, with a 3- to 8-fold increase in MDR-TB detection and testing worldwide (Albert et al., 2016). In 2017, the next-generation GeneXpert MTB/RIF Ultra assay (Ultra) was developed with improved TB detection capabilities and more definitive identification of RIF susceptibility and resistance (Chakravorty et al., 2017). The Ultra assay showed a 6.5% increase in sensitivity in clinical sputum samples (81 versus 87.5%) and a 13% increase in sputum smear-negative samples (78.9 versus 66.1%) when compared to the GeneXpert. Changes to the cartridge design, thermal cycling parametres, mutation detection chemistry and semi-quantitative categories are available (Chakravorty et al., 2017). The Ultra is currently being rolled-out in

South Africa in a phased approach, moving from Gauteng into additional provinces (WHO, 2017c).

While the GeneXpert is a major advancement in TB diagnostics, this assay still has several limitations. Firstly, it can only detect resistance to RIF. While RIF resistance serves as a marker for MDR-TB, the GeneXpert is unable to detect INH/RIF mono-resistance and increasing rates of RIF mono-resistance (RMR) have been reported (Mukinda et al., 2012, Dramowski et al., 2012, Sanders et al., 2006). The rise in RMR-TB requires that INH resistance needs to be confirmed phenotypically, if genotypic RMR-TB is detected (Mukinda et al., 2012). Lastly, the Xpert does not provide any information with regards to resistance to any other TB drugs, therefore it cannot replace conventional DST (at least in its current format) which requires a positive *M. tuberculosis* culture. For these reasons, culture remains the gold standard for TB diagnosis as it not only confirms the presence of viable *M. tuberculosis* organisms, but provides valuable material for down-the-line DST and speciation (Ryu, 2015). Additionally, the GeneXpert assay can only be used as an initial diagnostic tool and not for treatment monitoring as the system cannot differentiate between live and dead organisms (WHO, 2014d).

1.6.2.4 Other diagnostic assays

The urine lipoarabinomannan (LAM) assay is used to detect the mycobacterial antigen, LAM, in urine which serves as a biomarker for active TB disease. A systematic review and meta-analysis of seven studies reported a sensitivity and specificity range of 13 – 93 and 87 – 99%, respectively in microbiologically confirmed TB cases (Minion et al., 2011). The sensitivity was inversely correlated with CD4 count illustrating that the test is appropriate for individuals with advanced immunosuppression; however, the sensitivity was still suboptimal for clinical use (Minion et al., 2011). Whilst the LAM assay is an attractive tool for TB diagnosis in HIV-positive and paediatric patients due to its low-cost, ease of use and point-of-care features, it is currently not recommended as a screening tool for active TB disease. It can however be used to assist in the diagnosis in individuals a CD4 count of less than 100 cells per μL or HIV-positive patients that are extremely ill (WHO, 2015c, Lawn et al., 2009). Future promising diagnostic assays that require validation include the identification of volatile organic compounds in breath, sweat and urine (Zetola et al., 2017, Chambers et al., 2012, Banday et al., 2011) as well as blood-based host transcriptional profiles (Berry et al., 2010, Anderson et

al., 2014). For further information on other point-of-care diagnostics the reader is referred to an excellent review on the subject (Dheda et al., 2013).

1.7 TREATMENT

1.7.1 Drug-susceptible TB

Treatment of drug-susceptible TB consists of a six-month, four-drug combination regimen that was developed more than four decades ago (Zumla et al., 2013, Kerantzas and Jacobs, 2017). The first two months of treatment, termed the initial phase or early bactericidal phase, comprises administration of four-drugs including INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). The remaining four months of treatment, termed the continuation or sterilization phase, includes RIF and INH (Nahid et al., 2016). The treatment of TB with combination drugs is multifaceted and has three identifiable goals which include the following:

1. Target the actively replicating bacilli that exist within the lung and elsewhere. This will ultimately reduce the bacterial load, decrease the severity of disease and reduce the duration of infectiousness by achieving smear- and culture-negativity.
2. Prevent the emergence of drug-resistant strains. Treatment of TB with a single antibiotic may result in the selection of resistant strains leading to treatment failure and transmission of resistant strains within the community. Hetero-resistance, defined as the occurrence of both drug-susceptible and drug-resistance isolates within the same clinical sample has been described, and complicates treatment management (Kaplan et al., 2003, Post et al., 2004, Shamputa et al., 2004).
3. Achieve complete sterilization of the infection. This is achieved through the 'continuation phase' of therapy that aims to eliminate bacteria that are less metabolically active or 'sporadically multiplying' as these organisms carry the risk of potential relapse disease (Mitchison, 1985, Mitchison, 1979).

Figure 1.4 illustrates a hypothetical model for TB chemotherapy. Overall, the treatment success rates using this regimen are high amongst all new TB cases (86%) (WHO, 2015a). In South Africa, these rates vary across the country, with high success rates reported in Uthungulu, Kwazulu Natal (90%), Western Cape (83%) and Johannesburg (80%) and poor success rates

in Limpopo (58% at provincial level and 47% in Vhembe) (Budgell et al., 2016, Day et al., 2011).

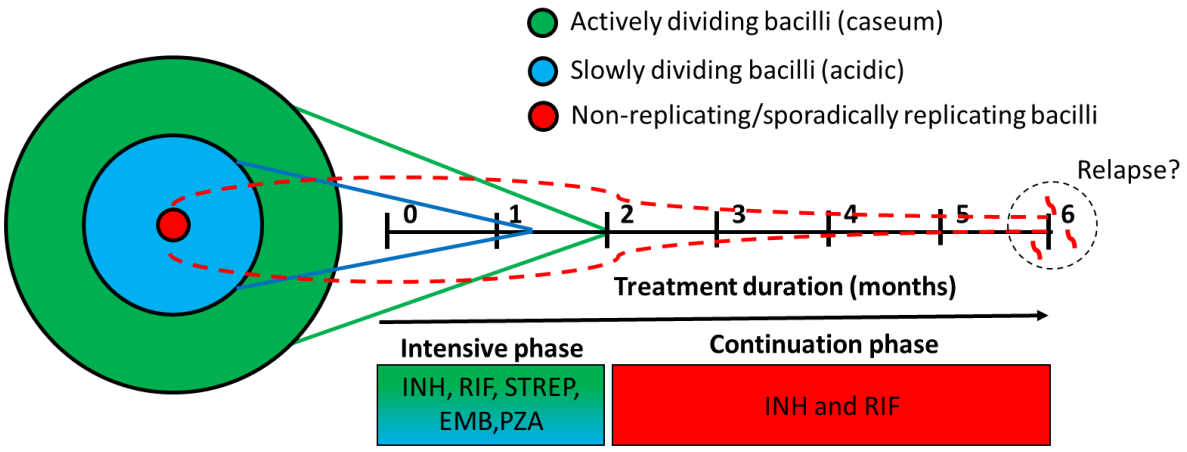


Figure 1.4 Hypothetical model of the bactericidal activity and sterilizing effect of first-line tuberculosis chemotherapy. The model depicts three populations of *M. tuberculosis* found in active disease. The green circle represents the bulk of the bacterial population. These actively replicating bacilli are present in the liquefied caseum of pulmonary cavities. The large numbers of organisms in this population may contribute to the acquisition of drug-resistant mutations in sub-optimal treatment conditions. The blue circle consists of bacteria that replicate more slowly due to stress factors including local acidic conditions, reduced oxygen concentration as well as the presence of reactive oxygen and nitrogen intermediates, amongst others. The clearance of organisms in population A and B during the 'intensive' phase of first-line treatment result in conversion to sputum smear- and culture-negative. Adapted from Iseman et al. (2000). Drawn by Amanda McIvor.

Whilst this regimen is referred to as 'short course', the treatment duration is long, making non-adherence amongst patients a likely possibility. A systematic review based on 14 studies concluded that default was most likely to occur after the two month intensive phase and later during the 6-month regimen, highlighting the urgent need to shorten the current regimen (Kruk et al., 2008). In the 1900's, directly observed therapy (DOTS) was implemented worldwide into TB control programs to encourage patient adherence and prevent resistance to RIF (WHO, 2002, WHO, 2003). DOTS is an internationally recommended approach to TB control and is endorsed by both the WHO and International Union Against Tuberculosis and Lung Disease (WHO, 2002, WHO, 2003). DOTs has five key components which include the following: (i) political commitment, (ii) case detection by smear microscopy, (iii) systems to ensure a regular and uninterrupted supply of antibiotics, (iv) standard recording and reporting systems and (v) standard short course chemotherapy that is administered where patient treatment is observed and supervised. Various studies have yielded conflicting results of the effectiveness of DOTS

(Zwarenstein et al., 1998, Wright et al., 2004, Walley et al., 2001, Volmink and Garner, 1997, Pope and Chaisson, 2003, Newell et al., 2006). Results from a systemic Cochrane review concluded that DOT had no important effect on TB treatment or cure when compared to self-administration of TB treatment (Volmink and Garner, 2007).

1.7.2 Drug-resistant TB

Treatment of MDR-TB is based on a standardised (DOTS-plus) approach and the recommended guidelines are outlined in Table 1.4. The regimen consists of treatment with at least four drugs for eight months (intensive phase) and a total treatment duration of 21 to 24 months (WHO, 2011). The recommended treatment duration for patients with newly diagnosed TB (i.e. patients not previously treated for TB disease) is 20 months and can be adjusted depending on patient response. Treatment of MDR-TB is toxic, (Loveday et al., 2015) poorly tolerated, prolonged, more expensive and has a poor treatment success rate compared to drug-susceptible TB (around 50%) (Pai et al., 2016, Liu et al., 2011, Pietersen et al., 2014, Dheda et al., 2014). The growing number of MDR-TB cases has led to the decentralization of treatment facilities to peripheral clinics which has resulted in better outcomes (Loveday et al., 2015). In contrast to the standardised treatment regimen employed for MDR-TB, treatment of XDR-TB is based on an individual approach whereby the DST results and the patients' medical history is taken into account, Table 1.4. In recent years, the emergence of incurable and/or totally drug-resistant TB (i.e. XDR treatment failures or resistance spanning beyond XDR-TB) has been reported in many counties including South Africa (Dheda et al., 2014). Due to the scarcity of long-stay treatment facilities, XDR treatment failure patients who are discharged from hospital can transmit untreatable strains in the community (Pietersen et al., 2014). Indeed, XDR-TB strains have been demonstrated to be highly transmissible (Shah et al., 2017a, Ghandi et al., 2006, Wells et al., 2007, Moro et al., 1998, Ritacco et al., 1997, Pearson et al., 1992). These findings have raised many ethical, legal and logistical predicaments and debates regarding patient long-term accommodation as well as individual rights regarding unrestricted travel and work (Dheda and Migliori, 2012). Interrupting the transmission of drug-resistant and untreatable TB strains within the community should be a top priority of national TB control programmes. Furthermore, efforts to reduce this treatment regimen, limit drug-toxicity and promote treatment adherence are urgently required.

Table 1.4 Recommended treatment regimens for MDR and XDR-TB

Treatment of MDR-TB	Treatment of XDR-TB
Use at least four drugs to which the strain is susceptible or likely to be susceptible (drugs that were included in a previous regimen for ≥ 3 months are not recommended; excludes PZA and EMB) (WHO, 2011)	Regimens should be based on individual DST profiles and similar principles as those outlined for MDR-TB, i.e. the use of at least four drugs is likely to be successful
Use a backbone of later-generation FQ (e.g. moxifloxacin or levofloxacin) and injectable drug (AMK, KAN or CAP) (WHO, 2011)	Adverse events are associated with CAP (renal failure, hypokalemia, hypomagnesia and hearing loss) (Müller et al., 2013)
Add any first-line drug or group four drug to which isolate is susceptible to a 'non-failing' regimen	Differential susceptibility to FQs can occur and genotypic susceptibility testing can be used to optimise the treatment regimen (clofazamine, linezolid and high dose-INH) (Müller et al., 2011, Jacobson et al., 2010).
Injectable drugs are to be used for 6 to 8 months, and 21 to 24 months total treatment is recommended (WHO, 2011)	Group 4 and group 5 drugs can be used, but their effectiveness is uncertain. (Dheda et al., 2014)
Oxazalidonones (linezolid) can be used for an effective regimen in the treatment of MDR-TB (is the isolate is resistant to fluoroquinolones) and XDR-TB. Monitoring for toxicity (neuropathy and bone marrow depression) is essential (Sotgiu et al., 2012, Lange et al., 2014, Chang et al., 2013)	
Bedaquiline and Delaminid can be added in cases of high toxicity or if high-grade resistance precludes the use of a four-or-more drug regimen that is likely to be successful. Monitoring is required since both of these drugs prolong QT interval* (WHO, 2014c, WHO, 2013c)	
Psychosocial and financial support are required for adherence	
Patients should be monitored for adverse reactions which are common with the use of second-line drugs (Müller et al., 2013)	
A single drug should not be added to a failing regimen	

Abbreviations: MDR-TB = multidrug-resistant TB; XDR-TB = extremely drug resistant TB; DST = drug susceptibility testing; INH = isoniazid; PZA = pyrazinamide; EMB = ethambutol; AMK = amikacin; CAP = capreomycin; KAN = kanamycin; * QT interval: In cardiology, a QT interval is defined as a measure of the time between the start of the Q wave and the end of the T wave in the hearts electrical cycle. Adapted from (Dheda et al., 2016)

1.7.3 New drugs

After many years of stagnation, promising new TB drugs are under clinical development and are summarised in Table 1.5. Two of these drugs, namely Bedaquiline and Delamanid, have been registered for the treatment of drug-resistant TB. Other drug candidates currently in clinical trials initially used to treat other infectious diseases have shown efficacy against *M. tuberculosis*. Examples of these repurposed drugs include the later generation fluoroquinlones (i.e. moxifloxacin, levofloxacin and gatifloxacin), the oxazolidinones, linezolid and sutezolid as well as clofazamine (initially used in the treatment of leprosy). Advances in new TB drugs, treatments and algorithms are reviewed extensively elsewhere (Zumla et al., 2013).

Table 1.5 New chemical entities for the treatment of tuberculosis

Antibiotic	Approval	Mechanism of action	Genes associated with resistance
Bedaquiline/ TMC 207 (class: diarylquinolone)	Yes USA (FDA), European Union (EMA), Russian Federation (Pharmstandard), South Korea (MFDS) and South Africa (MCC)	Inhibition of *M. tuberculosis* ATP synthase	*atpE* gene mutation encoding subunit C of ATP synthase; Mutations in Rv0678 coding for repressor of *M. tuberculosis* efflux pump
Pretomanid/ PA-824 (class: nitroimidazole)	None	Inhibition of mycolic acid biosynthesis and generation of mycobactericidal nitrogen oxide derivatives (dormant *M. tuberculosis*)	Mutation in *fbiA*, *fbiB* or *fbiC* lead to impaired coenzyme F420 synthesis; Mutation in Rv3547 coding for deazaflavin-dependent nitroreductase (inhibit activation of pro-drug)
Delamanid/ OPC 67683 (class: nitroimidazole)	European Union (EMA)	Inhibits mycolic acid biosynthesis	Mutation in mycobacterial Rv3547 prevents activation of drug
SQ-109 (class: diamines)	None	Inhibits mycobacterial cell wall synthesis, specifically targeting the transmembrane transporter, encoded by the *mmpl*3 gene	Possibly mutation in *mmpl*3 gene?

Abbreviations: FDA = Food and Drug Administration; EMA = European Medicines Agency; MFDS = Ministry of Food and Drug Safety; MCC = Medicines Control Council. Table adapted from Dheda et al. 2016.

1.7.4 Prophylaxis

Preventative treatment aims to eliminate LTBI before reactivation disease occurs and forms a significant component of the WHO's post-2015 strategy to eliminate TB disease (WHO, 2013a). In high burden settings such as South Africa, preventative therapy is generally restricted to high risk populations, including HIV-positive individuals and children under the age of five who have household contacts with active disease. In low burden countries, immigrants with LTBI are targeted for preventative therapy. Generally, low burden countries such as the USA, prefer preventative therapy than mass vaccination with BCG of new born infants which is practised in South Africa. The identification of biomarkers that are able to identify 'high risk' individuals with LTBI that are likely to develop active TB disease is an important area of research. The identification of these individuals would target them for preventative therapy ultimately reducing the pool of latently infected individuals and protecting the greater population. It is however important to rule out active TB disease before administering preventative treatment as this may result in the acquisition of drug resistance.

1.8 PREVENTION AND CONTROL IN SOUTH AFRICA

The national tuberculosis treatment program (NTP) was established in 1994 with the goal of integrating TB services into primary healthcare systems (Karim et al., 2009, Churchyard et al., 2014). The NTP was confronted with multiple challenges including the emergence of the HIV epidemic and the increasing burden of MDR and XDR-TB strains. To combat this dual epidemic of TB and HIV, the National Strategic Plan for HIV, sexually transmitted infections (STIs) was developed in South Africa (2012 – 2016) to halve the incidence and mortality due to TB disease by 2016 and to eliminate all new TB infections, death and stigma by 2032 (Churchyard et al., 2014). The three main strategies to achieve these goals include Find, Treat and Prevent (Churchyard et al., 2014). These are detailed further below.

1.8.1 Find

The identification of patients who are infectious is a key priority in the control of TB disease. To identify these patients, South Africa has an extensive network of microscopy centres and diagnostic laboratories, some of which are equipped with the facilities to perform culture and DST (Churchyard et al., 2014). In 2011, the GeneXpert was introduced with the intention of

ultimately replacing smear microscopy. In addition to identification of infectious individuals, an additional strategy specified by the CDC also includes the identification and treatment of persons who are non-infectious. Examples of patients who are non-infections include those with EPTB disease, primary pulmonary disease (in children), bacteriologically unconfirmed cases of pulmonary disease (i.e. subclinical disease) and LTBI (CDC, 1988). An additional component of 'Find' includes case finding and contact tracing (Palomino et al., 2007). In low TB prevalent countries, such as the USA, a great deal of emphasis is placed on contact tracing; however, in high burden countries such as RSA, contact tracing remains a significant challenge.

1.8.2 Treat

In South Africa, the introduction of community-based tracing teams has led to a significant improvement in the treatment success rates in both smear-positive and –negative/EPTB cases (79 and 76%, respectively) (Churchyard et al., 2014). The treatment success rates among retreatment cases is poorer at 66.3%. Loss to follow-up remains a major challenge where up to 25% of patients do not start treatment and continue to transmit *M. tuberculosis* in the community (Claassens et al., 2013). In addition to treating TB, it is important that HIV-infected patients are placed on anti-retroviral medication and these patients are not lost to follow-up (Dalal et al., 2008).

1.8.3 Prevent

The prevention of TB constitutes a neglected area of TB control in South Africa. Examples for the prevention of TB disease include the following: (i) screening of high risk individuals to improve case detection, (ii) treatment of LTBI with isoniazid preventative therapy, (iii) infection control, (iv) early ART initiation for HIV-positive individuals and (v) TB vaccination programmes.

1.9 KNOWLEDGE GAPS RELATED TO THIS PhD:

The long duration of therapy required to cure TB disease remains one of the core challenges to eradicating this disease. It is believed that this protracted treatment stems from the ability of *M. tuberculosis* to achieve a non-replicating state in the host, thereby establishing sub-populations of bacteria that are difficult to eradicate. As the majority of TB drugs are only effective against actively replicating organisms, these organisms can become phenotypically resistant to many of these drugs. In this thesis, the non-replicating of state of *M. tuberculosis* in patients with drug-susceptible, active pulmonary disease was investigated. Chapter two includes a literature review of these non-replicating states in *M. tuberculosis* as well as other bacterial organisms. Furthermore, other pathogenic organisms that are able to persist in the host are discussed. A further issue addressed in this thesis is the long duration of culture. To address this, methods to reduce the time to culture diagnosis, particularly in HIV-positive individuals, were investigated.

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BACKGROUND OVERVIEW

CHARACTERIZATION OF **DIFFERENTIALLY CULTURABLE** BACTERIA IN AXENIC CULTURE AND FROM TUBERCULOSIS PATIENTS

CHAPTER 1
Literature review of **Tuberculosis**

Topics and questions addressed in this chapter:

What is tuberculosis (TB)? Section 1.1. Introduction
What is the burden of disease? Section 1.2. Epidemiology
What causes TB? Section 1.3. Aetiology
How does TB disease develop? Section 1.4. Tuberculosis pathogenesis
What are the clinical outcomes of TB disease? Section 1.5. Clinical presentation
How is TB diagnosed? Section 1.6. Diagnosis
How is TB treated? Section 1.7. Treatment
How can TB be prevented? Section 1.8. Prevention and control in South Africa
What can be concluded? Section 1.9. Knowledge gaps related to this PhD

CHAPTER 2
Literature review of **Microbial Dormancy**

As differentially culturable organisms are closely related to dormancy and metabolic quiescence, Chapter 2 focuses on these concepts in other bacteria and more specifically in *Mycobacterium tuberculosis*

Topics and questions addressed in this chapter:

Are all bacteria in a population culturable? Section 2.1. The great plate count anomaly
What are common terms encountered in the literature? Section 2.2. Terminology and definitions
What are the stages of microbial dormancy? Section 2.3. Stages of microbial dormancy
How is dormancy related to disease? Section 2.4. Cellular quiescence and intracellular human pathogens
What about dormancy in *M. tuberculosis*?
Section 2.5. *Mycobacterium tuberculosis*: latency, active disease, the VBNC state and resuscitation
What is the scout hypothesis? Section 2.5.1. The scout hypothesis
What are the physical characteristics of non-replicating *M. tuberculosis?* Section 2.5.2. Phenotypic characteristics of non-replicating M. tuberculosis
What dormancy models are available? Section 2.5.3. *M. tuberculosis* models of non-replicating persistence
What are resuscitation promoting factors? Section 2.5.4. Resuscitation promoting factors

CHAPTER 2

MICROBIAL DORMANCY

"Counsel: I beg your pardon m'lud.

Judge: Well, I mean, your witness is dead.

Counsel: Yes m'lud. Er, well, er, virtually, m'lud.

Judge: He's not completely dead?

Counsel: No, he's not completely dead m'lud. No. But he's not at all well."

Monty Pythons Flying Circus, Episode 3, Court Scene

2.1 THE GREAT PLATE COUNT ANOMALY

In 1985, a concept known as the "great plate count anomaly" was described by scientists, Staley and Kanopka (Staley and Konopka, 1985). This phenomenon illustrated the discrepancies observed between the numbers of cells that can be viewed under a microscope, which are orders of magnitude greater than the number of viable organisms that can be cultured in the laboratory under certain conditions. These observations illustrate that bacterial organisms isolated in pure culture form are not representative of the true biodiversity that occurs in the natural environment. In fact, the organisms that are culturable represent less than 1% of all microbial species and have previously been described as the 'weeds' of the microbial world (Hugenholtz, 2002), Figure 2.1. These observations, in association with morphological, microbiological and molecular evidence, suggest that a large proportion of these organisms are in a metabolically inactive or dormant state when trying to recover them in the lab.

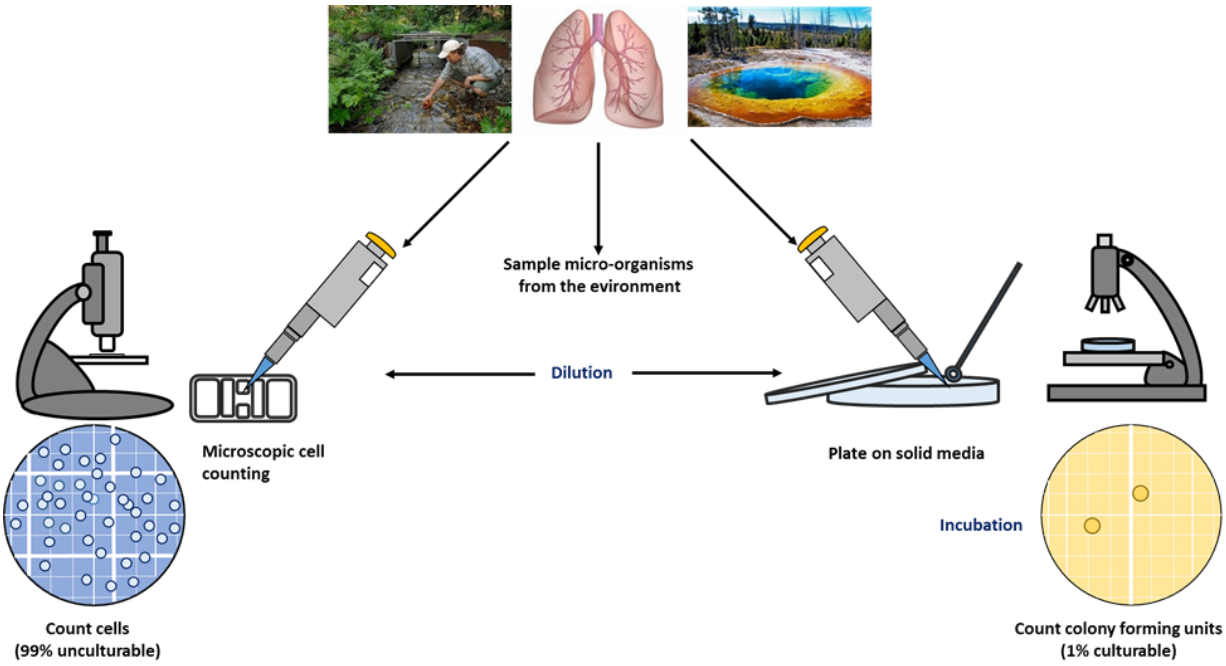

Figure 2.1 The great plate count anomaly. Plate cell counts obtained by cultivation (right) are orders of magnitude lower than cell counts observed under the microscope (left). Figure adapted from: https://commons.wikimedia.org/wiki/File:Great-plate-count-anomaly.svg. Figure drawn by Amanda McIvor.

2.2 TERMINOLOGY AND DEFINITIONS

In nature, harsh and unpredictable environments often result in conditions that are not favourable for growth and reproduction. When subjected to these conditions, many taxa, including microorganisms, have evolved mechanisms to ensure their survival by entering into a reversible state of low metabolic activity or dormancy to reduce energy expenditure and increase preservation of the genotype (Guppy and Withers, 1999). These metabolically inactive states are most likely responsible for the large microbial bio-diversity whereby repeated transitions to and from the 'seed bank' preserve the taxonomic richness of many ecosystems (Jones and Lennon, 2010, Lennon and Jones, 2011, Stevenson, 1977). Definitions of terms relating to these states of low metabolic activity in the laboratory and in disease and pathogenesis are commonly encountered in the literature are outlined in detail below:

2.2.1 Dormancy

Dormancy can be broadly defined as a 'reversible state of low metabolic activity in which cells can persist for extended periods without division' (Kell and Young, 2000). Dormancy is a phenotype of the bacilli and common term that is used across many disciplines, extending from botany (i.e. seeds and buds) (Soppe and Bentsink, 2016) to cancer research (Wheelock et al., 1981, Sosa et al., 2014), where tumours can enter into a state of dormancy and non-proliferation. In the microbial world, the spore is the most often cited form of dormancy. In some bacterial species, for example *Bacillus* spp. and *Clostridium* spp., asymmetric cell division can occur in response to growth-restrictive conditions whereby a hardy and inactive daughter cell, termed a spore, is produced (Stragier and Losick, 1996). Sporulation is a complex process that occurs in response to nutritional deficiency, inhibition of GTP synthesis or stationary growth where a defined and enriched medium is exhausted. Sporulation is tightly regulated and involves transcriptional and subsequent morphological changes that result in the formation of a biologically resilient and robust spore (Dworkin and Shah, 2010). A defining characteristic of the spore morphotype is a thick protective coat made of unique glycoproteins, equipping the organism with a 'damage-proof' phenotype, rendering the spore resistant to heat, radiation, desiccation, antibiotics and other forms of external pressure (Setlow, 2011). Spores can remain dormant for many years and possess the ability to re-enter a vegetative state when conditions that favour growth prevail. Much interest in germination of spores stems from food spoilage and foodborne disease, and their role in infectious disease (for e.g. *Bacillus anthracis* and pulmonary anthrax). Few reports have suggested that *M. tuberculosis* and close relatives are able to form spore-like structures (Ghosh et al., 2009, Csillag, 1961, Singh et al., 2010), although these results have not been reproducible (Traag et al., 2010). Consequently, the use of this term in the TB field is fraught with problems as there is neither strong evidence for or against the formation of specialist spore-like morphotypes in mycobacteria.

2.2.2 Microbial seed bank

Dormancy results in the generation of a seedbank, which has been defined as 'a reservoir of dormant individuals that can potentially be resuscitated in the future under different environmental conditions' (Lennon and Jones, 2011). From an ecological perspective, seedbanks are essential as they maintain biodiversity (i.e. allow for competing organisms to coexist), preserve community

stability, influence fundamental evolutionary processes and safeguard organisms from extinction following perturbation events (Lennon and Jones, 2011, Locey et al., 2017, Aanderud et al., 2015, Wang et al., 2015, Shoemaker and Lennon, 2018). While macroscale factors such as nutrient and resource limitation were understood to govern the transitions to and from dormancy (Lennon and Jones, 2011), microscale factors including the spatial structure of the environment and trophic complexity have also been implicated in seedbank dynamics (Locey et al., 2017).

2.2.3 The viable but non-culturable state and cellular quiescence

The 'viable but non-culturable state' (VBNC) or cellular quiescence are both terms used in the literature to describe cells that fail to grow under standard culture and laboratory conditions and is similar to dormancy however, some cases of VBNC have been associated with measurable metabolic activity, maintenance of membrane potential and no drastic morphological differentiation (Mukamolova et al., 2003, Rao et al., 2008, Gengenbacher et al., 2010, Rittershaus et al., 2013). This state is reversible and cells are able to resuscitate and resume normal metabolic activity when subject to a subset of conditions such as nutrient rich medium or culture filtrate (CF) from growing cells (Dworkin and Shah, 2010). There is however some controversy where the terms dormancy and VBNC are used interchangeably to describe the same physiological state. The VBNC state was first described in *Vibrio cholera* and *Escherichia coli* (Xu et al., 1982) and has subsequently been described in numerous bacterial human pathogens including *Campylobacter spp.* (Cappelier et al., 1999), *Listeria monocytogenes* (Besnard et al., 2000), *Helicobacter pylori* (Adams et al., 2003), *Mycobacterium tuberculosis* (Shleeva et al., 2002, Mukamolova et al., 2010), *Enterococcus faecalis* (Figdor et al., 2003), *Salmonella* spp. (Morishige et al., 2013, Cho and Kim, 1999, Waldner et al., 2012), *Legionella pneumophila* (Faulkner and Garduño, 2002), *Micrococcus luteus* (Mukamolova 1998) and *Vibrio vulnificus* (Whitesides and Oliver, 1997, Oliver and Bockian, 1995) to name a few.

2.2.4 Tolerance and persistence

Bacterial tolerance refers to the ability of cells to survive killing by antibiotics without expressing or using genetic resistance mechanisms (Lewis, 2007). In this case, bacteria may exist in a state where the drug target is not vulnerable but other areas of metabolism are active. Persister cells

were originally described in 1944 by Bigger, who made the observation that in a growing population of *Staphylococcus* spp., a subpopulation of cells that were not genetically penicillin-resistant, displayed increased tolerance in the presence of this antibiotic, while others died (Bigger, 1944). When transferred to fresh medium, the population expanding from these penicillin-tolerant organisms were now susceptible (Bigger, 1944). It was proposed that the penicillin-tolerant populations of *Staphylococcus* were dormant and non-dividing however, as the observation became more frequently reported, the term persister became widely used. Other terms commonly used to describe these populations include 'phenotypically resistant' as well as 'phenotypically tolerant'. In 1957, persistence was described in *M. tuberculosis* to two different antibiotics: INH and para-aminosalicylate (Hobby and Lenert, 1957). As the mechanism of action of these two compounds, along with penicillin, are unique (i.e. INH inhibits mycolic acid synthesis, para-aminosalicylate inhibits folate synthesis and penicillin targets peptidoglycan synthesis), persistence was shown to be independent from the antibiotic used and its associated pathway (Gold and Nathan, 2017). As persister cells constitute only a small percentage of the total population, there have been suggestions that persistence is a form of kin selection (Lewis, 2007, Lennon and Jones, 2011, Balaban et al., 2004, Avery, 2006, Gardner et al., 2007). Therefore, in the presence of a lethal agent, such as antibiotics, whereby most of the population is killed, these persister cells can propagate to restore the population containing the genome that they shared with their kin. For more detailed information, excellent reviews of the biology of persister cells and insights into their role in infectious diseases are available (Lewis, 2007, Gold and Nathan, 2017).

2.3 STAGES OF MICROBIAL DORMANCY

2.3.1 Initiation

There are two ways in which cellular dormancy can be triggered, namely responsive and spontaneous initiation (Lennon and Jones, 2011). In terms of responsive initiation, the transition to the dormant state occurs in response to environmental cues, such as changes in abiotic factors (i.e. temperature, pH, osmotic pressure and light) and/ or nutrient limitation/starvation. Microorganisms can detect changes in the environment (abiotic factors) via membrane-bound histidine kinase sensors (Lennon and Jones, 2011). These changes elicit an intracellular cascade of events that ultimately lead to alterations in gene expression and subsequent modifications in

protein synthesis (Mascher, 2006). Furthermore, nutrient limitation, involving intracellular depletion of amino acids, fatty acids and carbon sources, can trigger a stringent response (Aertsen and Michiels, 2004). Spore formation is an example of responsive dormancy initiation and involves a tightly regulated process that requires a great deal of energy investment. Sporulation has been described in members of the Bacillus and Clostridium genus as well as certain Fungi (Paredes-Sabja et al., 2011, Piggot and Hilbert, 2004, Olguín-Araneda et al., 2015, Sloan et al., 1960, Errington, 1993, Paredes-Sabja and Sarker, 2009). Briefly, the transition of *B. subtilis* from the vegetative state to form an endospore is controlled by the master transcriptional regulator, Spo0A. The transcriptional activity of SpoA in turn is activated by a 'phosphorelay' system consisting of five histidine kinases (KinA-E) (Tan and Ramamurthi, 2014). Ultimately, activation of Spo0A leads asymmetrical division of the cytoplasm resulting a mother cell and smaller forespore, both of which ultimately have different fates (Tan and Ramamurthi, 2014).

In some organisms, the switch from the vegetative state to the dormant state occurs spontaneously. It has been proposed that spontaneous switching is favoured under stable environmental conditional, whereas responsive switching is the preferred mechanism in fluctuating environmental conditions (Kussell and Leibler, 2005). Spontaneous switching has three distinct advantages over responsive switching: (i) organisms avoid the cost of active sensory machinery required for sensing the environment, (ii) these cells represent an "insurance policy" that allows for population survival in stochastic perturbation events and (iii) reduced growth rates of these sub-populations can indirectly benefit other bacteria by reducing the competition for limited nutrients or resources (Gardner et al., 2007, Avery, 2006, Dubnau and Losick, 2006). Persister cells are a good example of spontaneous responsiveness (Lennon and Jones, 2011).

2.3.2 The resting cell

A wide variety of dormant phenotypes exist in the microbial world, some of which have evolved drastic morphological changes such as spores (e.g. *Bacillus* and *Clostridium* spp.), conidia (e.g. *Aspergillus fumigatus*), cysts or akinetes (thick wall resting structures) (e.g. Cylindrospermum spp.). Additional phenotypes include a reduction in cell size to form 'dwarf' cells (Stevenson, 1977, Oliver, 2000, Porter et al., 1995), and coccoid forms (Shleeva et al., 2002, Boucher et al., 1994, Pascoe et al., 2014). While quiescent cells exhibit a wide array of morphological phenotypes,

these cells share some common features. One such feature is the accumulation of carbon stores (Rittershaus et al., 2013). For example, many bacterial species, including *M. tuberculosis*, store fatty acids such as triacylglycerides (TAGS) or wax esters (WE) in the VBNC or persistent state (Garton et al., 2008, Daniel et al., 2004, Kalscheuer et al., 2007). Carbon storage has also been reported in the yeast cell, *Saccharomyces cerevisiae* that accumulates carbon in the form of glycogen, trehalose and TAGS during low growth rates (Gray et al., 2004) and pathogenic *Vibrio cholera* that utilises glycogen stores to prolong survival under nutrient-poor conditions (Bourassa and Camilli, 2009). It is hypothesized that these carbon stores are important source of energy reserves for survival during dormancy and for regrowth when environmental conditions improve. Evidence for these carbon stores as a fuel source for re-growth following cellular quiescence has been demonstrated in the budding yeast, *S. cerevisiae*. Upon exit from quiescence, the trehalose stores that accumulated in stationary phase cultures are utilised as an energy source to initiate growth. Furthermore, Δ*tps1* mutants (i.e. cells that could not synthesize trehalose) displayed reduced growth rates and survival (Shi et al., 2010).

A noteworthy feature of quiescent cells is modification of the cell wall. Virtually all bacteria are surrounded by peptidoglycan (PG), an elastic-like meshwork that conserves the cellular integrity of the organism under changing environmental conditions. PG is a complex polymer that consists of long glycan strands of the repeating disaccharide *N*-acetyl glucosamine- *N*-acetyl muramic acid (NAG-NAM), which are cross-linked via penta-peptide stem chains. Numerous studies have shown alterations in the structural composition of PG in replicating versus non-replicating bacteria. For example, *E. coli* grown to stationary phase exhibits both an increase in absolute PG content (Mengin-Lecreulx and van Heijenoort, 1985) as well as an increase in pentaglycine bridges (cross-linking the glycan chains) (Dougherty and Pucci, 1994). Furthermore, in *E. coli*, changes in the type of cross-links in the PG stem peptides have been reported in declining growth rates where under logarithmic growth conditions, 4 → 3 inter-peptide linkages are produced but in late exponential phase, 3 → 3 inter-peptide linkages occur (Goffin and Ghuysen, 2002). Changes in the level and gradient of PG cross-linking during stationary phase has also been reported in *S. aureus* where fewer pentaglycine cross bridges are present, the cell-wall is significantly thicker than in log-phase cultures (Zhou and Cegelski, 2012). Changes in the cell-wall and the degree of PG cross-linking are also important in spore formation (Atrih et al., 1996). Cell wall modification in *M. tuberculosis* is discussed in detail in section 2.5.2.4.

Additional features of quiescent cells include reduced energy expenditure and primary metabolism. While bacterial endospores are metabolically inert (Setlow, 1995), quiescent cells require energy to support non-growth functions as well as to maintain their membrane potential for ATP synthesis (Lennon and Jones, 2011, Morita, 1982, Van Bodegom, 2007, Rao et al., 2008). Indeed, in non-replicating *M. tuberculosis* cells, ATP levels are maintained constantly at levels approximately 5-fold lower than those measured in actively dividing cells (Rao et al., 2008). Other features of quiescent organisms include macromolecular synthesis and mechanisms for stability and preservation of genome integrity (Rittershaus et al., 2013).

2.3.3 Exit from dormancy

Similar to adopting the dormant state, exit from dormancy can occur stochastically or in response to environmental cues. An increase in the availability of environmental resources allow endospores to undergo a process of germination. These so-called 'germinants', tend to be low-molecular-weight compounds (i.e. single amino acids, sugars or purine nucleotides) that bind to receptors in the inner membrane of spores (Setlow, 2003). This interaction leads to enzymatic activity and hydrolysis of the PG layer of the spore cortex, followed by vegetative outgrowth of the cell (Setlow, 2003, Rittershaus et al., 2013). Stochastic emergence has been reported in non-spore forming organisms including *E. coli* (Balaban et al., 2004), *M. smegmastis* (Buerger et al., 2012b), and to a lesser degree in the spore forming *B. subtilis* (Paidhungat and Setlow, 2000). In addition to sensing environmental cues, bacteria can also exit dormancy in response to signals released by growing bacteria (Votyakova et al., 1994, Kaprelyants and Kell, 1996, Dworkin and Shah, 2010) For example, *B. subtilis* germination occurs in response to muropeptides release from growing *B. subtilis* cells (Shah et al., 2008).

2.4 CELLULAR QUIESCENCE AND INTRACELLULAR HUMAN PATHOGENS

Treponema pallidum (Syphilis), *Borrelia borgdoferia* (Lyme disease), *Coxiella burnetii* (Q fever), *Chlamydia* spp. (range of chronic infections) and *M. tuberculosis* are examples of intracellular human pathogens that are able to persist within the human host for many years without causing disease. It is thought that the dormant intracellular state adopted by these organisms is responsible for the asymptomatic, latent stage of disease and also facilitates pathogenesis. For example, *B. borgdoferia*, the causative agent of Lyme disease, can exist in the form of spirochete round bodies when environmental conditions that are not conducive for growth (Sapi et al., 2011, Brorson et al., 2009). It is thought that these round bodies facilitate the persistence of this pathogen in the host. *Chlamydia* spp. are obligate human pathogens with a unique developmental lifecycle. These species can exist as inert, metabolically inactive elementary bodies (EB), facilitating their uptake by the host cell following infection. The EB remains in a host-derived vacuole (termed the inclusion) after which they are able differentiate into larger and metabolically active reticulate bodies (Hogan et al., 2004). Various line of evidence (for example, disease recurrence and *in-vitro* studies investigating persistence) suggest that these species are able to persist in altered states in the host during chronic infection, and it is supposed that the EB form is responsible for long-term infection. Indeed, EBs are induced following exposure to antibiotics and cytokines, as well as in response to nutrient depletion (Hogan et al., 2004, Harper et al., 2000).

Latency is a clinical term used to describe asymptomatic *M. tuberculosis* infection and was defined by Amberson as 'the presence of any tuberculous lesion which fails to produce symptoms in its presence' (Amberson Jr, 1938). LTBI is established by the arrest of mycobacterial growth prior to the onset of disease or the spontaneous resolution of primary TB disease (Gomez and McKinney, 2004). In most individuals, a cell-mediated immune response is able to arrest disease progression at the site of infection, i.e. the Ghon complex (Ghon, 1923). LTBI is the typical outcome for the majority of individuals exposed to *M. tuberculosis* and chronic infection is likely to persist in the absence of antibiotic intervention. Following years or decades of subclinical persistence, reactivation disease can occur in approximately 10% of cases resulting in active disease and possible transmission (Selwyn et al., 1989, Selwyn et al., 1992). As mentioned previously, it remains unclear whether LTBI is characterised by a population of non-replicating, dormant-like bacilli.

2.5 MYCOBACTERIUM TUBERCULOSIS: LATENCY, ACTIVE DISEASE, THE VBNC STATE AND RESUSCITATION

M. tuberculosis has immune-evading capabilities that enables this organism to survive and persist in the host environment for extended periods of time in the form of LTBI before converting to active, contagious disease (Peddireddy et al., 2017, Gengenbacher and Kaufmann, 2012). In the mammalian host, *M. tuberculosis* infection occurs via the inhalation of airborne droplet nuclei that end up in the lodged in the alveoli where infection is initiated (Kaufmann, 2001). Following infection, these bacteria are subsequently phagocytosed by alveolar macrophages which serve as the host's primary defence mechanism against invading pathogens (Russell et al., 2009). Inside the phagosome, *M. tuberculosis* is subjected to severe environmental conditions that include increased acidification, exposure to reactive oxygen and nitrogen species (ROS and RNS) as well as hydrolytic enzymes and antimicrobial peptides (Flannagan et al., 2009, Nathan and Shiloh, 2000, Huynh and Grinstein, 2007). Therefore, in order to ensure survival, the tubercle bacillus is equipped with a high metabolic plasticity that enables it to switch to an altered state of non-replicating persistence until conditions are favourable to revert to normal replication, resulting in reactivation disease. It is now generally well accepted that *M. tuberculosis* organisms co-exist at different stages of replication during both LTBI and during active disease (Barry et al., 2009). Evidence to support this heterogeneous state can be explained by the use of INH as a successful chemo prophylactic treatment of LTBI in various developed countries. The mechanism of action of INH involves the inhibition of cell wall synthesis and thus only targets replicating organisms. These observations imply that *M. tuberculosis* converts to an INH-susceptible state at some point during latent infection (Fox, 1999). Presently, the factors that control the progression from LTBI to active disease or the factors that maintain the equilibrium between these two states remains poorly understood. Various hypotheses have been suggested and are discussed in further detail below.

2.5.1 The 'scout' hypothesis

An attractive model termed the microbial 'scout' hypothesis describes the equilibrium between dormant and replicating organisms and their association with the transition from LTBI to active TB disease (Epstein, 2009b, Epstein, 2009a, Gengenbacher and Kaufmann, 2012). In LTBI, where

the vast majority of bacteria are hypothesized to exist in a dormant state, few organisms resuscitate stochastically and independent from environmental cues. These 'scouts' or newly active cells sense the environment for available resources and conditions conducive for growth. In adverse environments (i.e. chemotherapeutic treatment, host immunity or lack of available resources) the scout will die, thus preserving the vast majority of the population that will remain in a dormant state. In an on-going process, it appears that at random a small fraction of the dormant population will successively resuscitate in turn to become scouts. If a scout forms under conditions that are permissible for survival and growth, this organism will elicit biochemical signals, possibly resuscitation-promoting factors (Rpfs), that will alert the non-replicating population to re-emerge and multiply, thus achieving the ultimate goal of proliferation of the species (Epstein, 2009b, Chao and Rubin, 2010, Buerger et al., 2012b, Buerger et al., 2012a). The resuscitation and replication of these organisms will ultimately tip the scales of the balance, causing the transition from a 'controlled' LTBI to pathology and reactivation disease. In active disease, the majority of the population will actively divide; however, a small percentage of the population will remain dormant, which may explain the presence of a persister population that is tolerant, requiring longer periods for eradication (Lewis, 2010, Lewis, 2007). Following treatment completion, the remaining non-replicating population will be undetected by conventional culture methods suggesting clinical cure and that sterilization and eradication of *M. tuberculosis* has been achieved. The stochastic awakening of *M. tuberculosis* cells that survived treatment and were not eliminated by the immune system are possibly responsible for recurrent TB disease due to relapse. In light of the above, recurrent disease due to reinfection could also result in active TB disease and possibly a mixed strain infection, if residual bacteria remain viable after the first episode of TB. For example, the inhalation of a new *M. tuberculosis* strain into a host with LTBI may send signals to awake the remaining 'dormant' population, from a previous infection, leading to reactivation disease (McIvor et al., 2017). One line of evidence to support this is that disease attributable to reinfection after successful treatment completion is four times higher than that attributable to new disease (Verver et al., 2005). However, many factors contribute to this increased susceptibility to a second (or subsequent) episode of TB infection such as lung damage as well as the hosts' genetic predisposition. Further work is needed to associate this phenomenon with pre-existing, non-replicating bacteria. Experimental observations in support of the scout hypothesis were illustrated by the revival of environmental cells and spores [including *Bradyrhizobium* and *Mycobacterium*

spp. (non-spore forming) and *Bacillus* and *Streptomyces* (spore forming)] as well as in model microbial species (*Escheria coli and Mycobacterium smegmatis*) (Buerger et al., 2012b).

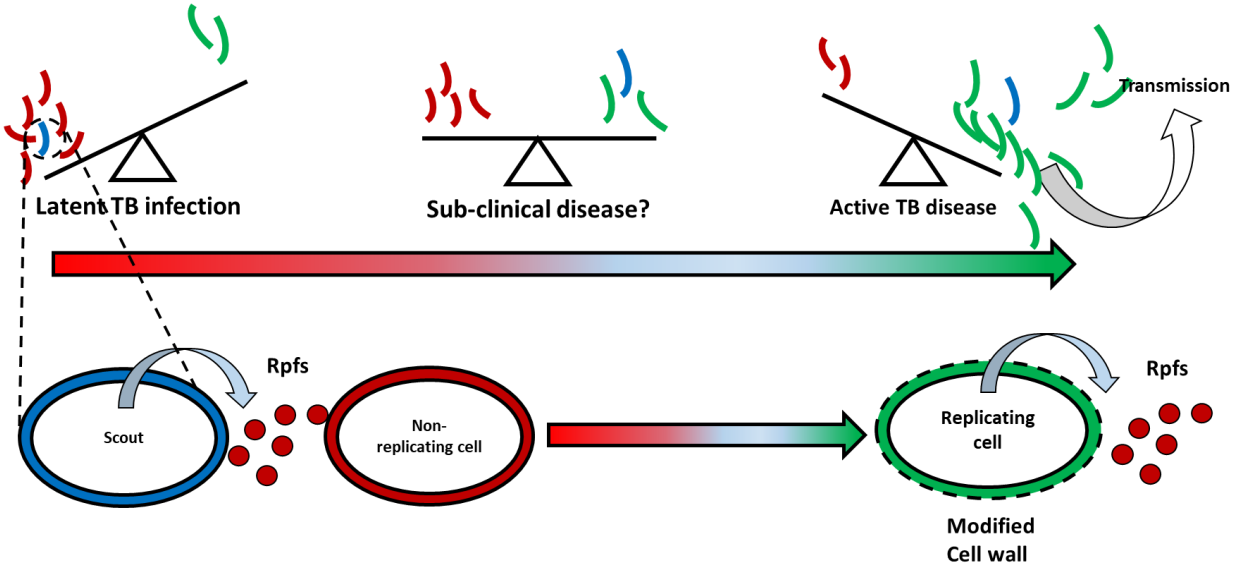

Figure 2.2 Schematic representation of the scout hypothesis. Latent tuberculosis infection (LTBI) is characterized by predominantly non-replicating bacilli (red). Few active scouts (blue) spontaneously resuscitate to sense whether environmental conditions are conducive for growth. Under favourable conditions, i.e. in caseous/necrotic granulomas, scouts release biochemical signalling molecules such as resuscitation-promoting factors (Rpfs), and possibly other molecules, that signal non-replicating bacilli to become active and in-turn, also release similar stimulator molecules. In active TB disease, the majority of the population are actively dividing (green); however, a small proportion of the population remain in a non-replicative and phenotypically tolerant state. These organisms are likely responsible for the long treatment period required to cure active TB disease. In active TB disease, bacilli are transmitted via high velocity exhalation manoeuvres such as coughing. The bio-aerosol cloud expectorated from a diseased individual possibly contains a mixture of actively dividing and non-replicating bacilli, of which the latter can be detected using specialised culture methods. Adapted from Gengenbacher and Kaufmann et al. 2013. Drawn by Amanda McIvor.

2.5.2 Phenotypic characteristics of non-replicating *Mycobacterium tuberculosis*

Non-replicating/persisting tubercle bacilli exhibit unique phenotypic characteristics that include tolerance to antibiotics, loss of acid fastness, inability to form colonies on solid media and the accumulation of TAG lipid droplets that may possibly be utilized as an energy source during dormancy and reactivation disease. These characteristics are discussed below.

2.5.2.1 Accumulation of lipid bodies

Auramine-Nile Red labelling has demonstrated the presence of lipid inclusion bodies in *M. tuberculosis* populations derived directly from patient sputum samples. The frequency of these lipid-body positive (LBP) cells ranged from 3 to 86% (2 to 8 lipid bodies per cell) in 82 patients from the Gambia and the UK (Garton et al., 2008). These results indicate that LBP tubercle bacteria are a commonly observed in smear positive patients. Consistent with this, the same study revealed that *M. tuberculosis* grown under hypoxic conditions enters into a drug tolerant, non-replicating state and accumulate lipid inclusion bodies (Garton et al., 2008). In a separate study, an *in-vitro*, multiple stress assay employing low oxygen (5%), high CO2 (10%), low nutrient (10% Dubos medium) and acid pH (5.0) resulted in the accumulation of storage lipids (both TAG and WE) in *M. tuberculosis cells* (Deb et al., 2009). Measurement of gene transcript levels of *M. tuberculosis* subjected to these stress conditions indicated the up-regulation of several TAG and WE genes, including *tgs1* (Deb et al., 2009). The *tgs1* deletion mutant failed to accumulate TAG, which was restored following complementation with *tgs1* under the same multiple stress conditions (Deb et al., 2009). The accumulation of these lipids suggests that they serve as an important carbon source during periods of non-replication and reactivation. Evidence for the use of TAGs for regrowth was shown in *M bovis* BCG, where entry and exit from hypoxia-induced dormancy was accompanied by the appearance and disappearance of intracellular lipid particles, respectively (Low et al., 2009). Furthermore, the deletion of both (but neither one alone) isocitrate lyase (ICL) genes (*icl1* and *icl2*) in *M. tuberculosis* resulted in complete impairment of intracellular replication in macrophages and rapid elimination from the mouse lung (Munos-Elias and McKinney, 2005). Since ICL is a key metabolic enzyme in fatty acid utilization, these findings provide further evidence that mycobacteria use fatty acids (as opposed to only carbohydrates) as a carbon source during infection. ICL inhibitors may therefore serve as a novel therapeutic in the treatment of TB.

In work involving clinical strains, the upregulation of DosR, the so-called “dormancy” regulon, along with the concomitant accumulation of TAGs in W-Beijing strains has been suggested as an adaptive strategy for these strains in the stressful environments encountered during infection (Reed et al., 2007). Indeed Beijing strains, that are globally distributed, have been associated with outbreaks, drug resistance and hyper-virulence (Cowley et al., 2008, Hanekom et al., 2007, Bifani et al., 2002). The link of Beijing 2 strains with relapse (Lan et al., 2003, Burman et al., 2009) may

therefore be associated with the ability of these strains to adapt non-replicating states, providing this organisms with an adaptive advantage against immune assault and/or drug treatment.

2.5.2.2 Drug tolerance

As previously discussed, the occurrence of sub-populations of non-replicating bacilli during active TB disease are thought to be responsible for the prolonged treatment period required. Indeed, non-replicating mycobacteria generated by environmental stressors (i.e. low oxygen, nutrient starvation or nitric oxide exposure) *in-vitro*, show tolerance to antibiotics (Gengenbacher et al., 2010, Rao et al., 2008, Xie et al., 2005, Hussain et al., 2009, Gold and Nathan, 2017). The phenomenon of phenotypic drug tolerance and its association with non-replicating tubercle bacilli were compared using ten anti-tuberculous compounds (Sarathy et al., 2013). With the exception of two compounds (i.e. mefloquine and thioridazine), a drastic decrease in drug susceptibility was observed in nutrient starved, non-replicating *M. tuberculosis* H37Rv cells when compared to replicating cells. In line with this, the accumulation of intracellular drug levels (measured by liquid chromatography coupled with mass spectrophotometry) was lower in non-replicating organisms for the majority of drugs tested (Sarathy et al., 2013). In a recent publication, *M. tuberculosis* bacilli residing in the cavity caseum from rabbits were shown to exhibit extreme tolerance to many first- and second-line drugs (Sarathy et al., 2018).

2.5.2.3 Loss of acid fastness

ZN microscopy is a widely utilised diagnostic for TB disease; however, many patients that are ZN negative still have clinical signs of disease or are positive for disease via other diagnostic methods (i.e. positive culture or NAAT). The loss of acid fastness in non-replicating organisms was demonstrated *in-vivo* in both C57BL/6 mice and samples from TB patients (Seiler et al., 2003). In experiments involving mice, ZN staining from lung sections after infection were analysed using two different detection techniques: (1) conventional, cell-wall dependent ZN staining and (2) cell-wall independent immunohistochemistry using a polyclonal rabbit anti–*M. bovis* Bacille-Calmette-Guérin serum (pAbBCG) (Wiley et al., 1990). Four weeks following infection, typical pink rod-shaped bacilli were observed using the conventional cell-wall dependent staining method; however, ten weeks after infection ZN-positive stains were gradually lost and barely no bacilli

were visible 39 to 40 weeks post-infection (Seiler et al., 2003). Broad staining of infected tissue was detectable using pAbBCG. In the same study, the results were confirmed using auramine, another staining method that is cell-wall dependent. Tissue sections from patients with acute or reactivated TB (five patients) were positive using both cell-wall dependent and pAbBCG staining. In contrast, samples from patients with persistent latent TB were positive by pAbBCG staining, but were ZN negative (Seiler et al., 2003). While the mechanism for loss of acid fastness is not fully understood, important metabolic processes such as the accumulation of TAG and changes to the cell wall are thought to be important (Vilcheze and Kremer, 2017).

2.5.2.4 Cell-wall remodelling and non-replicating persistence

As with many other bacterial organisms, changes in the PG cross-linking in the *M. tuberculosis* cell wall occurs during different growth states (Rittershaus et al., 2013, Lavollay et al., 2008). Analysis of PG content from *M. tuberculosis* H37Rv stationary phase cultures was performed using reverse phase high performance liquid chromatography and mass spectrophotometry to evaluate the contribution of D,D- and L,D-transpeptidases in the formation of 4→3 and 3→3 PG cross-links, respectively (Lavollay et al., 2008). The results revealed that approximately 80% these cross-links were 3→3, generated by L,D-transpeptidation. It was proposed that these cross-links are likely to play a pivotal role in the adaptation of *M. tuberculosis* to stationary phase (Lavollay et al., 2008). As 3→3 cross-links are insensitive to β-lactam antibiotics, a decrease in 4→3 cross-links may explain the reduction in susceptibility in non-replicating cells. To further investigate the role of PG and its susceptibility to anti-TB drugs, the L,D-transpeptidase protein, MT2594 in *M. tuberculosis*, was inactivated (Gupta et al., 2010). The loss of this protein led to altered colony morphology, reduced virulence and an increase in susceptibility to the β-lactam antibiotic, Amoxicillin (Gupta et al., 2010). These findings indicate that a combination of a L,D-transpeptidase and β-lactamase inhibitor may be an attractive treatment option to target non-replicating bacilli in TB disease (Gupta et al., 2010). Indeed, meropenem-clavulanate has been shown to be effective against drug-resistant *M. tuberculosis* (Hugonnet et al., 2009, Forsman et al., 2015).

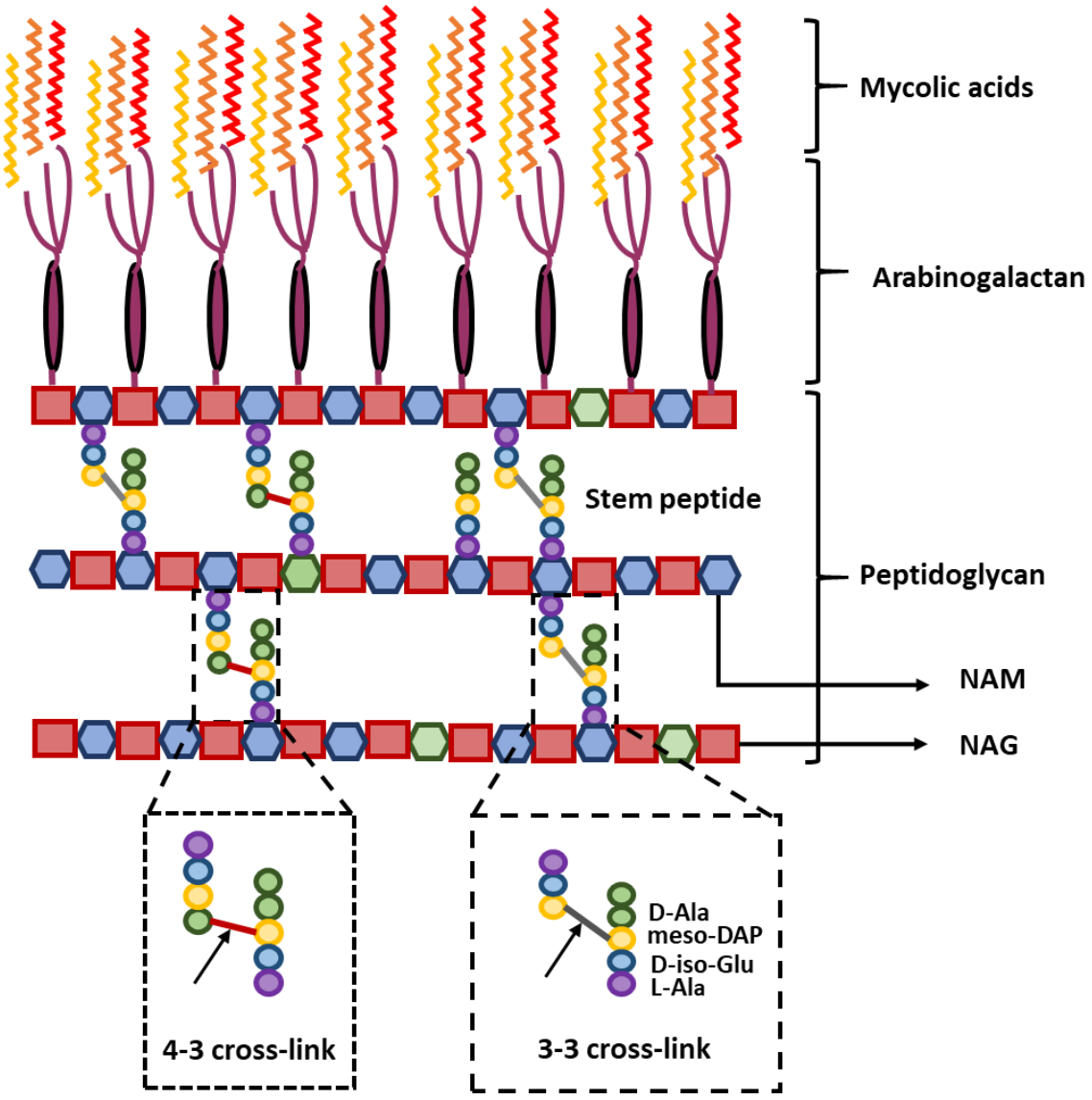

Figure 2.3 Mycobacterial cell-wall structure and remodelling. The mycobacterial cell wall is composed of three macromolecules, namely mycolic acids, arabinogalactan and peptidoglycan. The high density of lipids in the cell wall resist accurate gram staining; however, acid-fast stains such as the Ziehl-Neelson stain can be used. The peptidoglycan layer consists of repeating units of the disaccharide *N*-acetyl glucosamine– *N*-acetyl muramic acid (NAG–NAM) that are linked via peptide bridges. In stationary phase cultures 3→3 cross-links predominante (Lavollay et al., 2008).

In recent cell wall remodelling investigations, *in-vitro M. tuberculosis* subjected to hypoxic conditions (1% O_2 and 5% CO_2) led to the catabolic remodelling of cell-surface trehalose mycolates (Eoh et al., 2017). The break-down of cell surface-derived trehalose resulted in the accumulation of pentose phosphate pathway intermediates that are thought to be involved in PG biosynthesis (Eoh et al., 2017). These findings broadly implicate metabolically regulated pathways

for the transition of *M. tuberculosis* into altered growth states and these adaptations allow for an anticipatory response (Eoh et al., 2017). In a study published the previous year, a double deprivation model (i.e. O_2 and nutrient depletion) resulted in the generation on non-replicating TB bacilli after 728 days of latency. High resolution atomic force microscopy on filtered culture media (where cells adhered to silicon surfaces coated with antibodies), showed the presence of non-AFB that exhibited not only a change in morphology (round or oval shape) and reduction in size (150 – 300 μm in length), but also cell-wall deficiency (Velayati et al., 2016). Spoligotyping and WGS confirmed these cells as *M. tuberculosis*. The spoligotype pattern was identified as H37Rv; however, spacers 2, 14, 20, 21 and 33-36 were absent. While cell-wall deficient *M. tuberculosis* was previously hypothesized to exist (Khomenko, 1987, Stanford, 1987), this study provided the first-line of evidence for the existence of these persister cells (Velayati et al., 2016). Studies conducted using these cell-wall deficient cells may provide valuable insight regarding the biology, pathology and treatment of LTBI.

2.5.3 *M. tuberculosis* models of non-replicating persistence

Despite extensive investigation, the non-replicting state of the tubercle bacillus both *in vivo* and *in vitro* remains poorly understood. Several *in vitro* and *in vivo* models to generate and investigate these non-replicating persisters/ non-culturable organisms are outlined below:

2.5.3.1 *In vitro* dormancy models

A number of *in vitro* dormancy models for *M. tuberculosis* have been developed and are summarised in Table 2.1.

Table 2.1 Historic and currently used *in vitro* models to generate/study differentially detectable *Mycobacterium tuberculosis*

Dormancy Model	Principle	Reference
Corper and Cohn Experiment	Adaption to stationary phase in conventional culture	(Corper and Cohn, 1933)
Nutrient starvation	Carbon starvation	(Loebel et al., 1933)
The Wayne Model	Gradual oxygen depletion in sealed culture tubes with agitation	(Wayne and Hayes, 1996)
Nutrient starvation	Cultivation in nutrient low medium	(Betts et al., 2002)
Chemostat culture system	Growth under defined conditions and adaptation to static culture through carbon starvation	(James et al., 2002)
Nitric oxide-based model	Exposure to low-dose nitric oxide	(Voskuil et al., 2003)
***In vitro* granuloma model**	*Mycobacterium bovis* BCG strain carrying a luciferase (*lux*) gene and lung myofibroblasts mixed with fresh peripheral blood mononuclear cells to form a granuloma	(Puissegur et al., 2004)
100-day static culture	Prolonged cultures in stationary phase without agitation and generation of rifampicin tolerance	(Hu et al., 2006)
Hypoxic resazurin reduction assay	Culture aliquots in vacutainer tubes followed by adding of redox indicator and visual inspection	(Taneja and Tyagi, 2007)
Low-oxygen recovery assay (LORA)	Recombinant H37Rv expressing a dormancy luciferase gene from *Vibrio harveyii*	(Cho et al., 2007)
Whole-cell nitrate reductase assay	Development of Wayne model in microplate format and monitoring nitrate reductase activity	(Khan and Sarkar, 2008)
Multiple-stress dormancy model	Combines stresses of: low oxygen (5%), high carbon dioxide (10%), low nutrient (10% Dubos medium) and acidic pH (5.0)	(Deb et al., 2009)
Gradual acidification	Gradual acidification following the onset of stationary phase (pH 8.5 to 4.7)	(Shleeva et al., 2011)
Potassium limitation	Potassium limitation in nutrient-sufficient, aerobic conditions	(Salina et al., 2014)
Exposure to first-line antibiotics	Treatment of *Mycobacterium tuberculosis* cultures with first-line anti-TB drugs (RIF, EMB, INH and MEM)	(Loraine et al., 2016)
Nutrient starvation and exposure to RIF	Nutrient starvation (incubation in PBS) followed by exposure to high-dose RIF	(Saito et al., 2017)

RIF = rifampicin, EMB = ethambutol, INH = isoniazid, MEM = meropenem. Adapted from (Alnimr, 2015).

2.5.3.2 *In vivo* dormancy models

Two murine models of dormancy (or latency as the term is used in the literature) have been described and are detailed below. While these models cannot truly mimic LTBI in human disease, they provide valuable insight into the pathogenesis of LTBI (Flynn et al., 1998, Adams et al., 1995, MacMicking et al., 1997, Orme, 1988, Scanga et al., 1999).

2.5.3.2.1 The low-dose, chronic murine model

In the low-dose or 'untreated' mouse model of latent TB, mice are infected with a low-dose (5 to 10 CFU) of *M. tuberculosis* either via the aerosol route or infected intravenously. After approximately three months the, pulmonary bacillary burden plateaus between 3 to 4 log and the infection is contained solely by the host immune response – similar to LTBI in human disease (Sever and Youmans, 1957). After 15 to 18 months, the model results in a high bacillary burden and the mice succumb to TB disease. Whilst this model does not mimic true LTBI, it has been used as a model of persistent infection (Stead et al., 1968, Wayne and Sohaskey, 2001).

2.5.3.2.1 The Cornell model

The Cornell model (also known as the treated mouse model) is a historical murine model of LTBI (McCune and Tompsett, 1956, McCune et al., 1956). A schematic representation of this model is shown in Figure 2.2. In the original model, mice were infected intravenously with 1 x 10^6 to 3 x 10^6 viable *M. tuberculosis* H37Rv bacilli and treated subsequently for twelve weeks with two anti-TB chemotherapeutics, namely INH and PZA, to reduce the bacterial burden to undetectable levels. Following treatment cessation, reactivation disease was shown to occur either spontaneously (McCune and Tompsett, 1956) or was induced following immunosuppression with the administration of cortisone (McCune et al., 1966). Variations of the Cornell model have since been developed (Scanga et al., 1999). Reactivation disease was shown to occur in a modified Cornell model when the production of reactive nitrogen intermediates (RNI) was blocked by aminoguanidine, a nitric oxide synthase inhibitor, highlighting the role of RNI in preventing reactivation disease (Flynn et al., 1998). The Cornell model has been employed as a tool for testing treatment regimens (Brooks et al., 1999, Dhillon et al., 1998, Dhillon et al., 1996, Paramsivan et

al., 1998), vaccines (Dhillon and Mitchison, 1994, Lowrie et al., 1999) as well as the immunology and bacteriology of the proposed latent state (van Pinxteren et al., 2000, Rees and Hart, 1961, De Wit et al., 1995, Hu et al., 2000).

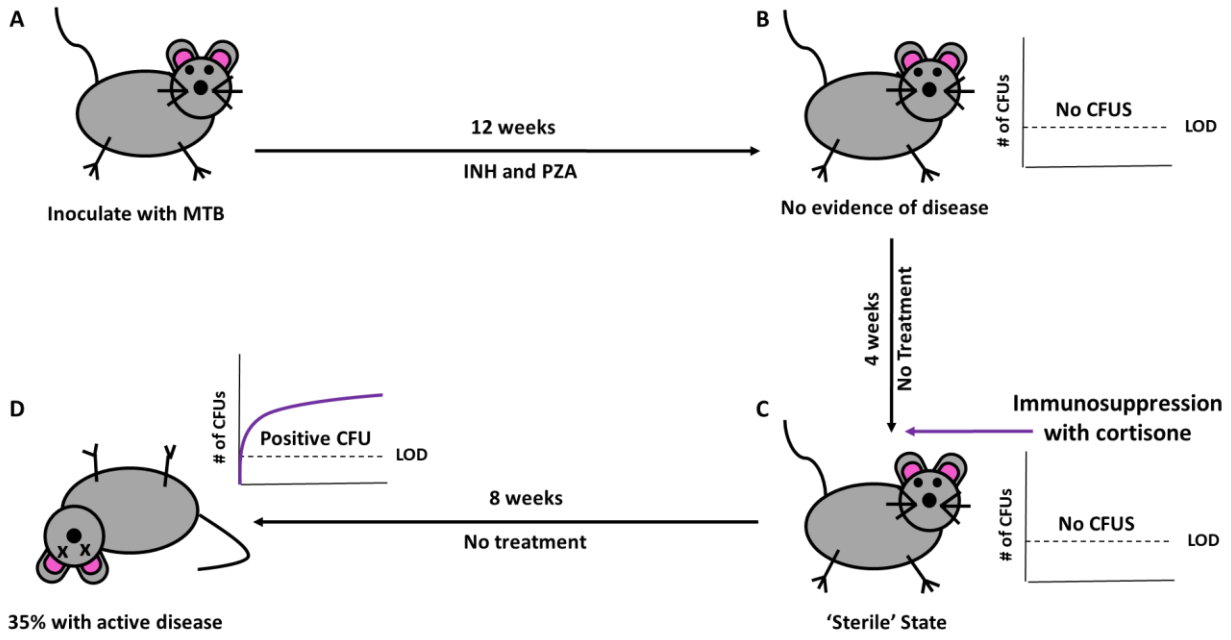

Figure 2.4 Schematic diagram of the Cornell model of dormant tuberculosis (TB). (A) Outbred mice are infected with ~ 10^5 colony forming units of *M. tuberculosis* H37Rv. (B) No evidence of disease is present following 12 weeks of treatment with both isoniazid (INH) and pyrazinamide (PZA). (C) Four weeks after cessation of treatment with INH and PZA, the mice appear to be well and TB infection cannot be measured by cultivation methods. This is termed the 'sterile' state of latent TB. In some models, mice are given cortisone as an immunosuppressant. (D) Mice are followed up for an additional eight weeks (12 weeks total following treatment cessation) and approximately 35% develop culture-positive, active TB disease. LOD = limit of detection, CFU = colony forming units. Figure adapted from (Parrish et al., 1998), Drawn by Amanda McIvor.

2.5.4 Resuscitation-promoting factors

It is now generally accepted that aged cultures of some bacterial species, including *M. tuberculosis*, contain a population of organisms that cannot form colonies on solid media, but can be resuscitated by the addition of CF from early stationary phase cultures (Sun and Zhang, 1999, Shleeva et al., 2002). Rpfs are a family of muralytic enzymes that have been implicated in the reactivation of non-replicating bacteria. Two decades ago, the role of Rpf and its involvement in the resuscitation

of non-culturable bacteria was described by Mukamolova et al. (1998), where the addition of this protein in pico-molar concentrations increased the viable cell count of 'non-culturable' *Micrococcus luteus* cells 100-fold (Mukamolova et al., 1998). Furthermore, it was demonstrated that extensive washing of *Mi. luteus* cells led them to be dependent on exogenously added Rpf for growth (Mukamolova et al., 1998). These findings illustrated that Rpf exhibits properties consistent with that of a cytokine or bacterial growth factor (Callard and Gearing, 1994, Mukamolova et al., 2002b).

The addition of purified *M. luteus* Rpf was also shown to stimulate the growth of slow growing mycobacteria, including *M. tuberculosis* (strains H37Ra and H37Rv), *M. avium*, *M. bovis* (BCG) and *M. kansasii.* (Mukamolova et al., 1998). Moreover, aged *M. tuberculosis* Academia strain (avirulent) cultures yielding small coccoid, non-culturable cells, were obtained by filtering clumped cultures grown in the absence of Tween 80. These cells could only be resuscitated following the addition of CF or recombinant Rpf (Shleeva et al., 2002). Following phagocytosis by macrophages, *M. tuberculosis* cells reportedly lose their ability to form colonies on solid media suggesting the adoption of the dormant state. Biketov et al. (2000) investigated this effect of Rpf on non-replicating *M.tuberculosis* Academia cells from murine peritoneal macrophages following infection *in vivo* (Biketov et al., 2000). The viable counts of cell suspensions derived from these macrophages were assessed by the most probable number (MPN) serial dilution assay and compared to the number of CFUs obtained from lysates. Bacterial viability measured by the MPN assay was often much higher than that obtained by CFUs (Biketov et al., 2000). In addition, cells isolated from macrophages had different surface properties compared to cells grown *in vitro.* This was evidenced by the inability of these cells to absorb the bacteriophage DS6A, a reduced tendency to form clumps in liquid media and loss of Rpf-mediated resuscitation following freezing/thawing (Biketov et al., 2000). It was concluded that *M. tuberculosis* cells isolated from macrophages were either damaged or in a non-replicating state, and these cells could be recovered by the addition of Rpf to liquid culture media. Whilst the biological function of these proteins is not understood, these findings suggest a putative role for these Rpf-like proteins in modulating bacterial growth during disease.

2.5.4.1 Characterization of Rpfs

Genes encoding Rpf-like proteins similar to that found in *Mi. luteus* are present among the G+C gram positive bacteria (i.e. Streptomyces, Corynebacterium and Mycobacteria), (Kell and Young, 2000). *M. tuberculosis* has five Rpf homologues designated RpfA to RpfE (http://genolist.pasteur.fr/Tuberculist), of which all 5 Rpf-like proteins are reportedly found in actively dividing cells *in vitro* and in mice (Mukamolova et al., 2002b, Tufariello et al., 2004). Furthermore, the expression of some of these genes have been detected in granulomas from lung tissue sections in patients with TB disease (Fenhalls et al., 2002). The presence of multiple homologues of these *rpf*-like genes led to the hypothesis that these proteins may have overlapping biological functions or may be partially or wholly redundant. Subsequent gene knock-out experiments were performed to determine the functional role of these Rpf-like proteins in *M. tuberculosis*. Growth and survival in mutant *M. tuberculosis* strains wherein one of the five *rpf* homologues was deleted, was not compromised, thereby confirming that none of the *rpf*-like genes are individually essential for growth *in vitro* (Downing et al., 2004, Tufariello et al., 2004) or in the mouse model of infection (Tufariello et al., 2004). The first line of evidence to show that these proteins serve some discrete biological function in *M. tuberculosis* came from subsequent gene knock-out experiments whereby mutants lacking three of the five *rpf* genes displayed differential growth defects *in vitro* and were unable to resuscitate spontaneously *in vitro* (Downing et al., 2005). Consistent with this, an *rpfB* defective mutant of the *M. tuberculosis* Erdman displayed delayed growth kinetics in mouse model of dormancy (Downing et al., 2005). Following these observations, a series of double mutants were constructed since functional redundancy among the Rpf family members may have obscured single knock-out phenotypes (Russell-Goldman et al., 2008). One double mutant, Δ*rpfAB*, displayed altered colony morphology and elicited an altered macrophage cytokine response. *In vivo*, this mutant also displayed a reactivation deficit in C57BL/6 mice that were treated with the nitric oxide synthase inhibitor (aminoguanidine). This reactivation deficit was more severe than that of the single Δ*rpfB* mutant in aminoguanidine-treated mice. Additional observations showed that this mutant exhibited a deficiency in persistence, a defect not observed in Δ*rpfA* and Δ*rpfB* single mutants. Together, these data provide evidence that RpfA and RpfB may modulate the host innate immune response to *M. tuberculosis* (Russell-Goldman et al., 2008). To further investigate the collective roles of Rpf, quadruple and quintuple mutants of *M. tuberculosis* H37Rv were constructed. In this case, all five *rpf*-like genes

were shown to be collectively dispensable for *M. tuberculosis* growth *in vitro*, again indicating functional redundancy (Kana et al., 2008). This is in contrast to *Mi. luteus*, where the single *rpf* gene was found to be essential for growth and survival (Mukamolova et al., 2002a). A quadruple mutant (containing only *rpfD*) and a quintuple mutant (lacking all five *rpf* genes) both displayed delayed colony formation and increased sensitivity to detergent (sodium dodecyl sulphate) in comparison to mutants containing *rpfB* or *rpfE*. Furthermore, quadruple mutants retaining the *rpfD* and *rpfE* genes, were attenuated for growth in mice. These experiments suggest 'a functional hierarchy within the *rpf* gene family, with *rpfB* and *rpfE* ranking above *rpfD*' (Kana et al., 2008).

2.5.4.2 Structure of the Rpf domain

Nuclear magnetic resonance imaging of the *M. tuberculosis* RpfB domain provided valuable information on the structure of Rpfs and yielded insight into the biological function of this protein family (Cohen Gonsaud et al., 2005). The structure of RpfB was described as a 'compact hybrid' bearing a structural resemblance to that of a soluble lytic trans glycosylase and a *c*-type lysozyme – both of which are involved in the cleavage of PG (Cohen Gonsaud et al., 2005). The active and conserved catalytic glutamate was also shown to be crucial for catalytic activity (Cohen Gonsaud et al., 2005). Consistent with these findings, studies analysing the crystal structure of a truncated form of RpfB suggest that it is a cell-wall associated protein owing to the presence of a 'comma-like shape formed by a lysozyme-like globular protein catalytic domain' as well as an elongated G5 domain (Ruggiero et al., 2009). The existence and role of a G5 domain in cell-wall associated proteins, cell-to-cell adhesion and biofilm formation, further suggest that that Rpf is a cell-wall associated protein (Rezwan et al., 2007). The muralytic activity of the Rpf from *Mi. luteus* cells has been confirmed through the degradation of fluorescamine-labelled *Mi. luteus* cell walls (Mukamolova et al., 2006). In addition, when the active glutamate residue was altered, Rpf activity was substantially attenuated (Mukamolova et al., 2006). Structural characterisation of RpfB in solution carried out using circular dichroism spectroscopy and light scattering experiments have provided further insight into the functional properties of the non-catalytic domains (Ruggiero et al., 2017). One of the findings was that the G5-linked-Ubiquitin domain has a possible role for the adhesion of this enzyme to polymeric PG (Ruggiero et al., 2017, Ruggiero et al., 2016).

2.5.4.3 Mechanism of action of Rpfs

Whilst the exact mechanism whereby Rpfs stimulate growth in *M. tuberculosis* (and other G + C rich gram positive bacteria) remains elusive, it is suspected that resuscitation stems from the ability of these enzymes to hydrolyze PG in the cell wall. Two mechanisms of growth stimulation whereby Rpfs remodel PG have been proposed. The first hypothesis of PG remodelling is based on the structure of Rpf outlined in section 2.5.4.2 whereby Rpfs directly interact with the cell wall by cleaving the β-1,4 glycosidic bond between NAM and NAG in PG. This cleavage may allow for the insertion of new PG units, ultimately leading to bacterial growth and cell division.

The second hypothesis is that Rpfs have an indirect interaction with the mycobacterial cell wall and are required for the release of signalling molecules that reactivate non-replicating bacteria through the activity of serine-threonine protein kinases (STPKs) (Shah et al., 2008, Dworkin and Shah, 2010). STPKs are transmembrane proteins consisting of an external sensor domain and cytoplasmic protein kinase domain. Following the detection of a cognate signal, the protein kinase phosphorylates target proteins leading to a signal cascade and subsequent cellular response (Kana and Mizrahi, 2010). The STPK, PrkC, in *B. subtillus* results in spore germination by sensing PG fragments through an external penicillin-binding protein and serine/threonine protein kinase (PASTA) domain (Shah et al., 2008). PknB, the homologue of PrkC in mycobacteria, has a high degree of structural similarity to other eukarytic STPKs, suggesting that this protein is also able to bind PG in a manner similar to PknB (Fernandez et al., 2006). It has been suggested that the combined action of Rpfs and other enzymes such as L,D-carboxypeptidases may generate the formation of a disaccharide tripeptide that can bind to PknB and trigger resuscitation in non-replicating bacteria in a manner similar to spore germination in *B. subtillus,* Figure 2.4 (Kana and Mizrahi, 2010). Yeast two-hybrid screening to identify potential binding proteins for Rpf reported an interaction between RpfB and RpfE with the mycobacterial endopeptidase, designated Rpf-interacting protein A (RipA) (Hett et al., 2007). The observation that both RipA and RpfB co-localize to the septa of dividing cells provides further evidence for a role for both these enzymes in bacterial growth and division (Hett et al., 2008). Figure 2.5 demonstrates a proposed model for the mechanism of action of Rpfs and RipA in the remodelling of PG in the mycobacterial cell wall as well as their possible involvement in host and bacterial signalling.

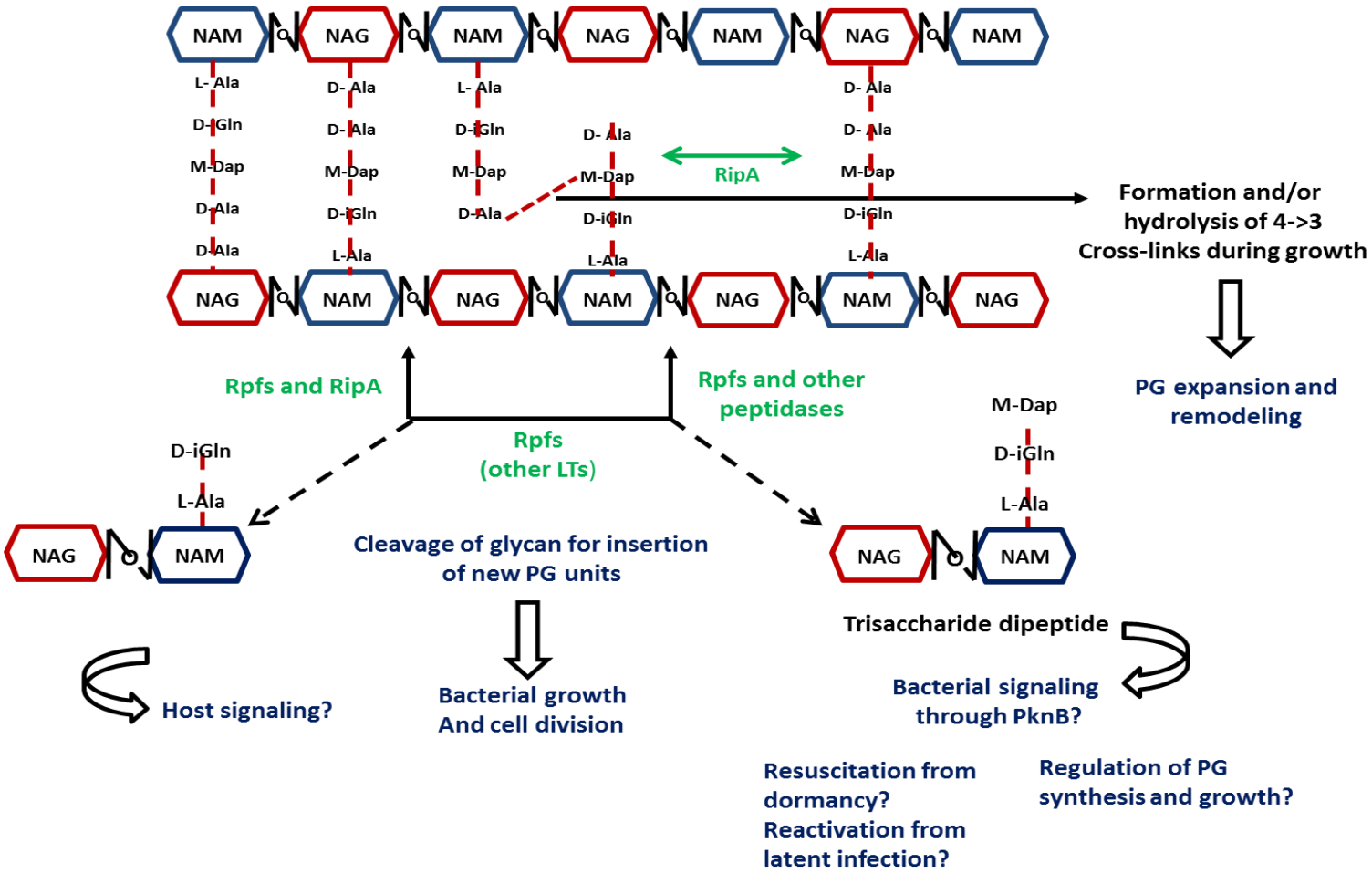

Figure 2.5 Proposed role of resuscitation-promoting factors in remodeling of peptidoglycan and production of muropeptide signaling molecules. Peptidoglycan (PG) is composed of both peptide and glycan strands. The glycan strands typically consist of repeating units of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG), which are cross-linked by peptide groups bound to the lactyl group on the NAMs from different glycan strands (Mahapatra et al., 2005). The stem peptide consists of D-iGLn, m-DAP, D-alanine and L-alanine residues (Kana and Mizrahi, 2010). Rpfs are through to cleave the β-1,4 glycosidic bond between NAM and NAG whereas endopeptidases, including RipA and carboxypeptidases, cleaves the bond between the peptide stems. The combined action of resuscitation-promoting factors (Rpfs) and other enzymes may generate muropeptides that have both bacterial and/or host signaling properties. Modified from Kana et al. 2010 and Hett et al. 2008. Drawn by Amanda McIvor.

In terms of host signalling, there is evidence to associate a role of bacterial PG fragments in pathogenesis (Bartoleschi et al., 2002, Boneca et al., 2007, Humann and Lenz, 2009). Figure 2.5 shows that the combined action of RipA and RpfB is speculated to generate a disaccharide tripeptide (with terminyl m-DAP residue) that may be detected by the mammalian protein, Nod2 (nucleotide-binding oligomerization domain) (Girardin et al., 2003, Kana and Mizrahi, 2010). Also, the combined action of RpfB and other peptidases could generate a disaccharide dipeptide that is detected by Nod1 (Girardin et al., 2003). It is speculated that pathogenic mycobacteria may utilize these muropeptides to modulate the host's innate immune response to their advantage (Jo, 2008, Nikitushkin et al., 2015, Wang et al., 2017).

2.5.4.4 Bacterial non-replicating states in TB disease

The clinical work investigating the phenomenon of Rpf dependency and non-replicating states in TB disease is extensively reviewed in the introductory sections of chapters 3, 4 and 6.

2.6 KNOWLEDGE GAPS RELATED TO THIS PHD

A great body of work has demonstrated the existence of bacterial organisms that cannot be cultured in the laboratory using routine culture methods. It is presumed that these cells are in a state of non-replicating persistence and are responsible for the phenotypic tolerance to antibiotics. In TB disease, it is speculated that non-replicating *M. tuberculosis* may underpin the phenomenon of clinical latency, the six month treatment period required to achieve clinical cure, as well as relapse disease following supposed 'sterilisation' and clinical cure. The ability to detect these non-culturable organisms will not only provide valuable insight into the heterogeneity (and the roles bacterial organism play) within a community, but will also have clinical implications in terms of diagnosing disease, monitoring treatment response and the risk of developing reactivation disease. Furthermore, early bactericidal activity (EBA) assays will also benefit from testing the sterilising efficacy of potential drug candidates on these differentially culturable populations. Previous work at the Centre of Excellence for Biomedical and TB Research (CBTBR) has demonstrated a spectrum of differencially culturable tubercle bacilli (DCTB) in tuberculous sputum from patients with drug-susceptible TB at baseline (i.e. before the commencement of anti-TB treatment) (Chengalroyen et al., 2016) (study reviewed in chapter 3). DCTB are defined as non-platable *M. tuberculosis* populations that emerge differentially depending on culture conditions. A key question that remains to be investigated

is how these organisms adapt or respond to anti-TB therapy. In this thesis, the following questions pertaining to these DCTB populations are studied and addressed:

- What are the proportions of non-culturable versus conventionally culturable *M. tuberculosis* bacteria in patients with active, drug-susceptible TB disease before treatment initiation?
- How do the proportions of these organisms vary between individuals with and without HIV coinfection?
- How do these DCTB respond to treatment?
- Can DCTB serve as biomarkers that are able to assess or predict how patients will respond to anti-TB treatment?
- How does the MPN assay, in its 48-well micro-titre format, relate to other culture methods such as CFU and MGIT? Could the MPN assay possibly be used in the future in EBA studies to monitor the effect of new TB drugs on both actively replicating and non-replicating populations?
- Can the addition of CF (as a source of Rpfs) to the BACTEC MGIT 960 assay reduce time to TB diagnosis?

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Detailed components of this PhD

Background and literature review

CHAPER ONE	CHAPTER TWO
TUBERCULOSIS	MICROBIAL DORMANCY

Original Research Chapters

KEY QUESTIONS?	KEY CONCLUSIONS?
CHAPTER 3 Do all TB patients harbor DCTB? What are the various DCTB populations? Are Rpfs solely responsible for unmasking DCTB? Does host immunity affect the quantum of DCTB?	**CHAPTER THREE** ?
CHAPTER 4 How do DCTB respond to first-line TB treatment compared to conventionally culturable bacteria? Are there potential biomarkers to predict patient response to treatment? Do DCTB remain at the end of treatment?	**CHAPTER FOUR** ?
CHAPTER 5 What is the relationship between the MPN assay and currently employed *M. tuberculosis* culture methods? Can the MPN possibly be used in EBA studies? Does CF improve the relationship between the MPN and MGIT TTP in clinical samples?	**CHAPTER FIVE** ?
CHAPTER 6 Does the addition of CF to the MGIT enhance diagnostic pick-up? Can modification of the MGIT by the addition of CF improve the time to culture positivity? Does a reduction in total MGIT volume improve TTP by enhanced quorum sensing?	**CHAPTER SIX** ?

CHAPTER 3

DETECTION AND QUANTIFICATION OF DIFFERENTIALLY CULTURABLE BACTERIA IN TUBERCULOSIS PATIENTS: A PRETREATMENT ANALYSIS

3.1 INTRODUCTION

The clinical presentation of TB disease is complex, often manifesting as a spectrum of disease (ranging from sterilizing immunity to active disease) within a single patient (Barry et al., 2009, Esmail et al., 2016, Schnappinger and Ehrt, 2016, Lenaerts et al., 2015). Furthermore, a growing body of evidence points to a high complexity in the population structure of *M. tuberculosis* within the host environment (Turapov et al., 2016, Mukamolova et al., 2010, Chengalroyen et al., 2016, Manina et al., 2015, Post et al., 2004). The success of this pathogen has often been attributed to differences in bacterial growth states which are thought to underpin latent infection, the long treatment period required as well as relapse disease. Furthermore, these altered growth states, which include non-replicating persistence/metabolic quiescence, are likely responsible for drug-tolerance since most anti-TB chemotherapeutics target actively replicating organisms. Additionally, these drug-tolerant organisms may serve as the basis for the development of genetically stable and drug-resistant strains (Loraine et al., 2016). It has been proposed that the dynamic interplay between these differential growth states and their association with host immunity will ultimately determine the outcome of disease. In light of this, the complexity of *M. tuberculosis* population dynamics in TB disease has become an area of intense study with the detection of non-replicating organisms (characterized by impaired culturability) being a leading focus in this area. The detection and quantification of these organisms will be of utmost importance in clinical trials monitoring the EBA of new drugs to determine the sterilizing efficacy of new treatments as well as in the diagnosis of TB (particularly in patients with paucibacillary disease), patient care and management and ultimately for the complete eradication of this disease.

Accumulating microbiological studies have provided further insight into this phenotypic plasticity. A decade ago, *M. tuberculosis* cells derived from pre-treatment sputum samples were demonstrated to harbour varying amounts of lipid inclusion bodies by combined Nile red and auramine staining (Garton et al., 2008). Additional *in vitro* experiments revealed that *M. tuberculosis* cells in non-replicating persistence, as described by Wayne and Hayes (Wayne and Hayes, 1996), accumulated lipid inclusion bodies at a frequency similar to those cells seen in

sputum (Garton et al., 2008). The authors proposed that the presence of lipid bodies from sputum samples could potentially serve as a biomarker for non-replicating bacteria. Furthermore, it has been hypothesized that the accumulation of TAG lipid droplets may possibly be utilized as an energy source during non-replicating persistence and reactivation disease (Daniel et al., 2004, Gomez and McKinney, 2004, Zhang, 2004, Daniel et al., 2016, Daniel et al., 2011). Consistent with this, the upregulation of the TAG synthase gene, *tgs*1, (as well as several other genes in the DosR regulon) in clinical W-Beijing strains coincided with the accumulation of TAG, providing evidence of the involvement of these genes in TAG synthesis and the adoption of a non-replicative state (Garton et al., 2008, Reed et al., 2007).

Rpfs were one of the first mycobacterial proteins to be implicated in the modulation of mycobacterial growth and have subsequently been linked to recrudescent disease in mice (Kana and Mizrahi, 2010). These secreted enzymes, in conjunction with other mycobacterial proteins such as RipA, are thought to enhance bacterial growth by the hydrolysis of the PG layer in the cell wall allowing for degradation of the septa in diving cells leading to cell separation (Hett et al., 2007, Hett et al., 2008, Nikitushkin et al., 2015). The presence of an Rpf-dependent population in human infection was first described by Mukamolova and colleagues in 2010 (Mukamolova et al., 2010). The authors reported that pre-treatment smear-positive sputum samples were dominated by a population of *M. tuberculosis* cells that could only be cultured by supplementation with recombinant RpfE or fresh *M. tuberculosis* CF (as a source of Rpfs) in a limiting dilution assay. This Rpf-dependent population was determined by comparing the number of bacterial cells isolated in the limiting dilution MPN assay to those detected by conventional standard agar CFU assays. The data illustrated that at least 80% of the bacterial population was undetected by conventional CFU assays, conducted on sputum samples obtained from 20 out of 25 patients (Mukamolova et al., 2010).

These observations were confirmed in a recent study conducted on sputum samples from 110 pre-treatment TB patients illustrating that 86% harboured a population of organisms that could only be recovered following the addition of CF (Chengalroyen et al., 2016). In this study, the authors define these non-platable bacteria as ‘differentially culturable tubercle bacilli’ (DCTB) as they emerge differentially depending on culture conditions. This study illustrated a greater complexity in *M. tuberculosis* populations than previously understood, whereby various phenotypically

distinct bacterial sub-populations were described in a single sputum sample, including a population of DCTB that emerged independently of Rpfs. The distinct populations of bacteria reported by Chengalroyen et al. (2016) and are depicted in Table 3.1. In addition to mixed populations of DCTB, which accounted for the majority (53.6%) of *M. tuberculosis* cells isolated from this patient cohort, 21 (19.1 %) patients harboured DCTB that could only be cultured in the presence of CF derived from the wild-type H37Rv strain (sub-population 2). In contrast, 13 (11.8 %) patients harboured a population of DCTB that could only be cultured by supplementation with CF derived from a mutant strain devoid of any Rpfs, suggesting that Rpfs may function as an inhibitor of growth in certain populations, (sub-population 3). Two patients (1.8%) harboured a population of cells that could only be cultured in the MPN assay which was not supplemented with CF (sub-population 4). Lastly, CFUs were obtained from 15 patients (13.6%) where no DCTB were present. Of note was the emergence of DCTB that could be cultured not only in the presence of CF containing Rpfs, but also by the supplementation with CF derived from an *M. tuberculosis* strain where all five *rpf* genes had been deleted. These observations suggested a role for molecules other than Rpfs in resuscitation of DCTB. Indeed, previous studies employing *in vitro* systems to generate DCTB have implicated other molecules such as cyclic AMP (cAMP), fatty acids, cholesterol, muropeptides and mycobactins as factors important for the resuscitation of metabolically quiescent strains (Shleeva et al., 2013, Zhang et al., 2001, Nikitushkin et al., 2013, Soto-Ramirez et al., 2017). However, a recent study reported the generation of DCTB after starvation in PBS and treatment with RIF and in this case, these organisms (termed differentially detectable tubercle bacteria) were able to resuscitate in liquid media without CF supplementation (Saito et al., 2017).

These findings contribute further insight into the complexity of TB disease and altered growth states that exist in pre-treatment sputum samples and highlight possible diagnostic challenges for TB as a single diagnostic assay may not be sufficient to detect all subpopulations in sputum (Dartois et al., 2016). Whilst *in vitro* experiments have demonstrated tolerance of Rpf-dependent organisms to first-line antibiotics, including, INH and streptomycin (STREP) (Turapov et al., 2016, Mukamolova et al., 2010), (further reviewed in chapter 4), the relative proportions and rates of decline of these populations during first-line treatment in patients was addressed in this PhD. These findings are presented in chapter 4. However, before reporting these, the prevalence of

DCTB in the patient cohort used for this study, prior to the initiation of treatment, will be described in this chapter.

Table 3.1 Distinct subpopulations of *M. tuberculosis* isolated from treatment-naïve patients

Subpopulation	CF+ MPN	Rpf- MPN	MPN no CF	CFU
1	●●●	●●●	○○○	○○○
2	●●○	○○○	○○○	○○○
3	○○○	●●○	○○○	○○○
4	○○○	○○○	●○○	○○○
5	○○○	○○○	○○○	●●○

Definition of abbreviations: CF = culture filtrate; MPN = most probable number assay, Rpf $^{-}$ = resuscitation-promoting factor negative; CFU = colony forming unit. The number of coloured circles represents the proportion of patients with differentially culturable tubercle bacilli or CFU as indicated: three, > 50%; two, 2 – 50%; one <2%. Adapted from Dartois et al. 2016

Variation in the relative proportions of CF/Rpf dependent organisms, or lack thereof, identified amongst TB patients suggest that host factors play a role in the generation of this phenotype. In this case, the stress imposed by the cell-mediated host immune response may drive bacteria into distinct differentially culturable states. To further support this hypothesis, Chengalroyen et al. (2016) reported that individuals with a comparatively better immune competency (i.e. HIV-negative individuals or HIV-positive individuals with CD4 T-cell counts greater than 200 cells/mm^3) harboured increased numbers of CF-dependent DCTB in their sputum (Chengalroyen et al., 2016). In further support of this hypothesis, it was previously shown that two weeks after infection with *M. bovis* BCG Glaxo, BALB/c mice harboured Rpf-dependent bacteria in their lungs, implicating the host environment in generating this population (Turapov et al., 2014). In contrast, 24 hours post infection, the numbers of CFUs and bacteria isolated from CF supplemented MPN assays were not significantly different illustrating the absence of an Rpf-dependent population after axenic subculture of organisms derived from mice. As the establishment of Rpf-dependency overlapped with the onset of the adaptive immune response in

mice (Vallerskog et al., 2010), it was proposed that a direct link existed between the two (Turapov et al., 2014). Furthermore, additional experiments suggest that Rpf-dependency occurs in the lung and not from the transition from the lung to sputum (Turapov et al., 2014). Evidence to implicate the host macrophage environment in generating Rpf-dependent bacteria has also been reported (Biketov et al., 2000). Currently the role of the host immune system in generating DCTB remains speculative and further investigation is required.

The presence of DCTB in sputum poses a significant challenge with regards to the diagnosis, treatment and management of TB disease. As these populations are the most likely responsible for the prolonged treatment of TB, the acquisition of acquired drug resistance, and relapse disease, an understanding of their biology is of utmost importance. Circumstantial evidence for the potential role of DCTB in relapse disease was demonstrated by Hu et al (2015) whereby deletion of the *hspx* gene (resulting in the eradication of Rpf-dependent bacilli) resulted in a two-fold increase in the elimination of *M. tuberculosis* bacilli and a significant decrease in the rates of relapse disease in the Cornell mouse model when treated with standard anti-TB drugs (Hu et al., 2015). These findings indicate that genes that are overexpressed during non-replicative phases/dormancy, such as *hspx*, may serve as important targets for anti-persister drugs that could be used as antibiotic adjuvants to enhance the effectiveness of conventional antibiotics and possibly shorten treatment duration.

As most forms of TB disease manifest in the lungs, diagnosis and management relies predominantly on sputum which can problematic for multiple reasons. Firstly, a poor quality sample consisting predominantly of upper airway secretions may not contain many bacilli leading to a false negative diagnosis (Datta et al., 2017). Secondly, some patients, particularly those that are HIV-infected or young children, are unable to produce a diagnostically useful sputum (Moore et al., 2017, Datta et al., 2017). In addition, the HIV epidemic has resulted in an increase in the numbers of patients with smear-negative disease due to disseminated or EP disease making diagnosis based on sputum a challenging and in many cases a process that delays engagement into care (Hargreaves et al., 2001). The presence of DCTB in patient sputum adds a further level of complexity regarding the diagnosis and management of TB disease due to the fact that these organisms cannot be cultured using standard methods. Hence, further understanding of DCTB,

mycobacterial growth and the mechanism of resuscitation may shed further insight on how TB diagnosis can be improved in the future.

In this chapter, the relative proportions of DCTB subpopulations as defined by Chengalroyen et al. (2016) were investigated in an independent patient cohort of drug-sensitive individuals at baseline. These individuals were then placed on standard TB chemotherapy to assess the response of DCTB to drug treatment. Results from this longitudinal component are analysed and conveyed separately in chapter 4. The aim and objectives for this chapter are detailed below.

3.2 HYPOTHESIS

Populations (and sub-populations as defined by Chengalroyen et al. 2016) of DCTB are present in a distinct cohort of treatment naïve TB patients. It is hypothesised that HIV-negative individuals will harbour a greater proportion of DCTB compared to their HIV-positive counterparts, due to greater functionality in the host immune system.

3.3 AIM OF STUDY

The aim of this study was to quantify the relative proportions of DCTB populations (by MPN) versus platable bacteria (by CFU) in sputum samples collected from patients infected with active pulmonary, drug-susceptible TB at baseline.

3.3.1 Specific objectives of the study

1. Quantify the CF-dependent, Rpf-independent, CF-independent and conventionally culturable bacterial populations by MPN and CFU assays, respectively.
2. Determine whether there is a variation in the distribution of DCTB between HIV-infected and -uninfected individuals.
3. If a difference is obtained in (2), ascertain whether the variation in this bacterial distribution is linked to host immunity. For this, patients will be stratified into two groups with CD4 T-cell counts less than and greater than 200 cells/mm^3.
4. Describe circulating *M. tuberculosis* strains within the study community.

3.4 MATERIALS AND METHODS

3.4.1 Patients/Sources of sputum samples

Ethics clearance for this PhD was obtained from the Human Research Ethics Committee (medical) of the University of the Witwatersrand, Johannesburg [Clearance certificate number: M161058 (Appendix 3A)]. One hundred and seventy four newly diagnosed patients presenting with drug-susceptible pulmonary disease were recruited into the study through the clinical platforms of the Perinatal HIV Research Unit (PHRU). Since the aim of the study was to assess the response of DCTB to drug treatment, baseline samples from treatment naïve patients were required. Previous TB patients were excluded to eliminate any previous influence of treatment on these populations. The initial 132 patients were recruited from primary health care clinics in and around the Soweto area, greater Johannesburg, South Africa, after which recruitment relocated to Klerksdorp, North-West Province, South Africa, where an additional 42 patients were recruited. Patients were approached to participate in the study on the basis of a positive GeneXpert MTB/RIF result that was obtained from the public sector [National Health Laboratory Service (NHLS)] before the commencement of treatment. Patients presenting with a RIF resistant GeneXpert result were excluded from the study. Once informed consent was obtained, both baseline overnight and spot sputum samples were collected. Previous studies on DCTB (or Rpf-dependent bacteria in sputum) used a single sample. To assess the robustness of measure of DCTB, we sought to take two independent samples from each patient at enrolment. When informed consent was administered, the participant was given a sputum collection vial to take home. Early the next morning and during the night, the participant expectorated sputum into this collection vial, which was brought to the clinic the next day – this was termed the overnight sample. Immediately upon arrival, the participant was asked to expectorate another sputum sample, this was referred to as the spot sample. Hence, two samples were collected from each participant at enrolment, Figure 3.1.

Biometric parameters (including weight and height) and socio-demographic data were taken at enrolment. In addition, blood, medical history, previous TB treatment as well as detailed antiretroviral treatment (if applicable) were recorded before the onset of TB treatment. Patients were subsequently placed on a standardized first-line TB regimen consisting of RIF, INH, PZA and EMB for two months followed by an additional four months of treatment with RIF and INH. HIV positive individuals co-infected with TB were started on ART treatment as soon as possible

after starting TB treatment. However, whilst adherence to TB treatment was closely monitored, ARV treatment was not monitored by DOT and was administered in the public health care system. Hence, limited data on ART usage was available. Further details for the longitudinal component for this study are set out in chapter 4, Section 4.4.

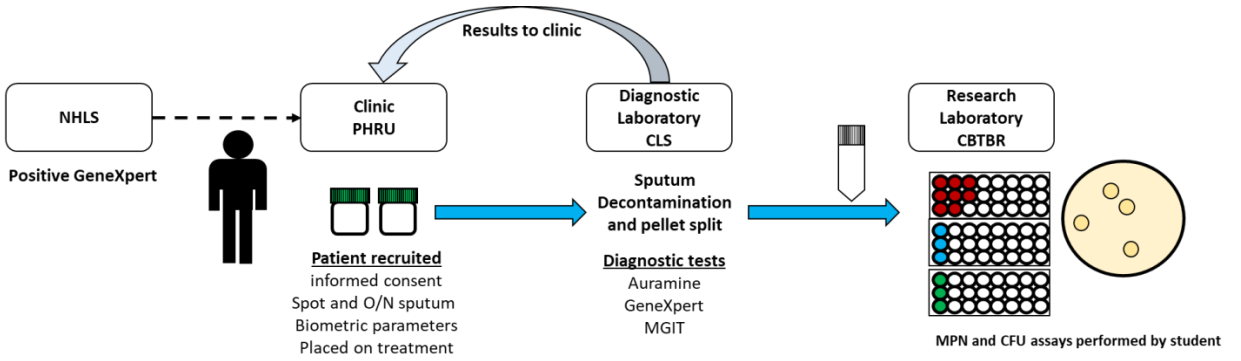

Figure 3.1 Patient recruitment and study methods map. Patients were recruited into the study at the Perinatal HIV Research Unit (PHRU) based on a positive GeneXpert result. Sputum samples collected at the clinic were transported that afternoon or the following morning to the Clinical Laboratory Services (CLS) for decontamination and processing. Following decontamination, the pellet was split. Approximately 1.5 mL of pellet was sent to the Centre of Excellence for Biomedical TB Research (CBTBR) for MPN and CFU assays (processed by PhD student and postdoctoral fellow), and 1.5 mL was kept for routine diagnostic tests. Results from these tests were communicated back to the referring doctor at PHRU.

3.4.2 Sputum processing and routine diagnostics

Following specimen collection, sputum samples were immediately transferred to the Clinical Laboratory Services (CLS) in Braamfontein. CLS is an accredited supplier of diagnostic services and was engaged in this project to provide MGIT, sputum smear and GeneXpert data for all samples. Sputum samples were decontaminated within 24 hours of sample collection and the pellet was split. Half of the sample was kept for routine processing and diagnostic testing while the remainder was used for MPN and CFU assays, Figure 3.1.

3.4.2.1 Sputum decontamination

Sputum samples were processed in the BSL3 laboratories of CLS and samples were decontaminated inside a class II A/B3 BSC using a *N*-acetyl-L-cysteine - Sodium Hydroxide

(NALC-NaOH) digestion-decontamination method. Briefly, an equal volume of 2% NaOH in NALC-NaOH-sodium citrate solution was added to the sputum in a 50 mL screw-cap plastic centrifuge tube (final concentration of NaOH is 1%). The 50 mL conical tube was then mixed on a vortexer for 15 to 30 seconds and then inverted to expose the entire surface area of the tube to the NALC-NaOH solution. The 50 mL tube was then left to stand for 15 to 20 minutes with occasional shaking to promote digestion. More NALC was added to the sample if it was still mucoid. Phosphate-buffered saline (PBS, p.H. 6.8) was then added to the 45 mL mark on the centrifuge tube. The solution was then spun down in a refrigerated centrifuge for 15 minutes at 3000 x *g*. The supernatant was decanted into a container containing tuberculocidal disinfectant, leaving only the pellet behind. Two to four mLs of PBS was then added to the pellet which was re-suspended by vortexing for five to ten seconds. 1.5 mL of the pellet was used for MPN and CFU assays and the remaining pellet was used for the routine laboratory tests. For methods on routine diagnostic tests run at CLS for sputum samples please refer to Appendix 3B. Currently, the effect of the decontamination procedure on the selection of certain bacterial sub-populations is not known and this is noted as a limitation. Further research is required in this regard.

3.4.3 Detection and quantification of differentially culturable tubercle bacilli and conventionally culturable bacilli

Assays to detect and quantify DCTB and conventionally culturable bacilli were carried out using MPN and CFU assays, respectively. These assays were performed by Amanda McIvor and are detailed below. As samples were processed in real time, the technical demands of having fresh CF available every day, and real time scoring of MPN assays necessitated assistance from a postdoctoral fellow for the MPN assays.

3.4.3.1 Most probable number assays

3.4.3.1.1 Preparation of culture filtrate

A fresh batch of 200 mL of CF from both the H37Rv and Rpf-deficient strain (strain BG1-Δ*rpfA* Δ*rpfB* Δ*rpfC* Δ*rpfD* Δ*rpfE* - referred to here on as BG1) was prepared on a daily basis for MPN processing. For this, two 50 mL cultures (in 550 mL culture flasks) were prepared from small dense log phase pre-cultures for each strain (in 50 mL culture flasks). Further details below.

Bacterial strains: For the pre-cultures, 1 mL of stock culture from frozen 1 mL aliquots of H37Rv and the BG1 strain (OD_{600nm} approximately = 0.6) was added to 50 mL flasks containing 8 mL of 7H9 media [supplemented with 0.05% Tween 80, 0.2% glycerol and 10% OADC enrichment (0,5 g oleic acid, 50 g albumin, 20 g dextrose, 0.04 g catalase in 1 L water)]. Prior to use, 7H9 media was incubated for 48 hours at 37 °C to ensure sterility. Each batch of media was labelled and dated and the results of sterility checks were recorded in a database. An example of the sterility checks for media batches used in this study are reported in Appendix 3C. The culture flasks were then transported in a secondary containment to the incubator and left to incubate at 37 °C for three days (OD_{600nm} 0.6 to 0.9).

Two 50 mL cultures for both the H37Rv and BG1 strain were prepared in 550 mL culture flasks from the pre-cultures for each day of the week. Two, four or eight mLs of pre-culture was added to 48, 46 or 42 mLs of 7H9 broth respectively depending on the final density of each culture that was required for sputum processing. The flasks were labelled 'Rv' or 'BG1' and with the appropriate day of the week on which the OD_{600nm} would be between 0.6 and 0.9. To streamline the process and for ease of use, a timetable was created for the daily preparation of bacterial pre-cultures, cultures and CF, Figure 3.2. For example, for CF required on Wednesday, pre-cultures were made the previous Friday. Eight mLs of pre-culture was subsequently sub-cultured into 42 mL of 7H9 both on the following Monday and left in the incubator at 37 °C until Wednesday morning at which the OD_{600nm} was measured. An OD_{600nm} between 0.6 and 0.9 was considered late exponential phase and used to obtain CF for MPN assays. Figure 3.3 illustrates the preparation of the two *M. tuberculosis* strains required for CF preparation.

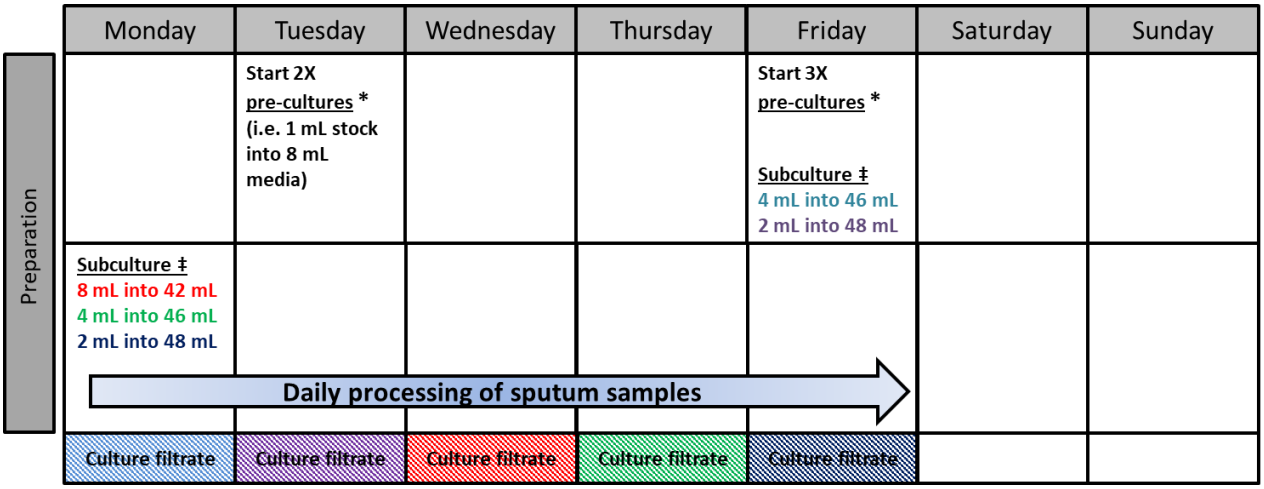

Figure 3.2 Calendar to illustrate streamlining and daily preparation of *M. tuberculosis* H37Rv and BG1 pre-cultures and cultures to obtain standardised culture filtrate for most probable number assays. Preparation of pre-cultures and cultures was standardised and optimised to obtain log-phase cultures for processing. The timetable above was constructed for ease of use. The different colours indicate separate 50 mL cultures prepared for a specific week day. For example, culture filtrate (CF) required for processing on Monday and Tuesdays required pre-cultures to be prepared the previous Tuesday by the addition of 1 mL of *Mycobacterium tuberculosis* H37Rv and BG1 (Δ*rpfA* Δ*rpfB* Δ*rpfC* Δ*rpfD* Δ*rpfE*) to 8 mL of standard media. After three days of growth, two large cultures were made on the previous Friday. CF for Monday and Tuesday was obtained from blue and purple cultures, respectively. * Pre-cultures were prepared every Tuesday and Friday, and ‡ large volume cultures from pre-cultures every Friday and Monday.

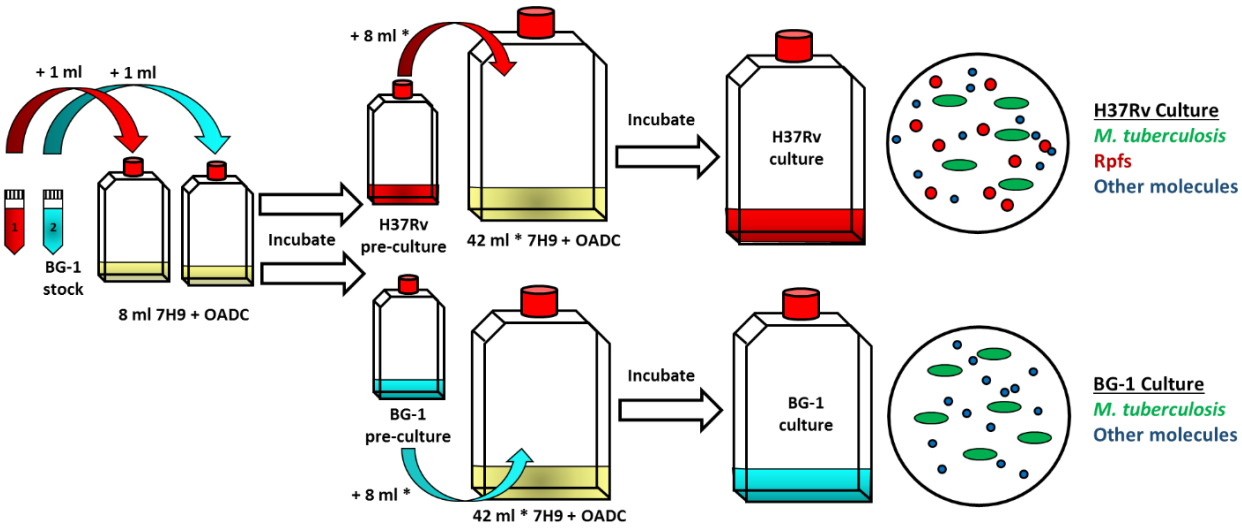

Figure 3.3 Preparation of *M. tuberculosis* bacterial strains, H37Rv and BG1. Pre-cultures from the two *M. tuberculosis* strains, H37Rv and BG1 (Δ*rpfA* Δ*rpfB* Δ*rpfC* Δ*rpfD* Δ*rpfE*) were prepared by the addition of 1 mL of freezer stock to 8 mL of standard media in 50 mL culture flasks. Following incubation, a calculated volume (denoted by *; also see Figure 3.2) was added to 42, 46 or 48 * mL of standard media in a 550 mL flask. The circles on the right give a simple illustration of the molecular components of cultures H37Rv and BG1. **Abbreviations:** OADC = oleic acid, albumin, dextrose, catalase; Rpfs = resuscitation-promoting factors.

Filtration: Both the H37Rv and BG1 cultures were removed from the incubator and placed in a container before transporting to the biosafety cabinet (BSC). One mL of each culture was removed using a Gilson pipette and placed in a cuvette for OD_{600nm} measurements. One mL of fresh media was added to another cuvette to serve as a blank control. The OD_{600nm} of both the H37Rv and BG1 cultures was read inside the BSC using a portable hand held spectrophotometer. After reading and recording the OD_{600nm}, the cuvettes were discarded in a container containing 4% agluseptin. Thereafter, 45 mL of the remaining cultures was poured into appropriately labelled 50 mL falcon tubes and the lids closed tightly. The 50 mL falcon tubes were placed in centrifuge buckets inside the biosafety cabinet ensuring that the 50 mL falcon tubes were evenly distributed and the centrifuge was balanced. The cultures were centrifuged at 3900 x *g* for five minutes to harvest the bacterial cells. Following this, the centrifuge buckets were opened carefully inside the safety BSC and the 50 mL tubes were removed with care to prevent dislodging of the pellet. The centrifuge cabinets were wiped with 70% ethanol before returning to the centrifuge. The plunger was removed from the 50 mL syringe and a 0.22 µm filter (Millipore, USA) was screwed onto the tip. The supernatant was then gently decanted into the syringe leaving the pellet behind and the plunger was reinserted into the syringe. Pressure was applied to filter and remove any remaining cells in the CF into a 250 mL culture flask. Following filtration, the 50 mL syringes were decontaminated by rinsing the syringe several times in a liquid waste bucket containing 4% agluseptin. Forty six mL of 7H9 media and 4 mL of the antimicrobial mixture, PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) was added to each culture bottle containing the filtered supernatant. The CF derived from the H37Rv and the BG1 strains were termed CF^{+} and Rpf^{-} CF, respectively.

For the media control, 4 mL of PANTA was added to a culture flask containing 96 ml of 7H9 media. Agluseptin (final concentration, 4%) was added to the 50 mL tubes containing the pellet cells before discarding and all equipment was wiped down with 4% agluseptin followed by 70% ethanol. Figure 3.4 illustrates the preparation of CF^{+} and Rpf^{-} CF from bacterial strains H37Rv and BG1, respectively.

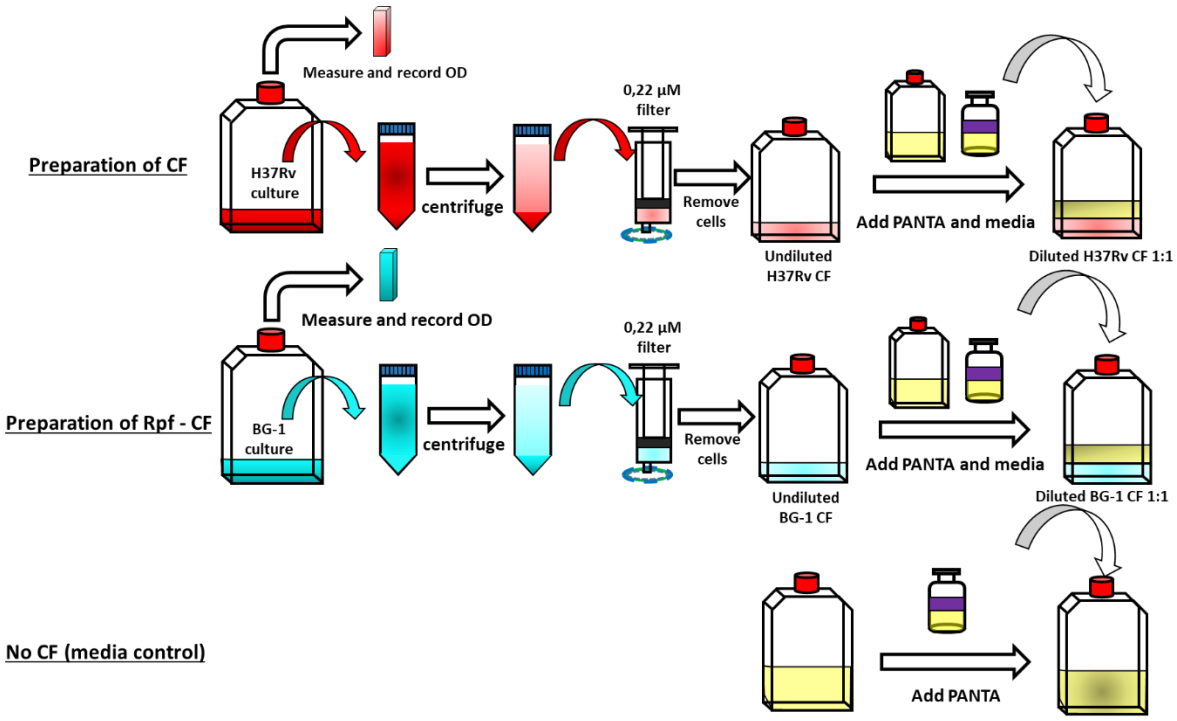

Figure 3.4 Preparation of culture filtrate and media control. Culture filtrate (CF) was prepared from bacterial strains, H37RV (red) and BG1 (Δ*rpfA* Δ*rpfB* Δ*rpfC* Δ*rpfD* Δ*rpfE*) (blue). To obtain CF, mycobacterial cultures were centrifuged at 3900 x *g* for 5 minutes. The resulting supernatant was filtered through a 0.22 µm syringe to remove any bacterial cells. The resulting CF was diluted two fold by the addition of 46 mL of 7H9 media and 4 mL polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) reconstituted in oleic acid, albumin, dextrose, catalase (OADC). For the media control, 4 mL of PANTA was added to 96 mL of 7H9 media.

3.4.3.1.2 Quality controls

Following the daily preparation of CF^+ and Rpf $^-$ CF, numerous quality controls were performed to ensure their sterility and that no cross contamination occurred during processing and the making of *M. tuberculosis* stocks. To ensure the sterility of the filtrate, a 1 mL aliquot of the CF^+, Rpf $^-$CF and media supplemented with PANTA was inoculated and spread out onto 7H11 plates and left to dry in the cabinet overnight. The following day, these plates were placed in the incubator at 37 ºC for six to eight weeks. The plates were checked on a weekly basis for the absence of contaminants and *M. tuberculosis* cells. An additional 1 mL aliquot from each filtrate was kept at 37 °C in 1 mL Eppendorf tube and monitored weekly to confirm the absence of bacterial growth. Liquid aliquots were kept for a minimum of eight weeks before being discarded. All results were recorded in an MS Excel spreadsheet for quality control (see Appendix 3D).

To ensure that the strain type was correct and no cross contamination of the CFs occurred during processing, a 1 mL aliquot of the CF^+ and Rpf^- filtrates were stored for PCR confirmation. It was assumed in this case that the CF derived from H37Rv and BG cultures contained DNA from the corresponding bacterial strain. Thus, PCR analysis, with primers targeting the *rpf* genes of the CF, would provide a means of confirming the genotype of the organism where from the CF was derived.

PCR assay and preparation*:* The stored CF^+ and Rpf^- CF was removed from the -20 °C freezer and left to thaw at ambient temperature (20 to 25 ºC). The primers were received in a powder form and were reconstituted using nuclease free water using instructions provided by the manufacturer. For the dNTPs, 10 µL of each nucleotide was added to 760 µL of nuclease free water. Primers are listed in Table 3.2.

The master mix was prepared by adding the following reagents to a 2 mL Eppendorf tube: 8.0 µL of nuclease free water (Hyclone), 8 µL of GC rich, 8 µL dNTPs, 5 µL 10X PCR buffer, 5 µL of primers -F1, -R1 and –M and 0.3 µL of Taq polymerase (total volume per sample 44.3 µL). Following preparation, 40 µL of mastermix was added to pre-labelled PCR tubes using a multichannel pipette. Ten µL of CF was then added to each corresponding PCR tube. The mixture was placed in a PCR machine (Eppendorf Mastercycler). The thermal cycler was programmed as follows: five minutes at 95 ºC as initial denaturation, followed by 35 cycles of 30 seconds at 95 ºC for denaturation, 30 seconds at 60 ºC for annealing and 30 seconds at 72 ºC for extension. Final elongation was set at 72 ºC for 10 minutes. The amplicons were stored at 4 °C until a gel was run to visualise the PCR products.

Agarose gel to detect PCR products: A 1 % agarose gel was made by weighing 1 g of agarose powder and mixed into 100 mL of fresh 1X TAE (Tris, Acetic acid, EDTA) buffer in a 500 mL Erlenmeyer flask. The agarose was placed in a microwave for about two minutes until it came to a boil. The agarose was then placed on a magnetic plate stirrer and left to cool until it reached approximately 55 °C. When the agar was sufficiently cooled, 10 µL of ethidium bromide was added to the agarose gel and it was placed back onto the stir plate for an additional minute. Two thin 15 slot combs (for 28 samples and two spaces for the DNA ladder) were placed on the gel casting container accordingly to form wells. Approximately 30 mL of cooled agarose gel was then poured into the gel casting container (15 x 7 cm UVTP tray) and left to set for 30 to 60 minutes.

After the gel had set it was transferred to a gel box (Sub Cell GT System, Biorad) and covered with 1X TAE buffer. As a DNA marker, 3 µL of DNA ladder (marker VI) was added to the first well of each row and 3 µL of loading dye was added to 5 µL of PCR product. The products were then added to the appropriate well on the gel. The power supply was set to 80V and the samples were left to run for approximately one hour.

Analysis: CF derived from the H37Rv and the BG1 mutant strain were assessed using two separate PCR reactions to ensure that the CFs were derived from the specified strains and that no cross contamination took place during filtration and CF preparation. For this, forward (F1), reverse (R1) and mutant (M) type specific primers were used to genotype both the *rpf*C and *rpf*E alleles. All three primers were added to the reaction mix to provide the opportunity to amplify the wild type or mutant bands. The CF from the H37Rv strain would yield band sizes of 371 bp and 357 bp for the *rpf*C and the *rpf*E wild type alleles, respectively. The CF from the BG1 strain on the other hand would yield PCR fragments of 556 bp and 575 bp for the *rpf*C and *rpf*E mutant alleles, respectively. As the mutant lacks the reverse primer 1 but has the region for the reverse primer 2 (designated M), the PCR product is larger, Figure 3.5. An example of a quality control analysis on a batch of CF is shown in Figure 3.6. This PCR analysis was done on all CF^+ and Rpf^- CF preparations from this study. If any inconsistencies were noted in CF preparations, the corresponding sputum samples were removed from the analysis. The results of the PCR checks on CF preparations is given in Appendix 3D.

Table 3.2 Primers used for the amplification of wild-type and mutant Rpf strains

Primer name	Primer sequence (5' – 3')	Application	Amplicon properties
RpfC-F1	CTCTATCAACGGGCCCTGAC	Forward, reverse and mutant type primers used for PCR genotyping of *rpfC* and Δ*rpfC* alleles	371 bp amplicon from wild type *rpf*C allele using RpfC-F1/ RpfC-R1 pair and 556 bp amplicon from mutant Δ*rpf*C allele using RpfC-F1/ RpfC-M pair
RpfC-R1	CACAGCAAACCCGAACTCAC		
RpfC – M	GAACTGCAGTCCGCCGTATT		
RpfE-F1	TTATCGTACGGTCCCCTTGG	Forward, reverse and mutant type primers used for PCR genotyping of *rpfE* and Δ*rpfE* alleles	357 bp amplicon from wild type *rpf*E allele using RpfE-F1/ RpfE-R1 pair and 575 bp amplicon from ΔrpfE allele using RpfE-F1/ RpfE-M pair
RpfE-R1	TCAGGATCGGCCAGGTCT		
RpfE-M	CGTCGGCATTGGCGATAC		

Abbreviations: Rpf = resuscitation-promoting factor; -F1 = forward primer; -R1 = reverse primer; -M = mutant
Adapted from Kana et al. 2008.

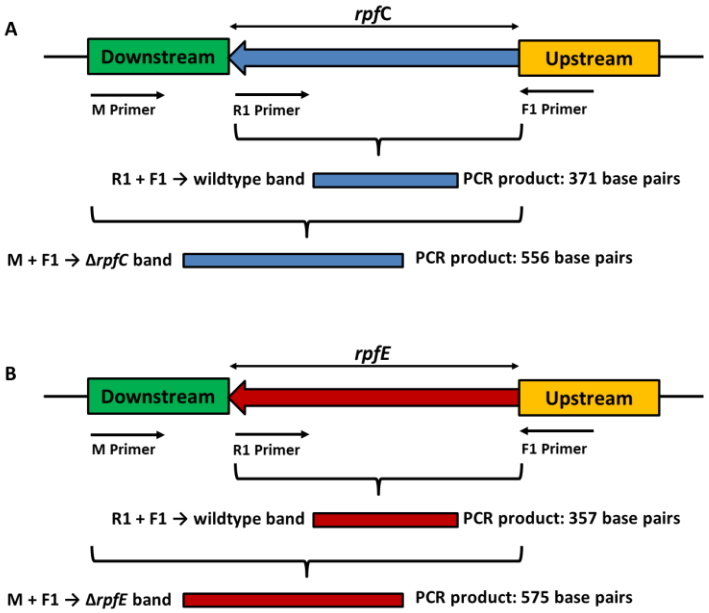

Figure 3.5 PCR-based genotyping of wild-type and BG1-ΔABCDE strains. The figure above illustrates the amplification of *rpfC* (A) and *rpfE* (B) alleles in the wild-type and mutant strains using the primers depicted in Table 3.2. The southern blot figures on the right show the expected fragment sizes: *rpfC*, 371 bp, Δ*rpfC*, 556 bp *rpfE*, 357 bp and Δ *rpfE*, 575 bp.

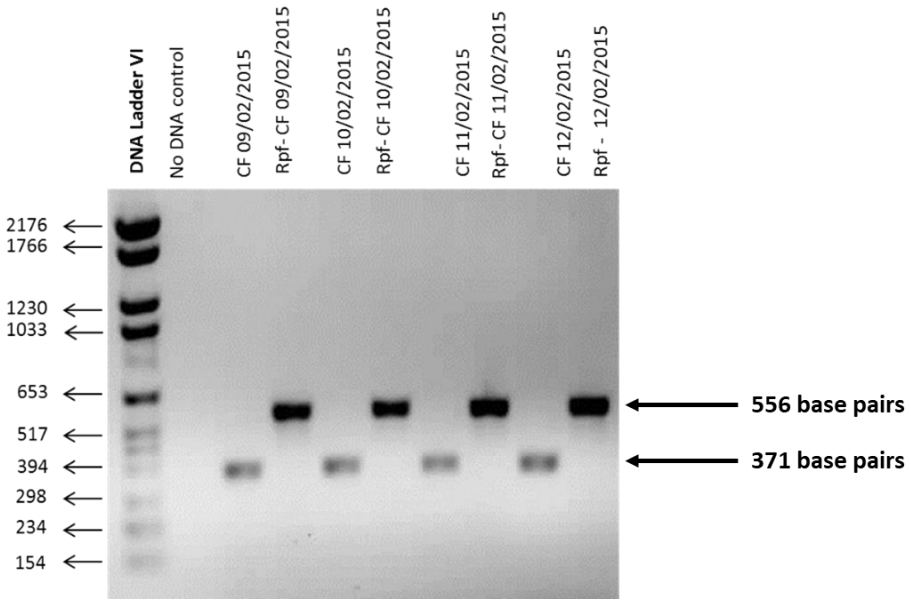

Figure 3.6 Example of PCR quality control. PCR was carried out using *rpfC* primers on culture filtrate derived from *M. tuberculosis* H37Rv and the BG1 quintuple mutant strain (Δ*rpfA* Δ*rpfB* Δ*rpfC* Δ*rpfD* Δ*rpfE*) for sample batches processed in February 2015. **Abbreviations:** CF = culture filtrate; Rpf resuscitation-promoting factor. The blot confirms that CF was derived from the H37Rv and BG1 *M. tuberculosis* strain types, respectively as well as the absence of CF cross contamination.

3.4.3.1.3 MPN plate set-up and execution

The MPN assay is based on the Poisson distribution and was designed to estimate the number of bacteria per mL based on serial dilution and presence-absence of growth in the resulting dilutions (McCrady, 1915). MPN assays were performed as previously described (Chengalroyen et al., 2016). These assays were carried out in triplicate for each sample to detect and quantify the amount of CF-dependent, Rpf-independent and CF-independent bacteria present in patient sputum samples. Figure 3.7 defines the various DCTB populations isolated in CF-supplemented and un-supplemented MPN assays. For the CF-dependent population, 450 µL of CF^{+} was added to the first three rows of a 48-well microtiter plates using a p1200 multichannel pipette. For the Rpf-independent population, 450 µL of Rpf^{-} CF was added to the last three rows of the first 48-well microtiter plate. As a control, 450 µL of fresh 7H9 media with no CF supplementation was added to the first three wells of an additional 48-well microtiter plate.

Following decontamination, 50 µL of sputum sample was added to all the wells in the first column on both MPN plates. Ten-fold serial dilutions were subsequently performed for each MPN assay by transferring 50 µL from the first column to each subsequent well until reaching the end of the plate. For this, both P1200 and P300 multichannel pipettes were used. Using the left hand, the decontaminated sputum sample and CF were mixed using the P1200 pipette (set to 400 µL) by gently pipetting up and down five to ten times. Thereafter, 50 µL of liquid was transferred using the P300 pipette in the right hand to the adjacent well for each row. Fifty µL from the last column was discarded into a large petri dish (150 X 15 mm) containing disinfectant and the tips were rinsed by slowly pipetting up and down before discarding in a 4L sharps container.

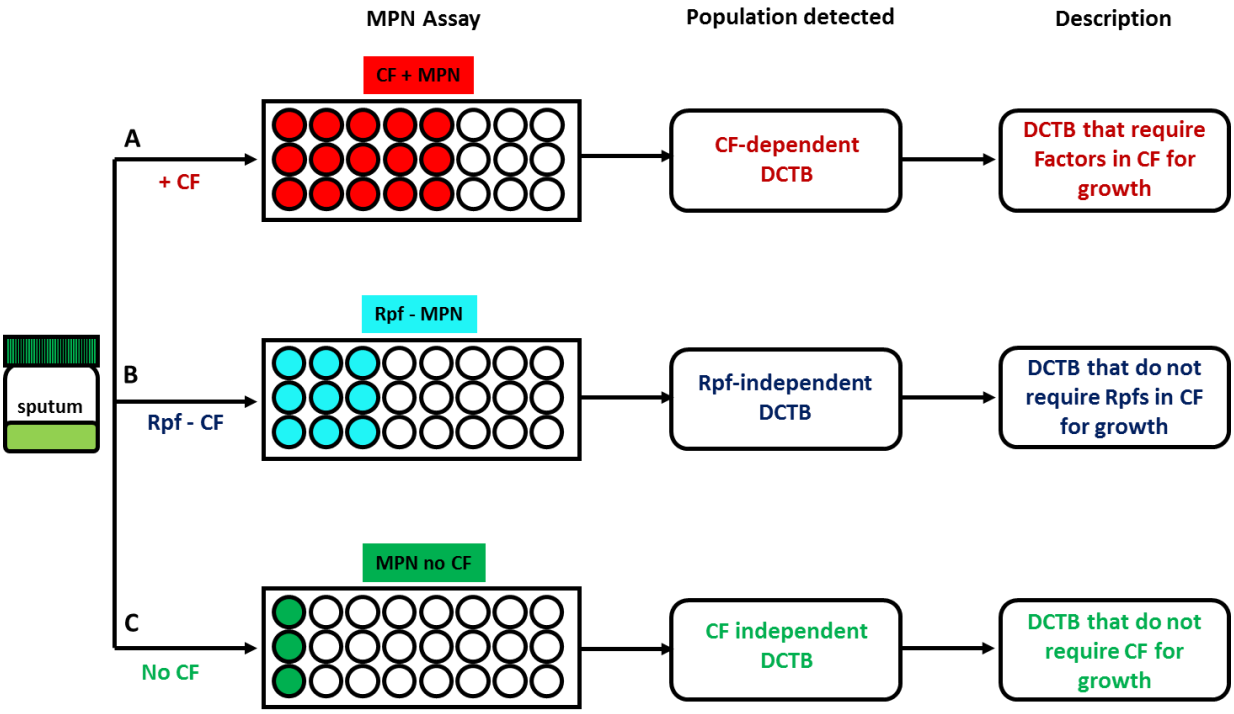

Figure 3.7 Set up of culture filtrate- supplemented and un-supplemented most probable number assays. (A and B) Most probable number (MPN) assays were set up with culture filtrate (CF^{+}) and resuscitation-promoting factor deficient (Rpf^{-}) CF supplementation of growth media. CF was isolated from wild type *Mycobacterium tuberculosis* H37Rv and Rpf^{-} CF from a quintuple *rpf* gene-knockout mutant, allowing for the detection of CF-dependent and Rpf-independent differentially culturable tubercle bacteria (DCTB), respectively. (C) To control for the CF effect, MPN assays with no CF supplementation were also performed allowing for the detection of CF-independent DCTB. A description of the population detected in each assay is also provided.

3.4.3.1.4 Incubation and plate scoring

Once all MPN plates were diluted for the processing day, plates were sealed using yellow biohazard tape and placed in the incubator at 37 °C in a sealed zip-lock bag. The plates were scored on a weekly basis using an MPN scoring sheet. The MPN of bacteria for each assay was calculated using specialized software (available from:

http://www.wiwiss.fu.berlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html).

3.4.3.1.5 Extraction and storage of MPN wells

Following six weeks incubation, the plates were kept aside for storage of positive culture in the MPN wells. DNA extraction for genotyping of *M. tuberculosis* strains was prepared simultaneously. The yellow biohazard tape to seal the plates was carefully removed to ensure no spillage of contents into the biosafety cabinet. Twenty µL of sample from the first and last well with visible growth was removed from each MPN experiment type (i.e. CF^+, Rpf $^-$ and No CF plates), and placed in a 1.5 mL Eppendorf tube containing 80 µL of TE buffer. The remainder of sample in the well was placed into a separate 1.5 mL Eppendorf tube for storage.

3.4.3.2 Colony forming unit assays

3.4.3.2.1 Set-up and execution

Ten-fold serial dilutions were performed by adding 100 µL of decontaminated pellet to 1.5 mL Eppendorf tubes containing 900 µL of 7H9 broth. Two serial dilutions were performed for each sample (10^1 to 10^2) and an additional dilution was performed for baseline and particularly mucoid samples (10^3). Six 7H11 plates were left on the bench top for approximately one to two hours to dry and these were pre-labelled with stickers containing the sample number as well as the dilution factor. Each dilution was performed in duplicate. Before plating, samples were vortexed for approximately ten seconds to ensure adequate dispersion of organisms in the suspension. Following this, 100 µL from the serial dilution tubes as well as the neat sample was then transferred to Middlebrook 7H11 plates and a disposable loop was used to streak out the solution on the 7H11 plate in order to obtain single colonies. For this, the plate was rotated for a minimum of four times after streaking was performed over the entire surface area of the 7H11 plate.

3.4.3.2.2 Incubation and plate reading

Following inoculation and streaking for single colonies, the six 7H11 plates were sealed in a gas permeable bag to prevent dehydration. The plates were placed in the incubator at 37 °C for a maximum incubation period of six weeks. The plates were examined every two weeks for growth. When single colonies were visible and at a size that was easy to count, the CFUs were recorded on a CFU scoring sheet.

3.4.4 Spoligotyping

Spoligotyping was performed on positive stored MPN wells to identify *M. tuberculosis* strains present in the populations studied.

3.4.4.1 Amplication of spacer DNA by PCR

Spacer oligonucleotide genotyping or 'spoligotyping' was performed using the primers DRa [(5'biotin) GGTTTTGGGTCTGACGAC] and DRb (CCGAGAGGGGACGGAAAC) to amplify the entire DR region.

3.4.4.1.1 Preparation of the mastermix

The primers for spoligotyping were ordered from Mapmygenome (Hyderabad, India) for spoligotyping. These were received in a powder format (100 µM stock) and were reconstituted by the addition of 1 mL of nuclease free water. Two hundred µL aliquots were prepared for future spoligotyping assays. Once reconstituted, the biotin primer (DRa) was stored at 4 ºC and the DRb primer was stored at -20 ºC. The amplification was performed in a 25 µL reaction volume consisting of: 6.5 µL nuclease free water (Hyclone), 12.5 µL ReadyMix™ Taq PCR reaction mix (Sigma-Aldrich), and 2 µL of DRa primer, 2 µL of DRb primer and 2 µL of template DNA. Following preparation, the PCR tubes were spun down to remove any bubbles that may have been present in the tube. The mixture solution was amplified by PCR machine (Eppendorf Mastercycler). The thermal cycle was programmed for three minutes at 95 ºC as initial denaturation, followed by 30 cycles of one minute at 94 ºC for denaturation, one minute at 55 ºC as annealing, 30 seconds at 72 ºC for extension, and final elongation at 72 ºC for 10 minutes. Once the run was completed, the PCR products were removed from the thermocycler and placed in the fridge at 4 °C until spoligotyping was ready to be performed.

3.4.4.2 Hybridization and detection

Hybridization of biotin-labelled PCR products to immobilised spacer-oligos (membrane-bound) were visualised on film as black squares following incubation in streptavidin-peroxidase and ECL detection reagent. The presence or absence of black squares yielded a fingerprint or known sequence to determine the *M. tuberculosis* strain genotype. The process is detailed out below.

3.3.4.2.1 Heat Denaturation of PCR products

Twenty µL of PCR product was added to the appropriate pre-labelled PCR tube containing 150 µL of 2X SSPE/ 0.1% Sodium Dodecyl Sulphate (2XSSPE/0.1% SDS) in the BSC. The diluted PCR products were subsequently transferred into a designated PCR rack designed to specifically fit into the PCR machine. The products were heat-denatured for 10 minutes at 99 ºC after which they were immediately transferred to ice. To prevent the tubes from popping open after transfer from the PCR machine to the ice, the lids were held firmly down using a paper towel.

3.4.4.2.2 Membrane preparation, hybridisation and detection

A commercially available spoligotyping membrane (Mapmygenome, India) was washed twice in 2X SSPE/0.1% SDS and placed in the oven at 60 ºC for five minutes. Following the wash step, the membrane was placed on a membrane cushion in a mini-blotter (Mapmygenome, India) with the slots running perpendicular to the line pattern on the membrane. In order to obtain the correct orientation, the membrane was placed on the mini-blotter with a ‘cut-out’ shape placed on the bottom left of the grooved surface, with the numbered wells on the top of mini-blotter running in the opposite direction. The membrane was subsequently covered with a membrane cushion, after which any excess fluid was removed by wiping with the cushion with a paper towel. The back of the mini-blotter was placed on top on the cushion, after which it was flipped over and sealed tightly with plastic screws. Any residual fluid was removed from the slots by aspiration. Following this, the slots were filled with diluted PCR product (approximately 150 to 160 µL). This was done in a slow and controlled motion to try and avoid the creation of air bubbles. The first and last wells were left ‘blank’ and filled with 2X SSPE/0.1% SDS and a H37Rv and BCG strain were included as controls. A no DNA control was also included for each run. The mini-blotter was transferred to

the hybridization oven and placed on horizontal surface at 60 °C for one hour. Following hybridization, the samples were removed from the mini-blotter by aspiration, after which the blotter was opened and the membrane was washed twice in 2X SSPE/0.5% for five minutes at 60 °C. For chemiluminescent detection, the membranes were labelled by incubation in roller bottles at 42 °C in 40 mL 2X SPPE/0.5% SDS containing 10 µL Streptavidin-peroxidase conjugate (500U/mL) for one hour. The membrane was covered with Saran-wrap and exposed to three sheets of light sensitive film for 20 minutes. The X-ray films were developed manually in the dark room. The *M. tuberculosis* genotypes were determined by entering the presence or absence of spacer sequences with a 1 or 0 using the following website: http://tbinsight.cs.rpi.edu/run_spotclust.html, last accessed 10 July 2017 (Vitol et al., 2006).

3.4.5 Data analysis

Bacillary load measures (i.e. CF^+, Rpf^-, MPN no CF and CFU assays) were compared between groups using the Wilcoxon matched-pairs signed-ranks test and data are reported as medians with interquartile ranges. To determine the various patient populations (i.e. mixed DCTB, no DCTB, CF-dependent, Rpf–independent and CF-indepdent), the resuscitation index (RI) was calculated [RI = log (MPN/CFU)] to determine the quantum of resuscitatable bacteria. Spearman's rank sum correlation was employed to further assess the relationship between CF-dependent and Rpf-independent populations. Patient demographics and TB diagnostic data were stratified by HIV-infection status. Absolute numbers and percentages were calculated for patient demographics (i.e. gender and BMI) and TB diagnostics (i.e. ZN smear status, qualitative Gene XPERT results and *M. tuberculosis* strain type) and the chi-squared test for proportions was used to compare the two groups. Demographics and TB diagnostics consisting of continuous variables (i.e. age, MGIT TTP, Gene XPERT Ct values) were compared using the students unpaired t-test. MPN and CFU data were compared between groups using the Mann-Whitney U test. To assess the role of host immunity on the generation of DCTB, the Mann-Whitney U test was used to compare patients by their HIV-infection status, as well as immune competency (HIV-positive patients grouped into CD4 T-cell count > or < 200 cells/mm^3). All statistical tests were two-sided and statistical significance was carried out using a 95% confidence interval.

3.5 RESULTS

3.5.1 Patients included in baseline analysis

A total of 174 patients were recruited into the study through the clinical platforms of the PHRU from TB diagnostic clinics in the Soweto (132 patients) and Klerksdorp (43 patients) regions. The participant disposition flowchart for these participants is given in Figure 3.8. From each cohort, the arrows depict patients that were removed from the study for various clinical and laboratory reasons. Screen failures denoted by ([a]) include patients that were initially screened for the study and then found to not meet the study requirements. Additional patients were terminated from the study and reasons for their removal are documented in Table 3.3 ([b]). Technical aspects denoted by ([c]) include MPN assays performed for patient samples that were incubated in Biolite 48-well plates (a brand of microtiter plate that inhibited growth in the MPN assay). This aspect is further discussed in section 3.5.2. An additional two patients were excluded as no sputum samples were received for baseline MPN and CFUs ([d]). Data from a total of 80 patients were available for baseline analysis.

Table 3.3 shows the 22 patients were that terminated from the study, denoted as ([b]) in Figure 3.8, and excluded from baseline analysis. In many cases, patients were excluded due to a previous TB infection (40.9%) or consent withdrawal (27.3%). The additional patients were excluded for reasons such as relocation (9%), death (1 patient), default (9%) or they were lost to follow-up (9%). In addition, data from the latter patients could not be used in baseline or early treatment analysis as they were also affected by technical issues (*), denoted as ([c]) in Figure 3.8, that affected the MPN results at baseline.

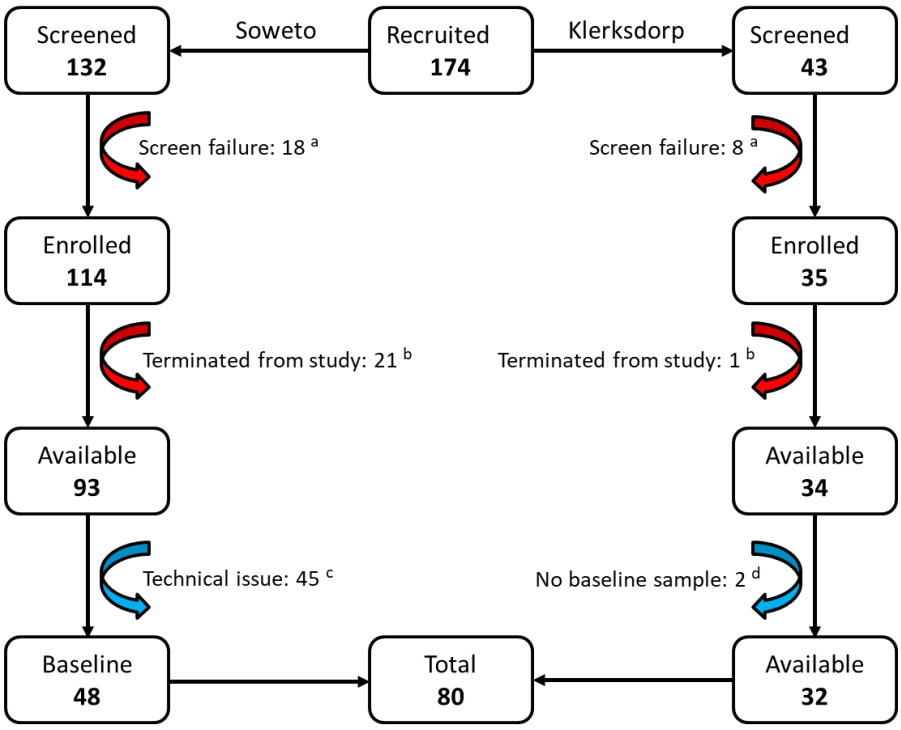

Figure 3.8 Participant disposition flow chart. A total of 174 patients were recruited into this study. These patients had a strong clinical suspicion for tuberculosis (TB) disease either through a positive smear or positive GeneXpert result. Of these, 132 and 43 were from clinics in Soweto and Klerksdorp, respectively. Twenty six (18 and 8 patients from each site) were classed as screen failures ([a]). Individuals classified as screen failures were initially enrolled into the study, but were later found to not meet the study criteria. An additional 22 (21 and one from each site) patients were terminated from the study ([b]). Reasons for study termination are provided in Table 3.3. An additional 45 patients were excluded from analysis due to technical issues ([c]) (see section 3.5.2). Two patients were excluded from analysis as no baseline sputum samples were collected ([d]). Baseline analysis was performed on a total of 80 patient samples.

Table 3.3 Reasons for patient termination from study

Reason for termination from study (n = 22)	Number of patients (%)	Patient ID
Relocation	2 (9.1)	59001, 59124*
Consent withdrawal	6 (27.3)	59061*, 59077*, 59116*, 59130, 59132, 57156
History of TB infection	9 (40.9)	59009, 59013, 59020, 59033, 59040, 59043, 59059*, 59079*, 59121*
Death	1 (4.5)	59117*
Treatment Default	2 (9.1)	59097*, 59111*
Lost to follow-up/Missed visits	2 (9.1)	59107*, 59101*

* Baseline patient data also affected by technical issues. These technical issues involved the use of growth inhibiting 48-well microtiter plates sealed with autoclave tape.

3.5.2 Technical issues leading to inhibition of growth on MPN assays

Data from 45 patients were excluded from baseline analysis due to a technical problem that occurred in the MPN assay. The brand of plates that were routinely used (Nunc from Thermo Scientific) were replaced with similar plates (Biolite plates from Thermo Scientific) due to cost saving. An assurance was provided by the supplier that bacterial growth will not be affected. However, MPN data for the samples were performed on Biolite 48-well plates yielded poor growth compared to other baseline samples processed on Nunc 48-well plates. As there was a general sense of baseline MPN data from a large number of patients already assessed at the CBTBR, it was hypothesized that the change of plates led to anomalies in the growth of bacteria. To test this, real-time analysis of patient data during the project revealed an inhibition of growth at baseline and during early treatment which became apparent when compared to the CFU data obtained. Initially, it was assumed that the first few patients affected belonged to subpopulation 5 described by Chengalroyen et al. (2016), whereby no DCTB were present and only CFUs could be obtained from these patients. However, these results occurred repeatedly with baseline patients. Figure 3.9, A, B, and C illustrates the inhibition of growth in three patients. In these patients, robust CFU counts were obtained at baseline and during the first few time points however, MPN data were consistently low.

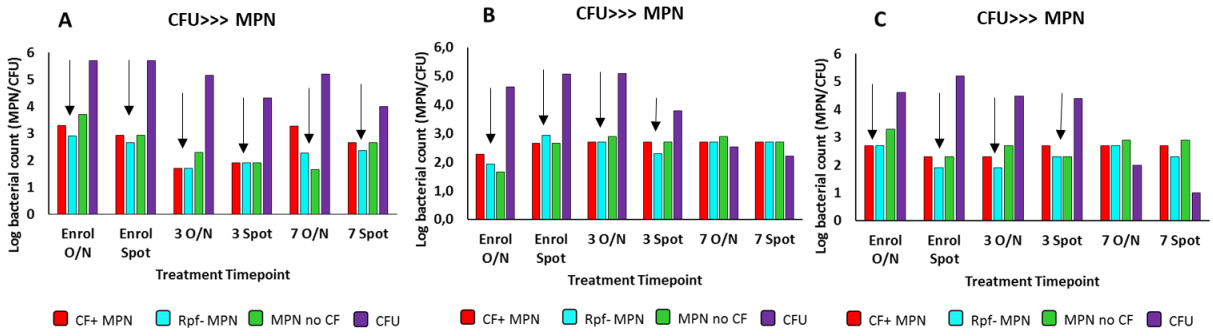

Figure 3.9 Examples of bacterial growth inhibition in the most probable number assay performed on Biolite 48-well plates in three patients. (A) Log bacterial counts for most probable number (MPN) assays set up with culture filtrate (CF^+) (red bars), resuscitation-promoting factor deficient (Rpf^-) CF (blue bars) and standard 7H9 media (green bars) are compared with log bacterial counts from standard colony forming unit (CFU) assays (purple bars) for patients (A) 59064, (B) 59066 and (C) 59072 up to 7 days post treatment initiation. The black arrows indicate unusually low MPN counts relative to the corresponding CFUs in several patient samples leading to the conclusion that the MPN assay was faulty and further investigation and troubleshooting was required.

Initially it was not clear as to why growth was inhibited in the MPN assay. Troubleshooting was performed to investigate possible reasons for the discrepancy between the MPN and CFU assay. This included the preparation of new media as well as Tween 80 as detergent can inhibit bacterial growth (Wayne, 1994). Following no improvement, the 48-well MPN plates were tested using axenic *M. tuberculosis* cultures to ensure that this phenomenon was due to growth inhibition in the plates per se and not due to DCTB subpopulations in sputum. In addition, two different types of tape (used to seal the MPN plates) were tested to assess if this could also be a potential confounder. For this, four experiments were performed: (1) MPN assays on NUNC plates sealed with biohazard tape, (2) MPN assays on NUNC plates sealed with autoclave tape, (3) MPN assays on Biolite plates sealed with autoclave tape and (4) MPN assays on Biolite plates sealed with biohazard tape. The results from this experiment indicated that optimal growth of *M. tuberculosis* was achieved using the NUNC/biohazard tape combination (1), Figure 3.10.

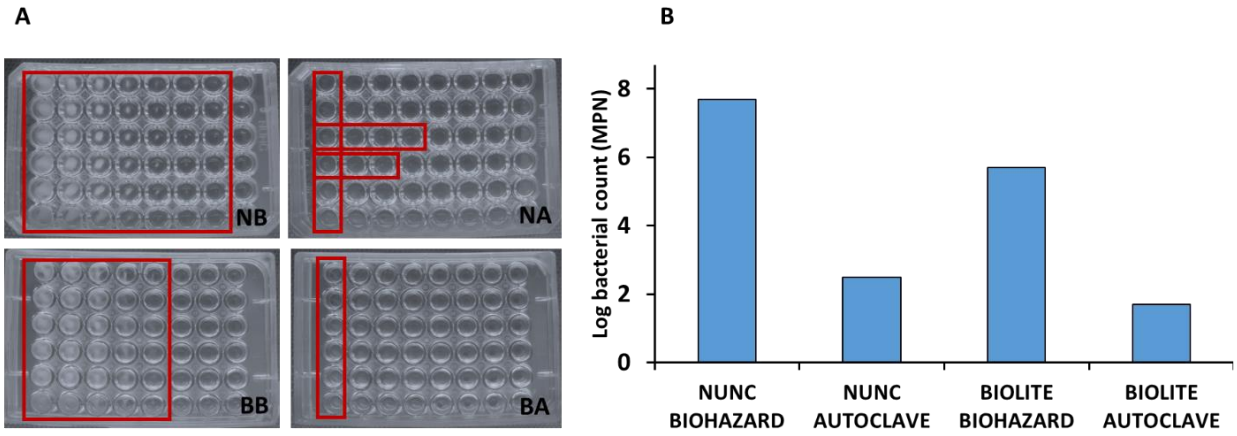

Figure 3.10 Discrepancies in bacterial growth detected using two different commercially available 48-well microtiter plates, NUNC and Biolite, sealed with biohazard tape or autoclave tape. (A) Photographs of MPN plates with *M. tuberculosis* H37Rv growth after incubation at 37 ºC for one week. (NB) Nunc 48-well culture plates sealed with yellow biohazard tape, (NA) Nunc 48-well culture plate sealed with autoclave tape, (BB) Biolite 48-well plate sealed with yellow biohazard tape and (BA) Biolite 48-well plate sealed with autoclave tape. (B) Graph illustrating differences in bacterial growth across the four experiments. **Abbreviations:** NB = Nunc/Biohazard, NA = Nunc/Autoclave, BB = Biolite/Biohazard and BA = Biolite/Autoclave.

MPN plates were observed after one and six week's incubation at 37 ºC. The NUNC and Biolite 48-well plates sealed with biohazard tape had growth in the 7^{th} and 5^{th} dilution wells following one week incubation, equivalent to 7.7 and 5.7 log bacteria per mL, respectively. The NUNC and

Biolite plates sealed with autoclave tape had growth in the first, and some additional central wells, equivalent to 2.3 and 1.7 log bacteria per mL, respectively. The MPN plates in Figure 3.10 were recorded after one week of growth, however, after six weeks incubation no further bacterial growth was present in wells that were previously scored as blank on NUNC and Biolite plates sealed with autoclave tape. These data suggested that sealing the MPN plates with autoclave tape, for as yet unknown reasons, inhibited the growth of bacteria. A potential reason for this observation may include the presence of growth inhibiting chemicals embedded in this particular autoclave tape. Alternatively, a more porous tape may have led to an increase in gas exchange or media evaporation, resulting in changes in the microenvironment and subsequent bacterial growth. Following these observations, all MPN experiments were performed only on NUNC plates sealed with biohazard tape for optimal recovery of bacteria. All data from Biolite plates were removed from further analysis. These data are reflected as patient samples that were removed from the analysis due to technical reasons in Figure 3.8.

3.5.3 Baseline analysis

3.5.3.1 The culture filtrate effect

In the overall patient population, positive cultures were obtained in 59/80 patients (73.8%) by CFU compared to 78/80 patients in the CF^+ MPN assay (97.5%). To further determine the effect of CF-supplementation on the recovery of *M. tuberculosis* from patient sputa, the log bacterial loads obtained in the CF^+, Rpf $^-$, no CF MPNs and CFU assays were compared.

Figure 3.11 represents a scatter plot illustrating the log bacterial loads obtained from sputum samples collected from the 80 patients included for analysis at baseline. In most cases, data from the O/N sputum samples was used. Preliminary analysis revealed a higher quantum of bacteria in these samples. In certain instances where data from the O/N MPN or CFU sample was contaminated or poor, data from the spot sample was used. Statistical significance between the various assays was conducted using the Wilcoxon matched-pairs signed-ranks test with a confidence interval set at 95%. The addition of CF (with and without Rpfs) was found to significantly increase the recovery of a population of *M. tuberculosis* cells that could not be detected by CF un-supplemented MPN assays and conventional CFU assays (a P value of <0.0001 was obtained for all comparisons between both CF supplemented MPN assays when compared to

the MPN no CF and CFU assays). These findings are in agreement with a number of previous publications that have demonstrated that sputum samples are dominated by a population of cells that cannot be cultured using conventional microbiological methods. In addition, a higher number of bacteria could be recovered in the CF-supplemented MPN assay when compared to the Rpf$^-$ MPN assays (median log 5.95 versus log 5.72, P = 0.0320). No significant difference was observed between the MPN no CF and CFU assays (P = 0.8005).

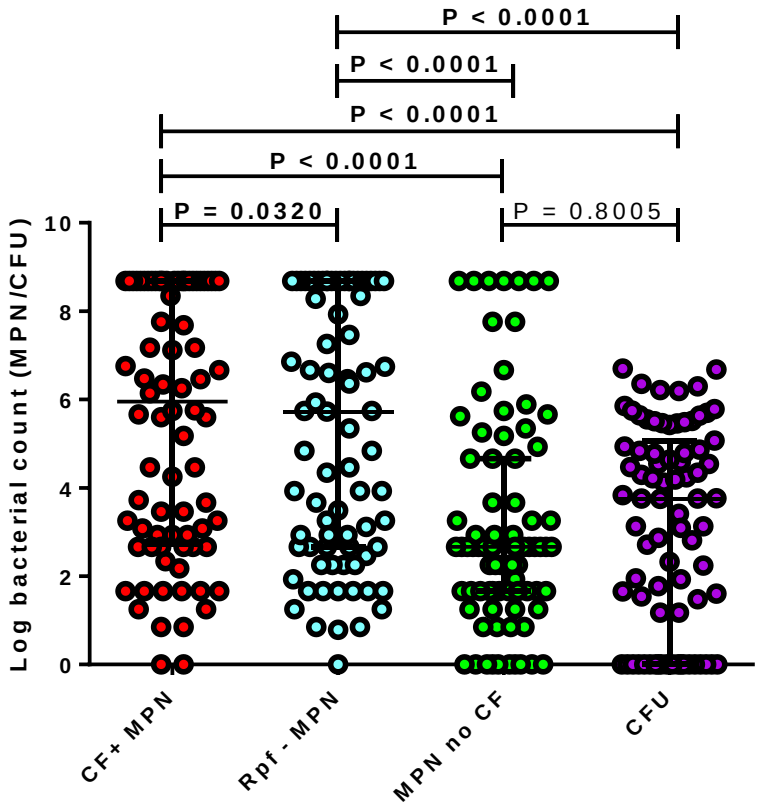

Figure 3.11 The effect of culture filtrate on the recovery of differentially culturable tubercle bacilli in treatment naïve patients. Scatter plots illustrate the bacterial load distributions obtained in 80 patients at baseline. Error bars represent medians and interquartile ranges. To determine statistical significance, the Wilcoxon matched-pairs signed-ranks test was used with a 95% confidence interval. CF$^+$ MPN (red), Rpf$^-$ MPN (blue), MPN no CF (green) and CFU (purple). CF = culture filtrate; MPN = most probable number assay. Significant differences are depicted in bold.

To further determine whether the resuscitative effect was restricted to Rpfs (and not due to stimulation via other molecules present in CF), resuscitation indices were calculated for both the CF$^+$ and Rpf$^-$ MPN assays. Spearman's rank sum correlation analysis revealed a positive correlation of 0.84 in baseline sputum samples between these two assays, Figure 3.12, confirming

that a high MPN count in the CF^+ assay was associated with a high count in the Rpf^- CF assay. These results suggest that, in most cases, factors other than Rpfs are also involved in the resuscitation of DCTB. Indeed, cAMP and various fatty acids, such as oleic acid which are also present in CF, have been implicated in the resuscitation of dormant bacteria (Shleeva et al., 2013). On average, the CF^+ MPN assay had a RI of 0.2 log greater than that observed in the Rpf^- MPN assay indicating a marginal benefit of Rpfs in resuscitation.

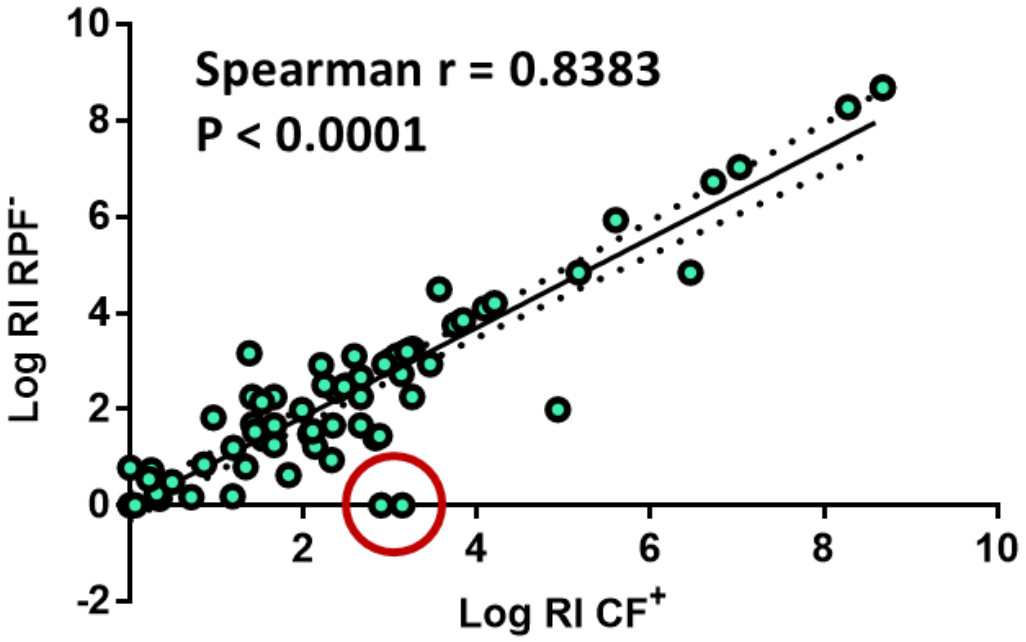

Figure 3.12 Correlation of resuscitation index (RI) between culture filtrate-supplemented most probable number assays with and without resuscitation-promoting factors (Rpfs). The resuscitative effect between most probable number (MPN) assays performed with culture filtrate (CF) derived from wild-type *Mycobacterium tuberculosis* H37Rv (CF^+) and from a quintuple *rpf* gene-knockout mutant (Rpf^-) was correlated to determine the resuscitative effect. A significant correlation was obtained between log RI Rpf^- and log RI CF^+ ($r = 0.8383$, $P > 0.0001$, Spearman rank-sum correlation). Red circles show the presence of differentially culturable organisms from two patients that were entirely dependent on Rpfs for growth.

3.5.3.2 Patient sub-populations at baseline

Of the 80 patient sputum samples analysed at baseline, 71 (88.8%) harboured populations of both CF-dependent and Rpf-independent DCTB, Figure 3.13. Within this mixed-population, 17 (24%)

patients displayed higher levels of Rpf-independent bacteria when compared to CF-dependent bacteria illustrating suppression of growth by Rpfs in this case. In contrast, 25 (35%) patients displayed higher levels of CF-dependent bacteria, whilst the same number of CF-dependent and Rpf-independent bacteria were obtained in 29 (41%) patients (see Figure 3.14). Of the remaining nine patient samples, three (3.8%) yielded CF-dependent DCTB with no detected Rpf-dependent DCTB, one patient harboured Rpf-independent DCTB, an additional patient had a negative MPN and no CFUs at baseline and five patients had CFUs with no DCTB (i.e. the number of organisms detected in the CF-supplemented MPN assays was lower than the CFU obtained). The patient with negative MPNs and no CFUs (57133) at baseline was included in the study as *M. tuberculosis* was cultured at subsequent time points (see chapter 4 for the longitudinal analysis). In addition, the baseline spot sample for this patient was positive for MTBC in the MGIT (positive culture after 30 days, AFB present on ZN strain, and positive for MTBC on HAIN MTBDR*plus*) and GeneXpert (Ct value: 30.6) assays.

No patients in this cohort had CF-independent DCTB (i.e. more organisms in MPN no CF assay than in CF-supplemented MPN assays and CFU). Figure 3.14 illustrates the subpopulations of *M. tuberculosis* isolated from the 80 patients analysed in this PhD. Furthermore, it highlights the variability within the mixed DCTB patient subpopulation.

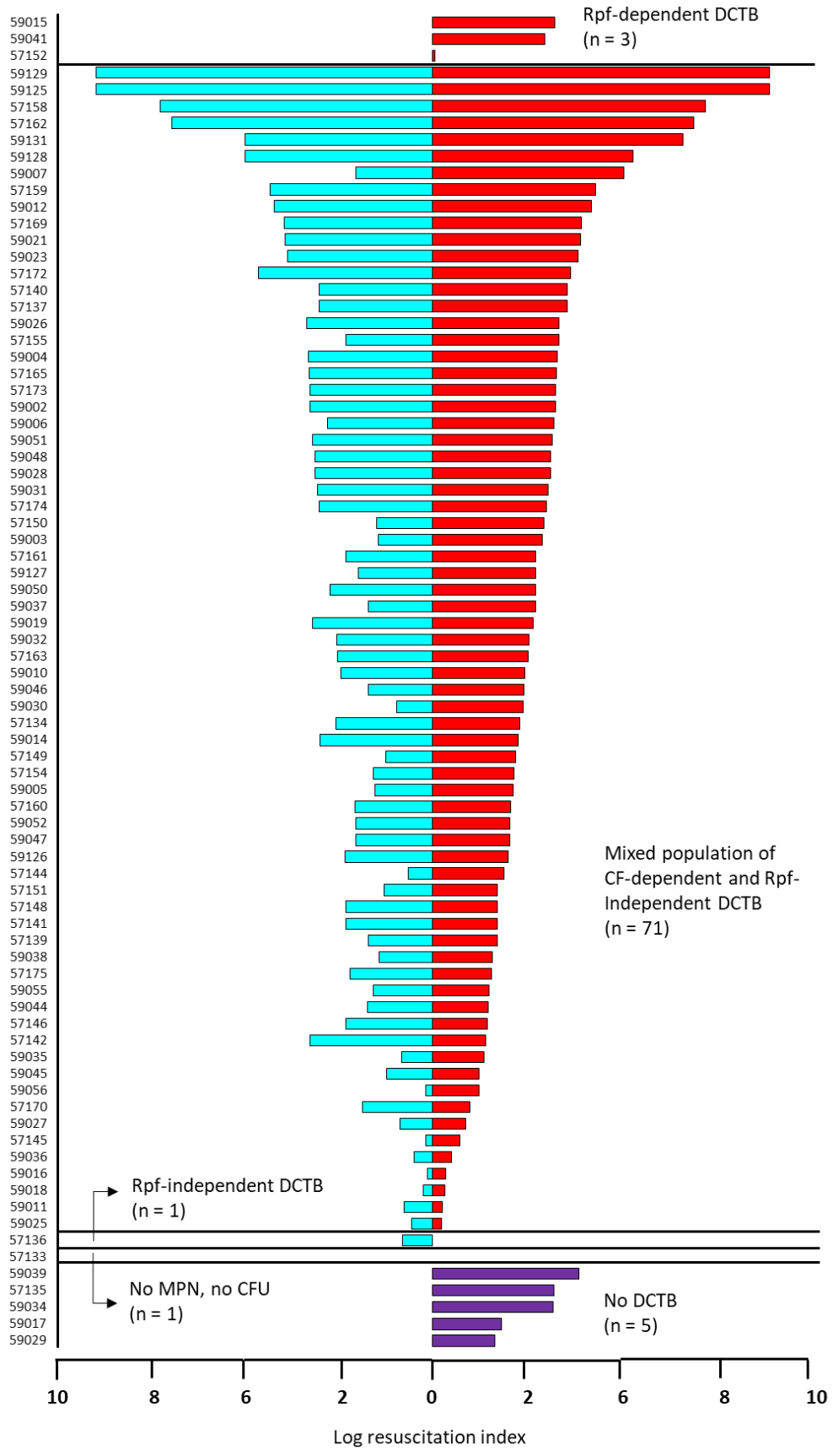
Rpf-dependent DCTB
(n = 3)
Mixed population of
CF-dependent and Rpf-
Independent DCTB
(n = 71)
Rpf-independent DCTB
(n = 1)
No MPN, no CFU
(n = 1)
No DCTB
(n = 5)
59015
59041
57152
59129
59125
57158
57162
59131
59128
59007
57159
59012
57169
59021
59023
57172
57140
57137
59026
57155
59004
57165
57173
59002
59006
59051
59048
59028
59031
57174
57150
59003
57161
59127
59050
59037
59019
59032
57163
59010
59046
59030
57134
59014
57149
57154
59005
57160
59052
59047
59126
57144
57151
57148
57141
57139
59038
57175
59055
59044
57146
57142
59035
59045
59056
57170
59027
57145
59036
59016
59018
59011
59025
57136
57133
59039
57135
59034
59017
59029
10
8
6
2
0
2
6
8
10
Log resuscitation index

Figure 3.13 Distribution of differentially culturable tubercle bacilli in treatment-naïve tuberculosis patients. The 80 patients included for analysis are listed on the y-axis with their relative proportions of differentially culturable tubercle bacilli (DCTB) [i.e. the quantum of bacteria that require resuscitation relative to the colony forming units on solid media, reported as the resuscitation index or RI where RI = Log_{10} (MPN/CFU)]. Culture filtrate (CF)-dependent bacteria, calculated as log (CF^+ MPN/CFU) are reflected in red on the right side of the y-axis, whilst Rpf-independent bacteria, calculated as log (Rpf^- MPN/CFU), are illustrated in blue on the left side of the y-axis. In cases where the CFU was zero, a value of 1 was used to reflect the absence of culturable bacteria, which indicates that the entire population in the MPN assay constituted DCTB. Patients with both red and blue bars harboured both CF-dependent and Rpf-independent DCTB populations. The absence of either red/blue or both bars indicate no detectable DCTB for the relevant population(s). The box depicts log CFU counts in samples with no detectable DCTB populations (purple bars). **Abbreviations:** MPN = most probable number; Rpf = resuscitation-promoting factor; CFU = colony forming unit.

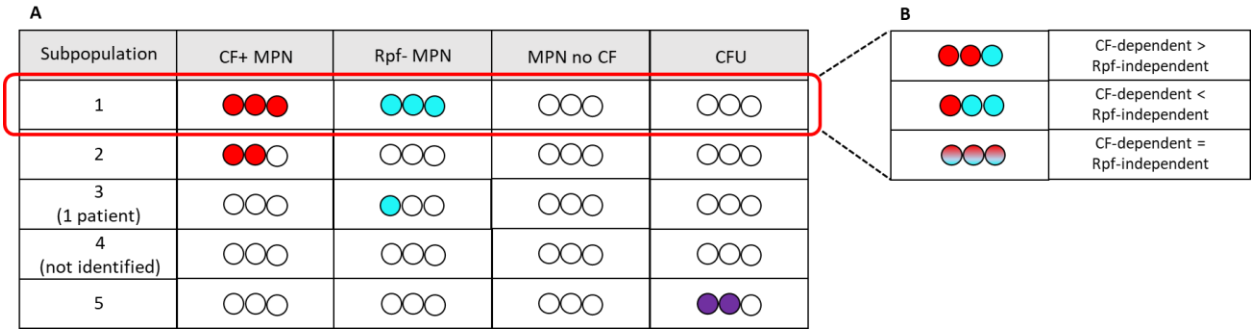

Figure 3.14 Distinct subpopulations of *M. tuberculosis* isolated from 80 treatment-naïve patients analysed in this thesis. (A) The number of coloured circles represents the proportion of patients with differentially culturable tubercle bacilli or CFU as indicated: three, > 50%; two, 2 – 50%; one <2%. (B) Within subpopulation 1 which consists of both CF-dependent and Rpf-independent bacteria, some patients exhibited a higher quantum of either CF-dependent or Rpf-independent DCTB, whilst others harboured equal numbers of bacteria that could be cultured by either CF^+ or Rpf^- CF. **Definition of abbreviations:** CF = culture filtrate; MPN = most probable number assay, Rpf^- = resuscitation-promoting factor negative; CFU = colony forming unit.

3.5.3.3 DCTB and host immunity

Chengalroyen et al. (2016) previously hypothesized and demonstrated that patients with compromised immunity have a lower quantum of DCTB when compared to individuals who are not HIV-infected. This observation was further investigated in a distinct cohort in this PhD. For this, patients were further stratified according their HIV status and HIV-positive patients were further divided according to their CD4 T-cell count. In addition, differences in patient demographics, routinely used TB diagnostics and strain type are reported, Table 3.4.

Of the 80 patients analysed at baseline, 68.8% were HIV-positive (n = 55), 68.8% were male and the median age was 36 years (IQR: 27 – 44 years). Many of these patients were underweight

(42.5%) and the median BMI was 19.1 (IQR: 17.9 – 21.5). The median age of the HIV-positive patient group was eleven years older than the HIV-negative TB group (P = 0.0558, students unpaired t-test). The majority of the population, both male and female, were HIV positive (61.8% and 84%, respectively). No significant differences were observed in BMI between the two groups. Lung pathology data was available for 34/48 patients analysed in the Soweto cohort only (see Appendix 3E). No chest X-ray data were available for any of the Klerksdorp patients. Analysis was carried out on the available data and no significant differences were observed between HIV-positive and –negative individuals in terms of pleural disease and infiltrates in both the left and right lungs. However, HIV-positive patients were more likely to present with lung adenopathy (P = 0.0283). Interpretation of this data is limited due to an incomplete data set and small sample size.

In terms of TB diagnostics, as expected, HIV-negative patients had higher rates of smear positivity (92%) than HIV-positive patients (67.3%) (P = 0.0179, chi-square test). Furthermore, HIV-negative patients had high bacterial loads, with the majority of patients (60%) having a P+++ smear grade. A large proportion of HIV-positive patients were smear negative (32.7%) and consistent with this, these patients had a higher GeneXpert cycle threshold (median 22.1 versus 18.2 in HIV-negative patients, P = 0.0644, unpaired t-test) and statistically had a significant lower qualitative score (P = 0.0256, chi-square test). The median MGIT TTP was also significantly elevated in HIV-positive patients at 9.0 days (IQR: 5.0 – 17.0) versus 6.0 days in HIV-negative patients (IQR: 3.5 – 7.5) (P = 0.0246, unpaired t-test).

Differences in bacterial burden, stratified by HIV-infection status is shown in Figure 3.15 (and Table 3.4). Sputum samples collected from the HIV-negative cohort at baseline had significantly higher numbers of both CF^+ and Rpf^- MPN counts when compared to their HIV-positive counterparts (log 8.3 versus 5.2 and log 7.9 versus 4.3, respectively; unadjusted P values: 0.0375 and 0.0276). Furthermore, HIV-negative individuals also had significantly higher levels of plateable bacteria as detected by CFU assays (log 4.8 versus log 2.8, P = 0.0037). These results suggest that patients with compromised immunity contain a lower quantum of both DCTB and culturable bacteria in their sputum possibly as a result of disseminated disease. Alternatively, since immunity is compromised, there may be less environmental pressures on these organisms to revert to these differentially cultured states. No differences were observed in the estimated numbers of bacteria cultured in the MPN no CF assay (log 3.0 versus 2.7, P = 0.2495).

Table 3.4 Demographics, immunology, microbiology and diagnostic data for tuberculosis patients categorized by HIV-infection status and C4 T-cell counts

Variable	Overall (n = 80)	HIV Negative (n = 25)	HIV Positive (n = 55)	P Value * †	CD4 <200 (n = 33)	CD4 >200 (n = 22)	P Value * †
Demographics							
Male, n (%)	55 (68.8)	21 (84.0)	34 (61.8)	**0.0473**	21 (63.6)	13 (59.1)	0.7739
Female, n (%)	25 (31.3)	4 (16.0)	21 (38.1)		12 (36.4)	9 (40.9)	
Age, yr, median (IQR)	36.0 (27.0- 44.0)	27.0 (22.5 -48.0)	38.0 (31.0 – 43.0)	0.0558	39.0 (31.5 – 43.0)	35.5 (29.8 – 45.3)	0.5987
BMI							
Underweight, n (%)	34.0 (42.5)	10.0 (40.0)	24.0 (43.6)	0.7604	14.0 (42.4)	10.0 (45.5)	0.8243
Normal, n (%)	37.0 (46.3)	12.0 (48.0)	25.0 (45.5)	0.8324	15.0 (45.5)	10.0 (45.5)	0.3265
Overweight, n (%)	8.0 (10.0)	2.0 (8.0)	6.0 (10.9)	0.6877	4.0 (12.1)	2.0 (9.1)	0.4788
Unknown, n (%)	1 (1.2)	1.0 (4.0)	-	0.1355	-	-	
Median (IQR), kg/m^2	19.1 (17.9 – 21.5)	19.1 (17.7 – 21.1)	18.9 (17.8 – 21.7)	0.4666	18.9 (17.8 – 22.2)	19.5 (17.9 – 21.6)	0.9732
Patient Immunology cells/mm3, median (IQR)	NA	NA	167.0 (93.0 – 310.0)	NA	101.0 (54.0 – 144.5)	340.0 (245.8 – 428.0)	**<0.0001**
Conventional TB diagnosis, n (%)							
Auramine smear							
Smear grade positive ††	60.0 (75.0)	23.0 (92.0)	37.0 (67.3)	**0.0179**	23.0 (69.7)	14.0 (63.6)	0.6389
Smear grade negative	20.0 (25.0)	2.0 (8.0)	18.0 (32.7)		10.0 (30.3)	8.0 (36.4)	
Scanty	8.0 (10.0)	2.0 (8.0)	6.0 (10.9)	0.6877	4.0 (12.1)	2.0 (9.1)	0.7240
+	12.0 (15.0)	2.0 (8.0)	10.0 (18.2)	0.2371	7.0 (21.2)	3.0 (13.6)	0.4755
++	11.0 (13.8)	4.0 (16.0)	7.0 (12.7)	0.6936	5.0 (15.2)	2.0 (9.1)	0.5088
+++	29.0 (37.5)	15.0 (60.0)	14.0 (27.3)	**0.0017**	7.0 (21.2)	7.0 (31.8)	0.3764
GeneXpert result							
High, n (%)	24.0 (30.0)	11.0 (44.0)	13.0 (23.6)	0.0654	6.0 (18.2)	7.0 (31.8)	0.2436
Medium, n (%)	22.0 (27.5)	10.0 (40.0)	12.0 (21.8)	0.0914	8.0 (24.2)	4.0 (18.2)	0.5939
Low, n (%)	19.0 (23.8)	2.0 (8.0)	17.0 (30.9)	**0.0256**	14.0 (42.4)	3.0 (13.6)	**0.0236**
Very low, n (%)	10.0 (12.5)	2.0 (8.0)	8.0 (14.5)	0.4119	4.0 (12.1)	4.0 (18.2)	0.6246
MTB not detected, n (%)	5.0 (6.3)	0 (0.0)	5.0 (9.1)	0.1368	1.0 (3.0)	4.0 (18.2)	0.3384
Median (IQR), Xpert cycle threshold‡	20.3 (15.4 – 25.4)	18.2 (15.0 – 20.8)	22.1 (15.5 – 26.2)	0.0644	22.6 (17.1 – 26.0)	20.8 (14.0 – 27.6)	0.5407
MGIT time t positivity, days, median (IQR)	7.0 (4.3 – 14.8)	6.0 (3.5 – 7.5)	9.0 (5.0 – 17.0)	**0.0246**	8.0 (5.5 – 15.0)	9.5 (4.0 – 21.0)	0.5113

MPN							
CF^{+} MPN, log median (IQR)	6.0 (2.7 – 8.7)	8.3 (3.9 – 8.7)	5.2 (2.7 – 8.7)	**0.0357**	5.2 (2.7 – 7.9)	5.0 (1.7 – 8.7)	0.8733
Rpf^{-} MPN, log median (IQR)	5.7 (2.7 – 8.7)	7.9 (3.6 – 8.7)	4.3 (2.3 – 8.7)	**0.0276**	4.5 (2.3 – 7.3)	3.6 (1.7 – 8.7)	0.8130
MPN no CF, log median (IQR)	2.7 (1.7 – 4.7)	3.0 (1.7 – 5.9)	2.7 (1.7 – 3.7)	0.2495	2.7 (1.7 – 4.1)	1.7 (0.6 – 3.6)	0.1608
CFU							
CFU, log median (IQR)	3.8 (0.0 – 5.1)	4.8 (3.0 – 5.6)	2.8 (0.0 – 4.5)	**0.0037**	3.1 (0.0 – 4.7)	2.3 (0.0 – 4.3)	0.7060

Definition of abbreviations: BMI = body mass index; CF = culture filtrate; IQR = interquartile range; MGIT = mycobacterial growth indicator tube; MPN = most probable number; CFU = colony forming unit; NA = not applicable; Rpf^{-} = resuscitation-promoting factor independent; TB = tuberculosis; n = number

*P value compares HIV-positive versus HIV-negative and HIV-positive patients with CD4 count above and below 200 cells/mm^3; Significant at P less than 0.05 (95% confidant interval) shown in bold.

† All the proportion comparisons by HIV status or CD4 T-cell count were conducted using the chi-square test.

†† Includes scanty, +, ++ and +++

‡ GeneXpert: *M. tuberculosis* was not detected in five patients (HIV-positive cohort). These patients were omitted from column statistics, i.e. HIV-negative n = 25; HIV-positive n = 50. Overall column statistics obtained from 75 patients.

These results are in agreement with that shown by Chengalroyen et al. (2016) and provides further evidence that compromised host immunity is associated with a reduction in DCTB as well as platable bacteria. In contrast to work previously reported, a significant difference was also seen in Rpf $^{-}$ MPN bacterial sub-populations between HIV-infected and –uninfected individuals (Chengalroyen et al., 2016). In recent work, host factors that may modulate DCTB in EP sites was investigated in 18 patients (Rosser et al., 2017). A significant correlation was found between host peripheral lymphocyte count and DCTB (P = 0.04), but no other host parameters (including HIV-infection status) were significantly associated with DCTB (Rosser et al., 2017).

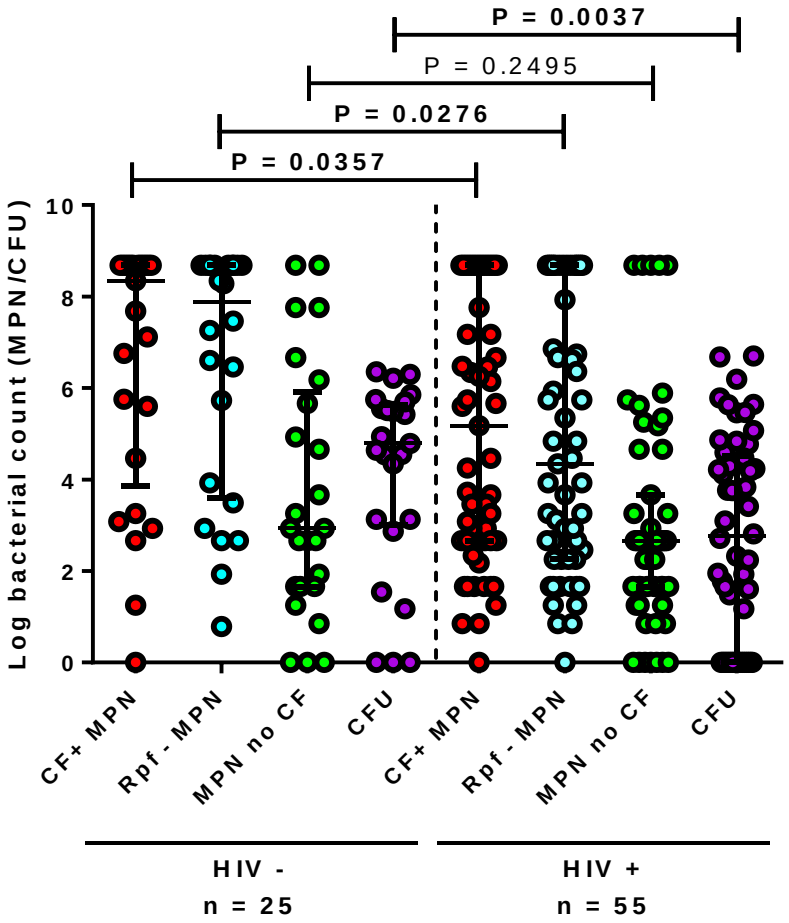

Figure 3.15 Measure of bacterial load straitified by HIV-infection status. Scatterplot depicting bacterial load distributions in HIV-infected/ uninfected individuals. Error bars represent medians and interquartile ranges. To determine statistical significance, the Mann-Whitney U test was used with a 95% confidence interval. CF^{+} MPN (red), Rpf $^{-}$ MPN (blue), MPN no CF (green) and CFU (purple). Depicted in bold are significant differences between the two groups with a 95% confidence interval. CF = culture filtrate; MPN = most probable number assay; Rpf = resuscitation-promoting factors; CFU = colony forming units; HIV = human immunodeficiency virus.

As HIV-positivity was associated with reduced bacterial load as determined by the CF^+ and Rpf^- supplemented MPN assays as well as the CFU assays, the involvement of the immune system was further explored by dividing patients into two sub-groups according to their CD4 T-cell counts, i.e. less than and greater than 200 cells/mm^3. This classification was used as acquired immunodeficiency syndrome (AIDS) is defined as all HIV positive individuals with a CD4 count of less than 200 cells per μL. The hypothesis was that patients with high CD4 T-cell counts will harbour more DCTB, similar to that observed in HIV-negative individuals. In contrast to a previous study, we found no significant differences between the two groups in the MPN and CFU supplemented assays (Figure 3.16 and Table 3.4).

It is unclear why this difference did not manifest in our cohort. In the present study, generally a higher quantum of DCTB (and wider spread of organisms between patients) was isolated in the CF^+ MPN assay compared to the previous study. For instance, in patients with a CD4 T-cell counts less than 200 cells/mm^3 the median bacterial load in the present study was log 5.2 (IQR: 2.6 – 7.9) compared to the previous study where the median bacterial load was log 1.9 (IQR: 1.3 – 3.3). The same pattern was seen in patients with CD4 T-cell counts greater than 200 cells/mm^3 [log 5.0 (IQR: 1.7 – 8.7), current study versus log 3.6 (IQR: 2.3 – 6.2), previous study]. It is likely that the differences in bacterial load accounted for the different conclusions drawn with respect to CD4 T-cell count and host immunity. The higher bacterial loads isolated in the present study may have occurred due to slight changes in protocol (i.e. splitting the sputum between CLS and the CBTBR for different assays versus splitting the pellet following decontamination for different assays). Other factors that may have contributed could include higher rates of smear positivity in this patient cohort, time prior to care seeking as well as the quality/quantity of sputum received. In the current study, sputum samples greater than 3 mL in volume were requested.

To further explore the association between host immunity and the presence of bacterial sub-populations, an alternative analysis was carried out and CD4 T-cell count was correlated with MPN (CF^+, Rpf^- and no CF) and CFU values obtained at enrolment. Using Spearman's rank sum correlation statistics, no significant correlation was found between baseline MPN/CFU and CD4 T-cell counts, Table 3.5.

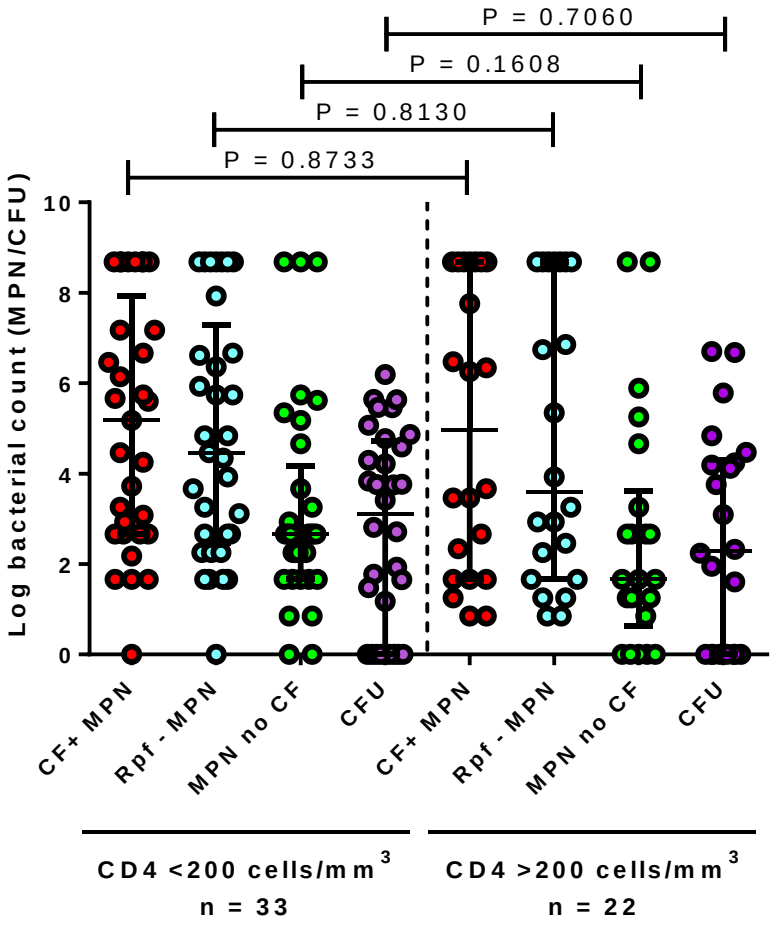

Figure 3.16 Measure of bacterial load straitified by CD4 T-cell counts. Scatterplot depicting bacterial load distributions in HIV-positive patients with advanced immune suppression (low CD4 T-cell counts, i.e. <200 cells/mm^3) versus patients with protective immunity (high CD4 T-cell counts, i.e. >200 cells/mm^3). Error bars represents medians and interquartile ranges. To determine statistical significance, the Mann-Whitney U test was used with a 95% confidence interval. CF^+ MPN (red), Rpf^- MPN (blue), MPN no CF (green) and CFU (purple). CF = culture filtrate; MPN = most probable number assay; Rpf = resuscitation-promoting factors.

Table 3.5 CD4 T-cell count is not correlated with MPN or CFU values at baseline

Spearman's correlation	CF^+ MPN	RPF^- MPN	MPN no CF	CFU
Correlation value (r_s)	-0.089	-0.100	-0.294	-0.115
P-value	0.520	0.466	0.029	0.406

Definition of abbreviations: CF = culture filtrate; Rpf = resuscitation-promoting factor; CFU = colony forming unit

3.5.3.4 Strain diversity

Spoligotyping was performed on positive MPN wells from the 80 patients at baseline to (1) gain a better understanding of the diversity within the study population, and (2) to determine whether certain strain types were more likely to respond to CF-supplementation. The *M. tuberculosis* strain distribution from Soweto and Klerksdorp regions are given in Figure 3.17.

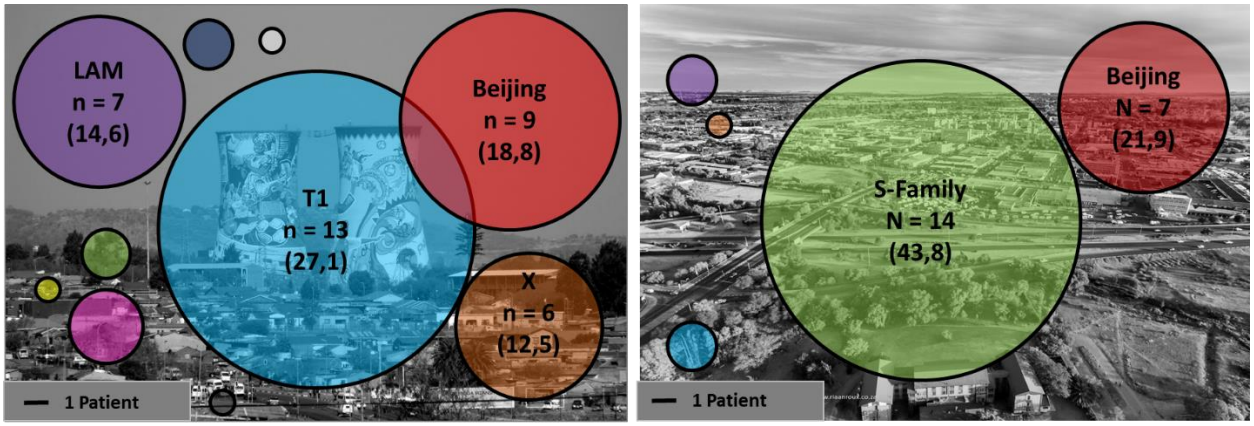

Figure 3.17 *Mycobacterium tuberculosis* strain distribution by geographic location. (A) *M. tuberculosis* strain distribution in the Soweto cohort. Strains from three patients could not be determined and are not reflected in the figure. Values for strain types identified in more than four patients are given. Values in parentheses illustrate overall percentages. Strains from patients that were not identified are included in the calculation of the overall percentage. T1 = blue, W-Beijing = red, LAM = purple, X-family = orange, CAS = pink (n =3) Haarlem = dark blue (n = 2), S-Family = green (n = 2), EAI = yellow (n = 1), T4 = black (n=1) and F36 = white (n = 1). (B) *M. tuberculosis* strain distribution in the Klerksdorp cohort. Strains from two patients could not be determined and are not reflected on the map. *M. tuberculosis* strains from an additional four patients were identified as H37Rv, are not reflected in the figure. This was most likely due to contaminating DNA from the culture filtrate. Values for strain types identified in more than four patients are given. Values in parentheses illustrate overall percentages. Strains from patients that were not identified or were identified as H37Rv are included in the calculation of the overall percentage. S-Family = green, W-Beijing = red, T1 = blue (n = 2), LAM = purple (n = 2) and X-Family = orange (n =1). **Abbreviations:** LAM = Latin American Mediterranean, CAS = Central Asian Strain, EAI = East-African Indian, F36 = Family 36. The size of the circles represent the relative proportions of each strain.

Table 3.6 *M. tuberculosis* strains isolated in Soweto and Klerksdorp regions

Strain typing, n (%)	Overall (n = 80)	Soweto region (n = 48)	Klerksdorp region (n = 32)	P-value
T1	15.0 (18.8)	13.0 (27.1)	2.0 (6.3)	**0.0172**
Beijing	16.0 (20.0)	9.0 (18.8)	7.0 (21.9)	0.7321
S-Family	16.0 (20.0)	2.0 (4.2)	14.0 (43.8)	**<0.0001**
Other*	28.0 (35.0)	21.0 (43.8)	7.0 (21.9)	0.0522
ND	5.0 (6.3)	3.0 (6.3)	2.0 (6.25)	1.0000

ND = not determined. * Includes the four strains that were identified as H37Rv in the Klerksdorp cohort

From the 80 positive MPN plates, spoligotyping results were obtained from 71 cultures. Of the nine patients where no results were obtained, five were not determined (i.e. blank on X-ray) and four were H37Rv, most likely from the CF derived from *M. tuberculosis* H37Rv. The predominant strains identified in the Soweto cohort belonged to the T1 (27.1%) and W-Beijing families (18.8%), followed by Latin American Mediterranean (14.6%, LAM, from sub-lineage IV), and X family (12.5%). Other strains identified included the Central Asian Strain (CAS, identified in three patients) Haarlem (identified in 2 patients), S-Family (2 patients), as well as the EA15 and F36 family (both strains identified in 1 patient). T4 was also identified in one patient. In the Klerksdorp cohort, the predominant strains identified were S-family (43.8%) and W-Beijing (21.9%). Other strain families identified included LAM (2 patients), T1 (2 patients), and X (one patient). When the two regions were compared with each other, the T1 family and S-family were significantly associated with the Soweto and Klerksdorp regions, respectively ($P = 0.0172$ and $P < 0.0001$). Whilst the sample sizes are small, the clustering of the strains, particularly the S-Family strain in Klerksdorp, may indicate recent transmission events (Mathema et al., 2006). No significant differences were observed between the other identified strains and region, although a greater diversity of strains were present in Soweto (other, $P = 0.0522$). Six possible mixed strain infections (8.5%) were identified in this patient cohort, where either low intensity banding patterns of a secondary strain were observed in the background, or a different strain isolated in a different MPN well from the same sample. Previous studies have reported mixed infection rates of up to 20% in pulmonary specimens alone (McIvor et al., 2017, Cohen et al., 2012). To determine whether strain type was associated with HIV status, strains were classified into T1, Beijing, S-family and other (Table 3.7). There was no significant difference in the distribution of these strains between HIV-infection status or in HIV-positive patients with CD4 counts less/ greater than 200 cells/mm^3. Patients with a CD4 T-cell count of less than 200 cells/mm^3 were more likely to be infected with 'other' *M. tuberculosis* strains. These strains included, H37Rv (13.6%), LAM (13.6%), CAS (9.1%), EA15, X1 and T4 (each strain identified in a single patient, 4.5%). When the H37Rv strains were removed from the analysis, these differences were no longer significant, $P = 0.1093$.

Table 3.7 *M. tuberculosis* strain type is not associated with HIV-infection status or CD4 T-cell count.

Strain type	Overall (n = 80)	HIV Negative (n = 25)	HIV Positive (n = 55)	P Value	CD4 <200 (n = 33)	CD4 >200 (n = 22)	P Value
T1	15.0 (18.8)	4.0 (16.0)	11.0 (20.0)	0.6709	8.0 (24.2)	3.0 (13.6)	0.3354
Beijing	16.0 (20.0)	6.0 (24.0)	10.0 (18.2)	0.5465	8.0 (24.2)	2.0 (9.1)	0.1535
S-Family	16.0 (20.0)	3.0 (12.0)	13.0 (23.6)	0.2278	7.0 (21.2)	6.0 (27.3)	0.6043
Other*	28.0 (35.0)	10.0 (40.0)	18.0 (32.7)	0.5273	7.0 (21.2)	11.0 (50.0)	**0.0258**
ND	5.0 (6.2)	2.0 (8.0)	3.0 (5.5)	0.6629	3.0 (9.1)	0.0 (0.0)	0.1458

First number is absolute number of patients with a particular strain type. Percentages are given in parentheses.
* Includes the four strains that were identified as H37Rv. Three of these strains were in the HIV-positive and CD4 >200 cells/mm^3 categories.

As the DCTB sub-populations were highly skewed with the majority of patients belonging to the mixed-DCTB population, assessing whether strain type was associated with any particular DCTB sub-population was difficult. In the CF-dependent population (three patients), the strains identified were W-Beijing, T1 and X1. In the single patient with an Rpf-independent bacterial population, a spoligotype of H37Rv was obtained and a clinical strain was not identified. Five of the patients analysed had no DCTB and Haarlem, Beijing and T1 (three patients) families were identified. Further research on a large patient cohort is required to establish the link between CF-dependency and strain type. Furthermore, research investigating the use of CF in unmasking mixed strain infections is required.

3.6. DISCUSSION

The detection and quantification of DCTB in patients with active TB disease has several important applications including assessing transmission dynamics, enhanced TB diagnosis, as well as monitoring of patient response to treatment and assessing risk of relapse disease. Furthermore, future studies to detect and quantify DCTB in latently infected patients or patients with assymptomatic sub-clinical disease may prove useful in identifying patients at risk for reactivation or progression to symptomatic disease, respectively. In this chapter, the relative proportions of conventionally culturable and differentially culturable bacterial populations in patients from

Soweto and Klerksdorp, South Africa, were described. Furthermore, the role of the immune system in generating DCTB was investigated by recruiting both HIV-positive and -negative individuals.

As previously reported, the results from this study support the findings that sputum obtained from treatment-naïve patients are dominated by DCTB (Mukamolova et al., 2010, Chengalroyen et al., 2016). Initially, it was proposed that Rpfs were key players in the resuscitation of non-culturable *M. tuberculosis* cells (Mukamolova et al., 2010). However, this finding was recently revised (Chengalroyen et al., 2016) where it was found that the addition of CF does not dictate the direct involvement of Rpfs in the resuscitation of DCTB populations. Other factors present in CF may be involved.

To further investigate this, the bacterial load in the CF-supplemented (CF^+ and Rpf^-) and -unsupplemented MPN assays were determined. A significant increase in bacterial load was observed in both CF-supplemented MPN assays when compared to CF-unsupplemented MPN and CFU assays (Figure 3.11), confirming that in sputum, the addition of CF does indeed enhance bacterial recovery. While substantive growth recovery was observed with Rpf^- CF, a significant difference was also observed between the bacterial load obtained in the CF^+ and Rpf^- MPN assays, with CF supplementation yielded an MPN of 0.2 log higher than Rpf^- CF supplementation ($P = 0.0320$). These observations suggest an added benefit of Rpfs (albeit marginal) in detecting DCTB populations but given the marginal nature of this effect, it is difficult to dissect further. Nevertheless, these results in conjunction with that observed by Chengalroyen et al. (2016) suggest a complex phenotypic diversity in sputum resident organisms. To further investigate this phenomenon, the proportion of resuscitated bacteria, relative to CFUs (a value referred to as the resuscitation index) was determined for both CF-dependent and Rpf-independent DCTB populations. The results illustrated that the majority of patients (88.8%) analysed in this study harboured mixed populations of DCTB, i.e. DCTB were unmasked following MPN supplementation with CF^+ and Rpf^- CF, providing further evidence for the role of other molecules in *M. tuberculosis* growth stimulation. A caveat here, is that whilst the Rpf^- CF does not contain an exogenous source of Rpfs, clinical *M. tuberculosis* strains are capable of producing their own Rpfs. Therefore the role of Rpfs in resuscitation cannot fully be excluded in experiments with the Rpf^- CF. To further understand the role of Rpf in bacterial resuscitation, the addition of Rpf-inhibitors to the Rpf^- MPN assay will more precisely define the presence of solely Rpf-dependent

as well a Rpf-independent bacterial populations. Nitrolphenylthiocyanates (NPTs) have been identified as low molecular weight compounds that inhibit the enzymatic and biological activity of Rpf proteins (Demina et al., 2009, Kaprelyants et al., 2012). The effect of NPT against exogenous Rpfs has been previously demonstrated where the growth stimulatory effect of recombinant RpfSm (a truncated version of *Mi. luteus* Rpf) on non-replicating *M. tuberculosis* cells was abolished following the addition of NPT (compound 5; 10 μg/ml) (Kaprelyants et al., 2012). This decrease in activity was measured by decreasing MPN counts relative to the number of CFUs.

Alternatively, the addition of recombinant Rpf to the MPN assay will impicate only Rpfs in bacterial resuscitation as CF has many molecules that be directly or indirectly involved in enhancing growth of *M. tuberculosis*. Whilst some analysis has been done on CF proteins/molecules, an exhaustive analysis of the molecular composition of *M. tuberculosis* CF is lacking (Sonnenberg and Belisle, 1997, Weldingh et al., 1998, Andersen et al., 1991). Further research employing state-of-the-art technologies such as mass spectometry would be useful in identifying biologically relevant compounds in CF that may be relevant in pathogenicity, phenotypic plasticity, growth inhibition and resuscitation. The identification of growth inhibitory/stimulatory molecules in CF could also be further explored in the the development of new drugs.

In contrast to that reported by Chengalroyen et al. (2016), the analysis revealed that a small number of patients harbored only CF-dependent or Rpf-independent DCTB (3.75% and 1.25% of the total patient population, respectively). Five patients (6.25%) also had no DCTB, but CFUs were isolated on solid media suggesting that growth in CF-supplemented liquid media is inhibitory to certain bacterial populations. Whilst the absolute proportions of various DCTB populations may have differed to those reported by Chengalroyen et al. (2016), it was encouraging to see that representative patterns from four out of the five previously reported populations were found in this study. The complexity of bacterial sub-populations detected in this chapter highlight the challenges and possible gaps in current TB diagnostic algorithms and that a single diagnostic may miss certain bacterial populations (Dartois et al., 2016).

The current study commenced in 2014 and was carried out according to an approved protocol that was designed at the CBTBR in accordance with previous published work. After the study was

largely complete, a new method of conducting the MPN to reduce increasing the risk of artifact was described (Saito et al., 2017). Previous studies have shown the addition of growth stimulatory factors to the MPN assay increase the bacterial yield 100-fold (Mukamolova et al., 2010), but this has been disputed in more recent work (Saito et al., 2017). In this study, the MPN was optimised using replicating axenic cultures so that the MPN result was equivalent to the CFU (i.e. DD/DCTB = 0). To achieve this, additional steps were employed. These steps included low speed centrifugation to break down large clumps. In addition, CFUs were calculated from the same dilution series used for the MPN assay (as opposed to a separate dilution series), the filter pipette tips were changed for each step in the dilution series and pentuplicate dilution series were performed in 96-well plates. The results from this study suggest that the reported MPN values in the current study could possibly be an overestimation. However, the inclusion of a MPN no CF control confirms the presence of DCTB in patient sputa. In addition, the CFUs obtained tended to be higher than the MPN no CF control suggesting that an artifactual increase may not have occurred. That said, we cannot exclude any artefacts but have endeavoured to apply robust methodology. Preliminary statistics investigating the confidence intervals and coefficience of variation in the triplicate assay illustrate precision in the MPN assay at baseline up until 14 days on treatment (see Appendix 3F). Pentuplicate assays were not carried out because of resource constraints and limited sputum. Future work to compare and quantify the DCTB in patient sputum samples using both MPN methods is required. As a separate comparator, other quantitative methods such as microscopy using a Petroff-Hauser chamber or flow cytometry should be included.

In chronic granulomatous TB disease, *M. tuberculosis* encounter numerous environmental stresses that are thought to drive these organisms into a non-replicating state. To test the hypothesis that host immunity serves as a driver for the establishment of DCTB, patients were stratified by their HIV-infection status and the distribution of DCTB amonst these two groups was compared. It was found that HIV-negative individuals harbour more DCTB than their HIV-positive counterparts, suggesting that host immunity is a determining factor in the generation of DCTB. However, when the HIV-positive group was further stratified according to immune competency (i.e. CD4 T-cell count greater or less than 200 cells/mm^3), no significant difference was observed between the distribution of DCTB. Furthermore, Spearman's rank sum correlation statistics revealed no significant correlation between baseline bacterial loads in the MPN and CFU with CD4 T-cell counts. These results are in contrast to those reported by Chengalroyen et al. (2016), where HIV-

positive individuals with a greater immune competency, i.e. CD4 T-cell count > 200 cells/mm^3, displayed higher CF^+ MPN values. Possible reasons for this discrepancy could be related to differences in disease pathology, or how the disease was acquired, i.e. recent transmission events versus reactivation disease. While analysis was attempted on the lung pathology data that was available, interpretation was limited due to an incomplete data set. Recent studies investigating the association between host immunity and DCTB from EP sites showed that host peripheral lymphocyte counts were correlated with DCTB (termed MPN_culture supernatant/CS bacillary counts) (Rosser et al., 2017). Other host factors analysed including HIV-infection status, diabetes millitus, vitamin D status, C-reactive protein levels, neutrophil or monocyte counts were not significantly correlated with MPN_CS counts (Rosser et al., 2017). However, due to the small sample size (19 samples fro 18 patients) multivariate analysis was underpowered in these studies and future work should investigate the impact of host-related factors on the generation of DCTB.

HIV-positive patients are more likely to develop disseminated disease (Palmieri et al., 2002, Gupta et al., 2015), resulting in lower bacillary load in sputum samples (Getahun et al., 2007, Hargreaves et al., 2001). Indeed, 15% of reactivation disease occurs in EP sites (Farer et al., 1979), and adipose tissue has been suggested as a site for *M. tuberculosis* persistence (Neyrolles et al., 2006, Beigier-Bompadre et al., 2017). Investigation into the quantification of DCTB in patients with EPTB disease has recently been investigated (Rosser et al., 2017). The authors concluded that CS-dependent DCTB are common in EPTB (present in 52% of patients), but to a lesser degree than that observed in sputum samples (Mukamolova et al., 2010, Chengalroyen et al., 2016).

The *M. tuberculosis* strains isolated in this study did not cluster with HIV serostatus, however the T1 and S-Family genotype were highly prevalent in patients recruited from Soweto and Klerksdorp regions, respectively. The samples size in this study was too small to make any meanginful comparison of strain prevalence by DCTB population. These limitations notwithstanding, clustering has been associated with recent transmission (Genewein et al., 1993, Mathema et al., 2006, Small et al., 1994), therefore large molecular epidemiological studies in these regions may be able to identify potential outbreaks.

A previous study investigating *M. tuberculosis* transmission in a community with a high prevalence of HIV showed that HIV-negative individuals were almost twice as likely to be a cluster index case compared to HIV positive individuals. These findings suggest that HIV-negative

individuals may be disproportionately responsible for TB transmission within a community (Middelkoop et al., 2015). Previous reports have shown that following *M. tuberculosis* infection, HIV-positive individuals advance more rapidly to active TB disease than HIV-negative individuals (Daley et al., 1992, Selwyn et al., 1989, Dooley et al., 1992). The rapid progression to active disease along with the clinical presentation of HIV-TB co-infection (i.e.paucibacillary disease), may explain the lower rates of transmission by HIV-positive individuals (Middelkoop et al., 2015). Given the above findings and that HIV-negative status is associated with an increased number of DCTB, it could be speculated that DCTB may play a role in the transmission of TB disease by HIV-negative individuals. The bio-aerosol cloud generated by these individuals through coughing may also contain a high number of these organisms. Therefore, this data may define a novel role for DCTB in the transmission of DCTB by HIV-uninfected to HIV-infected individuals within the community, Figure 3.18. Further investigation to prove this hypothesis is required. Also, one cannot discount the fact that the sheer numbers of HIV-infected TB cases in the endemic settings such as South Africa may be sufficient for these individuals to sustain transmision as a collective. The role of DCTB in transmission could possibly be achieved through the use of a cough aerosol sampling system, although it would be challenging if not impossible to determine which organisms are responsible for establishing the infection. For this, *in vitro* models to generate DCTB, methods to label these organisms for subsequent identification and animal based experiments may be required to shed light on these phenomena. It would also be interesting to establish whether DCTB and culturable organisms are linked to reactivation disease and recent transmission, respectively. Large molecular genotyping studies are required to shed further light on these and related topics.

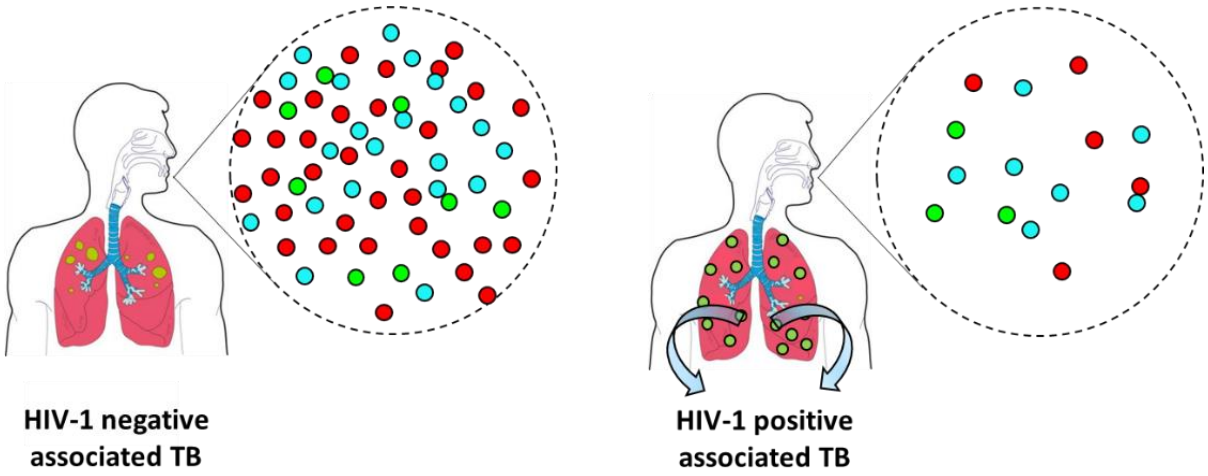

Figure 3.18 Are HIV-negative patients more infectious due to a higher quantum of differentially culturable tubercle bacilli in their sputum? A number of questions regarding differentially culturable tubercle bacilli (DCTB) and their supposed role in transmission dynamics requires further investigation. For instance, does the bio-aersosol cloud generated by immune-competent individuals consists of a higher quantum of culturable organisms (green dots) and DCTB (red and blue dots) than that generated by immune-compromised individuals? What is the role of DCTB (versus conventionally culturable bacilli) in transmission and progression of TB disease? Are DCTB more robust than conventionally culturable bacilli?, and if so, does this provide the organism with an adaptive advantage to survive host defense mechanisms? Are DCTB more likely to result in the establishment of infection and subsequent disease? Or are they more susceptible to damage than conventionally culturable bacteria?

3.7 FUTURE RESEARCH/ CHAPTER CONCLUSION

To conclude, the data illustrated herein confirm previous reports of the prevalence of DCTB in the sputum of treatment-naïve individuals in South Africa. Sputum from patients in the cohort reported herein also display a complexity in bacterial subpopulations with mixtures of CF-dependent and Rpf-independent DCTB. HIV-infected individuals appear to habour lower numbers of MPN responsive organisms when compared to their HIV-uninfected counterparts. Molecular typing of strains did not yield an intrinsic capacity for any given strain to adopt a differentially culturable state. Future research to further investigate these findings will include the following:

1. Fractionation of the CF using membranes that discriminate by molecular weight followed by MPN assays on axenic cultures and patient sputa using these different fractions to determine the size/nature of molecules involved in resuscitaion.

2. Previously MPNs were conducted with heat treated filtrate to eliminate enzymatic activity; however, in most cases, whilst growth stimulation was reduced, it was not completely

abolished (Chengalroyen et al., 2016). These results suggest that the enzymes involved in resuscitation are either heat stable or growth stimulation is also mediated by non-enzymatic effects. To further investigate the mechanistic basis, the CF can be treated with lipases, proteases (start with broad spectrum and then narrow down to individual) and glycosydases etc. MPN assays will determine whether certain molecules in CF stimulate or inhibit growth. Additionally, the CF can be subjected to p.H. and temperature adjustments.

3. Assessment of the association of DCTB with drug-resistant pumonary disease as well as EPTB disease. In light of this, two new large-scale studies have recently been launched at the CBTBR and patients are currenly being recruited.

4. Are DCTB more likely to occur in patients with history of TB disease (long standing latent infection) as opposed to recent transmission events? Large scale epidemiological studies employing molecular-based DNA fingerprinting techniques or whole genome sequencing would be required in these cases.

APPENDICES

APPENDIX 3A: ETHICS CLEARANCE CERTIFICATE

R14/49 Miss Amanda McIvor

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M161058

NAME: (Principal Investigator)	Miss Amanda McIvor
DEPARTMENT:	Centre of Excellence for Biomedical TB Research
PROJECT TITLE:	Characterisation of Differentially Culturable Bacteria in Axenic Culture and from Tuberculosis Patients
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M140265 and M110833 Dr Neil Martinson
SUPERVISOR:	Bavesh Kana
APPROVED BY:	Prof C Feldman, Co-Chairperson, HREC (Medical)
DATE OF APPROVAL:	20/12/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in October and will therefore be due in the month of October each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3B: DIAGNOSTIC TESTS PERFORMED AT CLS

MGIT culture (done by CLS laboratory personnel)

Inoculation

MGIT culture was performed on all samples using the BACTEC MGIT 960 detection system. For this, 800 µL of PANTA (reconstituted in 15 mL of growth supplement) was added to each labelled MGIT tube prior to inoculation. Thereafter, 0.5 mL of decontaminated sediment was then added aseptically to each tube using a 3 mL sterile disposable Pasteur pipette. The tubes were then capped and inverted several times before insertion into the detection system.

Removal of positive and negative cultures

Positive and negative MGIT cultures were removed from the detection system after the instrument signalled the presence of a positive or negative culture via light indicators on the drawers of the instrument. Samples were incubated for a maximum of 42 days after which, a negative result was recorded if no growth was obtained. Positive cultures were inoculated onto blood agar plates to check for contamination and a ZN stain was performed to confirm the presence of AFB. All positive MGIT cultures were also confirmed as MTBC by the HAIN MTBDR*plus* assay. All results were recorded using the CLS computational reporting system and provided with unique patient identifiers in an MS Excel Format.

Smear microscopy (done by CLS laboratory personnel)

A 20 µL drop of decontaminated pellet was transferred to a microscope slide (smear was approximately 1.0 cm by 2.0 cm in size), fixed for an hour at 65°C and stained with auramine fluorescent staining according to the laboratory SOP. For this, the slide was flooded with auramine-O and left to stain for 15 minutes. The slide was then rinsed with water and decolorized with 0.5% acid-alcohol for two minutes. After rinsing with water, the slide was counterstained with potassium permanganate for two minutes and rinsed with water. After air drying, the slides were examined using 20X and 40X objective for screening. A 100X oil immersion objective was used if needed to confirm the characteristic morphology of fluorescing bacilli. The number of fluorescing bacilli was counted for 100 fields and the mean number of acid fast bacilli (AFB) per field was

determined. The table below is taken from the TB alliance Protocol NC-002 Microbiology Manual (Page 29) and was used to report the smear grading for each sputum sample tested.

Determination of smear grade based on the number of AFB observed per field of view

Number of AFB (400X magnification)	Result
No AFB in at least 40 fields	No AFB
1 – 19/ 40 fields	Scanty
20 – 199/ 40 fields	1+
5 – 50/ field	2+
>50/ field	3+

GeneXpert (done by CLS laboratory personnel)

The GeneXpert (Cepheid, Sunnyvale, CA) was performed on all patient sputum samples received at the CLS. This system is able to integrate DNA extraction, genomic amplification and a semi-quantitative diagnostic detection of *M. tuberculosis* as well as RIF resistance due to mutations in the *rpo*B gene. One and a half mL of GeneXpert MTB/RIF sample reagent was added to a 50 mL conical tube containing 0.5 mL of decontaminated sediment using a sterile transfer pipette. The sample reagent and specimen was then mixed by vigorously shaking the tube 10 to 20 times after which it was left to incubate at room temperature for 15 minutes with shaking at every five minute interval. The liquefied sample was added to the cartridge using a sterile transfer pipette and the test was started within 30 minutes of adding the sample to the cartridge. The results were interpreted by means of the GeneXpert DX system by measurement of fluorescent signals and mathematical algorithms. The results were quantitatively displayed as high, medium, low or very low bacterial load depending on the cycle threshold (Ct) value obtained (Table 3.3). In addition, a quantitative Ct value was also recorded. The Ct value is defined as the number of cycles that are required for the fluorescent signal to exceed the threshold, i.e. exceed the level of background noise. The Ct value is indirectly proportional to the amount of nucleic acid present in the sample, therefore a low Ct value is indicative of a higher bacterial load. The table below illustrates the range of Ct values for each qualitative category.

Qualitative Xpert reporting system based on Ct value range

MTB Result	Ct Range
High	<16
Medium	16 – 22
Low	22 – 28
Very Low	>28

Adapted from GeneXpert package insert.

HAIN MTBDR*plus* assay (done by CLS laboratory personnel)

The Hain MTBDR*plus* line probe assay (Hain Lifescience, Nehren, Germany) assay was performed on all MGIT samples that flagged positive to confirm the presence of MTBC and susceptibility towards both RIF and INH through mutations in the *rpoB* gene as well as the *inhA* promotor and *katG* genes, respectively. The assay was carried out according to manufacturer's instructions. Briefly, crude DNA was extracted from 1 mL MGIT aliquots. The samples were heat killed at 95 ºC for 20 minutes, followed by ultra-sonification for 15 minutes. Subsequently, the samples were centrifuged at 13 000 x *g* for 5 minutes after which the supernatant was removed for PCR. In a separate clean room, the master mix was prepared by adding 35 µL primer nucleotide mix (PNM), 5 µL 10X PCR buffer, 2 µL $MgCl_2$, 0.2 µL Hot Star Taq DNA polymerase and 3 µL sterile, deionised water (total volume: 50 µL per specimen). Forty five µL of master mix was aliquoted into to 0.2 mL PCR tubes and 5 µL of sterile deionised water was added to the first PCR tube to serve as the amplification control. The PCR tubes were labelled and taken into a separate room for amplification. In the amplification room, 5µL of DNA was added to each corresponding PCR tube. The thermal cycle was programmed as follows:

i)	5 minutes at 95 ºC	1 cycle
ii)	30 seconds at 95 ºC, 2 minutes at 58 ºC	10 cycles
iii)	25 sec at 95 °C, 40 sec at 53 °C, 40 sec at 70 °C	30 cycles
iv)	Hold at 4 70 °C	

Once the run was completed, the PCR products were removed from the thermocycler and hybridised onto probe strips using a GT-blot and commercially available kit (Hain Lifescience, Nehren, Germany).

APPENDIX 3C: EXAMPLE OF MEDIA STERILITY CHECKS

Media	Media batch number	date checked for sterility	Sample numbers processed	Media	Media batch number	Date checked for sterility	Sample numbers processed
7H11	JP 060614	2014/06/06	BM0002-BM0003	7H9	GB 280514	2014/05/29	BM0002-BM0003
7H11	JP 060614	2014/06/06	BM0004-BM0005	7H9	GB 280514	2014/05/29	BM0004-BM0005
7H11	JP 060614	2014/06/06	BM0006	7H9	JP 110614	2014/06/11	BM0006
7H11	AM 090614	2014/06/09	BM0007	7H9	JP 110614	2014/06/11	BM0007
7H11	AM 090614	2014/06/09	BM0008	7H9	JP 110614	2014/06/11	BM0008
7H11	AM 090614	2014/06/09	BM0009-BM0013	7H9	JP 110614	2014/06/11	BM0009-BM0013
7H11	AM 090614	2014/06/09	BM0014-BM0018	7H9	JP 110614	2014/06/11	BM0014-BM0018
7H11	AM 090614	2014/06/09	BM0019-BM0022	7H9	JP 110614	2014/06/11	BM0019-BM0022
7H11	AM 090615	2014/06/09	BM0023-BM0024	7H9	JP 110615	2014/06/11	BM0023-BM0024
7H11	JP 240614	2014/06/24	BM0025-BM0028	7H9	JP 240614	2014/06/25	BM0025-BM0028
7H11	AM 090615	2014/06/09	BM0029-BM0032	7H9	JP 240614	2014/06/25	BM0029-BM0032
7H11	JP 240614	2014/06/24	BM0033-BM0036	7H9	JP 240614	2014/06/25	BM0033-BM0036
7H11	JP 240614	2014/06/24	BM0037 - BM0040	7H9	JP 240614	2014/06/25	BM0036 - BM0040
7H11	JP 030714	2014/07/03	BM0041-BM0042	7H9	JP 240614	2014/06/25	BM0041-BM0042
7H11	JP 030714	2014/07/03	BM0043-BM0050	7H9	JP 240614	2014/06/25	BM0043-BM0050
7H11	JP 030714	2014/07/03	BM0051-BM0052	7H9	JP 240614	2014/06/25	BM0051-BM0052
7H11	JP 030714	2014/07/03	BM0053-BM0054	7H9	AM 080714	2014/07/08	BM0053-BM0054
7H11	JP 080714	2014/07/08	BM0055-BM0061	7H9	AM 080714	2014/07/08	BM0055-BM0061
7H11	JP 080714	2014/07/08	BM0062-BM0069	7H9	AM 080714	2014/07/08	BM0062-BM0069
7H11	AM 100714	2014/07/10	BM0070-BM0073	7H9	AM 080714	2014/07/08	BM0070-BM0073
7H11	AM 100714	2014/07/10	BM0074-BM0077	7H9	AM 080714	2014/07/08	BM0074-BM0077
7H11	AM 100714	2014/07/10	BM0078-BM0086	7H9	AM 080714	2014/07/08	BM0078-BM0086
7H11	AP 150714	2014/07/15	BM0087-BM0092	7H9	AM 080714	2014/07/08	BM0087-BM0092
7H11	AP 150714	2014/07/15	BM0093-BM0096	7H9	AM 080714	2014/07/08	BM0093-BM0096
7H11	AP 150714	2014/07/15	BM0097-BM0104	7H9	AP 160714	2014/07/16	BM0097-BM0104
7H11	AP160714	2014/07/16	BM0105-BM0112	7H9	AP 16072014	2014/07/16	BM0105-BM0108
7H11	JP220714	2014/07/22	BM0113-BM0133	7H9	AM 11072014	2014/07/11	BM0109-BM0120
7H11	AM240714	2014/07/24	BM0134-BM0175	7H9	GB22072014	2014/07/22	BM0121-BM0126
7H11	AP010814	2014/08/01	BM0176-BM0181	7H9	AP 22072014	2014/07/22	BM0127-BM0133
7H11	AP040814	2014/08/04	BM0182-BM204	7H9	AM110714	2014/07/11	BM0134-BM0145

Table 3C is an example of the database and records kept for the media quality control for batches made between June 2014 to August 2014.

APPENDIX 3D: EXAMPLE OF CULTURE FILTRATE STERILITY CHECKS AND PCR

Date Processed	Sample No.	Solid Media	Liquid Media	QC Pass/Fail	PCR RV/BG	QC Pass/Fail
2014/06/05	BM0001	NG	NG	Pass	Yes	Pass
2014/06/09	BM0002 - BM0003	NG	NG	Pass	Yes	Pass
2014/06/11	BM0004-BM0005	NG	NG	Pass	Yes	Pass
2014/06/12	BM0006	NG	NG	Pass	Yes	Pass
2014/06/18	BM0007	NG	NG	Pass	Yes	Pass
2014/06/19	BM0008	NG	NG	Pass	Yes	Pass
2014/06/23	BM0009-BM0013	NG	NG	Pass	Yes	Pass
2014/06/24	BM0014-BM0018	NG	NG	Pass	Yes	Pass
2014/06/25	BM0019-BM0022	NG	NG	Pass	Yes	Pass
2014/06/26	BM0023-BM0024	NG	NG	Pass	Yes	Pass
2014/06/27	BM0025-BM0028	NG	NG	Pass	Yes	Pass
2014/06/30	BM0029-BM0032	NG	NG	Pass	Yes	Pass
2014/07/01	BM0033-BM0036	NG	NG	Pass	Yes	Pass
2014/07/02	BM0037 - BM0040	NG	NG	Pass	Yes	Pass
2014/07/03	BM0041-BM0042	NG	NG	Pass	Yes	Pass
2014/07/04	BM0043-BM0050	NG	NG	Pass	Yes	Pass
2014/07/07	BM0051-BM0052	NG	NG	Pass	Yes	Pass
2014/07/08	BM0053-BM0054	NG	NG	Pass	Yes	Pass
2014/07/09	BM0055-BM0061	NG	NG	Pass	Yes	Pass
2014/07/10	BM0062-BM0069	NG	NG	Pass	Yes	Pass
2014/07/11	BM0070-BM0073	NG	NG	Pass	Yes	Pass
2014/07/14	BM0074-BM0077	NG	NG	Pass	Yes	Pass
2014/07/15	BM0078-BM0086	NG	NG	Pass	Yes	Pass
2014/07/16	BM0087-BM0092	NG	NG	Pass	Yes	Pass
2014/07/17	BM0093-BM0096	NG	NG	Pass	Yes	Pass
2014/07/18	BM0097-BM0104	NG	NG	Pass	Yes	Pass

Table 3D is an example of the records kept for the CF quality control from June 2014 to July 2014. Contaminated BG CFs (determined by positive *M. tuberculosis* growth on solid and in liquid media) were obtained in samples 59125 (BM 1492; day 56), 57169 (BM2003; day 180), 57135 (BM1493 and BM1491; enrolment overnight and spot samples) – data not in Table 3D. In these samples, growth was observed in all the BG1 wells on the MPN plates. These samples were excluded from analysis (Rpf-independent data from BG1 only).

APPENDIX 3E. LUNG PATHOLOGY

Variable	Overall (n = 34)	HIV-Negative (n=12)	HIV-Positive (n=22)	P-value
Left lung cavities				
No (%)	15 (44.1)	3 (25.0)	12 (54.5)	0.0973
Yes (%)	19 (55.9)	9 (75.0)	10 (45.5)	
Right lung cavities				
No (%)	18 (52.9)	4 (33.3)	14 (63.6)	0.0907
Yes (%)	16 (47.1)	8 (66.6)	8 (36.4)	
Left lung infiltrates				
No (%)	11 (32.4)	3 (25.0)	8 (36.4)	0.4985
Yes (%)	23 (67.6)	9 (75.0)	14 (63.6)	
Right lung infiltrates				
No (%)	10 (29.4)	5 (41.7)	5 (22.7)	0.2468
Yes (%)	24 (70.6)	7 (58.3)	17 (77.3)	
Left lung adenopathy				
No (%)	27 (79.4)	12 (100.0)	15 (68.2)	**0.0283**
Yes (%)	7 (20.6)	0 (0.0)	7 (31.8)	
Right lung adenopathy				
No (%)	27 (79.4)	12 (100.0)	15 (68.2)	**0.0283**
Yes (%)	7 (20.6)	0 (0.0)	7 (31.8)	
Left lung pleural disease				0.1693
No (%)	33 (97.1)	11 (91.7)	22 (100.0)	
Yes (%)	1 (2.9)	1 (8.3)	0 (0.0)	
Right lung pleural disease				
No (%)	31 (91.2)	11 (91.7)	20 (90.9)	0.9407
Yes (%)	3 (8.8)	1 (8.3)	2 (9.1)	
Cavitation *				
1	17 (53.1)	4 (33.3)	13 (65.0)	0.2181
2	4 (12.5)	2 (16.7)	2 (10.0)	
3	11 (34.4)	6 (50.0)	5 (25.0)	
Extent of disease †				
A	10 (30.3)	2 (16.7)	8 (38.1)	0.2736
B	9 (27.3)	5 (41.7)	4 (19.0)	
C	14 (42.4)	5 (41.7)	9 (42.9)	

Data were available for 34 Soweto patients included for baseline analysis

* Data available for 32/34 patients (data from 2 HIV-positive patients were not available).

† Extent of disease data available for 33/34 patients (data from one HIV-positive patient was not available).

All comparisons were conducted using the chi-square test. Significance at P less than 0.05 (95% confidant interval) is shown in bold.

For cavitation, 1: absent, 2: single or multiple cavities with diameter of <4cm in aggregate and 3: single or multiple cavities with diameter ≥4cm in aggregate. For extent of disease, A: Limited – lesion(s) involving a total lung area less than one-quarter the area of the entire thoracic cavity as seen on PA or AP view. B: Moderate – lesion(s) greater that (A), but, even if bilateral involve a total lung area of less than one-half the area of the entire thoracic cavity as seen on PA or AP view. C: Extensive – lesion(s) involving a total lung area, equal to or more than half the area of the entire thoracic cavity as seen on PA or AP view.

APPENDIX 3F: INTRA-ASSAY VARIATION

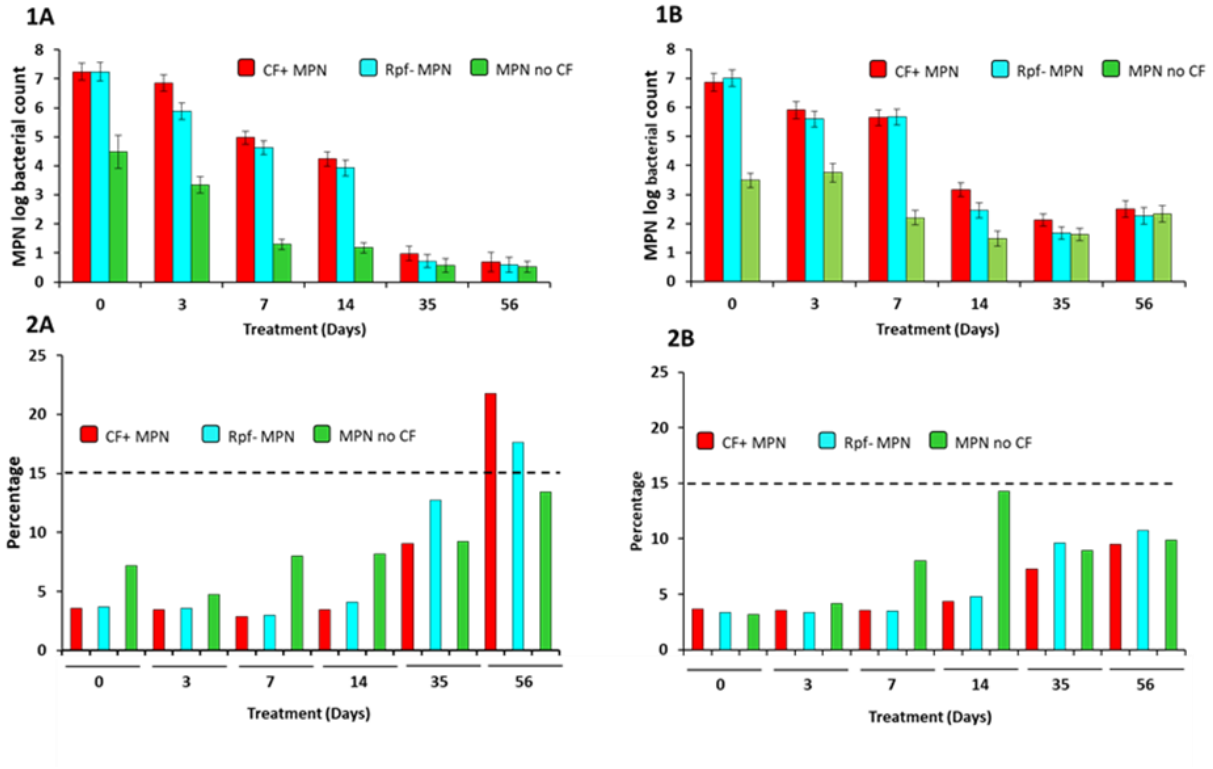

Figure 3F: Most probable number intra-assay variation in sputum samples from HIV-negative (A) and –positive (B) individuals. (1) The confidence intervals based on triple MPN dilution series from five HIV negative (left) and five HIV positive (right) individuals are depicted at different time points in the study. (2) The coefficient of variation was calculated to illustrate the extent of assay variability in relation to the mean. At earlier time points (i.e. days 0, 3, 7 and 14), the coefficient of variation was generally below 5% in the CF+ and Rpf- MPN assays indicating little variability between the replicates. A greater coefficient of variation was observed in the MPN no CF assays. At later time points (i.e. days 35 and 56), higher coefficients of variations are observed. These findings show that more variability is obtained in the MPN assay at later time points, possibly as a result of lower bacterial loads or an increase in artefact.

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Detailed components of this PhD

Background and literature review

CHAPER ONE	CHAPTER TWO
TUBERCULOSIS	MICROBIAL DORMANCY

Original Research Chapters

KEY QUESTIONS?	KEY CONCLUSIONS?
CHAPTER 3 Do all TB patients harbor DCTB? What are the various DCTB populations? Are Rpfs solely responsible for unmasking DCTB? Does host immunity affect the quantum of DCTB?	**CHAPTER 3** Four distinct sub-populations of DCTB observed amongst patients Rpf effect was marginal in unmasking DCTB HIV-negative patients harbored a higher quantum of DCTB at baseline CD4 T-cell count not associated with DCTB
CHAPTER 4 How do DCTB respond to first-line TB treatment compared to conventionally culturable bacteria? Are there potential biomarkers to predict patient response to treatment? Do DCTB remain at the end of treatment?	**CHAPTER FOUR** ?
CHAPTER 5 What is the relationship between the MPN assay and currently employed *M. tuberculosis* culture methods? Can the MPN possibly be used in EBA studies? Does CF improve the relationship between the MPN and MGIT TTP in clinical samples?	**CHAPTER FIVE** ?
CHAPTER 6 Does the addition of CF to the MGIT enhance diagnostic pick-up? Can modification of the MGIT by the addition of CF improve the time to culture positivity? Does a reduction in total MGIT volume improve TTP by enhanced quorum sensing?	**CHAPTER SIX** ?

CHAPTER 4

DETECTION AND QUANTIFICATION OF DIFFERENTIALLY CULTURABLE BACTERIA IN TUBERCULOSIS PATIENTS: A LONGITUDINAL ANALYSIS

"It is not the strongest of the species that survive,

nor the most intelligent, but the ones

most responsive to change."

Charles Darwin

4.1 INTRODUCTION

Numerous studies have demonstrated the presence of DCTB in pre-treatment patient sputum samples at baseline (Mukamolova et al., 2010, Chengalroyen et al., 2016). Furthermore, the detection of both CF-dependent and Rpf-independent populations in TB disease suggests a high complexity of bacterial populations within a single patient which is speculated to prevent the rapid clearance of these organisms during treatment. However, how these populations respond to treatment in human disease *in vivo* has not previously been investigated and is the focal point of study in this chapter of this thesis.

It has long been hypothesized that dormant bacilli (associated with LTBI) are more tolerant to TB drugs when compared to actively dividing organisms due to the physiological changes that these organisms incur (Gold and Nathan, 2017). Non-replicating *M. tuberculosis* generated by means of various *in vitro* models have been shown to exhibit an increased tolerance to RIF and INH compared to actively dividing organisms (Wayne and Hayes, 1996, Chao and Rubin, 2010, Deb et al., 2009, Sala et al., 2010, Salina et al., 2014, Wayne and Sramek, 1994, Saito et al., 2017, Gold and Nathan, 2017). Whilst these findings are significant, it has been demonstrated that Rpf-dependent bacteria can be generated in response to host-related factors pertaining to the *in vivo* environment (Turapov et al., 2014a, Manina et al., 2015). Considering this, and the demonstration of DCTB in sputum of treatment naïve individuals, it is therefore important to assess how these organisms respond to treatment. Few studies have investigated response to treatment and tolerance to chemotherapeutics of DCTB organisms isolated from patient sputum samples (Turapov et al., 2016, Mukamolova et al., 2010). These studies provide

anecdotal evidence to suggest that DCTB display tolerance to TB drugs. However, these studies have been conducted on a small number of patient samples (Mukamolova et al., 2010, Turapov et al., 2016) and further expansion of this approach to larger cohorts will provide much needed answers to key questions regarding bacterial persistence. Studies investigating these phenomena are discussed. For clarity, we use terminology for differentially culturable organisms as that cited in the respective studies. As an example, MPN assays stimulated with CF from wild type H37Rv yield a bacterial count that has been annotated as CF-dependent DCTB in our work. Other groups have termed these organisms Rpf-dependent bacteria. In this case, we use the nomenclature that is used in the article reporting the data. We have adopted this approach as other authors may contend our reannotation of their results and we have, as yet, not published an opinion piece on this to allow for an open debate on the nomenclature.

4.1.1 Studies investigating the effect of TB treatment on DCTB

One of the first studies investigating the impact of chemotherapy on DCTB was carried out by Mukamolova et al. (2010). Sputum samples from three individual patients were treated directly with RIF for one week after which MPN and CFU assays were carried out (Mukamolova et al., 2010). Following exposure to this antibiotic, no bacteria were detected in the CFU and CF un-supplemented MPN assays; however, the Rpf-dependent population, as detected by the CF supplemented MPN assay, remained essentially unchanged, thus demonstrating tolerance of this population to RIF. To further monitor the response to treatment of this population, four sputum samples were collected from patients between seven and 11 days following the start of treatment. It was determined that bacterial counts detected using CFU assays declined more rapidly than those detected in the MPN-supplemented assays. These results demonstrated that Rpf-dependent cells are eliminated at a much slower rate than actively dividing bacteria (approximated at 20 times slower) (Mukamolova et al., 2010). An alternative explanation could be that Rpf-dependent bacteria are generated, from the population of replicating bacteria, in response to chemotherapy, possibly as a mechanism to ensure survival. Consistent with this, Turapov et al. (2014b) showed that exposure of *M. bovis* BCG to antimicrobials that target cell wall biosynthesis under non-permissive growth conditions yielded enhanced survival of DCTB. The transcriptional regulator *RaaS* (i.e. regulator of antimicrobial-assisted survival) was associated with this phenomenon, where its induction resulted in reduced expression of ATP-dependent efflux pumps leading to the long term survival of DCTB. Furthermore, an in-frame deletion mutant (Δ*RaaS*) was unable to survive *in vitro* under non-permissive growth

conditions or during macrophage and mouse infection (Turapov et al., 2014b). These findings have clinically relevant implications with regards to TB treatment and targeting non-replicating bacilli *in vivo*. For example, efflux pump inhibitors have been recognised as a group of antimicrobials to prevent the emergence of drug resistance in replicating bacteria (Gupta et al., 2013). Caution should be taken when introducing these drugs into current algorithms as they could possibly promote the long term survival of non-replicating bacteria, leading to increased rates of treatment failure and recurrent TB disease due to relapse. In a separate study, mycobacterial cultures (*M. smegmatis* mc^2 155 and *M. tuberculosis* H37Rv) treated with first-line antibiotics led to the generation of organisms that could only be recovered with CF supplemented media (Loraine et al., 2016). The authors propose that this may serve as a useful *in vitro* model to generate DCTB to test the efficacy of new antimicrobials on Rpf-dependent bacteria (Loraine et al., 2016).

As previously mentioned, the effect of first-line anti-chemotherapeutic agents on *M. tuberculosis* isolated from patient sputa has been investigated (Turapov et al., 2016). For this, drug treatment was performed by incubation of decontaminated sputa in 10 mL of supplemented 7H9 media containing PANTA and the relevant antimicrobial for a week (the final concentration of each antimicrobial were reported as follows: STREP, 10 and 20 μg/mL; RIF, 1 and 5 μg/mL; EMB, 10 and 20 μg/mL; INH, 1 and 10 μg/mL and PZA, 40 and 100 g/mL). The numerically dominant Rpf-dependent populations from the sputum samples were reported to be more tolerant to both INH and STREP, whilst PZA had no substantial effect on this population and only a marginal effect was observed following incubation in EMB after seven days (Turapov et al., 2016). Interpretation of the results presented in this study may have been influenced by a number of limitations. Firstly, the conclusions made were from observations based on four sputum samples from four patients, this is a relatively small sample size and variation between patients can influence what bacterial populations emerged after drug treatment. The sputum samples were previously frozen at -80 ºC and selected on the basis of their high-volume. The freezing of samples may generate sub-populations of DCTB. The authors did attempt to address this limitation in their study by assessing the growth of culturable and Rpf-dependent populations in stored decontaminated homogenised sputum samples [sputum samples were stored in i) PBS, ii) 200mM trehalose in water and iii) 10% (vol/vol) glycerol in water]. The authors noted that in most cases there was an increase in the viable counts in both culturable and differentially culturable populations (Turapov et al., 2016). Additionally, the results may have been restricted by the range of drug concentrations chosen,

as well as the length of incubation in the presence of a particular drug. Nevertheless, these findings create the possibility for the assessment of future drug candidates using *M. tuberculosis* cells directly isolated from patient sputa. As TB is treated with a multi-drug combination regimen and not a single antibiotic, it would be interesting for future studies to assess how the different *M. tuberculosis* populations respond to treatment with multiple antibiotics.

In an interesting new development, a method to generate differentially culturable organisms involving nutrient starvation, following exposure to RIF has been described (Saito et al., 2017). The organisms emerging from this model were termed differentially detectable TB (DDTB). The investigators were unable to generate DDTB from a clinical isolate with an *rpoB* mutation, showing that RIF must engage with its canonical target to generate a differentially culturable phenotype. DDTB produced by this method were shown to be phenotypically tolerant to INH and a four drug combination (RIF, INH, EMB and PZA) (Saito et al., 2017). In this case, the DDTB did not require CF for growth recovery in MPN assays.

The results from the studies described above, suggest that measuring the bactericidal effects of currently used TB drugs may be problematic due to the reliance on CFU decline as a measure of drug efficacy. For example, EBA studies are used to monitor the efficacy of different chemotherapeutic regimens, for clinical advancement, by measuring the decline in CFU in sputum samples during early treatment (Sirgel et al., 2000, Jindani et al., 1980, Sirgel et al., 1993, Sirgel et al., 1997). The preservation (and possible accumulation) of DCTB would not be revealed using these current methods and this may skew the readout of drug efficacy. Therefore future EBA studies would benefit by monitoring the effect of antimicrobials on these various differentially culturable *M. tuberculosis* populations. New methods to quantify and assess DCTB during treatment are important as they could aid in developing bacterial biomarkers for treatment response. For example, monitoring DCTB during and after treatment could serve as a potential marker to identify patients that are likely to fail treatment or relapse. This could lead to greater responsiveness in treatment algorithms, i.e. if a particular regimen is not working, it could be changed according to DCTB profiles. Furthermore, the presence of DCTB at the end of treatment may result in that particular patient receiving preventative treatment, thus preventing relapse disease.

4.1.2 Proposed spectrum of differential culturability during treatment of TB disease

Prevailing evidence suggests that DCTB can emerge in response to drug treatment, stress conditions and can also be influenced by host immunity. Considering this, the spectrum of differentially culturable organisms, and the consequent diagnosis of TB, may change during treatment. Figure 4.1 is a hypothetical model illustrating the proposed spectrum of bacterial culturability before and throughout the six-month TB treatment regimen. The figure also proposes the microbiological outcome using conventional routine diagnostic tests for diagnosis of disease as well as treatment monitoring. Before treatment, bacteria exist in multiple growth states within the lung cavities (Mukamolova et al., 2010). It is hypothesized that these organisms arise from the expansion of small founding populations that are seeded to the lung following the initial transmission event. Whilst there is no direct evidence to corroborate this, it is speculated that these organisms may transition into a state of increased phenotypic heterogeneity in response to immune assault or antibiotic therapy. These DCTB populations require the addition of specific growth factors to be cultured in the laboratory. During early treatment, actively diving bacteria are killed first. These bacteria can be isolated using standard laboratory methods, such as CFU assays, accounting for the measurable decline observed in EBA studies during early treatment. Routine diagnostic culture-based tests will remain positive until all actively dividing bacilli are eradicated. There is also evidence to suggest that liquid-based culture systems may be able to detect a population of *M. tuberculosis* that will not be detected on solid media (Bowness et al., 2014, Chengalroyen et al., 2016). Smear and molecular-based tests may remain positive for longer depending on the sensitivity of the assay since DNA from dead bacteria may still be present in the sputum. Routine culture-based tests will most likely become negative towards the end of early treatment. During late treatment (i.e. continuation phase of treatment), less organisms can be isolated using CF-supplemented methods as bacteria are killed during treatment, albeit at a slower rate than their actively dividing counterparts. At treatment completion, the presence of a few survivors, where sterilisation was not achieved, may create the risk of relapse disease. The reliance of DCTB on CF or Rpfs may possibly also change during this period, Figure 4.1

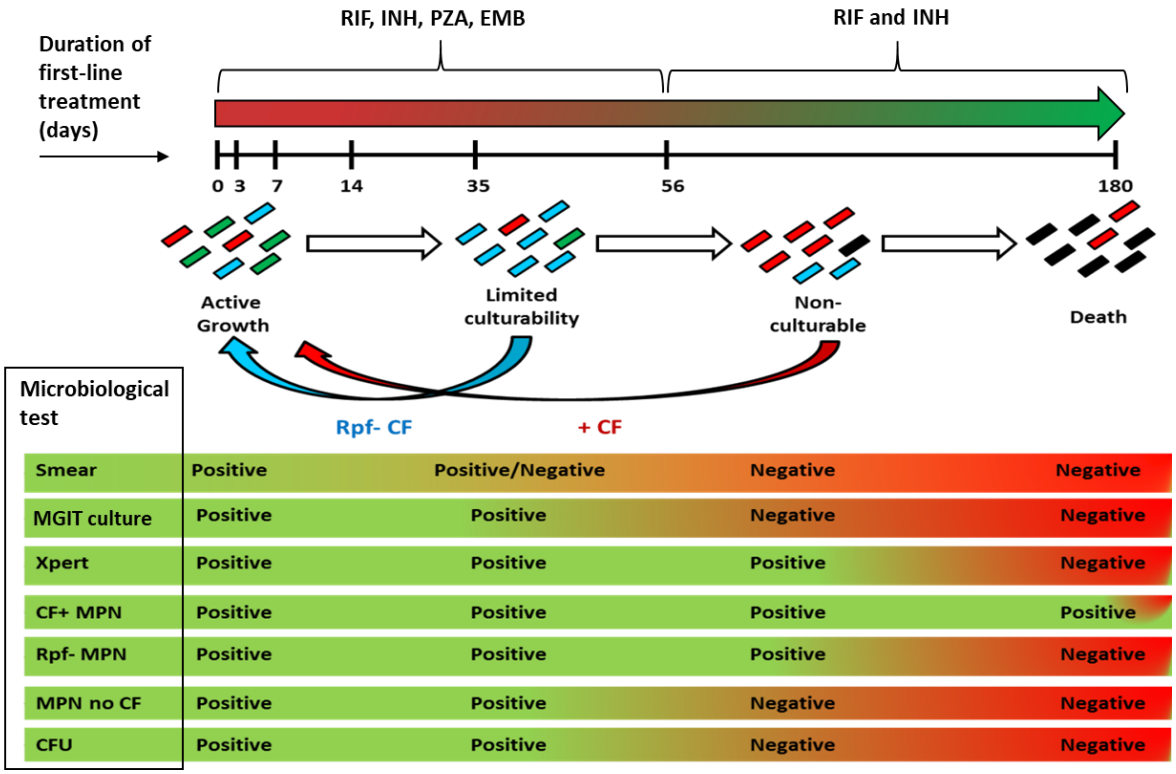

Figure 4.1 Hypothetical spectrum of differential culturability in pre-treatment and sputum samples collected throughout first-line TB treatment. In treatment-naïve patients, sputum samples consist of a mixture of actively diving tubercle bacilli (green) and differentially culturable tubercle bacilli (DCTB) that require resuscitation-promoting factors (Rpfs) (red) and/or other molecules present in culture filtrate (CF) (blue) for growth. Depending of the bacterial load in sputum, TB disease can be diagnosed using conventional diagnostic methods including smear microscopy, MGIT culture and PCR-based assays, including the GeneXpert. During treatment, actively diving organisms are either killed by these antibiotics or the pressures encountered by these bacilli drive them into variable states of limited culturabilty. These DCTB can be recovered in the laboratory using CF. Following prolonged treatment, the majority of these organisms will die, however, few organisms may remain, undetected by conventional diagnostic methods. It is speculated that these differentially culturable organisms may be responsible for relapse disease. RIF = rifampicin, INH = isoniazid, PZA = pyrazinamide and EMB = ethambutol.

4.2 HYPOTHESIS

DCTB are more tolerant to first-line TB antibiotics than conventionally culturable bacteria. During treatment, it is hypothesized that DCTB will decline at a slower rate or accumulate during the administration of anti-TB chemotherapy.

4.3 AIM OF THE STUDY

To determine the relative proportions of DCTB in sputum samples collected from patients infected with active pulmonary, drug-susceptible TB at specified time points during first-line TB treatment.

4.3.1 Specific objectives of the study

1. To compare rates/patterns of decline in DCTB (measured by MPN assays) versus conventionally culturable bacteria (measured by growth on agar plates) during treatment.
2. To assess if DCTB can serve as potential biomarkers to monitor response to treatment and/or relapse disease.
3. To determine whether certain patterns of decline are associated with certain patient demographics and HIV-infection status.
4. To determine if TB diagnostic data at baseline can be used as a biomarker to predict patterns of decline in DCTB.
5. To compare patterns of decline obtained using MPN and CFU assays with currently employed routine diagnostic tests.
6. To assess whether organisms detected at the end of treatment using the MPN assay are viable by other microbiological methods

4.4 METHODS

4.4.1 Patients/Sources of sputum samples

Sputum samples for the longitudinal analysis were recruited as set out in chapter 3, section 3.4.1 The 174 patients recruited for this study were followed up during the course of their treatment and were asked to return to provide both overnight and spot sputum samples at the following time points (acceptable deviations are enclosed in parentheses): 3 (2 – 4), 7 (5 – 10), 14 (12 – 19), 35 (30 – 40), 56 (50 – 65), 90 (80 – 100), 150 (140 – 160) and 180 days (170 – 190), Figure 4.2. For definitions of overnight and spot sputa, please refer to chapter 3 (see section 3.4.1, page 92). Following completion of treatment, the patients were followed up for an additional year to monitor for relapse disease (monitoring of relapse is not part of this thesis). Time points in this study were skewed towards early treatment and were selected based on data

from EBA studies where a rapid decline in bacterial load is observed in early treatment. Figure 4.2 (A) illustrates the time points at which patients were asked to return to the clinic to provide sputum samples.

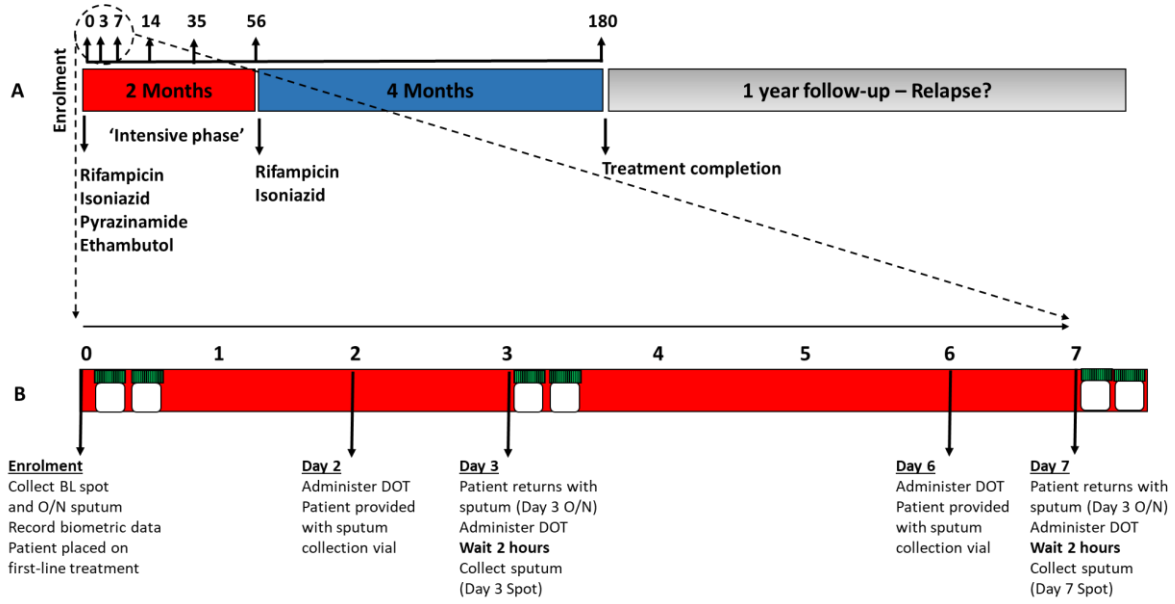

Figure 4.2 Study design, directly observed therapy and sputum collection. (A) Patients were recruited at baseline and placed on standard TB chemotherapy for six months. Spot and overnight sputum samples were collected at time points indicated. Patients were followed up for an additional year to monitor for relapse disease (analysis of relapse fell outside the scope of this thesis). (B) Whilst on TB treatment, study participants were required to come to the clinic the day prior to the study visit for a directly observed therapy (DOT) visit. A DOT visit was also conducted on the clinic visit day. DOT was conducted to ensure that any DCTB that emerged were not due to issues regarding adherence to treatment. In addition to DOT, one day prior to the scheduled time point visit, the patient was asked to come to the clinical and collect a sputum vial. The patient took that vial home, expectorated sputum into it overnight and early during the morning. When returning to the clinic the next day, the patient returned this vial, which was used as the overnight sputum. For the spot sample, sputum collection was carried out two hours after the second DOT visit. Additional sputum and blood samples were also collected for pharmokinetic analysis of drug concentrations (not part of this thesis), and frozen at -80 °C for future analysis.

The 80 patients analysed at baseline were included for longitudinal analysis; however, additional patients were lost/excluded at various time points throughout the study. Table 4.1 shows the number of patients analysed at each time point and reasons for exclusion from analysis for each particular time point.

Table 4.1 Number of patients analysed at each time point stratified by both HIV-infection status and site location.

Number of patients included for analysis							
Soweto Cohort			Klerksdorp Cohort				
Time Point	HIV +	HIV -	HIV +	HIV -	Total	Missed time point	Terminated from study
0	32	16	23	9	80	NA	NA
3	30	16	23	9	78	0	2
7	29	16	22	9	76	2	0
14	28	16	22	8	74	1	3 **(5)**
35	26	16	22	7	71	2	2 **(7)**
56	26	15	21	8	70	0	3 **(10)**
180	23	15	17	7	62	0	8 **(18)**

Patients removed from the study before or after day 56 were classified as early and late drop outs, respectively. Table 4.2 describes the various reasons for patient termination/ exclusion from analysis. In Table 4.1, the first value under the 'terminated from study' column represents the number of patients that were terminated from the study for that particular time point, i.e. the last sputum sample was received at the previous time point. The second figure in parenthesis represents the accumulated number of patients that were terminated at each time point. For decline analysis (section 4.5.3), patients who had completed at least 14 days of treatment were included (n = 75). A total of 62 patients were available for end of treatment analysis (sputum samples received 180 days following treatment initiation). The higher rate of HIV-positive versus HIV-negative drop-outs (27.3% versus 12%, respectively) at earlier time points can mainly be attributed to the severity of disease (i.e. patients were either hospitalised, could not produce sputum or died). At the end of treatment (i.e. day 180), the majority of HIV-positive patients had relocated or were lost to follow-up, which may be due to a number of factors.

Table 4.2 Reasons for patient termination

	Reason for termination	Number of patients terminated (%)	Last sputum sample received
Early drop-out	Hospitalisation	3 (30.0)	0, 7, 35
	Death	1 (10.0)	14
	Relocation	2 (20.0)	14, 35
	Could not produce sputum	3 (30.0)	0, 7, 7
	Treatment default	1 (10.0)	35
Late drop-out	No end-point sample received/ Lost to follow-up	6 (75.0)	56
	Relocation	2 (25.0)	56

The main reasons for 'early' study termination were hospitalisation (30%), unable to produce sputum (30%) and relocation (20%). One patient defaulted on their treatment and regrettably, one patient succumbed. The main reason for late termination was that patients were lost during follow-up or did not or were unable to attend their last clinic appointment at treatment completion. Two patients relocated to another area.

4.4.2 Processing of sputum samples for longitudinal analysis.

Sputum decontamination and routine TB diagnostic tests including AFB smear microscopy, GeneXpert, MGIT culture and HAIN MTBDR*plus* assays were performed on all received follow-up sputum samples as previously described in chapter 3 (please see Appendix 3B, page 134). In addition, MPN and CFU assays were performed on all follow-up sputum samples as previously described in chapter 3 (please see section 3.4.3, page 94). Spoligotyping was performed on selected follow-up samples to confirm the strain type identified at baseline. Spoligotyping was performed as described in chapter 3 (please refer to section 3.4.4, page 105).

4.4.3 Reflex assays

Towards the end of treatment, turbidity – albeit low – in the MPN assay was noted, which was scored as positive. To determine whether 'poor or limited growth' in the MPN assay was *M. tuberculosis* and not a contaminating organism or cellular debris, reflex assays were performed by inoculating material from the positive MPN well into small volume liquid cultures (450 μL), MGIT culture and onto solid 7H11 media. In addition, the HAIN MTBDR*plus* was performed

on positive wells to confirm the presence of *M. tuberculosis* complex. Approximately 400 µL from the three replicate MPN wells were pooled together giving 1200 µL of culture. The details of each assay are outlined below and illustrated in Figure 4.3.

4.4.3.1 Reflex into 450 µL culture

Due to the increased volume of the MGIT (approx. 8 mL) in comparison to the MPN well (450 µL), 50 µL of 'positive' culture was inoculated into 400 µL of freshly prepared standard 7H9 media in 48-well microtiter plates. This was performed in triplicate. Growth was monitored by visual inspection after 6 weeks incubation at 37 °C.

4.4.3.2 Reflex onto solid media

For reflex onto solid media, 100 µL from selected low 'positive' MPN wells were sub-cultured onto 7H11 plates. Plates were inspected for *M. tuberculosis* growth after 6 weeks incubation at 37 °C.

4.4.3.3 Confirmation of low positive wells using the HAIN MTBDR*plus* assay

After the pooled samples were reflexed into small volume cultures and onto solid media, the remaining sample (± 900 µL) was sent for further analysis to CLS (please refer to chapter 3 for a description of the CLS laboratory). The HAIN MTBDR*plus* assay was carried out to confirm the presence of *M. tuberculosis* complex. Approximately 500 µL of positive culture was required to carry out this assay. The assay was carried out according to the manufacturer's instructions.

4.4.3.4 Reflex into MGIT culture

The remaining 'positive' culture (± 400 µL) was reflexed into MGIT cultures. After addition of MPN residual culture, 800 µL of PANTA reconstituted in OADC was inoculated into each MGIT tube. MGITs were incubated in the BACTEC MGIT 960 instrument for up to 42 days to confirm culture negativity. Relevant confirmatory tests including AFB microscopy, blood agar and TBc ID tests were performed on positive MGIT cultures.

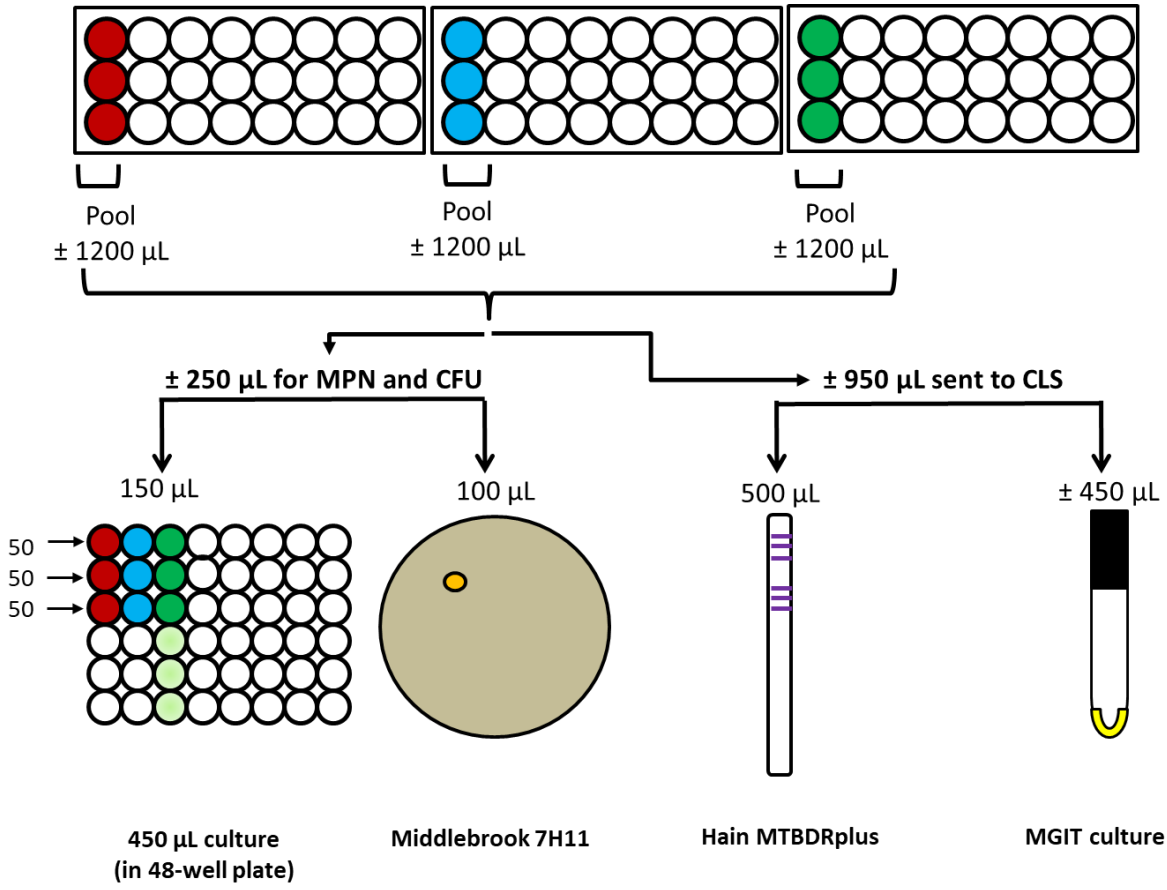

Figure 4.3 Reflex assays. Positive most probable number (MPN) assays exhibiting low or limited growth were reflexed into small volume cultures in 48-well microtiter plates, onto solid 7H11 media and into the mycobacterial growth indicator tube (MGIT). Furthermore, 500 µL of positive culture was sent for HAIN MTBDR*plus* to confirm the presence of *M. tuberculosis* complex. Hain and MGIT assays were carried out at CLS.

4.4.4 DMN-TRE staining of end-of-treatment samples

To confirm the presence of *M. tuberculosis* at the end of treatment, select positive MPN cultures were stained using a fluorogenic derivative of trehalose conjugated to the solvatochromatic dye 4-*N*,*N*-dimethylamino-1,8-napthalidimide, the reagent referred to here on as DMN-Tre (Kamariza et al., 2018). DMN-Tre gets incorporated into the mycobacterial cell wall and only fluoresces when in the mycomembrane. As a result, labelling requires mycomembrane biosynthesis and allows for not only the identification of *M. tuberculosis*, but also indicates that the organism is viable. This is in contrast to currently employed *M. tuberculosis* staining methods, including ZN and auramine stains, that cannot distinguish live from dead mycobacteria.

4.4.4.1 DMN-Tre labelling of positive *M. tuberculosis* culture

Select positive cultures from the reflex assay (see section 4.4.3) were labelled with DMN-Tre. These wells were selected on the basis on their turbidity, where wells displaying high, medium and low growth were chosen, Figure 4.4. For this, 40 µL of 10 mM DMN-Tre was added to 400 µL of culture (final concentration: 1 mM). The stained samples were incubated at 37 ºC overnight. The following day the samples were fixed in a final concentration of 2.5% glutaraldehyde. For this, the samples were centrifuged at 3000 X *g* for 10 minutes after which the supernatant was removed and substituted with 200 µL of 2.5% gluteraldehyde. The samples were left to incubate in the BSL3 for 90 minutes with occasional inversion of the tubes to ensure that all the surfaces were decontaminated. Following incubation, the samples were harvested at 3000 X *g* for three minutes, after which the supernatant was removed and the pellet was re-suspended in 100 µL PBS. The samples were subsequently removed from the laboratory for microscopy.

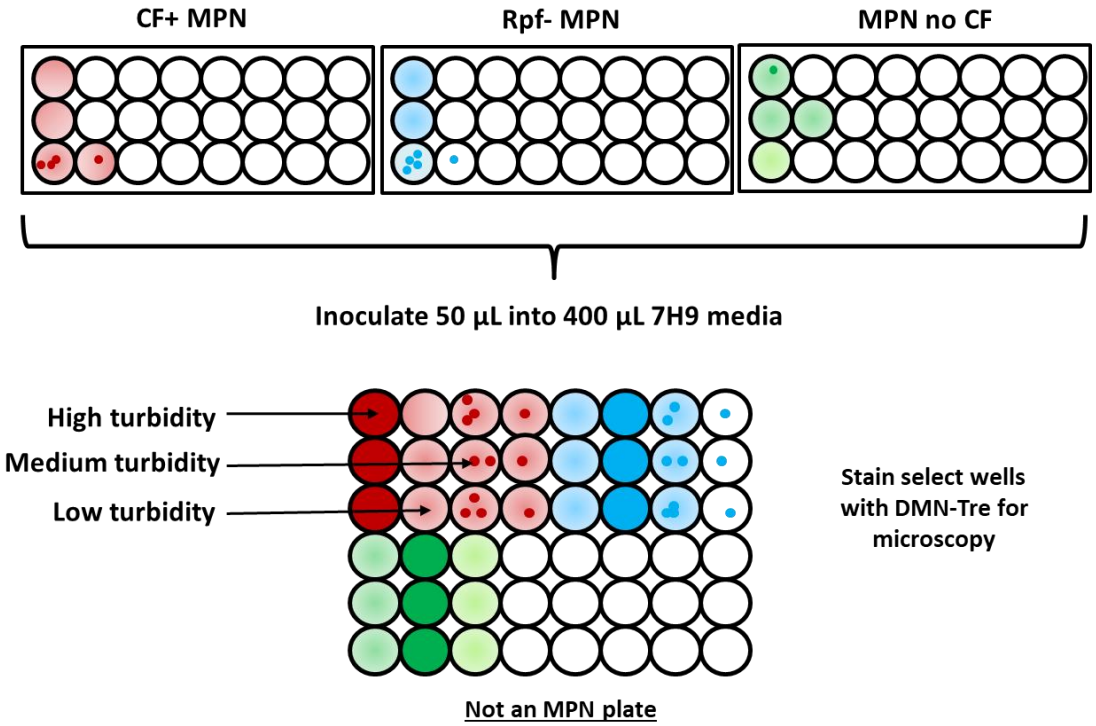

Figure 4.4 Reflex plate with high, medium and low growth for DMN-Tre labelling. Small 450 µL cultures reflexed from poor or limited growth MPN wells showed variation in growth ranging from high to low turbidity. Two wells were chosen from each turbidity (i.e. high, medium and low) and stained with DMN-Tre.

4.4.4.2 Preparation of slides

A 2% agarose gel was prepared by the addition of 1 g agarose powder to 50 mL of deionised water in a 200 mL Erlenmyer flask. The solution was placed in a microwave for about one minute until it came to a boil. The agarose was left to cool until it reached approximately 55 ºC. Approximately one mL of agar was placed on a microscope slide, after which a second microscope slide was placed on top of the initial slide to evenly distribute the agar. Following solidification of the agarose gel at ambient temperature, the top slide was carefully removed from the bottom slide. A 1 cm x 1 cm block of agar was cut out using a scalpel after which 20 µL of sample was added to the agarose and enclosed with a cover slip.

4.4.4.3 Fluorescent microscopy

Microscopy was performed using a Nikon A1R confocal microscope equipped with a Plan Fluor 60X oil immersion objective. Images were processed using the NIS-Elements AR software (Nikon, Inc). Multiple images were taken for each sample in both the DIC and FITC/GFP channels.

4.4.5 Data analysis

To determine the relationship between CF-dependent and Rpf-independent bacterial populations at the various time points throughout treatment, Spearman's rank sum correlation analysis was employed. Patients were stratified by their HIV-infection status and the Mann-Whitney U test was used to determine differences in bacterial load between these two groups at each time point analysed. To determine the rates of decline in the different bacterial populations (i.e. CF-dependent, Rpf-independent, CF-independent and conventionally culturable bacteria), locally weighted scatterplot smoothing (LOWESS) models were employed. Following the overall decline analysis, patients were sub-divided into four different decline profiles based on their graphs of bacterial decline. To determine whether patient demographic data or baseline TB diagnostic data were associated with a specific pattern of decline, absolute numbers and percentages were calculated. An additional group where no CFUs were obtained from patients at all treatment time points was included. The chi-squared test for proportions and analysis of variance (ANOVA) was used to make comparisons between all five groups. For comparisons between two groups, the chi-squared test for proportions,

unpaired student's t-tests and Mann-Whitney U tests were used. All statistical tests were two-sided and statistical significance was carried out using a 95% confidence interval.

4.5 RESULTS

4.5.1 The culture filtrate effect

In chapter three, analysis of pre-treatment sputum samples revealed that there was a direct correlation between the number of bacteria derived in the CF^+ supplemented MPN assay and Rpf^- supplemented MPN assay at baseline. This suggested that whilst the presence of Rpfs in CF provided a marginal benefit in bacterial recovery, the majority of the growth stimulatory effect was Rpf-independent. We hypothesized that this relationship between CF-dependent and Rpf-independent DCTB may change during treatment, with a greater dependency on Rpfs emerging as treatment progresses. To assess this, Spearman's rank sum correlation was employed to determine the relationship between the CF^+ supplemented and Rpf^- supplemented MPN assays at day 3, 7, 14, 35, 56 and 180. A positive correlation was observed at all time points illustrating that factors, other than Rpfs, present in CF can also resuscitate DCTB in sputum from patients throughout treatment. No significant dependency on Rpfs emerged during treatment.

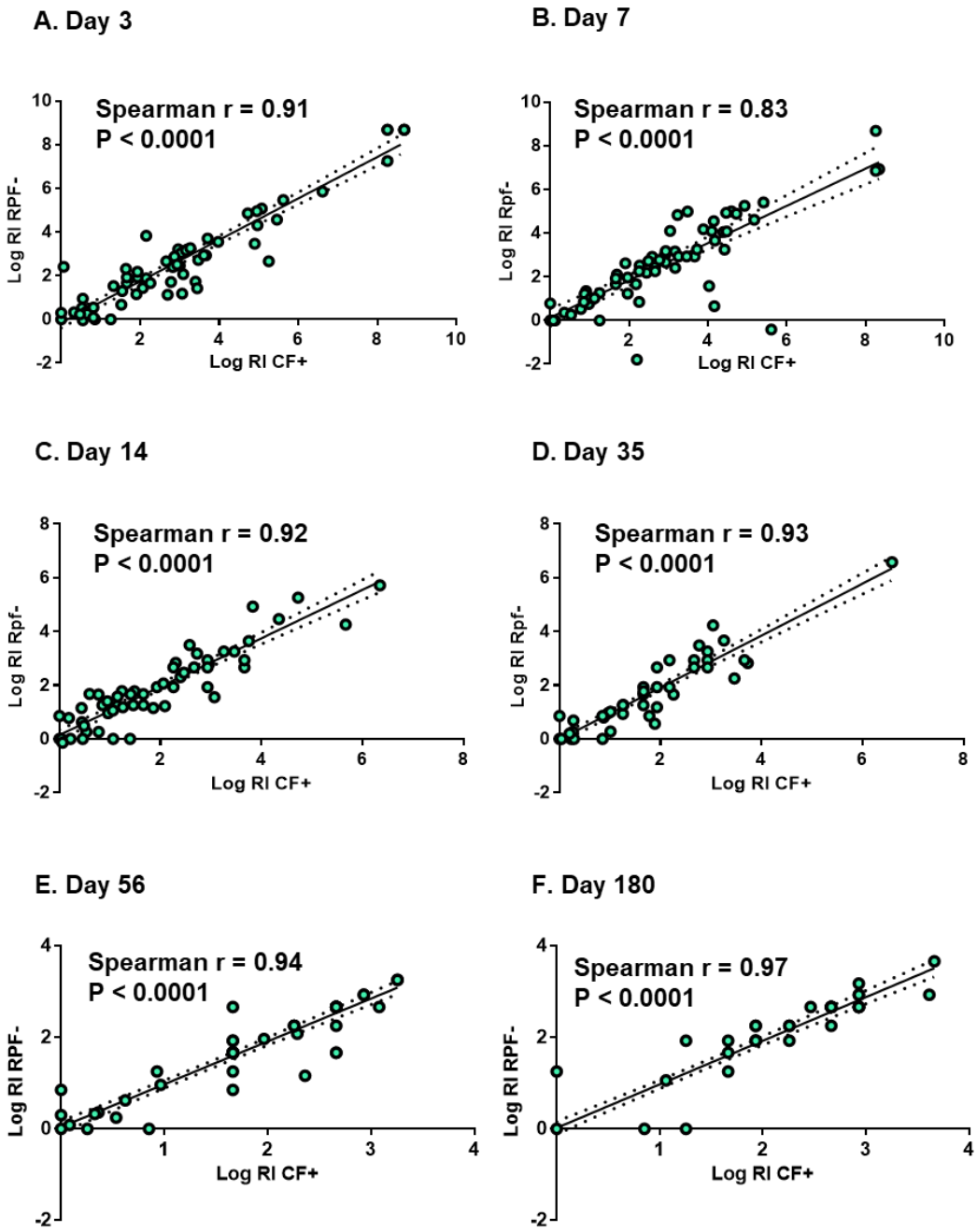

Figure 4.5 Correlation of resuscitation index between culture filtrate-supplemented most probable number assays with and without resuscitation-promoting factors. The resuscitative effect between most probable number (MPN) assays performed with culture filtrate (CF^+) derived from wild-type *M. tuberculosis* H37Rv and resuscitation-promoting factor ($Rpf)^-$ CF from a quintuple *rpf* gene-knockout mutant was correlated at each time point throughout treatment, i.e. day 3 (A), day 7 (B), day 14 (C), day 35 (D), day 56 (E) and day 180 (F). The resuscitation index (RI) was calculated using the following equation: RI = log (MPN/CFU). Culture filtrate (CF)-dependent bacteria, calculated as log (CF^+ MPN/CFU) are reflected on the x-axis, whilst Rpf-independent bacteria, calculated as log (Rpf^- MPN/CFU), are represented on the y-axis. In cases where the CFU was zero, the denominator was set to 1 to reflect the absence of culturable bacteria. In instances where there was no growth in any of the three MPN conditions or the CFU, patient data for that particular time point were all set to 0. If the CFU was greater than the MPN and a negative RI was obtained, the RI was set to 0 to indicate no resuscitation. In cases where contamination was present on solid media and a CFU could not be obtained, patient data for that particular time point was removed from analysis. Spearman's rank-sum correlation analysis was two sided and used with a 95% confidence interval.

4.5.2 HIV-negative patients harbour more DCTB than their HIV counterparts during early treatment

To determine whether DCTB are more prevalent in HIV-uninfected individuals, HIV-infected versus -uninfected groups were compared at each time point using the Mann-Whitney U test. Significant differences were observed in the CF-supplemented MPN assays conducted three, seven and 14 days following treatment initiation, with HIV-negative individuals harbouring more CF-dependent DCTB than their HIV-infected counterparts. Significant differences in the number of CFUs isolated from these two groups were also observed up to 35 days following treatment initiation. These results are in agreement with that observed in treatment-naïve patients. At later time points (i.e. sputum samples collected at days 35, 56 and 180 days post treatment initiation), no differences were observed in the quantum of DCTB in sputum regardless of HIV status, Figure 4.6. However, in all cases, MPN values were substantively higher than CFUS.

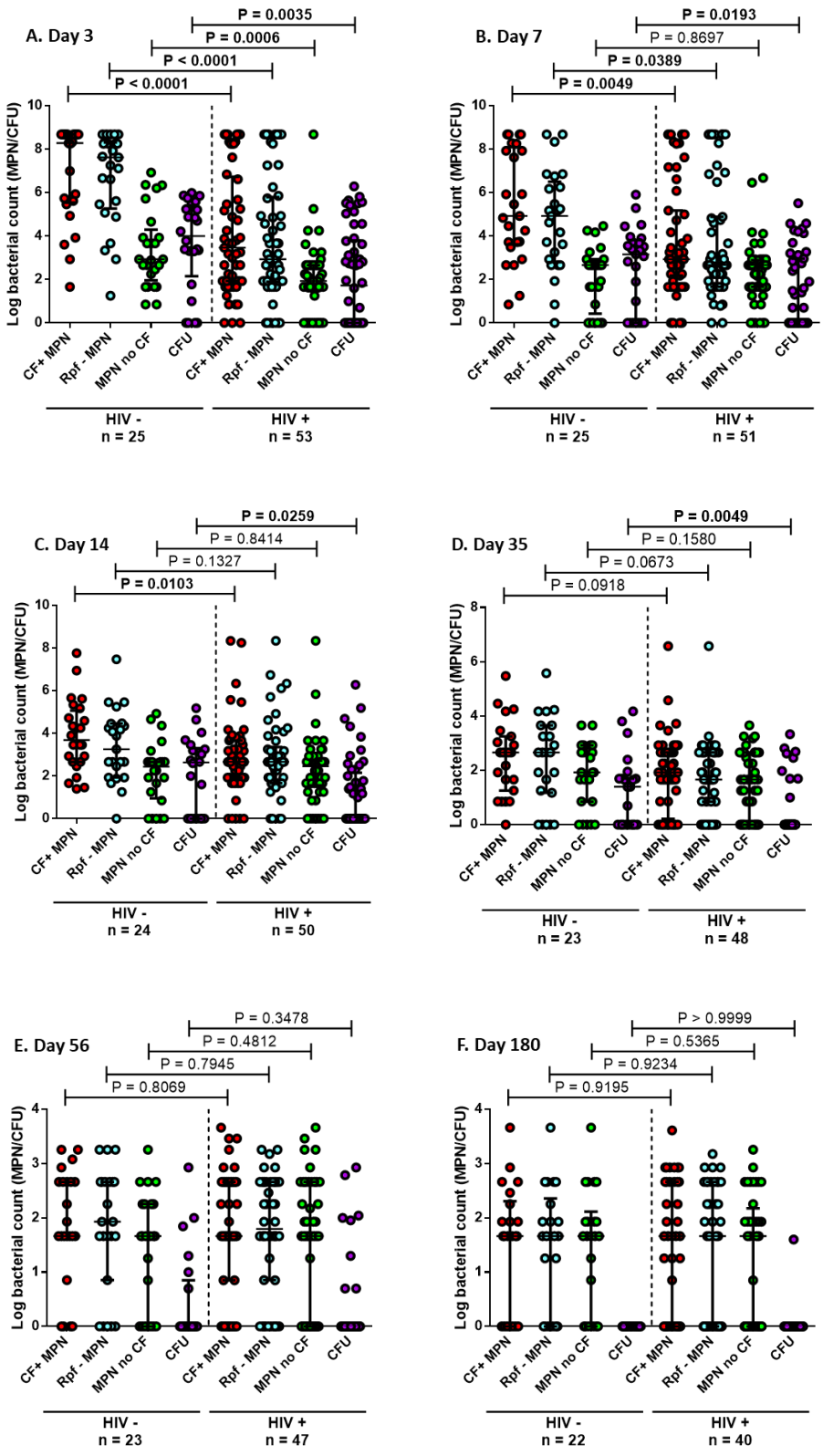

Figure 4.6 Measures of bacterial load stratified by HIV-infection status at selected time-points throughout first-line TB treatment. Scatterplots depicting bacterial load distributions in HIV-infected and -uninfected individuals at: (A) three (n = 78), (B) seven (n = 76), (C) 14 (n = 74), (D) 35 (n = 71), (E) 56 (n = 70) and (F) 180 (n = 62) days post-treatment initiation. Error bars represent medians and interquartile ranges. To determine statistical significance, the Mann-Whitney U test was used with a 95% confidence interval. CF^+ MPN (red), Rpf^- MPN (blue), MPN no CF (green) and CFU (purple).

4.5.3 Rates of decline

To determine the overall rates of decline in the DCTB populations, an analysis was carried out using linear mixed effects models on all patients. DCTB fluctuation curves were fitted using LOWESS. The analysis illustrated that CF-dependent DCTB populations declined at a marginally faster rate than CF-independent and conventionally culturable organisms (CFUs). The slopes obtained were -0,020, -0,010, and -0.007 for CF-dependent, Rpf-independent and CF-independent DCTB populations, respectively. A slope of -0.014 was obtained for the decline of CFU throughout first-line treatment.

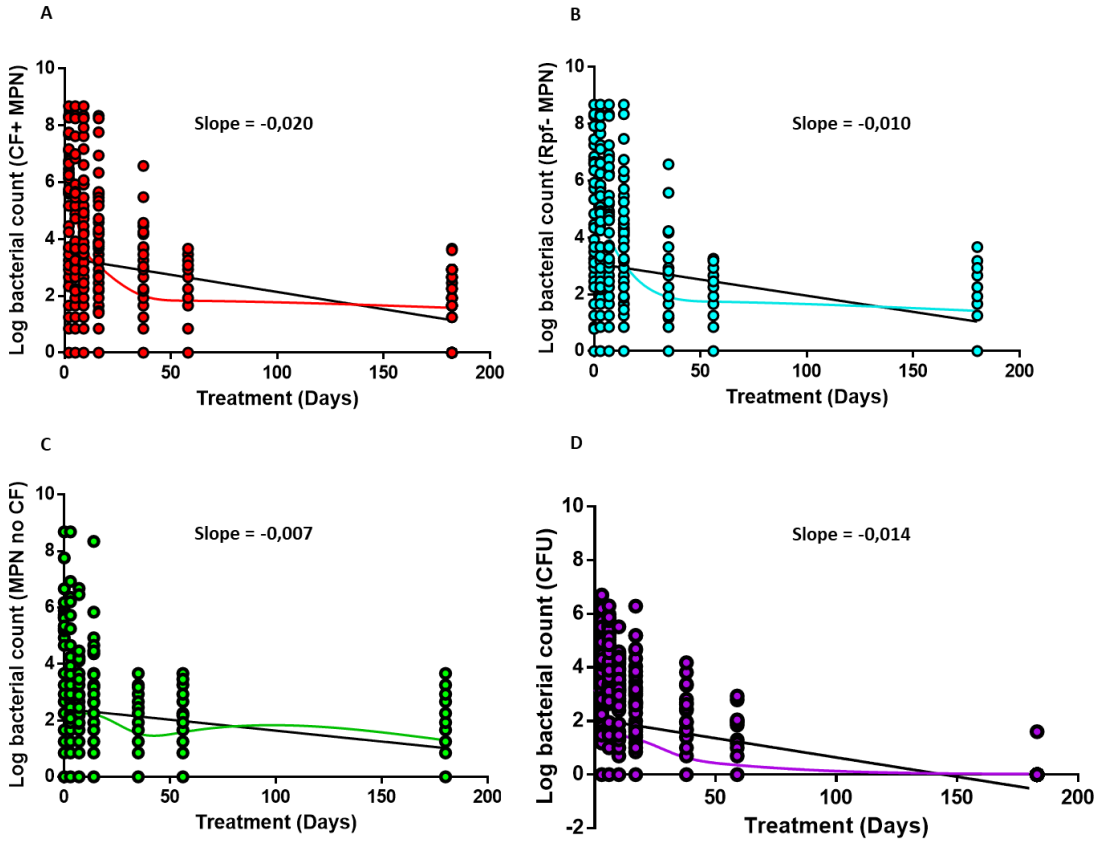

Figure 4.7 Rates of decline in differentially culturable and platable tubercle bacteria in 75 patients with drug-susceptible tuberculosis. Regression analysis was carried out using locally weighted scatterplot smoothing (LOWESS) model to determine the rate of decline for each bacterial population over six months of anti-TB treatment. Graphs are fitted with both LOWESS curves **(**colour lines) and non-linear regression (black lines). Culture filtrate (CF)-dependent DCTB are reflected in red (A), resuscitation promoting factor (Rpf) -independent DCTB in blue (B), CF-independent DCTB in green and colony forming units (CFU) in purple (D).

Overall analysis revealed that there was little material difference between the rates of decline in the four populations analysed, with the CF-dependent population declining at a slightly faster rate compared to the remaining three populations. The observed trends were in contrast to the initial hypothesis and previous reports demonstrating that DCTB are more tolerant to first-line anti-TB chemotherapeutics. To further investigate this, decline trends were studied in individual patients. It was clear that DCTB populations declined at different rates in different patients and that these trends were lost when the data for all patients were analysed as a collective. Hence, an alternate analysis was attempted. Patients were initially grouped into five major trends in DCTB behaviour. These included: (1) DCTB resist decline for three days, (2) DCTB resist decline for seven days, (3) accumulation DCTB, (4) decline in DCTB and (5) atypical DCTB behaviour. Examples of each of these five patterns are shown in Figures 4.8 to 4.12. Figures are represented up to seven days on anti-TB treatment.

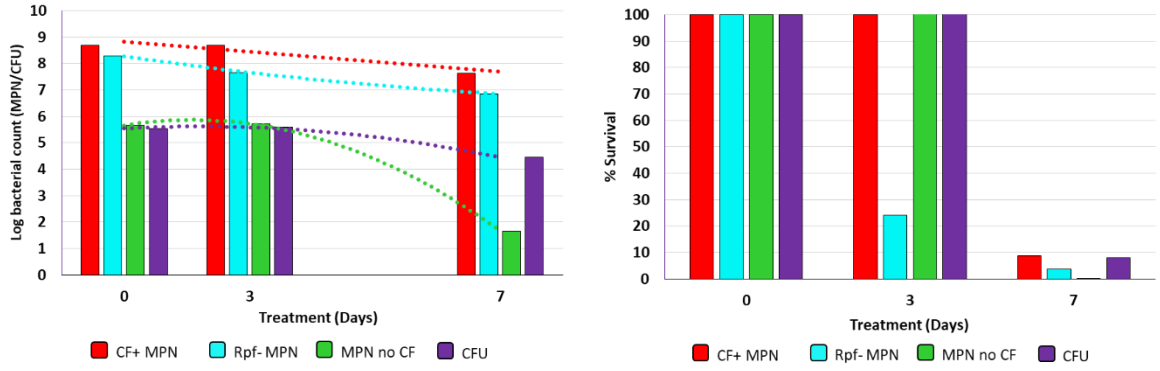

Figure 4.8 MPN and CFU analysis of DCTB populations during antimicrobial treatment reveal sub-populations of bacteria that resist decline during the first three days of treatment. (A) Log bacterial counts obtained using MPN and CFU assays are shown during the first week of anti-TB treatment in patient 59006 to illustrate pattern 1. Each assay has been fitted with a polynomial trend line to show the pattern of decline. (B) Bacterial counts expressed as percent survival compared to baseline were calculated using the following equation: [(viable count $_{baseline}$)/ (viable count $_{time\ \chi}$)] x 100. In this patient, pattern 1 shows no decrease in CF-dependent (or platable bacteria) during the first three days of treatment, but a rapid decline in all bacterial populations at day 7.

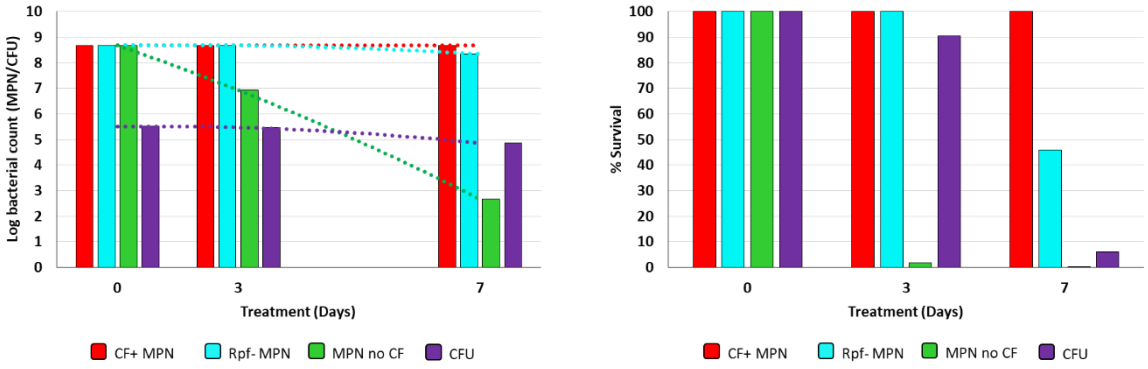

Figure 4.9 MPN and CFU analysis of DCTB populations during antimicrobial treatment reveal sub-populations of bacteria that resist decline during the first seven days of treatment. (A) Log bacterial counts obtained using MPN and CFU assays are shown during the first week of anti-TB treatment in patient 59002 to further illustrate pattern 2. Each assay has been fitted with a polynomial trend line to show the pattern of decline. (B) Bacterial counts expressed as percent survival compared to baseline were calculated using the following equation: [(viable count $_{baseline}$)/ (viable count $_{time\ \chi}$)] x 100. In this patient, pattern 2 shows a decrease in platable bacteria during the first seven days of treatment but no decrease in CF-dependent bacteria as detected by the MPN assay.

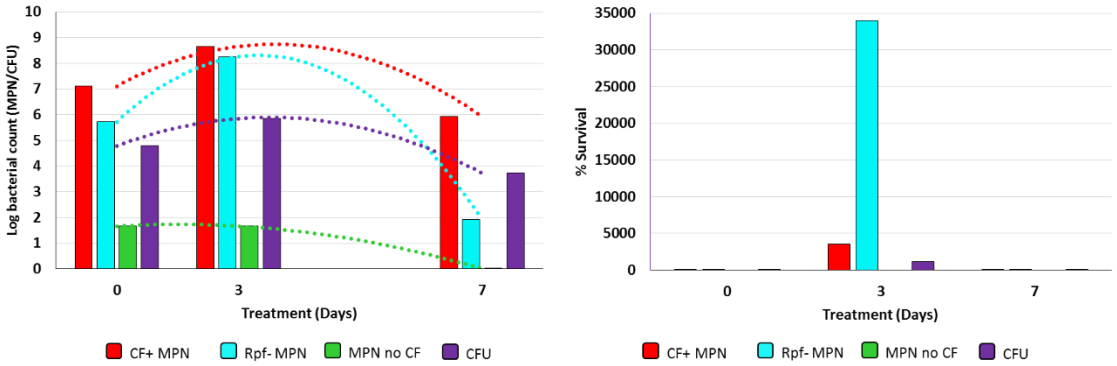

Figure 4.10 MPN and CFU analysis of DCTB populations during antimicrobial treatment reveal sub-populations of bacteria that accumulate during the first three days of treatment. (A) Log bacterial counts obtained using MPN and CFU assays are shown during the first week of anti-TB treatment in patient 59030 to further illustrate pattern 3. Each assay has been fitted with a polynomial trend line to show illustrate the increase in bacterial sub-populations. (B) Bacterial counts expressed as percent survival compared to baseline were calculated using the following equation: [(viable count $_{baseline}$)/ (viable count $_{time\ \chi}$)] x 100. In this patient, a drastic accumulation in Rpf-dependent bacteria, and to a lesser extent CF-dependent and conventionally culturable bacteria, is observed.

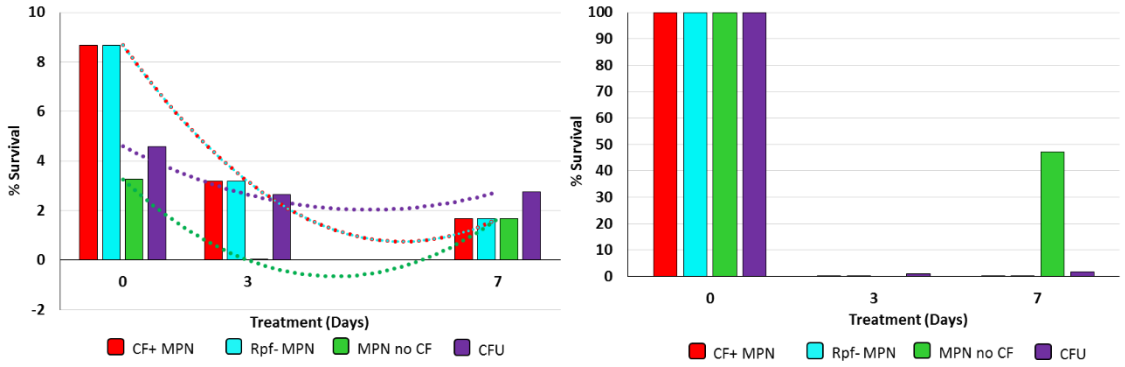

Figure 4.11 MPN and CFU analysis of DCTB populations during antimicrobial treatment reveal bacterial sub-populations that decline rapidly during the first week of treatment. (A) Log bacterial counts obtained using MPN and CFU assays are shown during the first week of anti-TB treatment in patient 59012 to further illustrate pattern 4. Each assay has been fitted with a polynomial trend line to best fit the pattern of decline. (B) Bacterial counts expressed as percent survival compared to baseline were calculated using the following equation: [(viable count $_{baseline}$)/ (viable count $_{time\ \chi}$)] x 100. A drastic decrease in all sub-populations is seen to occur during the first few days of treatment.

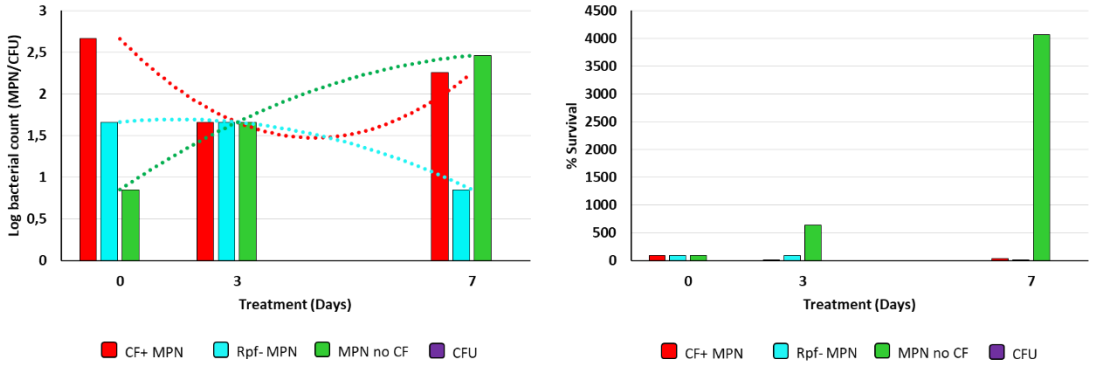

Figure 4.12 MPN and CFU analysis of DCTB populations during antimicrobial treatment reveal sub-populations of bacteria that exhibit an atypical pattern of decline. (A) Log bacterial counts obtained using MPN and CFU assays are shown during the first week of anti-TB treatment in patient 59037 to further illustrate pattern 5. Each assay has been fitted with a polynomial trend line to best fit the pattern of decline. (B) Bacterial counts expressed as percent survival compared to baseline were calculated using the following equation: [(viable count $_{baseline}$)/ (viable count $_{time\ \chi}$)] x 100. In general, patients exhibiting an atypical pattern had low starting bacterial loads that appear to persist throughout treatment. CFUs were not available for this patient at baseline and days 3 and 7 due to the CFU plates being contaminated.

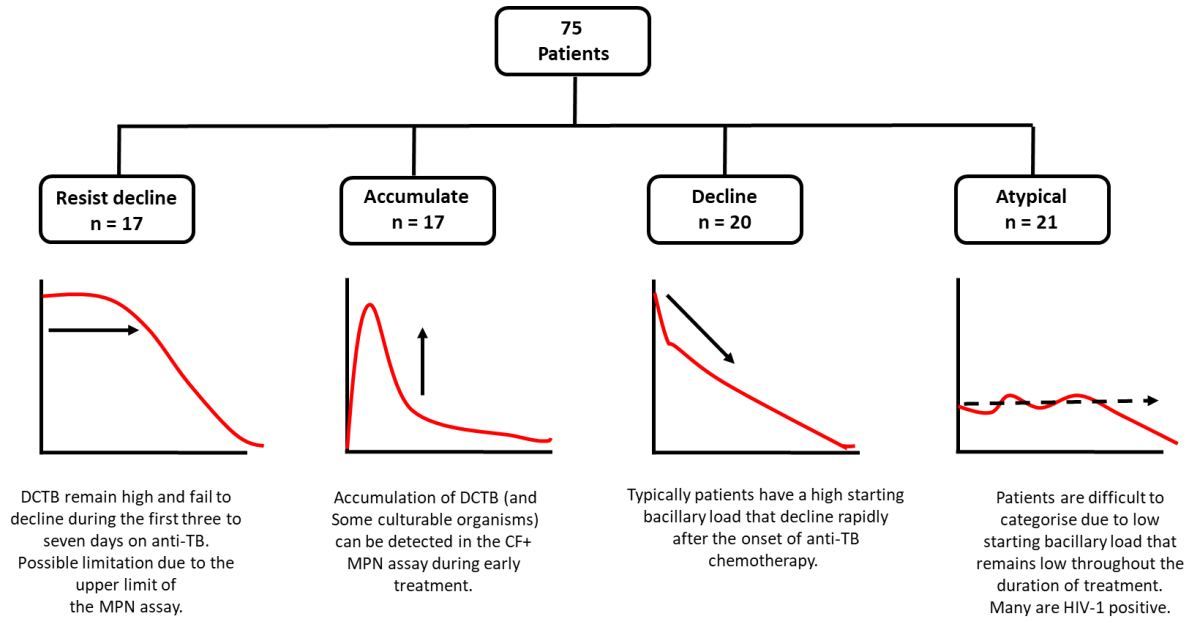

Figure 4.13 Patients categorized by defined patterns of decline. Seventy five patients that had completed at least fourteen days of anti-TB treatment were included for decline analysis. The four sub-categories that patients were assigned to included: resist decline (for three and seven days on anti-TB treatment), accumulate DCTB, decline in DCTB and atypical DCTB presentation. Patterns were defined according to the patterns observed in the CF-dependent population.

4.5.4 Patterns of DCTB decline

4.5.4.1 The 'drug tolerant' or 'resist decline' cohort

Data from 17 of the 75 patients analysed (approximately 23%) exhibited a pattern of decline that was characterized by the maintenance of DCTB levels three to seven days following the initiation of first-line anti-TB treatment. Nine and eight patients were identified in the three and seven day categories respectively. Figure 4.14 and 4.15 illustrates the decline in CFU along with DCTB and the decline in tubercle bacilli obtained via routine diagnostic data in 9 patients whereby DCTB do not decline within the first three days of treatment. Figure 4.16 and Figure 4.17 illustrates the same data in patients where DCTB fail to decline during the first week of anti-TB treatment. Routine DST to second line agents was not carried out in this study, therefore additional drug resistance (although unlikely) cannot exclusively be ruled out as a possible contributor to the 'resist decline' pattern in some patients.

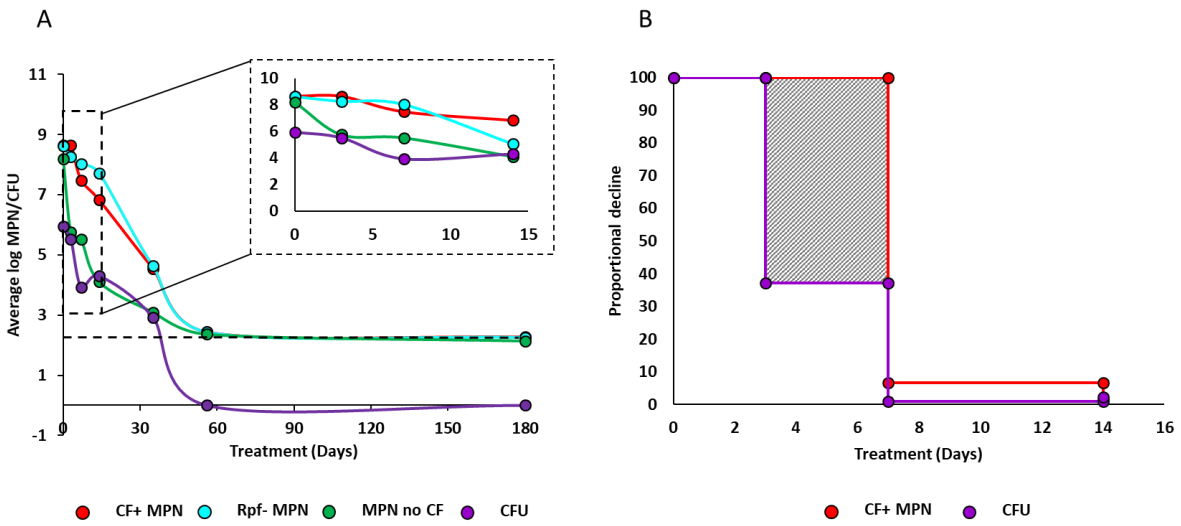

Figure 4.14 Decline in differentially culturable and conventionally culturable tubercle bacilli in nine patients exhibiting the drug tolerant/resist decline DCTB pattern during the first three days of standard first-line treatment. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition, the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line (at log 2.0) indicates possible background scored as positive growth in the MPN assay. This was determined by assessing residual turbidity remaining at later time points during treatment. To more clearly illustrate the drug tolerant/resist decline pattern, the box insert illustrates the decline in bacterial load during the first two weeks of treatment. (B) The proportional decrease in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB and platable bacteria to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline. The shaded grey area illustrates where the proportional decrease in the culturable population (as detected by CFU) was far greater than the CF-dependent population.

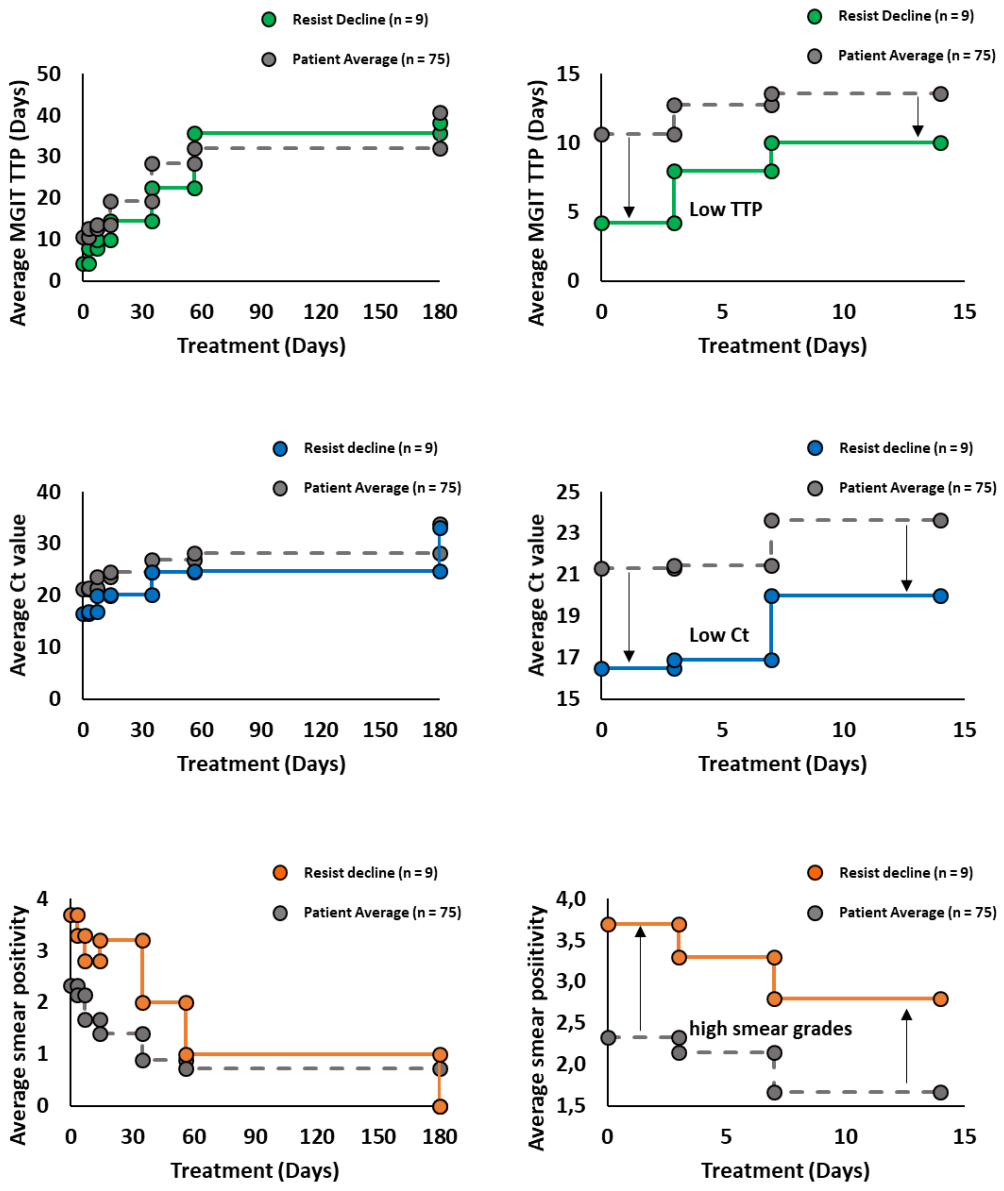

Figure 4.15 Average decline in bacterial load for the three-day drug tolerant/resist decline DCTB pattern throughout first-line treatment as determined by routine diagnostic assays. (A) The average time to positivity (TTP) (green) for the nine drug tolerant/resist decline patients obtained in the mycobacterial growth indicator tube (MGIT) is given for each of the seven time points throughout the six months of anti-TB treatment (left panel). The right panel illustrates the average increase in TTP during the first two weeks of anti-TB treatment, shown to illustrate the early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75, i.e. the average MGIT TTP for all DCTB decline sub-categories). (B) The average cycle threshold (Ct) value (blue) for the nine drug tolerant/resist decline patients obtained from the GeneXpert is given for each of the seven time points throughout the six months of TB treatment (left panel). The right panel illustrates the average increase in Ct value during the first two weeks of anti-TB treatment, to illustrate early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75 i.e. the average Ct value for all DCTB decline sub-categories). (C) The average smear positivity was calculated by providing the smear grading at each particular time point with a numerical value. P+++, ++, +, scanty and negative smears were scored as 4,3,2,1 and 0, respectively. The left panel illustrates the decline in smear grading over six months of TB treatment. The right panel illustrates the decrease in smear positivity during the first two weeks of anti-TB treatment, shown to illustrate early treatment response. The grey dotted line shows overall patient average (n = 75 i.e. the average smear positivity for all DCTB decline sub-categories).

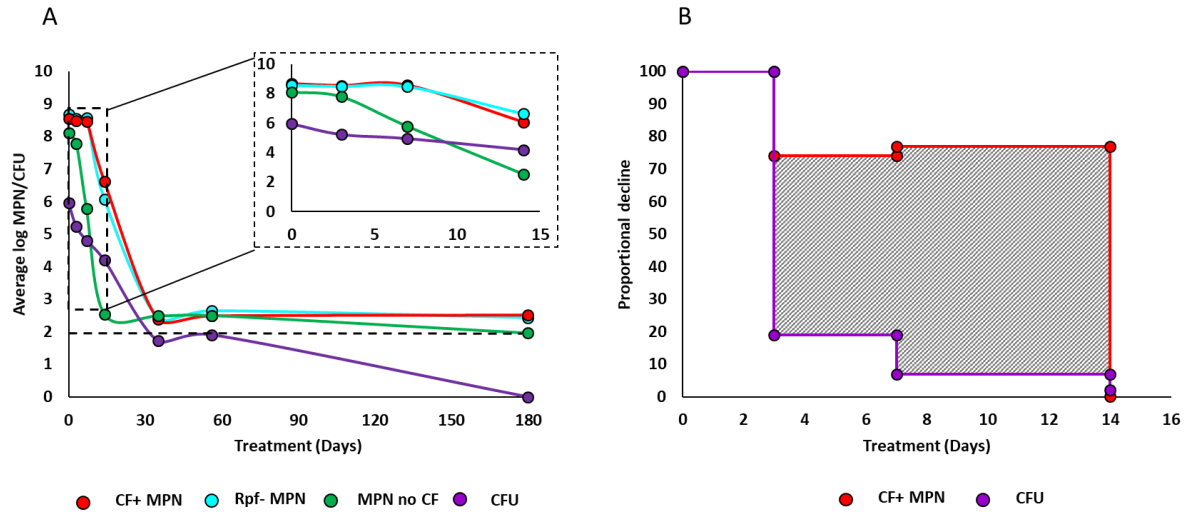

Figure 4.16 Decline in differentially culturable and conventionally culturable tubercle bacilli in eight patients exhibiting the drug tolerant/resist decline DCTB pattern during the first week of standard first-line tuberculosis treatment. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line (at log 2.0) indicates possible background scored as positive growth in the MPN assay. This was determined by assessing residual turbidity remaining at later time points during treatment. To more clearly illustrate the resist decline pattern, the box insert illustrates the decline in bacterial load during the first two weeks of treatment. (B) The proportional increase in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB (red) and platable bacteria (purple) to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline. The shaded grey area illustrates where the proportional decrease in the culturable population (as detected by CFU) was far greater than the CF-dependent population.

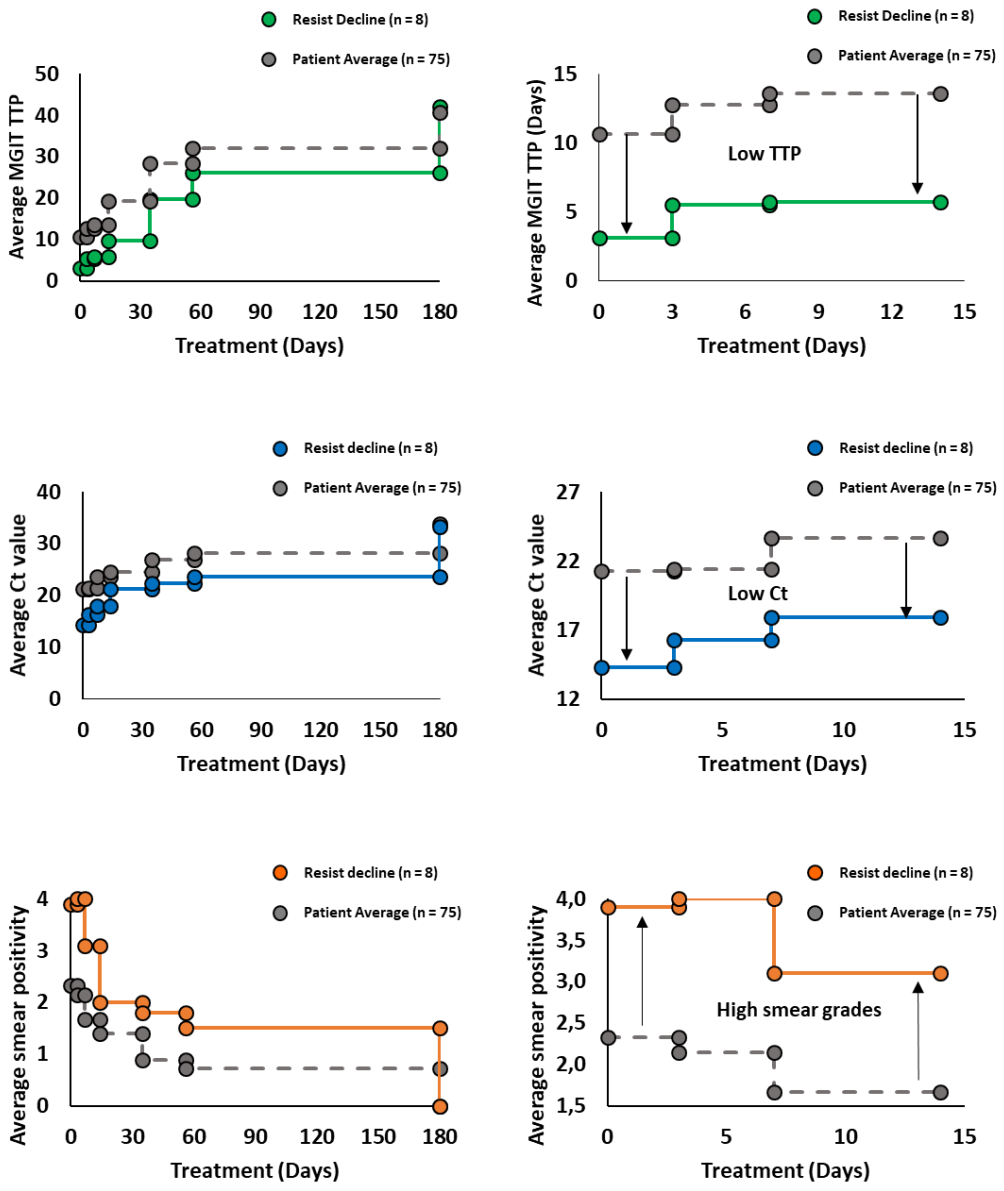

Figure 4.17 Average decline in bacterial load for the seven-day drug-tolerant/resist decline DCTB pattern throughout first-line treatment as determined by routine diagnostic assays. (A) The average time to positivity (TTP) (green) for the eight drug-tolerant/resist decline DCTB patients obtained in the mycobacterial growth indicator tube (MGIT) is given for each of the seven time points throughout the six months of anti-TB treatment (left panel). The right panel illustrates the average increase in TTP during the first two weeks of anti-TB treatment, shown to illustrate the early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75, i.e. the average MGIT TTP for all DCTB decline sub-categories). (B) The average cycle threshold (Ct) value (blue) for the eight drug tolerant/resist decline patients obtained from the GeneXpert is given for each of the seven time points throughout the six months of TB treatment (left panel). The right panel illustrates the average increase in Ct value during the first two weeks of anti-TB treatment, to illustrate early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75 i.e. the average Ct value for all DCTB decline sub-categories). (C) The average smear positivity was calculated by providing the smear grading at each particular time point with a numerical value. P+++, ++, +, scanty and negative smears were scored as 4,3,2,1 and 0, respectively. The left panel illustrates the decline in smear grading over six months of TB treatment. The right panel illustrates the decrease in smear positivity during the first two weeks of anti-TB treatment, shown to illustrate early treatment response. The grey dotted line shows overall patient average (n = 75 i.e. the average smear positivity for all DCTB decline sub-categories).

The drug tolerant/resist decline DCTB patient cohort was characterised by high sputum bacterial loads at baseline as evident by MPN, CFU and routine diagnostic assays. In the MPN assay, CF-dependent bacteria appear to be somewhat drug tolerant and resist decline during the first three to seven days of anti-TB treatment. It is important to note that interpreting this pattern of decline is limited due to the MPN assay having an upper limit of log 8.7. For instance, in patients where bacterial growth was present in all of the MPN wells at baseline (or at early treatment time points), the most probable number of bacteria could not accurately be established and a value of log 8.7 was used in the analyses; however, a value of >log 8.7 is more correct. It is therefore not clear how this particular DCTB population responds to treatment.

Analysis of the routine diagnostic data illustrated a decline in bacterial load at each subsequent time point. In the three day resist cohort, the average MGIT TTP increased from 4.4 days at baseline to 8 days and 10 days at the 3 and 7 day time points, respectively, illustrating a decrease in bacterial burden following treatment initiation. Similarly in the seven day resist cohort, the average MGIT TTP increased from 3.1 days at enrolment to 5.5 and 5.8 days at the 3 and 7 day time points respectively. An increase in Ct values were also observed in these patients at each subsequent time point following treatment initiation illustrating a decline in bacterial burden. This group of patients also had high smear grades at baseline and during early treatment. In all 17 patients (i.e. three and seven day resist decline combined, see Table 4C), 82.4% had P+++ grading's at baseline and at the day 3 time point.

4.5.4.2 The 'accumulate' cohort

Seventeen patients out of 75 (approximately 23%) exhibited the accumulate DCTB pattern. The proportional decline (or in this case, the accumulation during early treatment) of DCTB and conventionally culturable bacteria was calculated for each time point as a percentage of the baseline population, which was set to 100%. Patients exhibiting the accumulate DCTB profile showed an increase in DCTB during the first week of treatment (Figure 4.18 A and B). At day 3, the DCTB population increased to approximately 2800X (ca. 3 log) from that observed at baseline. In the proportional analysis, the number of CF-dependent DCTB isolated remained above that obtained at baseline until day 35 at which point it dropped to less than 1% of the population quantified at baseline.

During the first three days of treatment, the CFU increased to approximately 460X that observed at baseline but dropped to less than 1% of the initial population at day 7. At day 14 the CFU increased to 750X that observed at baseline. At day 35 and 56, the percentage of culturable bacteria relative to that observed at baseline was 6 and 0.3%. At the end of treatment, no culturable bacteria were isolated in this patient cohort.

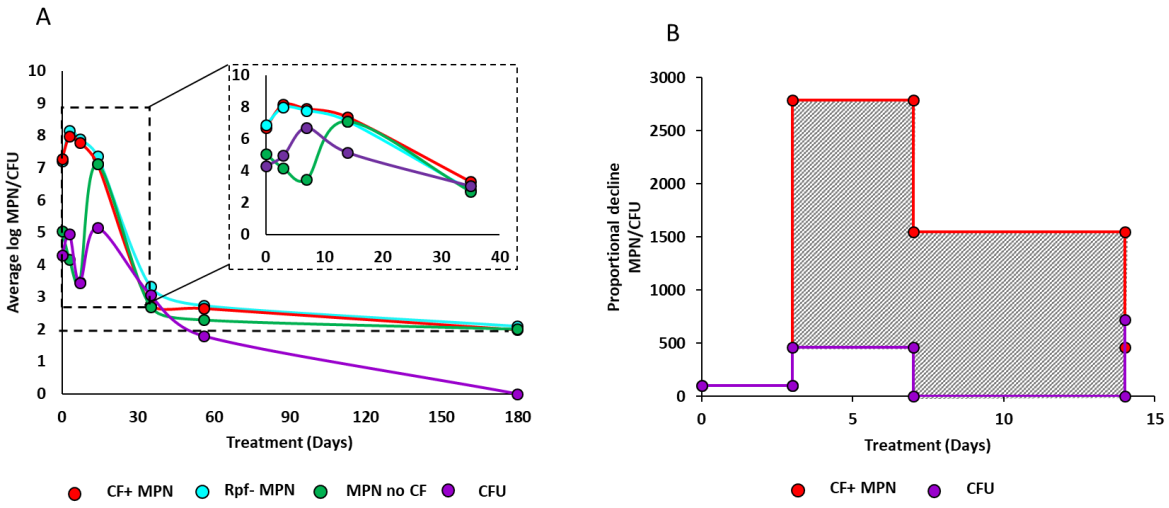

Figure 4.18 Decline in differentially culturable and conventionally culturable tubercle bacilli in seventeen patients exhibiting the accumulate DCTB pattern. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line (at log 2.0) indicates possible background scored as positive growth in the MPN assay. This was determined by assessing residual turbidity remaining at later time-points during treatment. To more clearly illustrate the accumulation of DCTB during early treatment, the box insert illustrates the decline in bacterial load during the first thirty five days of treatment. (B) The proportional increase in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB (red) and platable bacteria (purple) to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline. The shaded grey area illustrates where the proportional decrease in the culturable population (as detected by CFU) was far greater than the CF-dependent population.

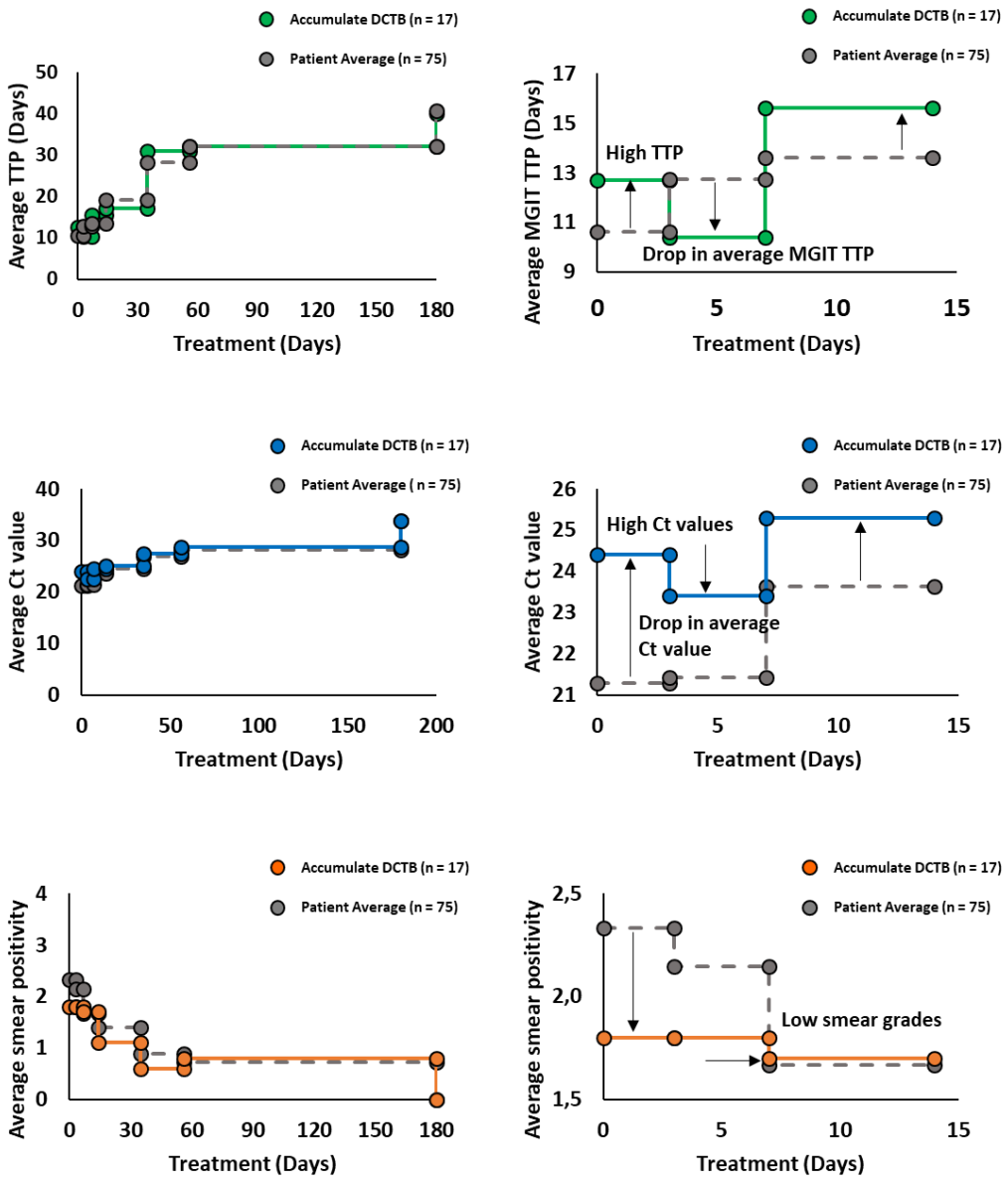

Figure 4.19 Average decline in bacterial load for the accumulate DCTB pattern throughout first-line treatment as determined by routine diagnostic assays. (A) The average time to positivity (TTP) (green) for the seventeen accumulate DCTB patients obtained in the mycobacterial growth indicator tube (MGIT) is given for each time point throughout the six months of anti-TB treatment (left panel). The right panel illustrates the average increase in TTP during the first two weeks of anti-TB treatment, shown to illustrate the early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75, i.e. the average MGIT TTP for all DCTB decline sub-categories). (B) The average cycle threshold (Ct) value (blue) for the seventeen accumulate DCTB patients obtained from the GeneXpert is given for each time point throughout the six months of TB treatment (left panel). The right panel illustrates the average increase in Ct value during the first two weeks of anti-TB treatment, to illustrate early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75 i.e. the average Ct value for all DCTB decline sub-categories). (C) The average smear positivity was calculated by providing the smear grading at each particular time point with a numerical value. P+++, ++, +, scanty and negative smears were scored as 4,3,2,1 and 0, respectively. The left panel illustrates the decline in smear grading over six months of TB treatment. The right panel illustrates the decrease in smear positivity during the first two weeks of anti-TB treatment, shown to illustrate early treatment response. The grey dotted line shows overall patient average (n = 75 i.e. the average smear positivity for all DCTB decline sub-categories).

Diagnostic assays conducted for the accumulate DCTB patient cohort were assessed to determine if similarities in trends (related to bacterial burden) could be linked to the estimated numbers of culturable and DCTB obtained in the CFU and MPN assays [Figure 4.19 and Tables 4A, 4B and 4C (see Appendices)]. In the MGIT, the TTP decreased during the first three days of treatment with average TTPs of 12.7 and 10.4 days reported at baseline and three days following treatment initiation, respectively. After seven and fourteen days, the average TTP increased to 15.6 and 17.1 days, respectively. The initial decrease in TTP is associated with an increase bacterial burden which is mirrored by both the number of bacteria obtained in the CF-supplemented MPN and CFU assays. The majority of patients (92.3%) from this cohort had negative MGIT cultures at the end of treatment, with 35.3% having two consecutive negative cultures. The pattern observed in the MGIT was reflected in the GeneXpert, whereby the Ct value decreased from 24.0 to 22.6 following three days of anti-TB treatment, indicating an increase in bacterial burden. Following seven and 14 days of treatment the average Ct values remained relatively stable with average reported Ct values of 24.5 and 24.3, respectively. Following 14 days of treatment, the Ct value declined at each consecutive time point. These results illustrate that the pattern of Ct values obtained from the GeneXpert assay was similar to the accumulate DCTB pattern, with the Ct value being qualitatively 'high' during the first two weeks of treatment. Auramine smear data also indicated an increase in bacterial burden during the first two weeks of anti-TB treatment, with the percentage of patients with a P+++ smear grading increasing from 17.6% at baseline to 29.4 and 23.5% at three and seven days post-treatment initiation, respectively. At fourteen days post treatment initiation, the percentage of patients with a P+++ grading decreased to 5.9% of patients. These results suggest that the CFU assay and accumulate DCTB pattern detected by the MPN assays are echoed by TTP obtained via the MGIT assay and the GeneXpert along with smear diagnostic assays, respectively.

4.5.4.3 The 'decline' cohort

One group of patients (26.7% of total population) was characterised by a continuous decline in both CF-dependent DCTB and conventionally culturable bacteria from baseline to the end of first-line treatment. Results showing the bacterial decline in MPN and CFU assays as well as routine diagnostic assays for this patient cohort are depicted in Figures 4.20 and Figure 4.21, respectively.

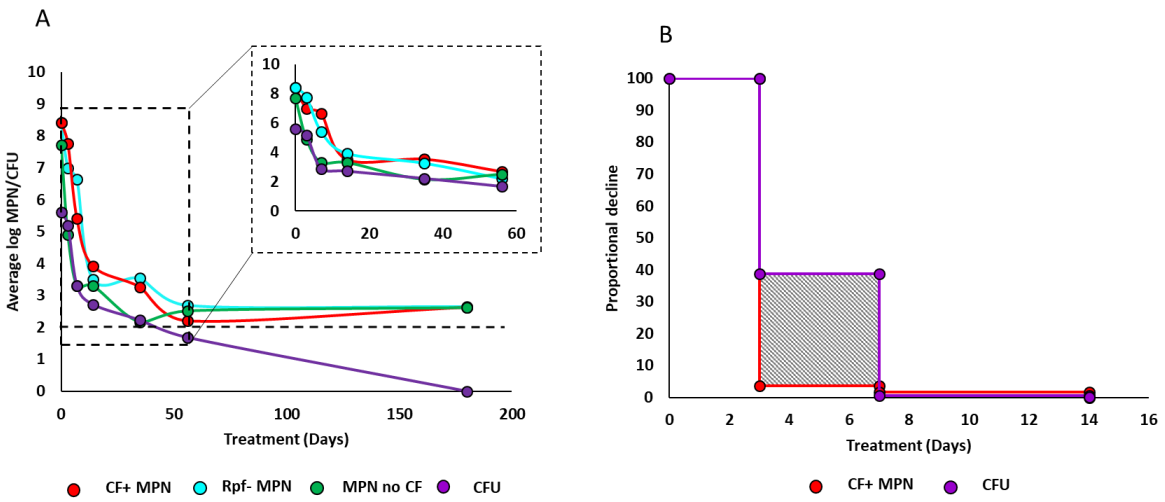

Figure 4.20 Decline in differentially culturable and conventionally culturable tubercle bacilli in twenty patients exhibiting the decline in DCTB pattern. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line (at log 2.0) indicates possible background scored as positive growth in the MPN assay. This was determined by assessing residual turbidity remaining at later time-points during treatment. The box insert illustrates the decline in bacterial load during the first fifty six days of treatment. (B) The proportional increase in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB (red) and platable bacteria (purple) to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline. The shaded grey area illustrates where the proportional decrease in the culturable population (as detected by CFU) was less than the CF-dependent population.

At baseline, the bacterial load obtained in the CF-supplemented MPN assays yielded approximately 3 logs more than that obtained in CFU assays. During treatment, there was approximately a 1 log difference (or less) between the MPN and CFU assays, whilst at the end of treatment, no viable organisms were present but approximately 2.5 log organisms were still obtained in the MPN assay, based on turbidity in the MPN assay. Proportional decline analysis conducted during the first two weeks of treatment revealed that DCTB decline rapidly during the first three days of treatment, with less than 5% of the initial organism load quantified in the CF^{+} MPN experiments at baseline. The conventionally culturable population, detected by viable colonies on solid media, initially declined at a much slower rate with 37% of initial organism load observed three days following treatment initiation. In both assays, less than 2% of the initial organism load was observed after seven days.

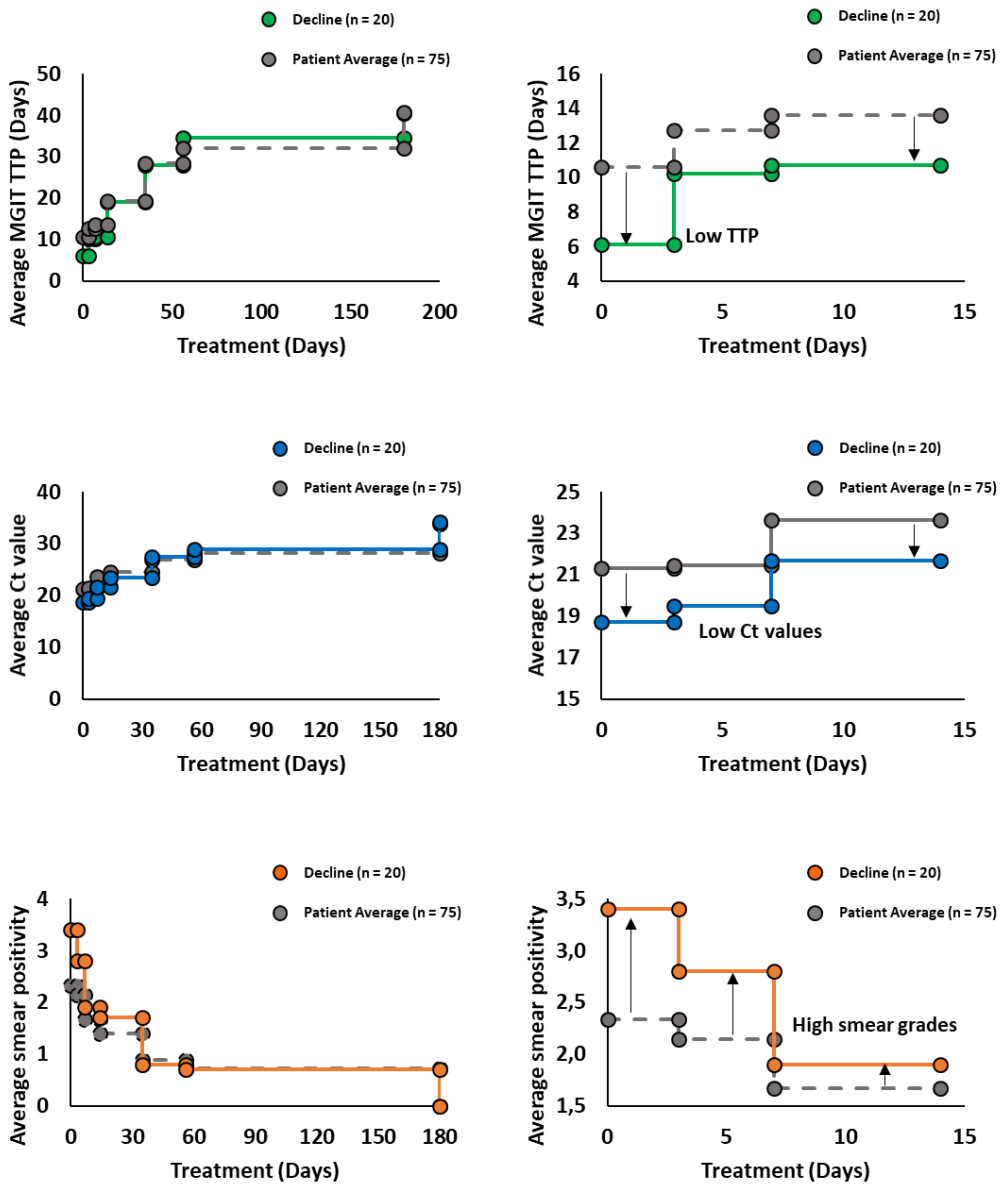

Figure 4.21 Average decline in bacterial load for decline in DCTB pattern throughout first-line treatment as determined by routine diagnostic assays. (A) The average time to positivity (TTP) (green) for the twenty decline in DCTB patients obtained in the mycobacterial growth indicator tube (MGIT) is given for each time point throughout the six months of anti-TB treatment (left panel). The right panel illustrates the average increase in TTP during the first two weeks of anti-TB treatment, shown to illustrate the early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75, i.e. the average MGIT TTP for all DCTB decline sub-categories). (B) The average cycle threshold (Ct) value (blue) for the twenty decline in DCTB patients obtained from the GeneXpert is given for each time point throughout the six months of TB treatment (left panel). The right panel illustrates the average increase in Ct value during the first two weeks of anti-TB treatment, to illustrate early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75 i.e. the average Ct value for all DCTB decline sub-categories). (C) The average smear positivity was calculated by providing the smear grading at each particular time point with a numerical value. P+++, ++, +, scanty and negative smears were scored as 4,3,2,1 and 0, respectively. The left panel illustrates the decline in smear grading over six months of TB treatment. The right panel illustrates the decrease in smear positivity during the first two weeks of anti-TB treatment, shown to illustrate early treatment response. The grey dotted line shows overall patient average (n = 75 i.e. the average smear positivity for all DCTB decline sub-categories).

Analysis of data from routine diagnostic assays carried out on this patient cohort indicated a rapid decline in bacterial load after the commencement and throughout TB treatment. Overall, these patients had a high starting bacterial burden when compared to the total patient cohort with low average TTP and Ct values and high corresponding smear grades. In the MGIT assay, the average TTP increased rapidly from 6.1 days to 10.2 days following the commencement of treatment. This increase in TTP was sustained throughout treatment (Table 4A). At the end of treatment, approximately 92% of these patients were culture negative, with 45% of patients having at least two consecutive negative MGIT cultures. As observed in the MGIT, the Ct value increased steadily throughout treatment in this patient cohort. At the end of treatment, *M. tuberculosis* was not detected by the GeneXpert assay in 92% of this population. With regards to smear microscopy, the observed decline in bacterial load was rapid. At enrolment, all of the patients from this cohort had a positive smear grade of P+++, P++ or P+ (60%, 25% and 15%, respectively). Three days following the commencement of treatment, 20% of this patient cohort had low (scanty) or negative smear grades. The decline and change in smear grade is given in Figure 4.21 (C) and Table 4C.

4.5.4.4 'Atypical' presentation

Lastly, 21 patients (approximately 28% of the analysed population) demonstrated an atypical DCTB pattern of decline in both the CF-supplemented MPN and CFU assays. The overall pattern of decline observed in these patients was characterised by an initial decline in DCTB, and to a lesser extent CFU, during early treatment, followed by an increase in both these populations at day seven (Figure 4.22A). The majority of the atypical DCTB patients were characterised by low MPNs or CFUs at diagnosis that persisted throughout treatment. Fifteen of the sixteen patients had no CFUs at the end of treatment; however, one of these patients still had 4 viable colonies emerge on solid media following 180 days of treatment. Furthermore, an average of 2.8 log organisms was observed in the CF-supplemented MPN assay at the end of treatment.

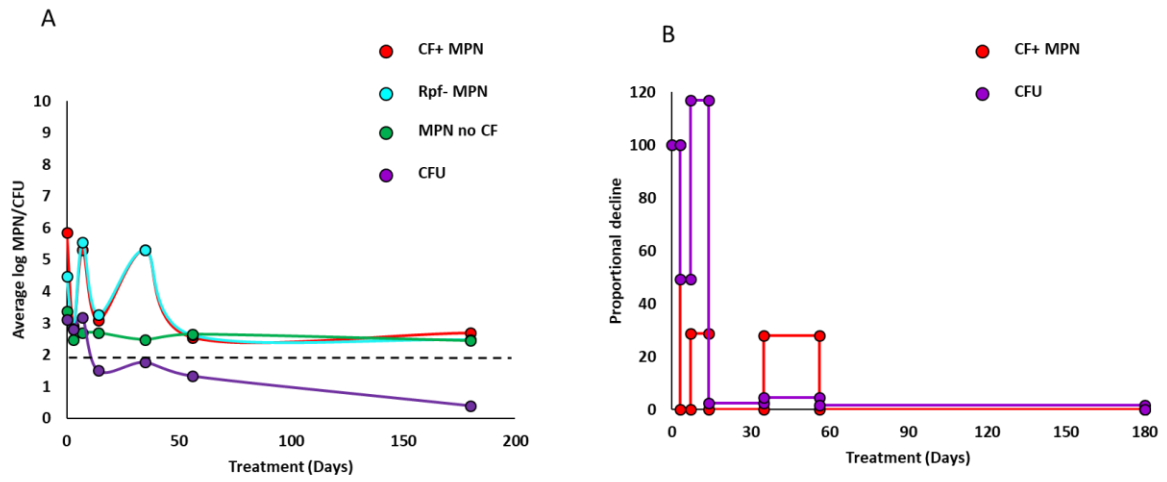

Figure 4.22 Decline in differentially culturable and culturable tubercle bacilli in twenty one patients exhibiting the atypical DCTB decline pattern. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line (at log 2.0) indicates possible background scored as positive growth in the MPN assay. This was determined by assessing residual turbidity remaining at later time-points during treatment. (B) The proportional increase in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB (red) and platable bacteria (purple) to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline.

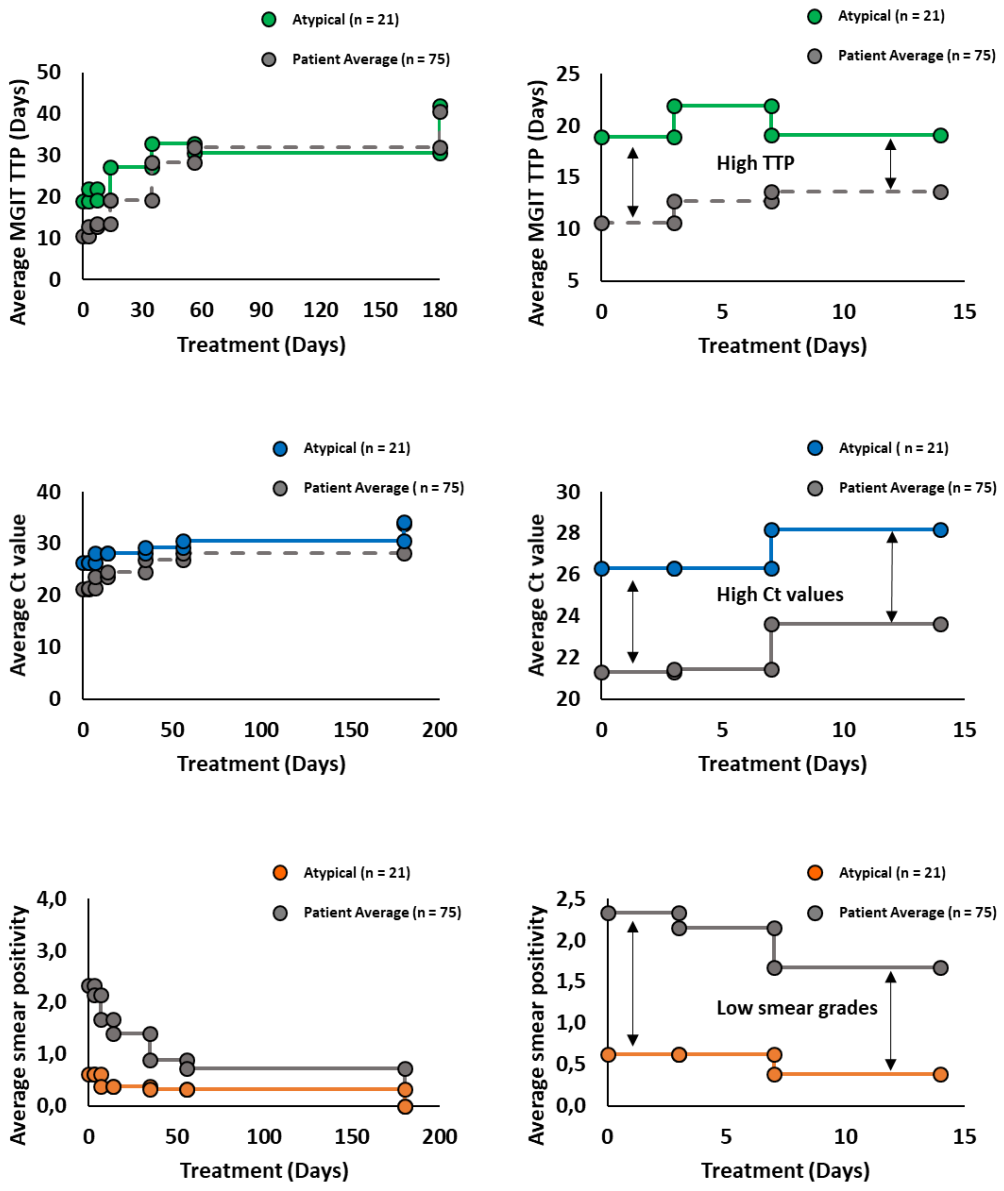

Figure 4.23 Average decline in bacterial load for atypical DCTB pattern throughout first-line treatment as determined by routine diagnostic assays. (A) The average time to positivity (TTP) (green) for the 21 atypical DCTB behaviour patients obtained in the mycobacterial growth indicator tube (MGIT) is given for each time point throughout the six months of anti-TB treatment (left panel). The right panel illustrates the average increase in TTP during the first two weeks of anti-TB treatment, shown to illustrate the early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75, i.e. the average MGIT TTP for all DCTB decline sub-categories). (B) The average cycle threshold (Ct) value (blue) for the 21 atypical DCTB behaviour patients obtained from the GeneXpert is given for each time point throughout the six months of TB treatment (left panel). The right panel illustrates the average increase in Ct value during the first two weeks of anti-TB treatment, to illustrate early treatment response. For comparison, the grey dotted line indicates overall patient average (n = 75 i.e. the average Ct value for all DCTB decline sub-categories). (C) The average smear positivity was calculated by providing the smear grading at each particular time point with a numerical value. P+++, ++, +, scanty and negative smears were scored as 4,3,2,1 and 0, respectively. The left panel illustrates the decline in smear grading over six months of TB treatment. The right panel illustrates the decrease in smear positivity during the first two weeks of anti-TB treatment, shown to illustrate early treatment response. The grey dotted line shows overall patient average (n = 75 i.e. the average smear positivity for all DCTB decline sub-categories).

Analysis of the routine diagnostic data obtained for the atypical DCTB patient cohort illustrated a similar pattern to that observed in the CF-supplemented MPN and CFU assays. In the MGIT assay, a decrease in bacterial load is observed during early treatment whereby the average TTP increases from 18.9 to 21.9 days. However, following one week of treatment, an increase in bacterial load is observed where a TTP of 19.5 days is observed. This increase in bacterial burden is short lived and followed by a gradual decline in TB bacilli as indicated by an increase in TTP at subsequent time points during the remainder of the treatment period, Table 4A. The results reflect the pattern of decline observed in the CF-supplemented MPN assay during early treatment. At the end of treatment, all of the atypical DCTB patients were MGIT culture negative. This is in contrast to the MPN assay where an average of 2.8 log bacteria were cultured. In the GeneXpert assay, the Ct value remained unchanged during the first three days of treatment (Ct = 26.3 at enrolment and day 3). In addition, the Ct value remained essentially unchanged from day seven to 14 (28.2 and 28.7, respectively). At the end of treatment, *M. tuberculosis* was not detected in 83.3% of this patient population. It is important to note that this group of patients are defined by low starting bacillary loads that can be observed in the MPN and CFU assays as well as all three diagnostic tests employed. Only one patient in this group had a higher starting bacillary load of log 7.2 (CF^+ MPN). When removed from analysis, the average starting bacillary load for this group of patients decreased from log 5.9 to log 3.2 in the CF^+ MPN assay. Following the removal of this single patient, the Figure depicting the decline in DCTB is relatively flat (see Appendix 4D 'Figure 4A', page 204).

4.5.5 Baseline factors associated with the various decline trends

Patient demographic factors associated with the various patterns of decline are shown in Table 4.3. The 'no CFU' column contained eleven patients whereby no CFU's were cultured at baseline or at any time point throughout treatment. The majority of these patients (approximately 82%) were HIV-positive. The atypical DCTB category also consisted predominantly of HIV-positive individuals (85.7%). Analysis was conducted between all five groups (Table 4.3) and between individual patient groups. P-values from statistical tests comparing individual groups are given in Appendix 4E (see Tables 4D, 4E, 4H and 4I). In terms of HIV-infection status, a significant difference was seen between the resist decline and atypical DCTB groups (P = 0.0108, chi-square test, Table 4H), where the atypical DCTB patient cohort were more likely to be HIV-positive (86% versus 47%). Age was found to differ significantly between most groups, Table 4.3 and Table 4D. Significant differences were also

obtained with regards to gender where the resist decline DCTB group differed significantly from the no CFU, accumulate and decline groups (Table 4H). Male gender was associated with the resist decline DCTB pattern characterised by high baseline bacterial loads. A significant difference was observed in BMI between the resist decline and atypical DCTB groups with the atypical group having a higher mean BMI (P = 0.0329, unpaired t-test, Table 4D). No significant differences were obtained between the individual DCTB decline groups for CD4 T-cell count, smoking status or the presence/absence of a BCG scar. These findings suggest that the patient's age, HIV-status and gender will most likely be predictive of the pattern of DCTB decline. For example, patients that were assigned to the atypical DCTB category were older (>39 years of age) and more likely to be HIV-positive (85.7%). Patients assigned to the resist decline DCTB sub-category were younger (<31 years of age) and more likely to be male (94.1%).

MPN and CFU assays along with commonly used TB diagnostic tests were analysed at baseline to determine whether one or multiple tests could serve as biomarker to predict the pattern of DCTB decline, Table 4.4. The resist decline and decline DCTB sub-categories were associated with a high bacterial burden in sputum at baseline. Again, it is important to note that the resist decline DCTB sub-category may in fact exhibit a decline pattern, but this cannot be assumed due to the upper limit of the current MPN assay/format. In the CF^{+} MPN assay, the median bacterial load was log 8.7 for both the resist decline and decline DCTB sub-categories. However, in the MPN no CF assay, a higher median bacterial load was present in the resist decline patient cohort (log 4.9 versus log 3.0). P-values from statistical tests comparing between individual groups are given in the Appendices (see Tables 4E, 4F, 4G and 4J). Overall, differences in bacterial burden obtained in all assays could serve as a potential marker for DCTB decline.

Table 4.3 Demographic factors associated with DCTB patterns of decline

	No CFU (n = 11)	Accumulate DCTB (n = 17)	Resist decline (n = 17)	Decline (n = 20)	Atypical (n = 21)	P-value
HIV status *						
Negative, n (%)	2 (18.2)	7(41.2)	9 (52.9)	5 (25.0)	3 (14.3)	0.0699
Positive, n (%)	9 (81.8)	10(58.8)	8 (47.1)	15 (75.0)	18 (85.7)	
CD4 Count †						
Mean (SD)	159.33 (193.8)	297.9 (396.4)	208.1 (132.0)	191.3 (127.7)	219.8 (189.5)	0.7041
Median (IQR)	90.0 (42.0; 130.0)	110.5 (61.5; 352.0)	171.0 (117.8; 271.0)	167.0 (102.0; 292.3)	171.0 (95.3; 315.0)	
Gender *						
Male, n (%)	7 (63.6)	9 (52.9)	16 (94.1)	12 (60.0)	15 (71.4)	0.0929
Female, n (%)	4 (36.3)	8 (47.1)	1 (5.9)	8 (40.0)	6 (28.6)	
Age †						
Mean (SD)	40.3 (9.7)	38.9 (11.5)	28.2 (6.0)	37.7 (10.6)	44.1 (8.4)	**<0.0001**
Median (IQR)	40.0 (34.0; 46.0)	38.0 (31.0; 47.3)	27.0 (24.0; 31.0)	35.5 (27.8; 48.0)	43.0 (39.0; 50.5)	
BMI †						
Mean (SD)	21.9 (4.1)	20.3 (2.7)	19.0 (2.6)	19.6 (3,5)	20.8 (3.8)	0.2005
Median (IQR)	19.8 (18.9; 24,6)	20.1 (18.8; 22.3)	18.3 (17,9; 19,8)	18.9 (17,8; 20,2)	19.6 (17.8; 20.2)	
BCG scar *						
Yes, n (%)	9 (81.8)	11 (64.7)	14 (82,4)	14 (70,0)	14 (66,7)	0.6956
No, n (%)	2 (18.2)	6 (35.3)	3 (17,6)	5 (25,0)	7 (33,3)	
Unknown, n (%)	-	-	-	1 (5,0)	-	
Smoking status *						
Yes, n (%)	6 (54.5)	4 (23.5)	6 (35.3)	7 (35.0)	10 (47.6)	0.4446
No, n (%)	4 (36.3)	13 (76.5)	11 (64.7)	11 (55.0)	10 (47.6)	
Unknown, n (%)	1 (9.1)	-	-	2 (10.0)	1(4.8)	

Abbreviations: n = number of patients; CFU = colony forming units; DCTB = differentially culturable tubercle bacilli; SD = standard deviation; IQR = interquartile range; BMI = body mass index and BCG = Bacille Calmette Guerin. * Significance was determined between all five groups using the chi-squared test for proportions with a 95% confidence interval; † P-values were determined between all five groups using analysis of variance (ANOVA) with a 95% confidence interval. Significance between groups is depicted in bold.

Table 4.4 Baseline TB culture and other diagnostic factors associated with patterns of decline

	No CFU (n = 11)	Accumulate DCTB (n = 17)	Resist decline (n = 17)	Decline (n = 20)	Atypical (n = 21)	P-Value
Log CF^+ MPN						
Mean (SD)	3,5 (2,8)	4,7 (2,4)	8,7 (0,1)	7,5 (1,5)	2,5 (1,5)	**<0.0001**
Median	2,7	4,5	8,7	8,7	2,7	
(IQR)	(1,7; 4,7)	(2,9; 6,8)	(8,7; 8,7)	(6,1; 8,7)	(1,7; 3,1)	
Log MPN no CF						
Mean (SD)	2,2 (1,9)	2,5 (2,1)	5,4 (2,7)	3,8 (2,4)	1,5 (1,2)	**<0.0001**
Median	2,0	2,7	4,9	3,0	1,7	
(IQR)	(1,0; 2,7)	(0,9; 3,3)	(2,7; 8,7)	(2,1; 5,3)	(0,9; 2,7)	
Log CFU						
Mean (SD)	-	2,4 (2,0)	5,3 (1,4)	4,2 (1,7)	1,3 (1,5)	**<0.0001**
Median	-	2,3	5,6	4,3	0,6	
(IQR)		(0,0; 4,5)	(5,5; 5,9)	(3,8; 5,2)	(0,0; 2,7)	
GeneXpert Ct value						
Mean (SD)	25,0 (6,1)	24,4 (6,6)	15,4 (3,3)	18,7 (4,2)	26,0 (6,8)	**<0.0001**
Median	26,1	23,5	14,3	18,4	26,1	
(IQR)	(22,4; 27,6)	(18,7; 29,3)	(13,4; 16,2)	(15,6; 21,5)	(21,2; 30,4)	
MGIT TTP (days)						
Mean (SD)	16,1 (13,5)	12,7 (11,7)	3,7 (1,6)	6,1 (3,2)	18,9 (12,5)	**<0.0001**
Median	11,0 (6,0;	8,0 (6,0; 11,0)	4,0 (3,0; 5,0)	6,0 (4,0; 7,3)	15,0 (11,0;	
(IQR)	19,0)				19,0)	
Smear *						
% Positive	47,1	70,6	100	100	42,9	**<0.0001**
% Negative	52,9	29,4	0	0	57,1	

Abbreviations: CFU = colony forming unit, DCTB = differentially culturable tubercle bacilli, MPN = most probable number, CF = culture filtrate, SD = standard deviation, IQR = interquartile range, MGIT = mycobacterial growth indicator tube. Ct = cycle threshold. P-values were determined between all five groups using analysis of variance (ANOVA) with a 95% confidence interval. * Significance was determined between all five groups using the chi-squared test for proportions with a 95% confidence interval Significance between groups is depicted in bold.

4.5.6 Hetero-resistance / acquired drug-resistance

In four of the 80 patients analysed, different DST profiles (as determined by the HAIN MTBDR*plus* assay) were observed at different time points during this study, Table 4.5. These differences in drug-susceptibility profiles may either have occurred as a result of hetero-resistance or acquired/secondary drug-resistance. Hetero-resistance, defined as the presence of both drug-susceptible and drug–resistant organisms in the same clinical sample, may arise in a

single infection where *M. tuberculosis* undergo genetic change through a mutation in genes associated with drug resistance (Rinder et al., 2001). Acquired drug resistance is defined as the development of resistance by spontaneous chromosomal mutations that occur under selective pressures of drug treatment (Zhang et al., 2005). From the time points at which susceptible and mono-resistant strains were isolated, hetero-resistance is most likely in patients 59045 and 59050, whereas a previously susceptible *M. tuberculosis* strains possibly acquired drug-resistant mutations in patients 59051 and 57172. Further work is required to determine whether these observations are accurate and whether these mixed resistance patterns are cause by different *M. tuberculosis* strains (i.e. mixed strain infections). Hetero-resistance has been reported in a number of previous studies (Post et al., 2004, Kaplan et al., 2003, Shamputa et al., 2004, van Rie et al., 2005) and mixed strain infections have been associated with unfavourable treatment outcomes (Theisen et al., 1995, Niemann et al., 2000, Baldeviano-Vidalon et al., 2005, Kamakoli et al., 2017, van Rie et al., 2005, McIvor et al., 2017).

Table 4.5 Mixed drug-susceptibility patterns identified in four patients during treatment

Patient #	Susceptibility pattern #1	Time point and sample	Susceptibility pattern #2	Time point and sample
59045*	INH resistant RIF susceptible	0 O/N and spot 3 O/N and spot 7 O/N	INH susceptible RIF susceptible	14 O/N
59050**	INH susceptible RIF resistant	0 O/N	INH and RIF susceptible	0 spot 3 O/N and spot
59051†	INH susceptible RIF susceptible	0 spot 3 spot 7 O/N and spot 35 spot	INH resistant RIF susceptible	Unscheduled (day 90 O/N)
57172‡	INH susceptible RIF susceptible	0 O/N and spot 3 spot 7 O/N and spot 14 spot 180 spot	INH resistant RIF resistant	Day 35 spot

Abbreviations: INH = isoniazid, RIF = rifampicin, O/N = overnight sputum sample, spot = spot sputum sample, MGIT = mycobacterial growth indicator tube. Sputum samples were received for all five patients up until the last time point (i.e. day 180). * For patient 59045, MGIT cultures (from both O/N and spot samples) were negative at days 35, 56 and 180. MGIT cultures were contaminated for the day 7 spot and 14 spot samples. ** MGIT cultures for patient 59050 were negative at days 7 (spot sample only), 14, 35, 56 and 180 (O/N and spot samples). The MGIT culture for the day 7 O/N sample was contaminated. † For patient 59051, the following MGIT samples were culture negative: 0 O/N, 3 O/N, 14 spot, 35 O/N, 56 (O/N and spot) and 180 (O/N and spot). No HainMTBDR*plus* data was available for 14 O/N. ‡ For patient 57172, MGITs were culture negative for the following samples: 3 O/N, 14 O/N, 35 O/N and 56 spot. MGITs were contaminated for the day 56 and 180 O/N samples. The Hain MTBDR*plus* was not performed on contaminated or culture negative MGITs.

4.5.7 Mixed infection with *M. tuberculosis* and non-tuberculosis Mycobacteria

Mixed infection with *M. tuberculosis* and NTM were identified in four and eight patients from the Soweto and Klerksdorp cohorts, respectively. The four NTM identified in the Soweto cohort included *M. peregrinum*, *M. gordonae*, *M. intracellulare* and *M.scroffulaceum*. The NTM identified in the Klerksdorp cohort included *M. intracellulare* (isolated from five patients), *M. fortuitum* (isolated from two patients), *M. scrofulaceum* and *M. avium-intracellualre*. This data along with the time-point at which each NTM was identified is given in Table 4.6. The isolation of NTM tended to occur once-off, with the corresponding MGIT received for that particular time point (i.e. spot or O/N) being positive for MTBC on the HAIN MTBDR*plus* test. Indeed, the isolation of NTM with pulmonary TB is not uncommon, but tends to occur as a result of colonisation, transient infection or contamination of the specimen (Jun et al., 2009, Kim et al., 2004, Kobashi et al., 2002).

Table 4.6 Non-tuberculosis mycobacteria isolated in 13 patients during the study

Patient #	Time point and sample	NTM identified
59005	Unscheduled Month 5 spot and Day 180 O/N (corresponding MGITs were culture negative)	*M. peregrinum,*
59045	Day 180 spot (Day 180 O/N was MGIT culture negative)	*M. gordonae*
59048	Day 180 O/N, Post day 180 (Day 180 spot was MGIT culture negative)	*M. scroffulaceum* *M. intracellulare*
59052	Day 35 spot (MTBC in 56 O/N)	*M. intracellulare*
57136	Day 35 O/N (MTBC in spot sample)	*M. intracellulare*
57139	Day 35 O/N (spot culture was negative)	*M. intracellulare*
57146	Day 14 spot (MTBC in O/N sample)	*M. intracellulare*
57152	Day 56 O/N (MTBC in spot sample)	*M. scroffulaceum*
57155 57155	Day 3 O/N * Day 14 O/N (MTBC in spot sample)	*M. intracellulare* *M. fortuitum*
57159	Day 14 spot (MTBC in O/N MGIT)	*M. fortuitum*
57173	Day 180 O/N (spot culture was negative)	*M. avium-intracellualre*
57175	Day 14 spot (MTBC in O/N MGIT)	*M. intracellulare*

* Day 3 spot sample was not received

It is quite possible that the presence of an NTM and mixed *M. tuberculosis* infection in a sputum sample may create a confounding factor in CFU and MPN enumeration. In cases where an NTM is identified in the MGIT, additional microbiological tests should be carried out to determine if the NTM is a contaminating organism (i.e. isolated only in the MGIT and not in the MPN or CFU assay) or whether the NTM was originally present in the sputum sample. In the latter case, data from a second sputum sample can be used (i.e. spot sample instead of overnight sample), or the data for that particular time point should be omitted. Since NTMs were isolated at a single time point in only one sputum sample, suggests that these organisms are most likely to be contaminants from the environment. This can be confirmed by performing species identification tests, such as the HAIN CM assay, directly on CFU or MPN culture positive samples.

4.5.8 End-of-treatment analysis

4.5.8.1 Positive diagnostic and microbiological assays at the end of treatment

For end of treatment analysis, 62 patients that had completed 180 days of anti-TB treatment and provided study samples were included for analysis. *M. tuberculosis* was detected in 10 patients by the GeneXpert assay and three patients were still MGIT culture positive. None of the patients had positive smears at the end of treatment. In microbiological assays, one patient still had CFUs emerge on solid media. In MPN assays, 41, 39 and 38 patients were positive in CF^+, Rpf^- and CF -unsupplemented assays, respectively (Table 4.7 and Figure 4.24).

Table 4.7 Number of patients positive for *M. tuberculosis* by diagnostic and microbiological assays.

Diagnostic/ Microbiological assay	Number of positive patients, n (%)
CF^+ MPN	41 (66.1)
Rpf^- MPN	39 (62.9)
MPN no CF	38 (61.3)
CFU	1 (1.6)
MGIT culture	3 (4.8)
GeneXpert	10 (16.1)

Abbreviations: CF = culture filtrate, MPN = most probable number, Rpf = resuscitation-promoting factor, CFU = colony forming unit, MGIT = mycobacterial growth indicator tube.

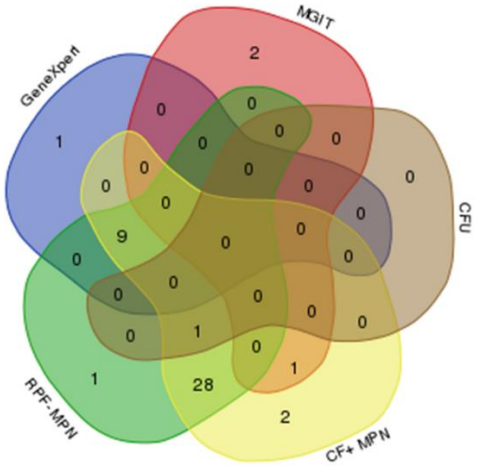

Figure 4.24 Venn diagram illustrating *M. tuberculosis* positivity at the end of treatment in different diagnostic and microbiological assays. The Venn diagram illustrates the number of patients that were positive for *M. tuberculosis* at the end of treatment by a particular assay**.** The diagnostic assays included were the GeneXpert (blue) and MGIT (red). No patients were positive at the end of treatment by smear microscopy. The microbiology assays included culture filtrate supplemented MPN assays [CF^+ (beige) and Rpf^- (green)] as well as colony forming unit (CFU) assays. Venn diagram was creating using the following URL: http://bioinformatics.psb.ugent.be/webtools/Venn.

Table 4.8 Detection of *M. tuberculosis* at end-of-treatment in multiple and single assays.

Diagnostic/microbiological test	Total positive
CF^+ MPN and GeneXpert	9
CF^+ MPN and MGIT	1
CF^+ MPN and CFU	1
GeneXpert	1
MGIT	2
CF^+	30

The high overlap observed between the CF^+ MPN assay and GeneXpert assay in detecting *M. tuberculosis* positive patients at the end of treatment suggests that the GeneXpert may be able to detect the presence of DNA from DCTB at the end of treatment (Figure 4.24 and Table 4.8). However, the GeneXpert assay is not currently recommended for treatment monitoring and is only recommended for initial diagnosis (WHO, 2014).

4.5.9 Reflex assays

To further assess whether the organisms isolated at the end of treatment were *M. tuberculosis* and not precipitation of drugs, cellular debris or contamination, reflex assays were performed

on a subset of samples. The MPN is based on a visual scoring system and the growth of these assays varied from minimal to high. Initial reflex assays were performed on samples with robust and poor growth ranging from day 14 to day 180. For 'end-of-treatment' samples, reflex assays were performed on both frozen (-80 °C) stored positive MPN wells and in real-time on samples from the last few patients enrolled in the study. In few cases, samples collected at unscheduled time points or after treatment completion, i.e., subsequent to the day 180 time point, were analysed.

4.5.9.1 Real-time analysis of treatment samples

A total of 88 samples from 30 patients were reflexed from low positive MPN wells during treatment. These samples were collected between 14 to 180 days following treatment initiation. In the MGIT only 4 samples were positive (4.5%) compared to the 450 µL culture where 39 samples were positive (43.3%). In the MGIT, the majority of the samples reflexed were negative (53.4%) and many were contaminated (42%). CFUs were cultured from 7 samples (8%). In the HAIN MTBDR*plus* assay, approximately 60% of samples were positive for MTBC. These findings are suggestive of possible background in the MPN assay.

Table 4.9 Proportions of positive, negative and contaminated samples obtained in the reflex assays.

	MGIT	450 µL culture	CFU	HAIN MTBDRplus®
Positive	4 (4.5)	39 (43.3)	7 (8.0)	52 (59.1)
Negative	47 (53.4)	48 (54.5)	73 (83.0)	36 (40.9)
Contaminated	37 (42)	1 (1.1)	8 (9.0)	NA

4.5.9.2 Reflex analysis performed on stored MPN wells

Following these results, reflex assays were performed on a single frozen sample from 31 patients at the end of treatment (day 180 samples only). Growth on the reflex MPN assay was observed in two patients only (6.5%). No CFUs were obtained from any of these samples. Negative and contaminated reflex MPN assays were observed for 23 (74.2%) and 6 (19.4%) patients, respectively. MGIT culture and HAIN MTBDR*plus* reflex assays were not performed on frozen end-of-treatment samples.

4.5.9.3 Real-time analysis of end-of-treatment and post-treatment samples

Seventy samples from 19 patients displaying low growth on MPN plates at the end of treatment (9 patients at the end of treatment, i.e. 180 days) or following treatment completion (samples from 10 patients received after 180 days) were reflexed into a separate 450 µL cultures. Positive growth was observed in 67.1% of cases. An example of a positive reflex plate is given in Figure 4.25.

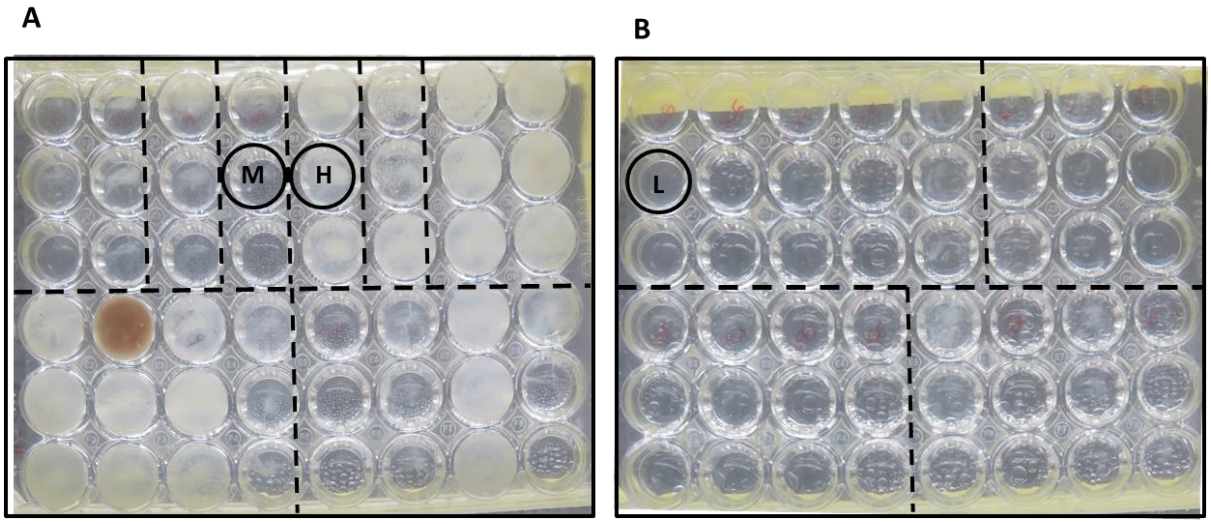

Figure 4.25 Example of 'positive' growth observed in reflex assay in patients 180 days after first-line TB treatment. (A) Reflexed MPN plate showing predominantly medium and high turbity growth. (B) Reflexed MPN plate showing low growth. Dotted lines divide samples reflexed from individual patients. Circles show wells that were labelled with DMN-Tre for microscopy. H = high turbity, M = medium turbity and L = low turbidity.

4.5.10 DMN-Tre staining

To assess whether the growth seen at the end of treatment (scored as positive on the MPN assay) was true *M. tuberculosis* growth, DMN-Tre staining was performed on end-of-treatment samples for a total of six patients. As previously mentioned, the morphology in the MPN wells varied greatly between samples during and at the end of treatment with some illustrating thick and robust growth adhering to the bottom of the MPN well and others showing poor growth that ranged from an opaque suspension in the media to tiny clumps at the bottom of the well. Two patients were selected from three growth categories displaying, high, medium and low growth. A positive control of H37Rv culture was included in the analysis.

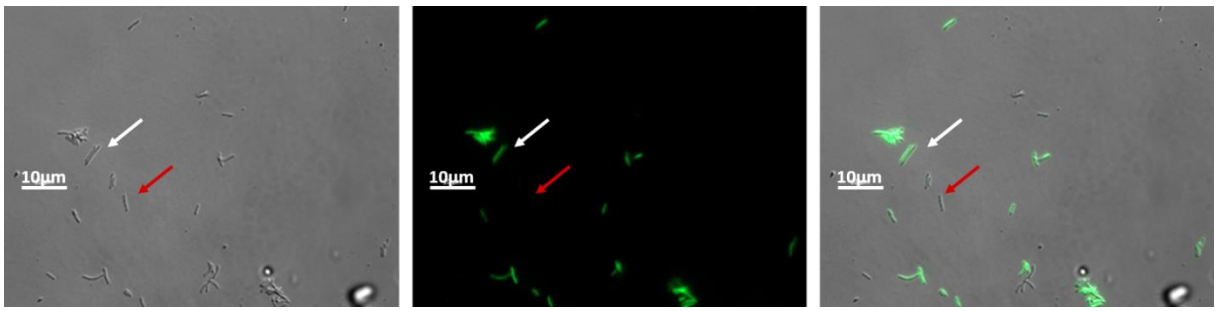

Figure 4.26 DMN-Tre labelling of *Mycobacterium tuberculosis* H37Rv positive control. *M. tuberculosis* H37Rv culture was stained with trehalose conjugated to the solvatochromatic dye 4-*N*,*N*-dimethylamino-1,8-napthalidimide (DMN-Tre). Images from left to right represent DIC, FITC/ GFP and merged images. White arrow illustrates a *M. tuberculosis* cell that is visible in both the DIC and FITC channel showing successful uptake of the DMN-Tre reagent. Red arrow shows a *M.tuberculosis* cell that is only visible in the DIC where DMN-Tre uptake did not occur. **Abbreviations:** DIC = differential interference contrast, FITC = fluorescein isothiocyanate and GFP = green fluorescent protein.

Figure 4.26 illustrates DMN-Tre staining of *M. tuberculosis* H37Rv culture. Fluorescent bacilli visible in both the DIC and FITC channels (shown by white arrows) depict the successful uptake of the DMN-Tre reagent, confirming organism viability. Unstained *M. tuberculosis* that are only visible in the DIC channel (red arrow) shows cells that are most likely dead.

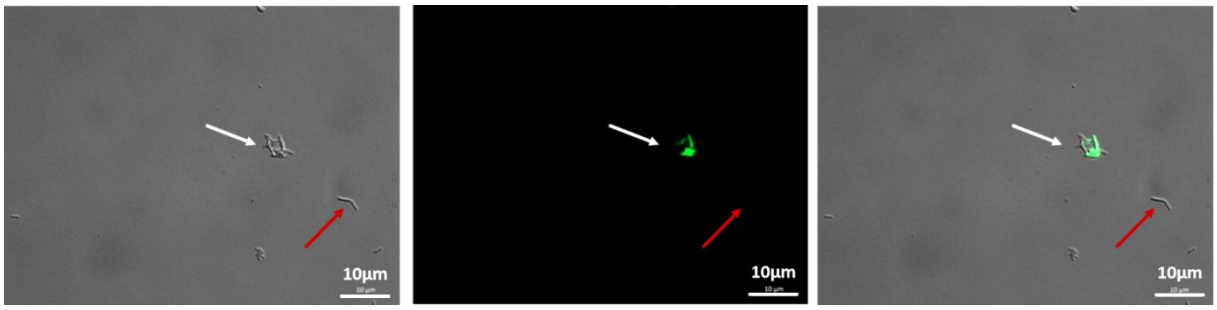

Figure 4. 27 DMN-Tre labelling of end-of-treatment reflex culture exhibiting high turbidly/growth. A high-growth reflex culture from patient 57160 growth was stained with trehalose conjugated to the solvatochromatic dye 4-*N*,*N*-dimethylamino-1,8-napthalidimide (DMN-Tre). Images from left to right represent DIC, FITC/ GFP and merged images. White arrow illustrates *M. tuberculosis* cell that is visible in both the DIC and FITC channel showing successful uptake of the DMN-Tre reagent. Red arrow shows a *M.tuberculosis* cell that is only visible in the DIC channel where DMN-Tre uptake did not occur. **Abbreviations:** DIC = differential interference contrast, FITC = fluorescein isothiocyanate and GFP = green fluorescent protein.

DMN-Tre staining of an end-of-treatment sample (Figure 4.27) confirms the presence of both live and dead *M. tuberculosis* cells isolated from a high turbidity culture. The culture was reflexed from a positive MPN well (containing the Rpf⁻ CF) from patient 57160 following 180

days of anti-tuberculosis treatment. In terms of the routine diagnostic data, both MGIT cultures (from day 180 O/N and spot samples) were culture negative. A low positive GeneXpert (Ct value, 29.3) was obtained in day 180 spot sample. The O/N sample was GeneXpert negative.

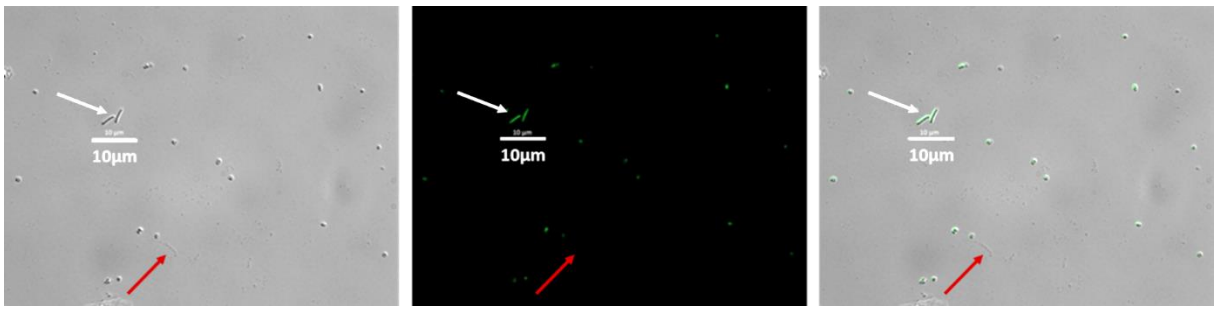

Figure 4. 28 DMN-Tre labelling of end-of-treatment reflex culture exhibiting medium turbidly/growth. A medium-growth reflex culture from patient 57139 was stained with trehalose conjugated to the solvatochromatic dye 4-*N*,*N*-dimethylamino-1,8-napthalidimide (DMN-Tre). Images from left to right represent DIC, FITC/ GFP and merged images. White arrow illustrates *M. tuberculosis* cell that is visible in both the DIC and FITC channel showing successful uptake of the DMN-Tre reagent. Red arrow shows a *M.tuberculosis* cell that is only visible in the DIC where DMN-Tre uptake did not occur. A mixture of cocci and bacilli are present. **Abbreviations:** DIC = differential interference contrast, FITC = fluorescein isothiocyanate and GFP = green fluorescent protein.

Figure 4.28 illustrates the presence of live *M. tuberculosis* bacilli isolated from a medium turbidity culture. This culture was reflexed from a positive MPN well (un-supplemented, no CF) from patient 57139 following 180 days of anti-tuberculosis treatment. When compared to the *M. tuberculosis* H37Rv control and the high turbidity well from patient 57160, the fluorescence in these images are less intense. Indeed, it has been demonstrated that stationary phase *M. smegmatis* cultures do take up the dye, but to a lesser extent than actively growing cells (Kamariza et al., 2018). The red arrow illustrates the presence of what would appear to be a 'ghost form' which is visible in the DIC channel but not in the FITC channel. In addition to bacilli, the presence of stained ovoid forms are also visible. The presence of ovoid/coccoid cells may indicate the presence of non-replicating organisms (Shleeva et al., 2002). Alternatively, these cells could illustrate the presence of a contaminant that is able to incorporate DMN-Tre (for example, Corynebacterium). Routine diagnostic data from this patient was GeneXpert negative and the end of treatment. Both MGIT cultures were reported as contaminated, but it is clear from the microscopy that *M. tuberculosis* was also present.

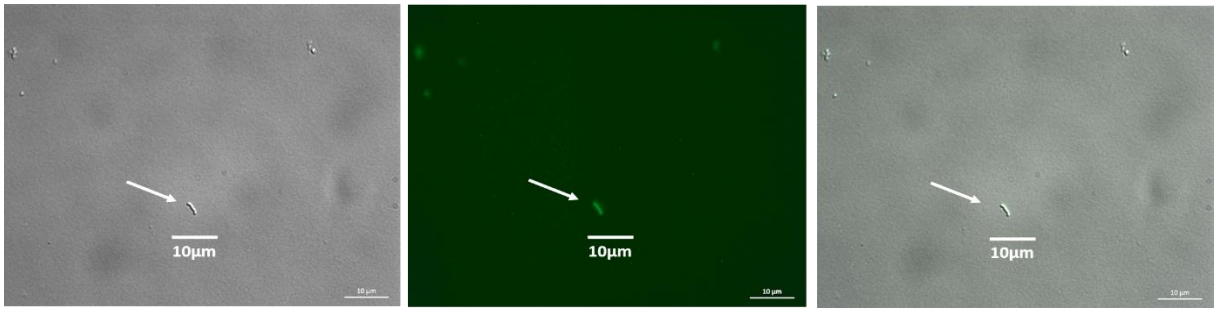

Figure 4.29 DMN-Tre labelling of end-of-treatment culture exhibiting low turbidly/growth. A medium-growth reflex culture from patient 57134 was stained with trehalose conjugated to the solvatochromatic dye 4-*N*,*N*-dimethylamino-1,8-napthalidimide (DMN-Tre). Images from left to right represent DIC, FITC/ GFP and merged images. White arrow illustrates *M. tuberculosis* cell that is visible in both the DIC and FITC channel showing successful uptake of the DMN-Tre reagent. **Abbreviations:** DIC = differential interference contrast, FITC = fluorescein isothiocyanate and GFP = green fluorescent protein.

Figure 4.29 shows the presence of one live *M. tuberculosis* cell isolated from a culture with very low turbidity. The culture was reflexed from a positive MPN well from Patient 57134 (CF^+ MPN) following 180 days of anti-tuberculosis treatment. Out of the two end of treatment MGIT cultures, one was contaminated and the other was culture negative. Both end-of-treatment GeneXpert samples were negative for *M. tuberculosis*. DMN-Tre staining of positive MPN wells confirms the presence of viable *M. tuberculosis* organisms at the end of treatment suggesting that complete sterilization of *M. tuberculosis* is not occurring in all patients.

Figure 4.30 illustrates the average number of organisms detected in MPN and CFU assays in 62 patients at the end of treatment. Positive growth was detected in 41 (55%), 39 (52%) and 38 (51%) patients at the end of treatment in CF^+, Rpf^- and no CF MPNs, respectively. One patient had a positive CFU at the end of treatment. While DMN-Tre microscopy suggests the presence of residual organisms at the end of treatment, this analysis was only conducted on six patients and further investigation is required to determine whether all positive MPN patients harbour organisms at the end of treatment. Given the results from the reflex assays, possible background in the form of cellular debris or contaminating oral flora may present in the MPN assay. This is evident by the high number of negative and contaminated MGIT cultures from low growth MPN wells during and at the end of treatment (see Table 4.9). In the contaminated cultures, it is possible that a mixture of *M. tuberculosis* and other organisms are present. Since the HAIN MTBDR*plus* was positive for MTBC in 60% of reflexed samples, this is likely the case. The addition of CF may also stimulate the growth of other organisms whose growth is ordinarily suppressed by PANTA in the MGIT (see chapter 6). Furthermore, the limit of detection in the MPN assay remains to be established. Nevertheless the presence of positive MPN wells at the

end of treatment led to the identification of residual *M. tuberculosis* bacilli following six months of anti-TB treatment.

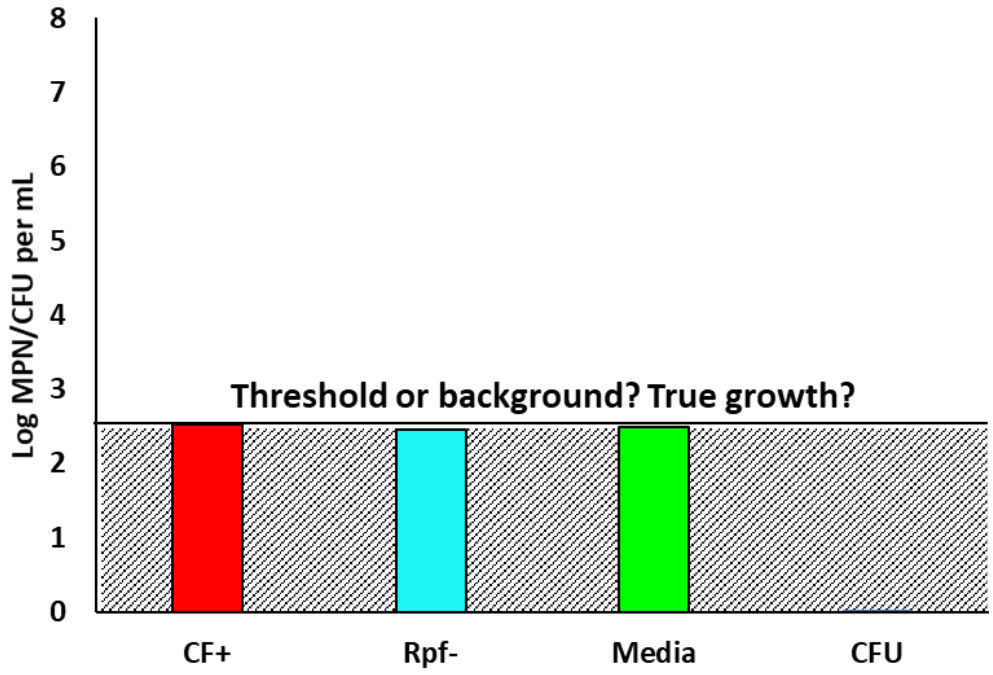

Figure 4.30 Growth detected at the end of treatment in MPN assays. Positive growth was detected in 41, 39 and 38 patients in CF^+ (red), Rpf^- (blue) and no CF (green) MPN assays. On average, about 2.5 log growth was detected in all three MPN assays. Reflex assays to determine whether this positive growth was due to *M. tuberculosis* and not contaminating organisms was performed.

4.6 DISCUSSION

Previous work has demonstrated the phenomenon of DCTB in TB patient samples prior to treatment (Mukamolova et al., 2010, Chengalroyen et al., 2016). Furthermore, evidence from studies conducted *in vitro* or on *ex vivo M. tuberculosis* clinical strains, suggest that these organisms are more tolerant to TB drugs (Wayne and Sramek, 1994, Wayne and Hayes, 1996, Deb et al., 2009, Mukamolova et al., 2010, Sala et al., 2010, Turapov et al., 2016, Loraine et al., 2016). However, how these populations respond to anti-TB chemotherapy has not been investigated *in vivo*.

In pre-treatment samples it was found that the prevalence of CF-dependent and Rpf-independent DCTB populations correlated, suggesting that factors, other than Rpfs, present in CF are involved in bacterial growth stimulation. Spearman's rank sum correlation analysis was carried out at all time points during treatment and significant correlations were observed,

confirming the results obtained in baseline samples. In addition, pre-treatment analysis revealed that HIV-negative individuals harbour more DCTB than their HIV-positive counterparts. Indeed, analysis of all treatment time points revealed that HIV-negative individuals harbour a significantly higher quantum of DCTB and conventionally culturable bacteria up to 14 and 35 days following treatment initiation, respectively. These results suggest that following one month of anti-TB treatment, HIV-negative individuals may still be more infectious than HIV-positive individuals. Indeed at 35 days post-treatment initiation, 56.3% of the overall patient cohort was still positive by MGIT culture (mean TTP 28.5 days, Table 4A), suggesting that these patients are still capable of transmitting TB. While it was previously though that infectiousness rapidly diminishes once treatment is initiated (Rouillon et al., 1976), there is evidence to suggest that patients can still transmit TB after two weeks on anti-TB chemotherapy (Fitzwater et al., 2010, Schwartzman and Menzies, 2000).

Regression analysis was conducted on 75 patients using LOWESS models to determine the rates of decline for the four populations studied (i.e. CF-dependent, Rpf-independent, CF-independent and CFU). In contrast to the initial hypothesis, the CF^{+} population declined at a faster rate when compared to the CF-independent and platable bacteria, suggesting that these organisms are more susceptible to anti-TB chemotherapy. However, the differences in rates of decline were marginal and provided no clarity on the response of DCTB to drug treatment. Individual patient analysis illustrated a far greater complexity with respect to the response of DCTB to first-line treatment, with a great deal of variation observed between patients. Four broad categories of response to treatment were defined, including (i) patients that harbour DCTB that are somewhat tolerant to treatment and fail to decline during the first three to seven days of treatment, (ii) patients that accumulate DCTB during the onset of early treatment, (iii) patients with DCTB that decline rapidly throughout treatment and (iv), patients that exhibit an atypical pattern of DCTB decline throughout treatment.

Approximately 23% of the patients analysed in this study were assigned to the tolerant pattern of decline. In these patients, bacterial decline was only seen following one to two weeks of treatment. It is important to note that the trend of DCTB response characterised by this group of patients is likely to occur due to the MPN assay having an upper limit of log 8.7. Many of these patients displayed an MPN of 8.7 log during early treatment because all MPN wells were positive in 48-well plates. Hence a more accurate description of the MPN bacterial load in this case is >8.7 log. However, as this could not be plotted, a value of 8.7 was used in graphs and for statistical analysis. Therefore, the 'true' number of organisms at baseline is not known and

this upper limit may have masked the true pattern of DCTB, which could have taken the pattern of accumulation or decline. Indeed, this pattern of persistence was not backed up by routine diagnostic tests and on average these tests tended to show a decline in bacillary load during early treatment, Figure 4.15, 4.17 and Tables 4A, 4B and 4C. However, at 56 and 180 days after treatment initiation, this group had the lowest rates of culture negativity at 40.0% and 86.7%, respectively. These results were reflected in GeneXpert assays and smear microscopy, where only 6.3% and 78.6% were *M. tuberculosis* not detected according to the GeneXpert and only 33.3% of this population were smear negative by day 56. These findings suggest that patients with DCTB that exhibit some form of antibiotic tolerance during early treatment are at higher risk of having a positive TB diagnostic at the end of treatment. Preliminary investigations at the CBTBR employing a combined method of flow cytometry and DMN-Tre staining on stored decontaminated pellets from four patients indicated that approximately 75% of these patients accumulated bacteria during treatment, while in 25% of patients, bacillary load declined rapidly following the onset of treatment (unpublished data, Peters et al). Analysis of baseline demographics and bacillary load measures showed that young age (less than 31 years), male gender and high sputum bacillary burden were associated with this pattern of decline. The high bacillary load observed in this patient cohort throughout treatment can be potentially linked to reduced drug activity *in vivo*. Indeed, lower concentrations of anti-TB drugs in the plasma have been linked to clinical failure and drug resistance (Kimerling et al., 1998, Sahai et al., 1997). Examples of factors associated with variations in drug absorption concentrations include HIV infection status, gender and previous TB disease (McIlleron et al., 2006). These findings suggest that drug level monitoring should be considered to endure optimal absorption of anti-TB agents and so that dosage can be adjusted accordingly if required. In the current study, additional sputum and blood plasma samples were collected at each time point and are being stored at -80 °C with the intention of conducting future pharmokinetic analysis to link the concentration of plasma and sputum drug levels to bacillary load and rate of decline.

Approximately 23% of the patients analysed accumulated DCTB during the first week of anti-TB treatment. These results are in agreement with previous observations, where treatment of axenic *M. tuberculosis* cultures or clinical strains in patient sputa illustrated tolerance to first-line antibiotics (Wayne and Hayes, 1996, Chao and Rubin, 2010, Deb et al., 2009, Sala et al., 2010, Turapov et al., 2016, Mukamolova et al., 2010). The observations from this patient category suggests that stress exerted on these organisms in the form of antibiotic pressure

results in a metabolic shift in these organisms into CF-dependent state. These organisms can be recovered following the addition of stimulatory molecules in CF to the culture medium. It is interesting to note that on average, this group of patients had a lower starting bacterial load as detected by CF-supplemented MPN and CFU assays (approximately log 4.7 and log 2.4, respectively), compared to the resist decline and rapid decline patterns. Thirty percent of these patients were smear negative at baseline, and these patients had higher MGIT TTPs and GeneXpert Ct values than the other groups analysed (with the exception of the atypical group). It remains to be investigated as to how transmissible these DCTB populations are and whether they are able to cause infection and disease in a new host. There are two possibilities: either the deficiency in culturability may imply damage that will render the organism unable to establish infection in a new host or ii) the metabolic changes associated with non-culturability may provide the organism with a selective advantage allowing it to be protected from the host immune system until conditions are favourable (i.e. following host immunological impairment).

The second highest number of patients (roughly 27%) exhibited the same pattern of decline as depicted in the LOWESS model for the overall dataset, i.e. a rapid pattern of decline in all bacterial populations, particularly the CF-dependent populations, following the onset of treatment. This pattern of decline is commonly observed in EBA studies, where bacterial decline (measured by CFUs) is rapid following the onset of treatment. Seventy five percent of patients in this category were HIV-positive with a median CD4 T-cell count of 171 cells/mm^3. In terms of TB diagnostics, these patients were all smear positive at baseline and had relatively low MGIT TTPs (6.1 ± 3.2 days) and GeneXpert Ct (18.5 ± 4.1) values. These data were accompanied by high baseline MPNs and CFUs (mean, log 7.5 and log 4.2, respectively). After 56 days of treatment, this patient group had the highest percentage MGIT culture negativity (66.7%) and *M. tuberculosis* not detected by GeneXpert (29.4%), suggesting that these patients respond well to anti-TB treatment. An important question to consider is why are DCTB in one subset of patients tolerant to first-line treatment, whereas in other patients this population declines faster compared to the culturable populations? These DCTB may be more susceptible to the bactericidal action of the drugs compared to the culturable population, possibly due to damage inflicted by the host immune system. Alternatively, the levels of drug concentrations and drug absorption in these patients may be more toxic to these bacilli than that in patients in other sub-categories, thus leading to effective killing of these populations. Further work looking at the drug concentrations in patient blood and sputum is required to understand this.

A substantive number of the patients analysed in this study (28%) were characterized by an atypical DCTB response to treatment. In general, these patients had a low starting bacterial load, as detected by MPN and CFU assays along with routine diagnostic assays that remained low throughout treatment. Most of the patients in this cohort were HIV positive (85.7%). Furthermore, this group of patients were significantly older than patients assigned to the other three DCTB decline groups [median age 43 years (IQR: 39 to 50.5)]. The low bacterial loads obtained in these patients may be due to poor quality sputum samples received as a results of EPTB disease (Silva et al., 2014). No data was collected on EPTB and hence, no further conclusions can be drawn here. In terms of baseline TB diagnostics, these patients had the highest median MGIT TTP [15 days (IQR: 11.0; 19.0)], GeneXpert Ct value [27.0 (IQR: 26.3; 30.2)] and the highest percentage of smear negative individuals (57.1%). While all these patients were culture negative at the end of treatment by MGIT, the majority of these patients harboured high proportions of DCTB (approximately 20% relative to their baseline counts, see Figure 4A) at the end of treatment.

Overall, the results from the decline analysis showed that various patterns of *M. tuberculosis* decline are present in different patients. Possible biomarkers to predict how patients may respond to treatment were identified using patient demographics (i.e. age) and baseline diagnostic tests. However, these deductions are speculative and more detailed analysis in larger cohorts is required to investigate this. While reasons for different responses to treatment remain to be established, Figure 4.31 proposes why different patterns of decline may be observed amongst TB patients.

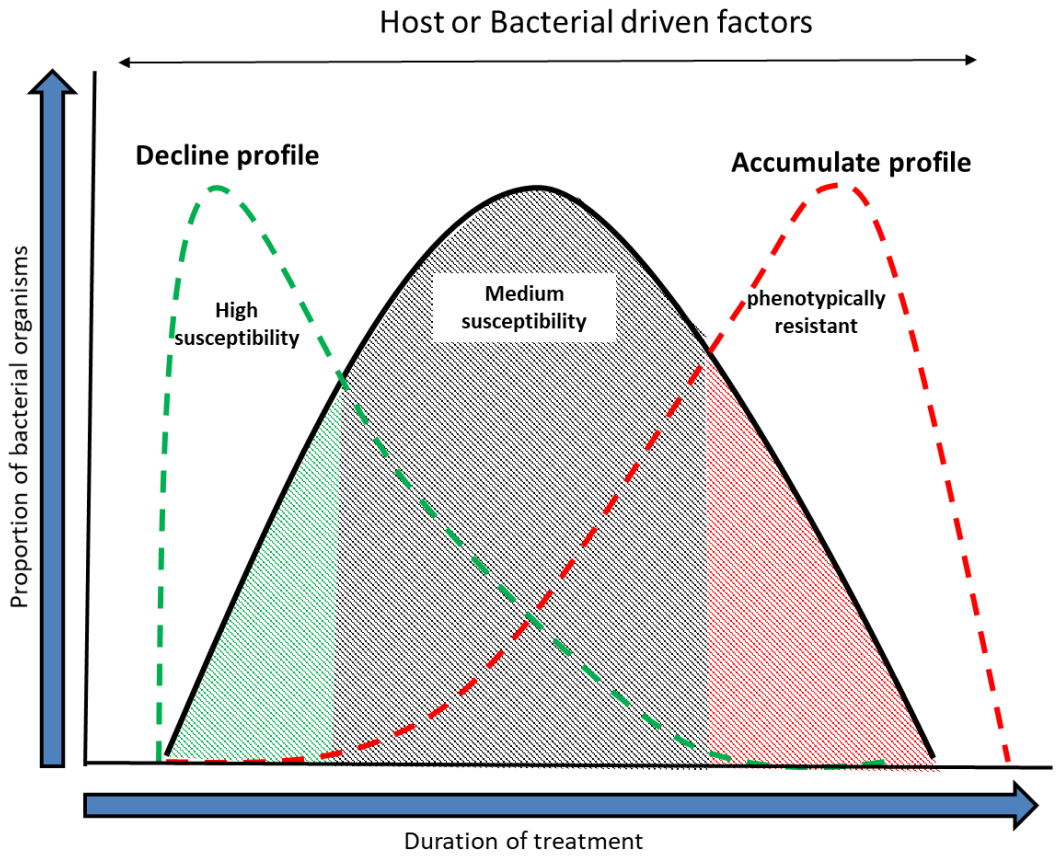

Figure 4.31 Proposed model of varying patterns of DCTB decline. In a normal and hypothetical *M. tuberculosis* population distribution (black line), a mixture of organisms displaying high, medium and low susceptibility to anti-TB drugs would exist, possibly due to altered growth states. However, various external factors (i.e. host immunity and drug treatment) in combination with inherent bacterial/genetic factors (i.e. bacterial strain type etc.), may shift the proportions of these populations to assume altered distributions. For example, damage inflicted by host immunity may damage the organisms making them more susceptible to anti-TB chemotherapy. These organisms will decline quickly following the administration of TB treatment and a decline profile will be apparent (dotted green line). In another scenario, external pressures/factors may drive these organisms into a non-culturable state making them phenotypically tolerant to anti-TB medication. In this case, DCTB may accumulate during early treatment or a resist profile may be obtained (dotted red line).

For the end of treatment analysis, 13 (21.0%) of patients still had a positive diagnostic test [10 (16.1%) positive by GeneXpert and 3 (4.8%) positive by MGIT culture]. MPN assays supplemented with CF revealed that 41 (66.1%) of patients still has positive cultures at the end of treatment. In one patient (1.6%), *M. tuberculosis* colonies were present at the end of treatment on solid media. To confirm whether the high the number of positive cultures obtained in the CF^+ MPN at the end of treatment were *M. tuberculosis*, fluorescent microscopy was performed on select reflex cultures using DMN-Tre staining of live organisms. Indeed, positive bacilli were present at the end of treatment in five out of six patient samples analysed. At the end of treatment, *M. tuberculosis* was not detected in samples from patient 57139 using the

GeneXpert assay, but both MGIT cultures were contaminated with no AFB present on ZN smears. These findings suggest that DMN-Tre may not be exclusive to *M. tuberculosis* and other bacteria harbouring a mycomembrane, such as members of the *Corynebacterium* genus, may also incorporate DMN-Tre. Alternatively, these organisms may be metabolically quiescent. For this, further work is required to determine whether DMN-Tre is capable of labelling non-replicating organisms. This can be achieved by labelling of corresponding positive and negative CF^+ and no CF MPN wells (at the same dilution factor), respectively. Furthermore, research is required on a larger number of patients to confirm the presence of non-replicating *M. tuberculosis* at the end of treatment. Currently, this is being carried out at the CBTBR using a combination of flow cytometry and DMN-Tre labelling.

Nevertheless, observations from end of treatment analysis suggests that patients still harbour and are able to expectorate live *M. tuberculosis* at the end of treatment. The majority of these organisms can only be detected using specialised culture methods, such as CF^+ MPN assays. Since these organisms are present in expectorated sputum, it is important for future studies to establish whether these organisms are infectious and/or capable of causing relapse disease. As these patients were clinically well at the end of treatment, it can be speculated that the majority of these patients may now have reverted from active disease to LTBI. Indeed, these patients are currently being followed-up for five years after treatment completion to monitor for recurrent TB disease. Molecular-based typing techniques will be employed to differentiate between relapse and re-infection. It is generally expected that relapse disease will occur within a year following treatment completion, whereas re-infection is more likely to place after one year (Marx et al., 2014).

This study has several limitations. Firstly, as discussed in chapter three, no recombinant Rpf work was carried out. Therefore, the observed growth stimulatory effect cannot exclusively be attributed to Rpfs and other molecules in CF may stimulate the growth of non-replicating organisms. In addition, it is not known whether (or to what extent) growth inhibitory molecules are present in CF. Since this work and previously published work has shown that CF can both increase as well as decrease the detection of DCTB, the identification of these molecules and a mechanistic understanding of the bacterial growth processes is important to confirm that the apparent 'rise and fall' in DCTB is due to a genuine biological change and not a result of varying factors present in CF. However, it must be noted that all efforts were made to standardise the preparation of the CF to limit such variability. A second limitation was that the quantification of DCTB was limited by the MPN assay in its current format. Future work

to determine the 'true' number of DCTB at baseline may involve performing MPN assays over two to three 48-well microtitre plates in order to obtain wells with no growth. Alternatively, samples received at baseline and during early treatment (i.e. up to day 7) will require dilution following decontamination before MPN assays are carried out. The problem with the second method is that sputum samples with a low starting bacillary load may be diluted out (for example, patients in the atypical cohort), giving a negative baseline (or early treatment) MPN assay. Other methods to quantify DCTB, such as flow cytometry may provide a more attractive means of detecting DCTB in patient sputum. Lastly, further work is required to determine the limit of detection in the MPN assay.

4.7 CHAPTER CONCLUSION AND FUTURE RESEARCH

Interpreting how mycobacteria respond to treatment in clinical patient samples is difficult since two independent biological systems (i.e. bacterium and host) are in a battle for survival. This work demonstrates that the DCTB present in treatment naïve sputum responds in complex ways upon the initiation of treatment. Four distinct patterns of DCTB behaviour were described, namely a drug tolerant DCTB population, DCTB that accumulate, DCTB that decline rapidly as well as an atypical DCTB presentation that seems to persist at low levels throughout treatment. Various factors such as host immunity, nutrition, drug absorption, pathology, bacterial strain type and other genetic predispositions, may influence how these organisms and the host will respond to anti-TB treatment. An additional important finding of this work was the presence of live *M. tuberculosis* at the end of treatment. This finding implies that the current TB regimen is not sterilizing in all TB patients. The results presented in this chapter define a potential and novel role for DCTB in assessing patient response to treatment. In addition, the presence of DCTB following treatment completion may serve as a biomarker for relapse disease and allow DCTB positive patients to be targeted for INH-preventative therapy. Further work is required in this regard. Additional future work will include the analysis of stored sputum and blood samples for drug concentrations by HPLC and/or mass spectrophotometry.

APPENDICES

APPENDIX 4A: MGIT TTP FOR LONGITUDINAL ANALYSIS

TABLE 4A: Average MGIT TTP for all patients and defined patient groups in longitudinal analysis

	All (n = 75)	**3 & 7 Day Resistance (n = 17)**	**Accumulate DCTB (n = 17)**	**Decline (n = 20)**	**Atypical (n=21)**
Baseline	**n =75**	**n = 17**	**n = 17**	**n = 20**	**n = 21**
Mean (SD)	10,6 (10,6)	3,7 (1,6)	12,7 (11,7)	6,1 (3,2)	18,9 (12,5)
Median (IQR)	7,0 (4,0; 12,5)	4,0 (3,0; 5,0)	8,0 (6,0; 11,0)	6,0 (4,0; 7,3)	15,0 (11,0; 19,0)
Negative (%)	6,7	0	5,9	0	19,0
Two consecutive negative (%)	NA	NA	NA	NA	NA
Day 3	**n = 75**	**n= 17**	**n = 17**	**n = 20**	**n = 21**
Mean (SD)	12,8 (10,4)	6,8 (3,7)	10,4 (6,8)	10,2 (7,4)	21,9 (13,2)
Median (IQR)	9,0 (6,0; 18,0)	7,0 (5,0; 8,0)	9,0 (7,0; 11,0)	8,0 (6,0; 12,0)	19,0 (11,0; 29,0)
Negative (%)	6,7	0	0	0	23,8
Two consecutive negative (%)	0	0	0	0	0
Day 7	**n = 74**	**n =17**	**n = 17**	**n = 20**	**n = 20**
Mean (SD)	13,6 (10,2)	7,9 (4,4)	15,6 (13,5)	10,7 (4.4)	19,5 (11,5)
Median (IQR)	11,0 (8,0;16,8)	7,5 (6,0; 10,3)	11,0 (9,0; 17,0)	11,0 (8,8; 13,0)	17,5 (10,8; 23,0)
Negative (%)	8,1	0	17,6	0	15,0
Two consecutive negative (%)	0	0	0	0	0
Day 14	**n = 74**	**n = 17**	**n = 17**	**n = 20**	**n = 20**
Mean (SD)	19,3 (12,5)	12,3 (6,1)	17,1 (11,6)	19,1 (11,2)	27,2 (14,5)
Median (IQR)	15,0 (11,0; 22,8)	13,0 (9,0; 13,0)	13,0 (11,0; 21,0)	17,0 (12,8; 20,8)	26,5 (12,8; 42,0)
Negative (%)	17,6	0	11,8	15	40,0
Two consecutive negative (%)	1,25	0	0	0	4,8
Day 35	**n = 71**	**n = 17**	**n = 16**	**n = 19**	**n= 19**
Mean (SD)	28,5 (13,9)	22,4 (13,3)	31,6 (14,0)	27,1 (14,3)	32,8 (12,6)
Median (IQR)	31,0 (16,0;42,0)	17,0 (13,0; 36,0)	42,0 (20,5; 42,0)	22,0 (15,5; 42,0)	42,0 (22,5; 42,0)
Negative (%)	43,7	23,5	56,3	42,1	52,6
Two consecutive negative (%)	7,5	0	5,9	5	19,0
Day 56	**n = 68**	**n = 15**	**n = 15**	**n = 18**	**n = 20**
Mean (SD)	32,1 (13,3)	30,2 (13,3)	31,5 (13,5)	35,0 (12,2)	31,3 (14,5)
Median (IQR)	42,0 (21,0;42,0)	31,0 (25,5; 42,0)	40,0 (19,0, 42,0)	42,0 (35,5; 42,0)	42,0 (19,3; 42,0)
Negative (%)	54,4	40,0	46,7	66,7	57,9
Two consecutive negative (%)	21,25	11,8	23,5	25	28,6
Day 180	**n = 55**	**n = 14**	**n = 13**	**n = 13**	**n = 15**
Mean (SD)	40,7 (5,7)	39,9 (8,0)	40,1 (6,9)	40,6 (5,0)	42,0 (0,0)
Median (IQR)	42,0 (42,0;42,0)	42,0 (42,0; 42,0)	42,0 (42,0; 42,0)	42,0 (42,0; 42,0)	42,0 (42,0; 42,0)
Negative (%)	94,6	86,7	92,3	92,3	100
Two consecutive negative (%)	37,5	29,4	35,3	45	47,6

% negative calculated using 'n' as denominator for each particular time point. % for two consecutive smear negative samples was calculated using 'n' as denominator for total number of patients from group, not time point. TTP was excluded from analysis if no acid fast bacilli were present on ZN examination. **Abbreviations**: n = number of patients analysed; SD = standard deviation; IQR = interquartile range; DCTB = differentially culturable tubercle bacilli

APPENDIX 4B: GENEXPERT CT VALUES FOR LONGITUDINAL ANALYSIS

Table 4B. Average XPERT Ct values for all patients and defined patient groups in longitudinal analysis

	All (n = 75)	3 & 7 Day Resist decline (n = 17)	Accumulate DCTB (n = 17)	Decline (n = 20)	Atypical (n = 21)
Baseline	**n = 75**	**n = 17**	**n = 17**	**n = 20**	**n = 21**
Mean (SD)	21,3 (6,9)	15,4 (3,3)	24,0 (6,5)	18,5 (4,1)	26,3 (6,8)
Median (IQR)	20,6 (15,5; 26,0)	14,3 (13,4; 16,2)	21,9 (18,7; 29,3)	18,5 (15,3; 21,2)	27,0 (26,3; 30,2)
MTB Not detected (%)	6,7	0	11,8	0	14,3
Day 3	**n = 74**	**n = 16**	**n = 17**	**n = 20**	**n = 21**
Mean (SD)	21,5 (6,4)	16,7 (4,1)	22,6 (6,5)	19,1 (3,7)	26,3 (6,4)
Median (IQR)	20,2 (16,0; 25,7)	16,0 (14,0; 18,8)	23,2 (17,5; 25,9)	19,8 (15,8; 21,2)	28,6 (28,6; 31,0)
MTB Not detected (%)	5,4	0	5,9	0	14,3
Day 7	**n = 73**	**n = 16**	**n = 17**	**n = 20**	**n = 20**
Mean (SD)	23,6 (6,5)	19,0 (3,6)	24,5 (7,5)	22,0 (4,2)	28,2 (6,4)
Median (IQR)	21,1 (18,9; 29,5)	18,8 (16,5; 20,5)	20,5 (19,4; 32,1)	20,8 (19,5; 23,6)	29,8 (29,7; 34,2)
MTB Not detected (%)	11,1	0	17,6	0	25,0
Day 14	**n = 72**	**n = 16**	**n = 16**	**n = 20**	**n = 20**
Mean (SD)	24,5 (5,8)	20,6 (3,5)	24,3 (6,0)	23,4 (4,0)	28,7 (6,2)
Median (IQR)	23,3 (20,4; 28,8)	19,6 (18,3; 22,9)	23,5 (21,0; 25,7)	22,6 (21,5; 26,4)	30,0 (30,0; 34,5)
MTB Not detected (%)	8,5	0	12,5	0	20,0
Day 35	**n = 70**	**n = 16**	**n = 15**	**n = 19**	**n = 20**
Mean (SD)	26,8 (5,9)	23,4 (4,7)	26,9 (6,3)	27,2 (5,6)	29,5 (5,5)
Median (IQR)	27,2 (22,7; 31,2)	24,0 (19,0; 27,2)	25,2 (23,0; 32,7)	27,5 (22,6; 29,2)	30,1 (30,0; 35,0)
MTB Not detected (%)	14,3	0	13,3	10,5	30,0
Day 56	**n = 68**	**n = 16**	**n = 16**	**n = 17**	**n = 19**
Mean (SD)	28,2 (5,4)	24,2 (4,3)	28,1 (5,7)	29,6 (4,6)	30,5 (5,2)
Median (IQR)	26,9 (24,3; 35,0)	24,5 (21,5; 25,7)	26,3 (24,1; 34,9)	28,0 (26,5; 35,0)	33,7 (33,3; 35,0)
MTB Not detected (%)	26,5	6,3	25,0	29,4	42,1
Day 180	**n = 60**	**n = 14**	**n = 15**	**n = 13**	**n = 18**
Mean (SD)	33,9 (2,8)	33,2 (4,0)	33,9 (3,0)	34,5 (1,9)	33,9 (2,6)
Median (IQR)	35,0 (35,0; 35,0)	35,0 (35,0; 35,0)	35,0 (35,0; 35,0)	35,0 (35,0; 35,0)	35,0 (35,0; 35,0)
MTB Not detected (%)	83,9	78,6	86,7	92,3	83,3

% MTB not detected calculated using 'n' as denominator for each particular time point

Abbreviations: n = number of patients analysed; SD = standard deviation; IQR = interquartile range; DCTB = differentially culturable tubercle bacilli

APPENDIX 4C: SMEAR DATA FOR LONGITUDINAL ANALYSIS

Table 4C. Smear data reported as percentage for all patients and stratified by defined sub-categories for longitudinal analysis

	All (n = 75)	3 & 7 Day Resistance (n = 17)	Accumulate DCTB (n = 17)	Decline (n = 20)	Atypical (n = 21)
Baseline (%)	**n = 75**	**n = 17**	**n = 17**	**n = 20**	**n = 21**
P+++	37,3	82,4	17,6	55,0	0
P++	14,7	11,8	17,6	25,0	4,8
P+	14,7	5,9	23,5	20,0	9,5
Scanty	10,7	0	11,8	0	28,6
Negative	22,7	0	29,4	0	57,1
Day 3 (%)	**n = 75**	**n = 17**	**n = 17**	**n = 20**	**n = 21**
P+++	36,0	82,4	29,4	40,0	0
P++	12,0	11,8	5,9	25,0	4,8
P+	10,7	0	11,8	15,0	14,3
Scanty	13,3	0	23,5	10	19,0
Negative	28,0	5,9	29,4	10	61,9
Day 7 (%)	**n = 75**	**n = 17**	**n = 17**	**n = 20**	**n = 21**
P+++	16,0	41,2	23,5	0	4,8
P++	18,7	29,4	11,8	35,0	0
P+	16,0	17,6	17,6	30,0	0
Scanty	14,7	5,9	5,9	25,0	19,0
Negative	34,7	5,9	41,2	10,0	76,2
Day 14 (%)	**n = 74**	**n = 17**	**n = 17**	**n = 20**	**n = 20**
P+++	6,8	23,5	5,9	0	0
P++	21,6	41,2	11,8	30,0	5,0
P+	16,2	17,6	17,6	25,0	5,0
Scanty	14,9	11,8	11,8	20,0	15,0
Negative	40,5	5,9	52,9	25,0	75,0
Day 35 (%)	**n = 71**	**n = 17**	**n = 16**	**n = 19**	**n = 19**
P+++	2,8	5,9	0	5,3	0
P++	11,3	35,3	6,3	5,3	0
P+	15,5	23,5	18,8	10,5	10,0
Scanty	12,7	11,8	0	26,3	10,0
Negative	57,7	23,5	75,0	52,6	8,0
Day 56 (%)	**n = 68**	**n = 15**	**n = 15**	**n = 18**	**n = 20**
P+++	0	0	0	0	0
P++	7,1	13,3	13,3	5,6	0
P+	15,7	33,3	13,3	22,2	0
Scanty	17,1	20,0	13,3	5,6	30,0
Negative	60,0	33,3	60,0	66,7	70,0
Day 180 (%)	**n = 62**	**n = 16**	**n = 15**	**n = 13**	**n = 18**
P+++	0	0	0	0	0
P++	0	0	0	0	0
P+	0	0	0	0	0
Scanty	0	0	0	0	0
Negative	100	100,0	100	100	100

% *M. tuberculosis* not detected calculated using 'n' as denominator for each particular time point

Abbreviations: n = number of patients analysed; SD = standard deviation; IQR = interquartile range; DCTB = differentially culturable tubercle bacilli.

APPENDIX 4D: DECLINE IN DCTB IN ATYPICAL PATIENT COHORT

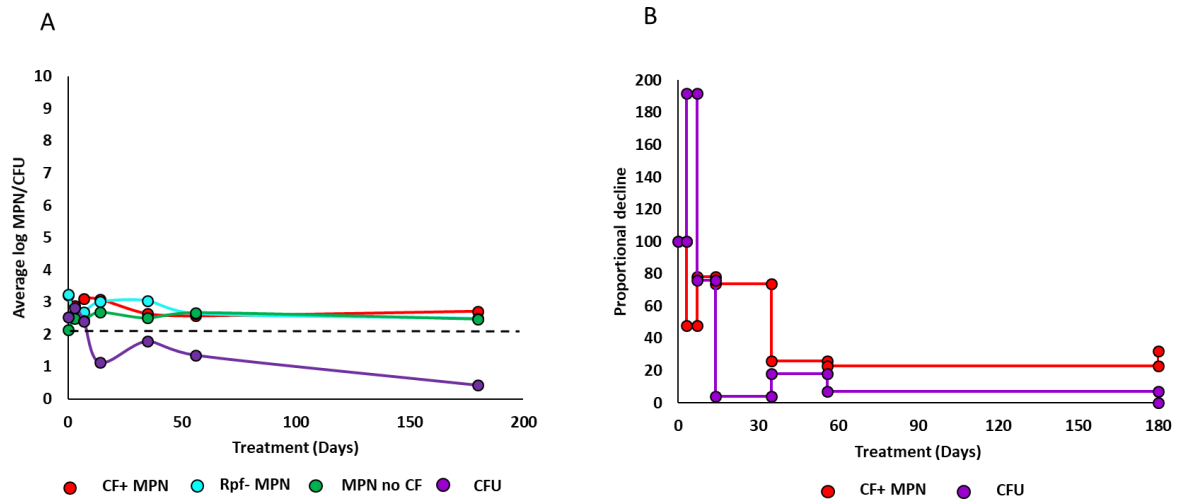

Figure 4A. Decline in differentially culturable and culturable tubercle bacilli in twenty patients exhibiting the atypical DCTB decline pattern during standard first-line tuberculosis treatment. (A) The average number of culture filtrate (CF) -dependent bacteria (red), and resuscitation-promoting factor (Rpf) –independent bacteria (blue) obtained in the CF-supplemented most probable number (MPN) assays were plotted for each selected time point during the six months of anti-TB treatment. In addition the CF-independent bacteria (green) obtained in the un-supplemented MPN assay and the average number of platable bacteria (purple), obtained by viable colony forming units (CFU) on solid media were plotted for each selected time point during the six months of anti-TB treatment. The dotted line indicates possible background scored as positive growth in the MPN assay. (B) The proportional increase in DCTB during the first two weeks of anti-TB treatment was calculated by setting the average bacterial load obtained at enrolment in both the CF-dependent DCTB (red) and platable bacteria (purple) to 100%. The average number of bacteria cultured at each subsequent time point was calculated as a proportion of the average number of organisms obtained at baseline.

APPENDIX 4E: STATISTICAL ANALYSES

Table 4D. Statistical significance between decline sub-categories for age and BMI.

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.2238	**0.0004**	0.5120	0.2550
Accumulate	0.4662		**0.0240**	0.4986	**0.0073**
Resist	**0.0329**	0.0902		**0.0026**	**<0.0001**
Decline	0.1265	0.3146	0.5384		**0.0385**
Atypical	0.4749	0.9924	0.0978	0.3029	

Statistical comparisons between each category were conducted using unpaired T-tests. The top part of the table shows the pairwise comparison between sub-categories according to age (blue) and the bottom part shows the pairwise comparison of each category according to patient BMI (orange). According to age, six comparisons differed significantly. According to BMI, the resist decline and no CFU group differed significantly.

Table 4E. Statistical significance between decline sub-categories for CD4 count and CFU

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.3556	0.5586	0.6301	0.4453
Accumulate	**0.0014**		0.5499	0.3386	0.4841
Resist	**<0.0001**	**<0.0001**		0.7686	0.8766
Decline	**<0.0001**	**0.0154**	**0.0334**		0.6236
Atypical	**0.0225**	**0.0440**	**<0.0001**	**<0.0001**	

Statistical comparisons between each category were conducted using unpaired T-tests (CD4 T-cell count) and Mann-Whitney U tests (CFU). The top part of the table shows the pairwise comparison between sub-categories according to CD4 T-cell count (blue) and the bottom part shows the pairwise comparison of each category according to CFU (orange). While no groups differed according to CD4 T-cell count, all groups differed significantly by CFU.

Table 4F. Statistical significance between decline sub-categories for MGIT and GeneXpert.

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.3339	**0.0010**	**0.0029**	0.8325
Accumulate	0.5152		**0.0036**	**0.0197**	0.1264
Resist	**<0.0001**	**<0.0001**		**0.0103**	**<0.0001**
Decline	**0.0002**	**0.0038**	**0.0152**		**<0.0001**
Atypical	0.7383	0.2804	**<0.0001**	**<0.0001**	

Statistical comparisons between each category were conducted using unpaired T-tests. The top part of the table shows the pairwise comparison between sub-categories according to MGIT (blue) and the bottom part shows the pairwise comparison of each category according to patient GeneXpert (orange). According to MGIT culture, seven out of ten comparisons differed significantly. According to GeneXpert, seven out of ten comparisons differed significantly. The significant and non-significant groups were the same amongst the MGIT and GeneXpert.

Table 4G. Statistical significance between decline sub-categories for CF+ MPN and MPN no CF

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.5084	**<0.0001**	**0.0001**	0.1459
Accumulate	0.9351		**<0.0001**	**<0.0001**	**0.0068**
Resist	**0.0088**	**0.0031**		**0.0013**	**<0.0001**
Decline	0.1714	0.0969	0.0786		**<0.0001**
Atypical	0.1275	0.1448	**<0.0001**	**0.0002**	

Statistical comparisons between each category were conducted using the Mann-Whitney U test. The top part of the table shows the pairwise comparison between sub-categories according to CF+ MPN (blue) and the bottom part shows the pairwise comparison of each category according to patient MPN no CF (orange). Eight and four comparisons were statistically different by CF^+ MPN and MPN no CF assays, respectively.

Table 4H. Statistical significance between decline sub-categories for HIV-1 sero-status and gender

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.2032	0.0659	0.6640	0.2883
Accumulate	0.5765		0.6872	0.2949	0.0612
Resist	**0.0397**	**0.0065**		0.0807	**0.0108**
Decline	0.8424	0.6658	**0.0159**		0.3869
Atypical	0.6515	0.2401	0.0728	0.4405	

Statistical comparisons between each category were conducted using chi-squared test for proportions. The top part of the table shows the pairwise comparison between sub-categories according to HIV-1 serostatus (blue) and the bottom part shows the pairwise comparison of each category according to gender (orange). The atypical and resist groups differed significantly according to HIV sero-status. According to gender, the resist group was significantly different to the no CFU, accumulate and decline groups.

Table 4I. Statistical significance between decline sub-categories for BCG scar and smoking status

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.9789	0.9712	0.4718	0.3652
Accumulate	0.0944		0.2435	0.7317	0.8992
Resist	0.3147	0.4516		0.3828	0.2749
Decline	0.2913	0.4468	0.9851		0.8187
Atypical	0.7097	0.1258	0.4442	0.4123	

Statistical comparisons between each category were conducted using chi-squared test for proportions. The top part of the table shows the pairwise comparison between sub-categories according to the presence or absence of a BCG scar (blue) and the bottom part shows the pairwise comparison of each category according to smoking status (orange). No significant differences were found between any groups.

Table 4J. Statistical significance between decline sub-categories for smear status

	No CFU	Accumulate	Resist	Decline	Atypical
No CFU		0.3869	**0.0022**	**0.0010**	0.5291
Accumulate	0.3869		**0.0155**	**0.0091**	0.0874
Resist	**0.0022**	**0.0155**		NA	**0.0002**
Decline	**0.0010**	**0.0091**	NA		**<0.0001**
Atypical	0.5291	0.0874	**0.0002**	**<0.0001**	

Statistical comparisons between each category were conducted using chi-squared test for proportions. Significant differences were observed comparing six groups. No differences were observed between atypical and no CFU as well as accumulate. NA: not applicable – since one row (no patients were smear negative in resist or decline categories), chi-square analysis could not be calculated.

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Detailed components of this PhD

Background and literature review

CHAPER ONE

TUBERCULOSIS

CHAPTER TWO

MICROBIAL DORMANCY

Original Research Chapters

KEY QUESTIONS?	KEY CONCLUSIONS?
CHAPTER 3 Do all TB patients harbor DCTB? What are the various DCTB populations? Are Rpfs solely responsible for unmasking DCTB? Does host immunity affect the quantum of DCTB?	**CHAPTER 3** Four distinct sub-populations of DCTB observed amongst patients Rpf effect was marginal in unmasking DCTB HIV-negative patients harbored a higher quantum of DCTB at baseline CD4 T-cell count not associated with DCTB
CHAPTER 4 How do DCTB respond to first-line TB treatment compared to conventionally culturable bacteria? Are there potential biomarkers to predict patient response to treatment? Do DCTB remain at the end of treatment?	**CHAPTER 4** Four patterns of decline were described An atypical DCTB presentation was associated with HIV-positive patients DCTB were detected in most patients following treatment completion and DMN-Tre staining confirmed the presence of DCTB
CHAPTER 5 What is the relationship between the MPN assay and currently employed *M. tuberculosis* culture methods? Can the MPN possibly be used in EBA studies? Does CF improve the relationship between the MPN and MGIT TTP in clinical samples?	**CHAPTER FIVE** ?
CHAPTER 6 Does the addition of CF to the MGIT enhance diagnostic pick-up? Can modification of the MGIT by the addition of CF improve the time to culture positivity? Does a reduction in total MGIT volume improve TTP by enhanced quorum sensing?	**CHAPTER SIX** ?

CHAPTER 5

HOW DOES THE MPN RELATE TO OTHER TB CULTURE METHODS?

5.1 INTRODUCTION

New antibiotics are urgently required to shorten the duration of treatment and to treat the increasing number of drug-resistant *M. tuberculosis* strains (Connolly et al., 2007). Methods to monitor response to treatment are important in this regard. The determination of EBA is regarded as the first step in the clinical evaluation of a new TB drug (Donald and Diacon, 2008). Since the introduction of antibiotics, various techniques have been employed to evaluate response to chemotherapy (Mitchison, 1950, Crofton et al., 1958, Joiner et al., 1953). One of the first studies to formally evaluate this was carried out by the Wissenschaftliche Arbeitsgemeinschaft für die Therapie von Lungenkrankheiten by quantifying the decrease of AFB in sputum (WATL, 1969). The investigators compared the effectiveness of the anti-TB drugs, para-aminosalicylic acid (PAS) and thiocarlide, with each other and also with bed rest in 29 patients with pulmonary TB disease. It was found that PAS was the most effective, followed by thiocarlide and lastly bed rest (WATL, 1969). In a separate early study, monotherapy with RIF, PAS and EMB was studied in 23 TB patients previously treated for pulmonary TB disease. In this study, the results indicated that RIF and EMB were equally effective and both were more effective than PAS (Schütz, 1968). Since the early 1980s, EBA studies have been used to monitor the effectiveness of anti-TB drugs, stemming from the ground breaking study conducted by Jindani et al. (1980). In this study, 27 anti-TB drugs and regimens were evaluated in 124 African patients with smear-positive, pulmonary TB disease. This was achieved by enumeration of CFU before treatment and every two days thereafter for two weeks (Jindani et al., 1980). Valuable information was generated from this study as well as a greater understanding of the actions of anti-TB drugs during early treatment. EBA has since been defined as " the fall in counts/mL sputum/day during the first two days of treatment" (Mitchison and Strum, 1997).

EBA studies, involving CFU counts on solid media, are currently used for the direct quantification of *M. tuberculosis* to assess treatment response to a particular drug (Johnson et al., 2006, Donald et al., 2003, Donald and Diacon, 2008, Jindani et al., 1980, Jindani et al., 2003, Sirgel et al., 2000, Sirgel et al., 1993, Sirgel et al., 1997). In addition, EBA also allows for the assessment of the efficacy of a drug with respect to dosage, pharmokinetics and toxicity (Diacon et al., 2012). Solid media, such as Löwenstein–Jensen, has been traditionally used for

the culture of *M. tuberculosis*; however, this culture method is slow requiring up to four weeks for smear-positive samples and up to eight weeks for smear-negative samples (Ryu, 2015). Liquid culture, on the other hand, is significantly faster with a reported average TTP of ten days in smear-positive TB patients (Pfyffer et al., 1997, Kolibab et al., 2014). Furthermore, liquid culture has been shown to be more sensitive and can detect a population of bacteria that cannot be cultured on solid media (Bowness et al., 2014, Mitchison and Coates, 2004, Mukamolova et al., 2010, Chengalroyen et al., 2016, Dhillon et al., 2013). With the development of new anti-TB drugs, methods to replace CFU that offer a faster turnaround and can be performed in basic laboratory set-ups are being investigated. Recently, automated liquid culture systems (including the BACTEC MGIT 960 and BACTEC 460 TB systems) have been suggested as viable alternatives to CFU (Pheiffer et al., 2008, Diacon et al., 2012) . Previous experiments investigating the relationship between TTP and CFU/mL have reported strong negative correlations (Shin et al., 2007, Bark et al., 2013, Diacon et al., 2010b, Kolibab et al., 2014, Bowness et al., 2014, Pheiffer et al., 2008) and certain studies have shown a relationship between MGIT TTP and treatment outcome (Epstein et al., 1998, Wallis et al., 2000).

Over the past decade, a great deal of interest has been directed towards the study of variable populations of *M. tuberculosis* cells in patients with pulmonary TB disease that may exist in altered growth states. As previously discussed, *M. tuberculosis* cells in artificial culture accumulate lipid inclusion bodies as they enter stationary phase (Garton et al., 2002). These LBP cells are also present in *M. tuberculosis* isolated from patient sputum samples (Garton et al., 2002, Garton et al., 2008). Previous work has shown that these cells are unable to form colonies on solid media (but may grow in liquid media) (Dhillon et al., 2013) and require the addition of recombinant Rpf or CF for growth (Mukamolova et al., 2010). Recently, studies conducted at the CBTBR in Braamfontein have used the MPN assay to determine the numbers of conventionally culturable as well as DCTB in clinical samples (Chengalroyen et al., 2016). The relationship between the 48-well MPN assay and currently employed culture methods such as traditional CFU on solid media or more recently used automated liquid systems such as the BACTEC MGIT 960 system has not been described.

5.2 HYPOTHESIS

The MPN assay, in its currently used 48-well format, correlates with other TB culture methods such as CFU assays and the BACTEC MGIT 960, and thus has the potential to be used as an EBA tool to assess the response of both conventionally cuturable and DCTB to anti-TB drugs.

5.3 AIM OF STUDY

To explore the relationship between the MPN assay in the currently described 48-well format with MGIT TTP and CFU assays in both axenic cultures and clinical TB samples throughout first-line treatment.

5.3.1 Specific objectives of study

1. Determine the relationship between the MPN assay with currently employed culture methods, namely the BACTEC MGIT 960 assay as well as CFU on solid 7H11 media in axenic cultures
2. To describe the relationship between the BACTEC MGIT 960 assay and CFU in serial dilutions from *M. tuberculosis* axenic cultures. Is this finding consistent with that obtained in previous studies?
3. Determine whether MGIT TTP can be predicted based on MPN or CFU counts and vice versa?
4. Determine the relationship between CF-supplemented and un-supplemented MPN assays with the TTP obtained in the BACTEC MGIT 960 assay in clinical samples collected and analysed in chapter 4.
5. Determine the relationship between CFU obtained on solid 7H11 agar with the TTP obtained in the BACTEC MGIT 960 assay in clinical samples collected and analysed in chapter 4.

5.4 METHODS

5.4.1 Preparation of axenic *M. tuberculosis* cultures

Duplicate *M. tuberculosis* H37Rv (Johannesburg strain) cultures were grown to log-phase (OD_{600nm} = 0.67) in standard 7H9 broth supplemented with 10% OADC. Ten-fold serial dilutions ranging from 10^3 to 10^{12} were performed by the addition of 200 µL of undiluted stock culture to pre-labelled 2.0 mL Eppendorf tubes containing 180 µL of 7H9 broth.

5.4.2 Colony counts on solid media

The number of viable *M. tuberculosis* cells in each dilution factor was determined using standard plate counting. One hundred microliters from each dilution (10^3 to 10^{12}) was plated out onto two 7H11 agar plates supplemented with OADC. Colony counts were determined after the incubation of agar plates at 37 ºC after 4 weeks.

5.4.3 MPN assays

MPN assays were performed for each dilution factor (10^3 to 10^{12}) as previously described (See section 3.4.3.1, page 94). Briefly, 450 µL of 7H9 media was added to 48-well microtiter plates. Following MPN plate preparation, 50 µL of each dilution factor was subsequently added in triplicate to the first well of the plate after which additional 10-fold dilutions were performed. MPN plates were incubated at 37 °C for 42 days after which the assays were scored and data analysed.

5.4.4 BACTEC MGIT 960 assay

MGIT tubes were inoculated with 800 µL PANTA reconstituted in OADC. The tubes were inverted several times after which 800 µL of media was removed from each tube and replaced with 800 µL of each serial dilution factor. The tubes were incubated at 37 °C in a MGIT 960 instrument and removed once the instrument signalled positive. ZN microscopy was performed for each positive MGIT to confirm the presence of AFB. Furthermore, 100 µL from each tube was plated onto blood agar and incubated for up to 48 hours to confirm the absence of a contaminant. The experiment was performed in duplicate. Due to time constraints, a third biological replicate was not completed and this remains a limitation.

5.4.5 Data analysis

The relationship between MGIT TTP (obtained using the BACTEC MGIT 960 assay), log CFU and log MPN were examined. Previous work has reported both the relationship between log-transformed as well as untransformed TTP with log CFU (Bowness et al., 2014, Perrin et al., 2010). Since a better fit was obtained using untransformed MGIT TTP, the relationship between this data and log MPN and log CFU are reported. Pearson's and Spearman's rank correlation analysis and linear regression analysis were employed to describe the relationship between these assays in axenic work and clinical work.

5.5 RESULTS

5.5.1 The relationship between MPN, MGIT TTP and CFU assays in axenic *M. tuberculosis* cultures

Absolute bacterial counts for MPN and CFU assays were determined using a statistical MPN calculation program (obtained from: http://www.wilrich@wiwiss.fu-berlin.de) and manual colony counting, respectively. Serial dilutions of an axenic cultures, grown to approximately OD_{600nm} = 0.6 to 0.7, were used as starter cultures. TTP was plotted against both log MPN and log CFU which yielded a negative relationship. Linear regression analysis (Figures 5.1 and 5.2) yielded R^2 scores of 0.93 and 0.94 for MPN and CFU assays, respectively. Pearson's and Spearman's rank correlation analysis showed significant negative correlations between MGIT TTP and bacterial burden, Table 5.1 [r_p = -0.97 and for both MPN and CFU assays ($P < 0.0001$ and $P = 0.0003$ for MPN and CFU, respectively); r_s = -1.00 for both MPN and CFU assays ($P < 0.0001$ and $P = 0.0004$ for MPN and CFU, respectively)]. These results illustrate that MGIT TTP decreases with an increase in bacterial load in either the MPN or CFU assay.

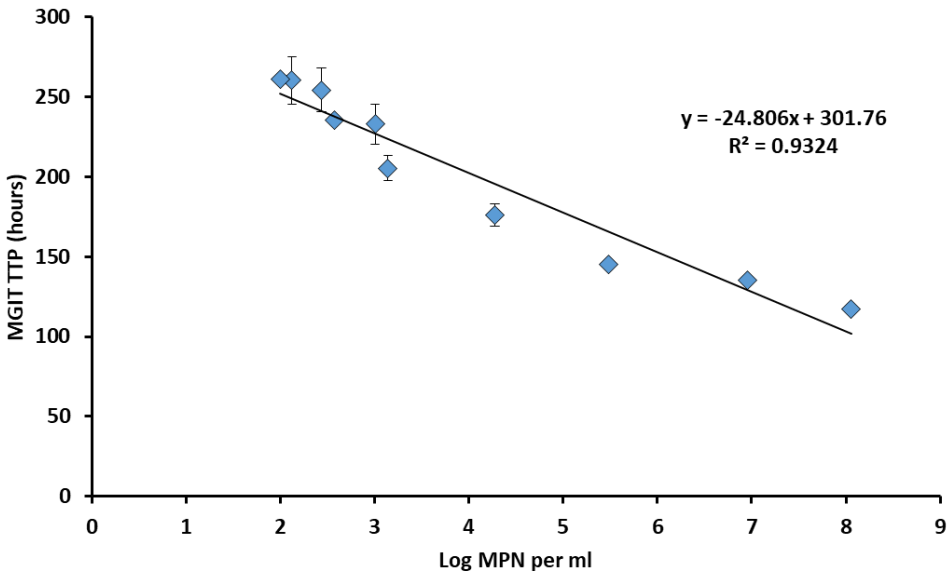

Figure 5.1 Generation of standard curve for *M. tuberculosis* using the BACTEC MGIT 960 assay plotted against most probable number assay. Ten-fold serial dilutions from log-phase H37Rv *Mycobacterium tuberculosis* cultures were inoculated both into the mycobacterial growth indicator tube (MGIT) to yield a time to positivity (TTP) as well as into 48-well micro-titre plates to perform the most probable number (MPN) assay. On the x-axis, the MPN is represented as log- transformed data. On the y-axis, the TTP is represented in hours. Data are represented as mean ± standard deviation.

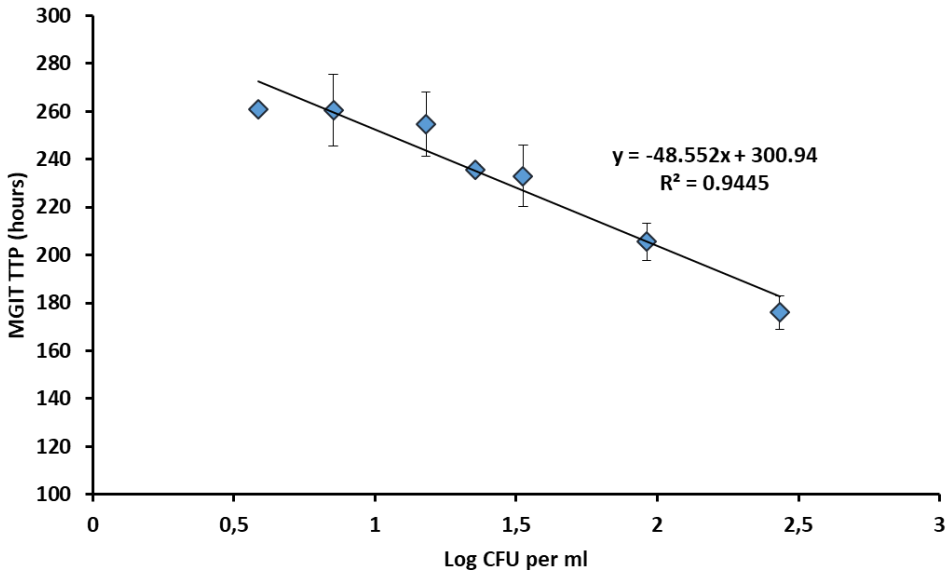

Figure 5.2 Generation of standard curve for *M. tuberculosis* using the BACTEC MGIT 960 assay plotted against CFU assay. Ten-fold serial dilutions from log-phase H37Rv *Mycobacterium tuberculosis* cultures were inoculated both into the mycobacterial growth indicator tube (MGIT) to yield a time to positivity (TTP) as well as into solid 7H11 media to determine the number of colony forming units (CFU). On the x-axis, the CFU is represented as log- transformed data. On the y-axis, the TTP is represented in hours. Data are shown as mean ± standard deviation

Table 5.1 Correlation between MGIT TTP with MPN and CFU assays in axenic cultures

	MPN	CFU
Pearson's correlation		
Correlation value (r_p)	-0.9659	-0.9719
P-value	**<0.0001**	**0.0003**
Spearman's rank correlation		
Correlation value (r_s)	-1.0000	-1.0000
P-value	**<0.0001**	**0.0004**

Abbreviations: MPN = most probable number; CFU = colony forming unit; r_p = Pearson's correlation coefficient; r_s = Spearman's correlation coefficient. P value significant at 0.05 (95% confidence interval), shown in bold.

Next, the relationship between the MPN assay and CFUs was explored. A positive linear relationship was obtained where $R^2 = 0.94$, Figure 5.3. Pearson's and Spearman's rank correlation analysis revealed a significant positive relationship where $r_p = 0.97$ (P = 0.0004) and $r_s = 1.00$ (P = 0.0004). These results indicate that an increase in the number of CFUs directly correlated with an increase in the number of bacteria obtained in the MPN assay.

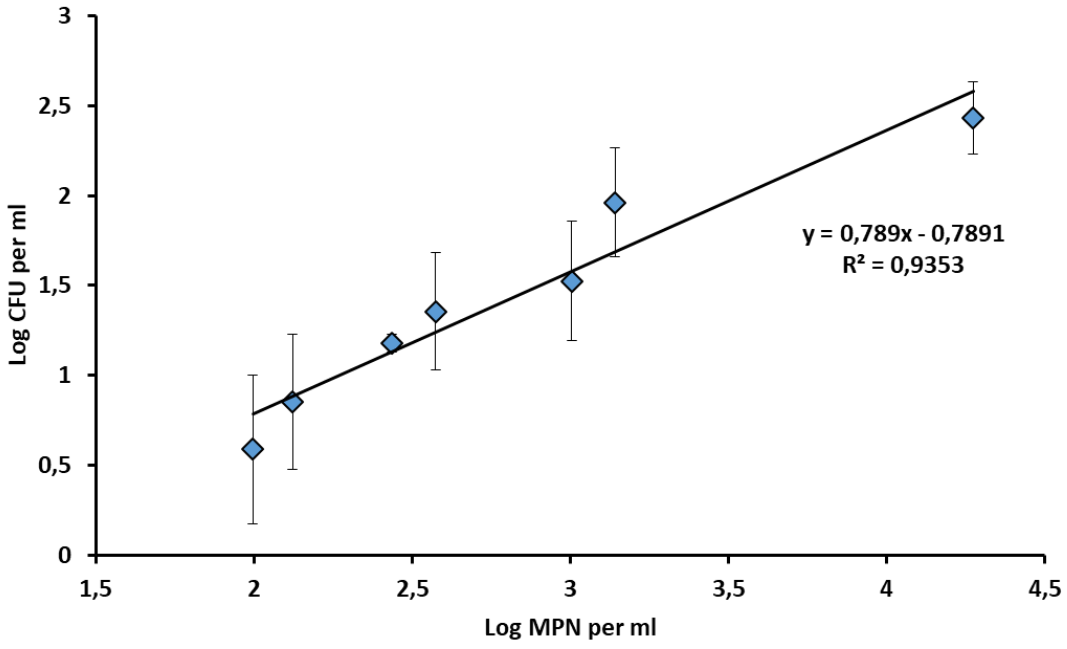

Figure 5.3 Plot of log CFU per mL against log MPN per mL in axenic *M. tuberculosis* cultures. Ten-fold serial dilutions from log-phase H37Rv *Mycobacterium tuberculosis* cultures were inoculated both onto the solid 7H11 media to determine the number of colony forming units (CFU) as well into 48-well micro-titre plates to determine the most probable number (MPN). On the x-axis, the MPN is represented as log- transformed data. On the y-axis, the CFU is represented as log transformed data. Data are represented as mean ± standard deviation.

Table 5.2 shows the average log bacterial counts obtained in the MPN and CFU assays with the corresponding MGIT hours to positivity. Furthermore, estimated TTP's were calculated based on bacterial numbers using the MGIT-MPN or MGIT-CFU correlation equations, Table 5.3.

Table 5.2 Log bacterial counts (MPN and CFU) and MGIT TTP (hours) obtained for each *M. tuberculosis* 10-fold serial dilution factor

Serial dilution factor	Mean log MPN (SD)	Mean log CFU (SD)	Mean MGIT TTP, hours (SD)
10^3	7.61 (1.03)	TNTC	117.5 (0.7)
10^4	6.82 (0.50)	TNTC	135.5 (2.12)
10^5	5.30 (0.60)	TNTC	145.5 (0.7)
10^6	4.20 (0.38)	2.43 (0.20)	176 (7.07)
10^7	3.02 (0.49)	1.96 (0.30)	205.5 (7.78)
10^8	2.80 (0.65)	1.52 (0.33)	233 (12.73)
10^9	2.56 (0.14)	1.36 (0.32)	235.5 (2.12)
10^{10}	2.3 (0.52)	1.18 (0.05)	254.5 (13.43)
10^{11}	2.09 (0.23)	0.85 (0.37)	260.5 (14.85)
10^{12}	1.76 (0.71)	0.59 (0.41)	261 (ND)

Abbreviations: MPN = most probable number; CFU = colony forming units; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation; TNTC = too numerous to count; ND =not determined.

Table 5.3 Predicted MGIT TTP based on MPN or CFU counts

Log MPN	Log CFU	Predicted MGIT TTP (days)
0.5	0	>12.06
1	0	11.54
2	0.79	10.51
3	1.58	9.47
4	2.37	8.44
5	3.16	7.41
6	3.94	6.37
7	4.73	5.34
8	5.52	4.30
9	6.31	3.27

Abbreviations: MPN = most probable number; CFU = colony forming units; MGIT = mycobacterial growth indicator tube; TTP= time to positivity.

Results from serial dilutions performed on axenic *M. tuberculosis* cultures indicated a strong linear relationship between all three culture assays, i.e. MGIT TTP, MPN and CFU assays. These results illustrate that the MPN assay performed on NUNC-plates in the 48-well format provides a highly sensitive method to determine the number of tubercle bacilli in axenic culture. Furthermore, the generation of standard curves from the analysis allowed for the prediction of bacterial load in the MGIT culture based on the TTP, or vice versa. In addition, the number of CFUs could be determined based on the MPN or vice versa. It is important to note that the numbers of bacteria obtained in the MPN assay were higher than those obtained in the CFU assay at all dilution factors, providing evidence for a population of bacteria in axenic culture that can only be grown in liquid media. Indeed, in the prediction analysis (Table 5.3), no CFUs can be cultured when the MPN is log 1 or less (i.e. *M. tuberculosis* growth is present in approximately one or two MPN wells but not on solid media). Furthermore, in samples where the MPN is less than log 1 (i.e. 10 organisms or less), the predicted MGIT TTP is greater than 11 days. Therefore the TTP in low MPN samples can vary anywhere between 11 and 41 days. Since it is difficult to accurately count single colonies in samples where bacillary load is high (and multiple serial dilutions and plating are required to determine a CFU), the MPN or MGIT TTP may be a more attractive method for quantifying bacteria in axenic samples.

5.5.2 The relationship between *M. tuberculosis* MGIT TTP with CFU and MPN assays in sputum samples

The relationship between MGIT TTP and MPN as well as CFU assays in patient sputum samples was investigated. For this, the relationship from the MGIT TTP for sputum samples received from each patient sub-category identified in chapter 4 (i.e. resist decline, accumulate, decline and atypical presentation of DCTB) was plotted against the equivalent MPN and CFU counts for all treatment time points, Table 5.4. The overall relationship between MGIT TTP and MPN/CFU for all patient samples (i.e. n = 75) is also reported. Spearman's rank and Pearson's correlation analysis was performed. Generally, Spearman's rank correlation analysis yielded slightly better r values (with the exception of the atypical group); however, the r values were very similar in both tests and the directions were the same, Table 5.4. For this reason, the correlation values from the Spearman's test are reported in the text. In the overall patient analysis, 485 matched MGIT-MPN (CF^+ and no CF) pairs were available for analysis. In addition 484 and 471 MGIT-MPN (Rpf-) and MGIT-CFU matched pairs were available for analysis, respectively. A significant negative correlation was obtained between MGIT TTP and

MPN assays in all 75 longitudinal patient samples, where r_s = -0.61, -0.60 and -0.35 for CF^+, Rpf^- and MPN no CF assays, respectively. A significant negative relationship was also obtained between MGIT TTP and CFU, where r_s = -0.55 (P < 0.0001). For linear regression analysis, the R^2 values were 0.33 (for both CF^+ and Rpf^- MPN assays), 0.15 for MPN no CF and 0.28 for CFU, see Figure 5A in the Appendices (page 233). The correlation results from the different sub-categories are discussed in separate sections below.

Table 5.4 Correlation between MGIT TTP with MPN and CFU assays in sputum samples

Category	Correlation value (r_p or r_s)	CF^+	P-value	Rpf^-	P-value	No CF	P-value	CFU	P-value
Resist decline	r_p	-0.70	**<0.0001**	-0.68	**<0.0001**	-0.44	**<0.0001**	-0.68	**<0.0001**
	r_s	-0.72	**<0.0001**	-0.69	**<0.0001**	-0.48	**<0.0001**	-0.68	**<0.0001**
Accumulate	r_p	-0.60	**<0.0001**	-0.57	**<0.0001**	-0.33	**0.0004**	-0.45	**<0.0001**
	r_s	-0.65	**<0.0001**	-0.60	**<0.0001**	-0.35	**0.0002**	-0.48	**<0.0001**
Decline	r_p	-0.62	**<0.0001**	-0.62	**<0.0001**	-0.38	**<0.0001**	-0.55	**<0.0001**
	r_s	-0.72	**<0.0001**	-0.70	**<0.0001**	-0.42	**<0.0001**	-0.61	**<0.0001**
Atypical DCTB presentation	r_p	-0.12	0.1768	-0.14	0.1020	-0.01	0.9079	-0.22	**0.0117**
	r_s	-0.08	0.3633	-0.09	0.3148	-0.02	0.8479	-0.17	0.0631
Overall	r_p	-0.58	**<0.0001**	-0.57	**<0.0001**	-0.34	**<0.0001**	-0.53	**<0.0001**
	r_s	-0.61	**<0.0001**	-0.60	**<0.0001**	-0.35	**<0.0001**	-0.55	**<0.0001**

Abbreviations: CF^+ = culture filtrate derived from *M. tuberculosis* H37Rv; Rpf^- = resuscitation-promoting factor deficient CF derived from quintuple *rpf* gene-knockout mutant; CFU = colony forming units; r_p = Pearson's correlation coefficient; r_s = Spearman's correlation coefficient. Significant at P less than 0.05 (95% confidant interval) shown in bold.

5.5.2.1 The relationship between MGIT TTP and MPN/CFU in the resist decline cohort

Log MPN and log CFU were correlated with MGIT TTP across all time points for patients in the resist decline sub-category. One hundred and thirteen matched log CFU and TTP pairs and 112 matched log MPN (CF^+, Rpf^- and no CF) and TTP pairs were available for analysis. Spearman's rank sum correlation analysis revealed a significant negative relationship between bacterial loads as detected by CF-supplemented and un-supplemented MPN assays as well as CFU assays when compared to MGIT TTP. The r_s values were -0.72, -0.69 and -0.48 for CF^+, Rpf^- and MPN no CF MPN assays, respectively ($P < 0.0001$). The r_s value obtained for the log CFU and TTP correlation was -0.68 ($P < 0.0001$). Linear regression analysis was also performed, Figure 5.4. The R^2 values were 0.50, 0.46 and 0.20 for the CF^+, Rpf- and no CF MPN assays, respectively. These results indicate that the MGIT correlates better with MPN assays that are supplemented with CF. It was hypothesized that the improved correlation could be due to the revival of organisms in the MPN assay that cannot be cultured using standard media. The association between DCTB and TTP was investigated in chapter 6 by the addition of CF to the MGIT. The results indicated that in the MGIT assay, CF did not stimulate the growth DCTB, but rather accelerated the growth of few viable organisms. However, this may not be the case in the MPN assay. The R^2 value obtained on solid media was 0.47, similar to that obtained in the CF^+ and Rpf^- MPN assay, suggesting that MGIT TTP can be predicted based on the number on CFUs obtained, or vice versa.

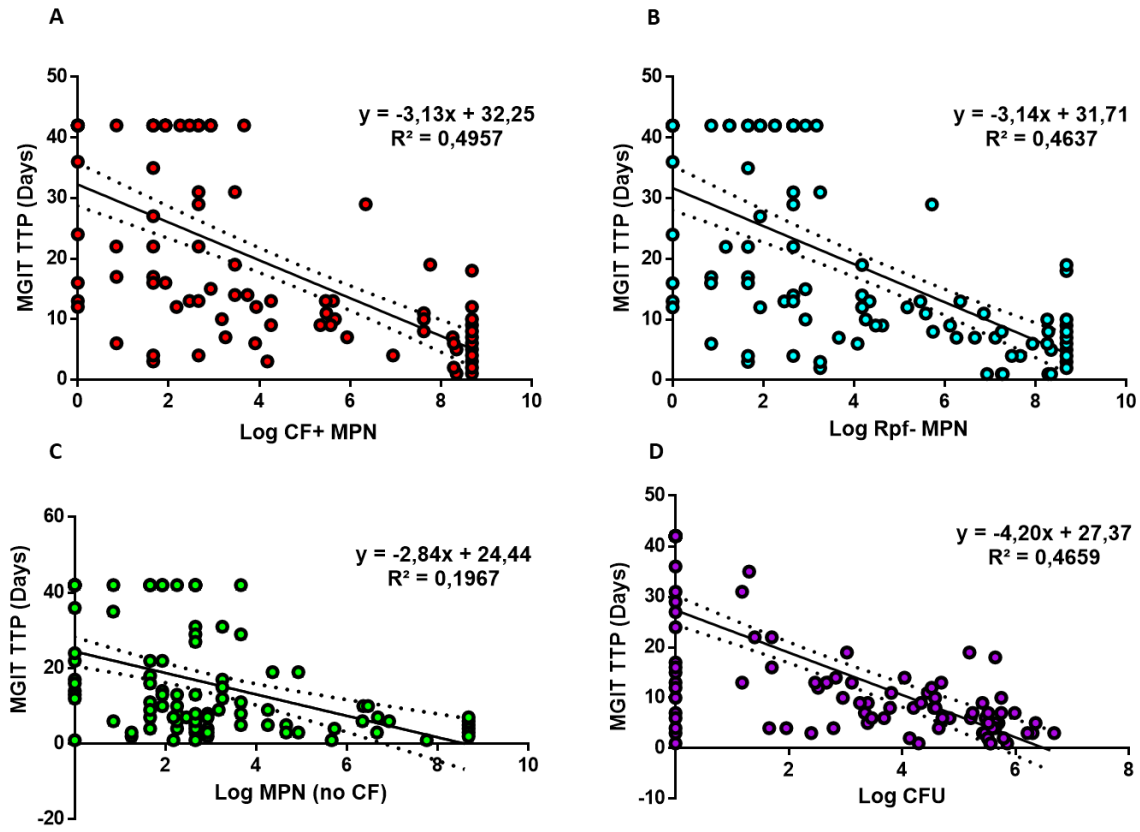

Figure 5.4 The linear relationship between the number of bacteria obtained in most probable number and colony forming unit assays with mycobacterial growth indicator tube time to positivity in patients from the resist decline cohort. The number of bacteria obtained by most probable number (MPN) or colony forming unit (CFU) assays was correlated with the mycobacterial growth indicator tube (MGIT) time to positivity (TTP) obtained for 17 patients in the resist decline DCTB cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the MPN/CFU is represented as log- transformed data. On the y-axis, the MGIT TTP is represented as the number of days to culture TTP. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green) and (D) CFU assays (purple).

5.5.2.2 The relationship between MGIT TTP and MPN/CFU in the accumulate cohort

For the accumulate DCTB patient sub-category, one hundred and ten matched log CFU and TTP pairs as well as log MPN (CF^+, Rpf^- and no CF) and TTP pairs were available for analysis. Similar to the resist decline DCTB group, Spearman's rank sum correlation analysis showed a significant negative correlation between MGIT TTP and the quantity of bacterial organisms detected using MPN and CFU assays. When correlated with MGIT TTP, the r_s values were -0.65, -0.60, -0.35 and -0.48 for the CF^+ MPN, Rpf^- MPN, MPN no CF and CFU assays, respectively ($P < 0.0001$ for CF^+, Rpf^- and CFU; $P = 0.0002$ for MPN no CF). In addition, linear regression analysis was carried out. The respective R^2 values were 0.37, 0.33, 0.11 and 0.20, illustrating poor linear relationships, Figure 5B (see page 234 in the Appendices).

5.5.2.3 The relationship between MGIT TTP and MPN/CFU in the decline cohort

Log MPN and log CFU were correlated with MGIT TTP across all time points for patients in the decline sub-category. One hundred and twenty nine matched log MPN Rpf^- and 128 matched log MPN (CF^+ and no CF) pairs were available for analysis. For log CFU versus MGIT TTP, 122 pairs were available for analysis. In this cohort, Spearman's rank sum correlation analysis revealed a significant negative relationship between MGIT TTP and bacterial loads obtained by all three MPN (i.e. CF^+, Rpf^-, no CF) and CFU assays with the following scores: r_s = -0.72, -0.70, -0.42 and -0.61, respectively ($P < 0.0001$). Linear regression analysis was also performed, Figure 5C (see page 235 in the Appendices). The R^2 values obtained were 0.39, 0.38, 0.14 and 0.30 for MPN (CF^+, Rpf^-, no CF) and CFU assays, respectively.

5.5.2.4 The relationship between MGIT TTP and MPN/CFU in the atypical DCTB presentation cohort

Log MPN and log CFU were correlated with MGIT TTP across all time points for the 21 patients in the atypical sub-category. One hundred and thirty five matched log MPN (CF^+ and no CF) and MGIT TTP pairs were available for analysis. One hundred and thirty three log MPN Rpf^- and MGIT TTP pairs were available for analysis. For log CFU and MGIT TTP, 126 pairs were available for analysis. The atypical cohort displayed no correlation between the MPN and the MGIT. The Spearman's rank scores were: r_s = -0.07, -0.09 and -0.02 for the CF^+, Rpf $^-$ and no CF MPN assays ($P = 0.3633$, 0.3148 and 0.8479), Table 5.4. In addition, no correlation was observed between the number of CFUs and MGIT TTP (r_s = -0.17; $P = 0.0631$). Linear regression analysis was also performed and is shown in Figure 5.5.

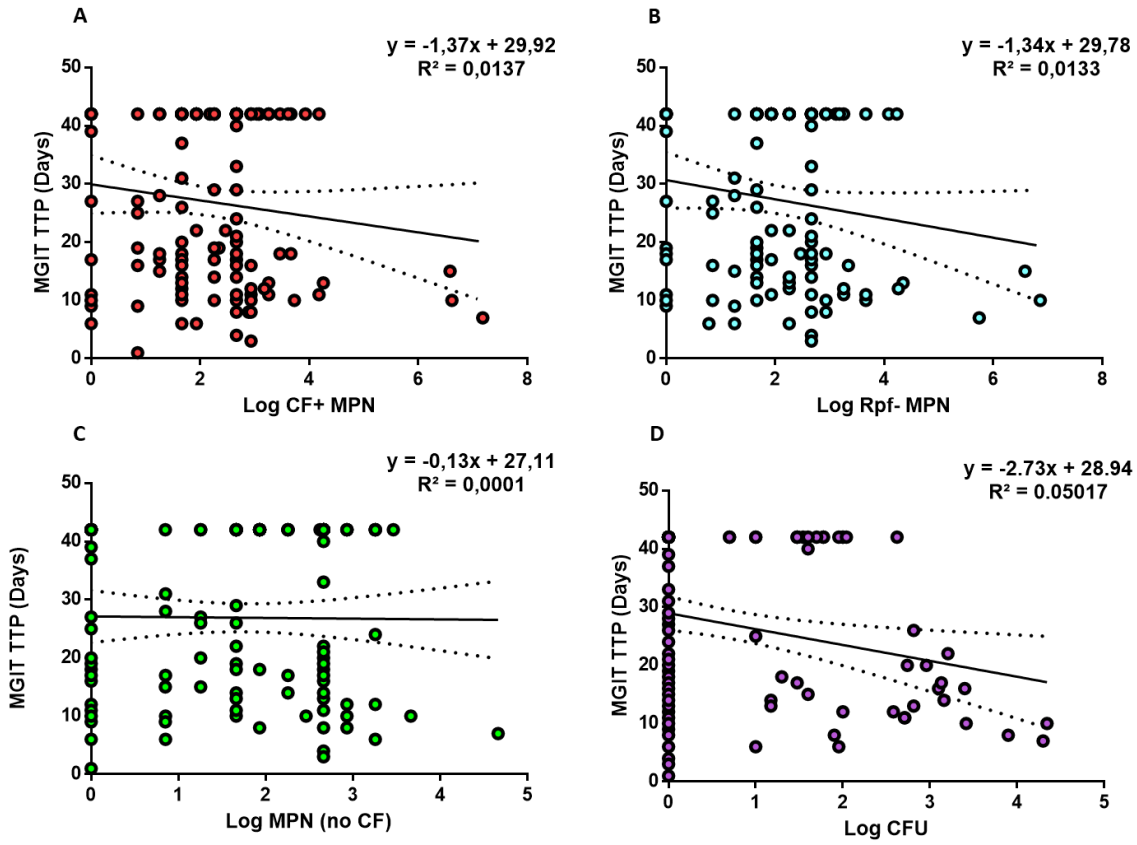

Figure 5.5. Correlation between the number of bacteria obtained in most probable number and colony forming unit assays with mycobacterial growth indicator tube time to positivity in patients from the atypical cohort. The number of bacteria obtained by most probable number (MPN) or colony forming unit (CFU) assays was correlated with the mycobacterial growth indicator tube (MGIT) time to positivity (TTP) obtained for 21 patients in atypical DCTB cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the MPN/CFU is represented as log- transformed data. On the y-axis, the MGIT TTP is represented as the number of days to culture TTP. (A) CF+ MPN assay (red), (B) Rpf- MPN assay (blue), (C) MPN no CF (green) and (D) CFU assays (purple).

The poor correlation observed in the atypical cohort can be due to numerous factors. Firstly, as the MPN is based on a visual scoring system, the growth recorded in the MPN may have been due to a contaminant that may appear similar in morphology to *M. tuberculosis*. Indeed, in chapter 6, a non-specific resuscitative effect was observed following the addition of CF to the MGIT (see chapter 6, section 6.5.2.1.1, page 262). As the poorest correlation was observed between the MGIT and the CF-supplemented MPN assay in this cohort, the MPN may have been contaminated in some patients that would have led to an over estimation of bacterial load in the MPN assay at a particular time point. Indeed, in 51 samples a positive MPN was obtained when the MGIT sample was culture negative at 42 days. The overall average MPN positivity in these samples was low at log 2.36 (IQR: 1.66 – 2.80). It is plausible that some of these wells may have been false positives due to drug precipitation, cellular debris or bacterial

contamination. The number of positive MPN wells with respective negative MGIT cultures was higher at later time points, suggesting that false negative MPN results are more likely to occur during late treatment, Table 5.5. In contrast, seven MGITs were positive when the CF^+ MPN assays were negative (average TTP 17.0 days, IQR: 9.5 – 22.0). The lack of correlation is possible also due to the low bacterial load in this patient cohort throughout treatment. The axenic work illustrated that a MPN of log 0.5 should yield a MGIT TTP of >12.06 days; therefore, in a positive MPN plate with only one of two positive wells, the TTP can vary anywhere between 12 and 41 days. Therefore, the range of possible values in MGIT TTP is much larger than observed in the MPN assay at low bacterial loads. It is not surprising that the atypical category displays no correlation between MPN and CFU as this group of patients was characterised by low MPN (and high MGIT TTP) values throughout treatment.

Table 5.5 MPN positive/MGIT negative sample pairs in the atypical patient cohort at treatment time-points.

Time point	No. samples MPN positive/ MGIT negative (%)
0	4 (7.8)
3	5 (9.8)
7	1 (2.0)
14	8 (15.7)
35	10 (19.6)
56	11 (21.6)
180	12 (23.5)

Abbreviations: MPN = most probable number; MGIT = mycobacterial growth indicator tube

5.5.2.5 The relationship between MGIT TTP and MPN/CFU throughout treatment

In line with the observations made in section 5.2.2.4 for the 'atypical' cohort, previous work investigating the relationship between MGIT TTP and CFU showed a decrease in the strength of the association throughout therapy (Bark et al., 2011). The decrease in correlation shows that CFU is more strongly predictive of MGIT TTP at high bacterial loads. The same appears to be true for the MPN assay. In this analysis, Spearman's rank sum correlation of MGIT TTP with MPN and CFU (conducted on 75 patients from all four sub-categories) at selected time points also showed a decrease in correlation throughout treatment, Table 5.6 and Table 5.7. A significant correlation was observed up to 14 days of anti-TB treatment. At later time points, the correlation between MGIT TTP and MPN/CFU was no longer significant. This suggests

that current measures of bacterial load are unable to detect some organisms as treatment progresses.

Table 5.6 Correlation between MGIT TTP and the CF^+ MPN at treatment time-points

Culture day	No of paired samples	Correlation	P-value
0	75	-0.8088	**<0.0001**
3	75	-0.5909	**<0.0001**
7	72	-0.5603	**<0.0001**
14	74	-0.3118	**0.0068**
35	69	-0.1307	0.2844
56	66	-0.03926	0.7543
180	54	0.2156	0.1174

Table 5.7 Correlation between MGIT TTP and CFU at treatment time-points

Culture day	No of paired samples	Correlation	P-value
0	73	-0.6805	**<0.0001**
3	71	-0.5061	**<0.0001**
7	70	-0.2743	**0.0216**
14	72	-0.3471	**0.0278**
35	66	-0.1862	0.1344
56	66	0.1178	0.3463
180	53	0.03395	0.8093

5.5.3 The relationship between CFU and MPN assays in sputum samples

Next the relationship between CFU and MPN was investigated. For this, the relationship from the MPN for sputum samples received from each patient sub-category identified in chapter 4 (i.e. resist decline, accumulate, decline and atypical presentation of DCTB) was plotted against the equivalent CFU counts for all time points, Table 5.8. The overall relationship between MPN and CFU for all patient samples (i.e. n = 75) is also reported. In contrast to the MGIT versus MPN/CFU correlation analysis, Pearson's correlation analysis yielded slightly better r values. The r values were very similar in both tests and the directions were the same, Table 5.8. For consistency, correlation values from the Spearman's test are reported in the text. Overall, a significant positive correlation was obtained between the MPN and CFU assays, where r_s = 0.64, 0.60 and 0.37 for CF^+, Rpf^- and MPN no CF assays, respectively ($P < 0.0001$). For linear regression analysis, the R^2 values were 0.54, 0.50 and 0.26, Figure 5D (page 235 in the

Appendices). The results from the different sub-categories are discussed in separate sections below.

Table 5.8 Correlation between MPN and CFU assays in sputum samples

Category	Correlation coefficient	CF^+	P-value	Rpf^-	P-value	No CF	P-value
Resist decline	r_p	0.8052	**<0.0001**	0.7807	**<0.0001**	0.6158	**<0.0001**
	r_s	0.8028	**<0.0001**	0.7406	**<0.0001**	0.5712	**<0.0001**
Accumulate	r_p	0.6744	**<0.0001**	0.6231	**<0.0001**	0.4423	**<0.0001**
	r_s	0.6027	**<0.0001**	0.5421	**<0.0001**	0.3067	**0.0010**
Decline	r_p	0.6890	**<0.0001**	0.6673	**<0.0001**	0.4119	**<0.0001**
	r_s	0.6527	**<0.0001**	0.5908	**<0.0001**	0.2946	**0.0009**
Atypical	r_p	0.3439	**<0.0001**	0.3722	**<0.0001**	0.2616	**0.0030**
	r_s	0.2312	**0.0089**	0.2516	**0.0047**	0.2188	**0.0134**
Overall (n = 75)	r_p	0.7324	**<0.0001**	0.7106	**<0.0001**	0.5098	**<0.0001**
	r_s	0.6372	**<0.0001**	0.6002	**<0.0001**	0.3705	**<0.0001**

Abbreviations: CF^+ = culture filtrate derived from *M. tuberculosis* H37Rv; Rpf^- = resuscitation-promoting factor deficient CF derived from quintuple *rpf* gene-knockout mutant; CFU = colony forming units; r_p = Pearson's correlation coefficient; r_s = Spearman's correlation coefficient. Significant at P less than 0.05 (95% confidant interval) shown in bold.

5.5.3.1 The relationship between MPN and CFU in the resist decline cohort

One hundred and fourteen MPN and CFU pairs were available for analysis. Spearman's rank sum correlation revealed a significant positive relationship between bacterial loads detected by CFU and MPN assays. The respective r_s values were: 0.80, 0.74 and 0.57 for CF^+, Rpf^- and MPN no CF assays, respectively ($P < 0.0001$). Linear regression analysis was also preformed, Figure 5.6. The R^2 values were 0.65, 0.61 and 0.38 for CF^+, Rpf^- and MPN no CF, respectively.

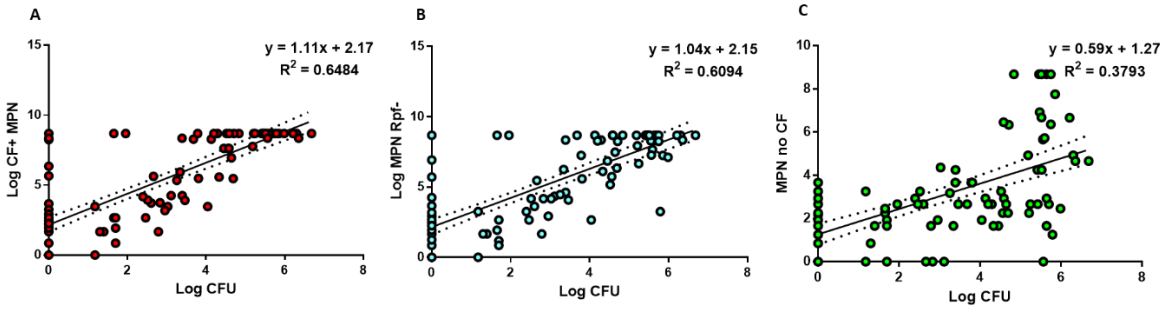

Figure 5.6 Correlation between the number of bacteria obtained in colony forming unit assays with most probable number assays in patients from the resist decline cohort. The number of bacteria obtained by colony forming unit (CFU) assays was correlated with the number of bacteria obtained from most probable number (MPN) assays for 17 patients in the resist decline cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the CFU is represented as log-transformed data. On the y-axis, the MPN is represented as log-transformed data. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green).

5.5.3.2 The relationship between MPN and CFU in the accumulate cohort

The association between MPN and CFU was investigated for the accumulate cohort. One hundred and thirteen CF^+/MPN no CF and CFU pairs were available for analysis. For Rpf^- and CFU, 112 pairs were available. A strong positive correlation was observed between the CFU and MPN assays in this patients cohort where $r_s = 0.60$, 0.54 and 0.31 ($P < 0.0001$ for CF^+ and Rpf^- and $P = 0.0010$ for MPN no CF), Table 5.8. Linear regression analysis was also performed on all samples throughout treatment, Figure 5E (see page 236 in the Appendices). The R^2 values were 0.45, 0.39 and 0.20, for CF^+, Rpf^- and MPN no CF, respectively.

5.5.3.3 The relationship between MPN and CFU in the decline cohort

One hundred and twenty four MPN and CFU pairs were available for analysis. A positive significant relationship was observed between CFU and MPN assays where $r_s = 0.66$, 0.59 and 0.29 for CF^+, Rpf^- and MPN no CF assays throughout treatment ($P < 0.0001$ for CF+ and Rpf- and $P = 0.0009$ for MPN no CF). Linear regression analysis was carried out where $R^2 = 0.47$, 0.45 and 0.17 for CF^+, Rpf^- and no CF MPN assays, Figure 5F (see page 236 in the Appendices).

5.5.3.4 The relationship between MPN and CFU in the atypical cohort

Spearman's rank sum correlation analysis was employed to determine the relationship between CFU and MPN in the atypical patient cohort. In contrast to that observed between the MGIT and MPN assay (where no association was apparent), a significant positive correlation was still observed in this cohort. The scores were r_s = 0.23, 0.25 and 0.22 (P = 0.0089, 0.0047 and 0.0134) for CFU correlated with the CF^+, Rpf $^-$ and no CF MPNs, respectively. The linear relationship between CFU and MPN assays for the atypical cohort is shown in Figure 5.7. These results illustrate that the MPN can more accurately predict CFU (and vice versa) at low bacterial loads than the MGIT. These results also suggest that the MPN assay (supplemented with CF) may be a more favourable assay for future EBA studies than the MGIT for patient samples with low bacterial burden. Indeed, correlation analysis performed at each time point (for all patients, i.e. sub-categories combined), showed a positive correlation up to 56 days on treatment, Table 5.9.

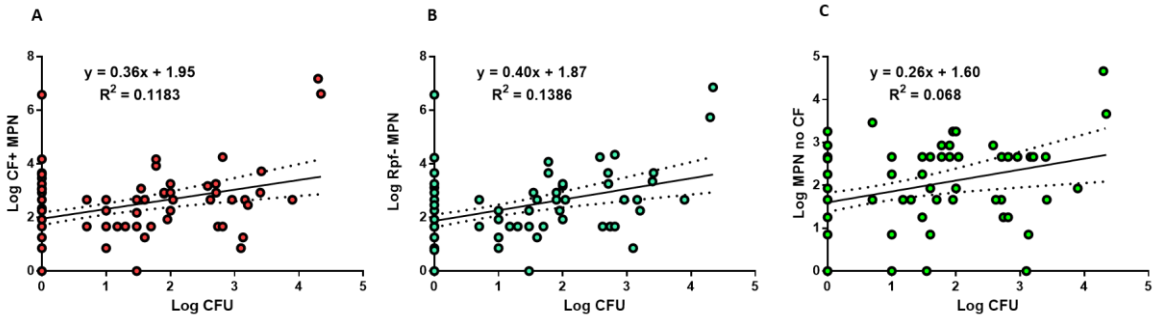

Figure 5.7 Correlation between the number of bacteria obtained in colony forming unit assays with most probable number assays in patients from the atypical cohort. The number of bacteria obtained by colony forming unit (CFU) assays was correlated with the number of bacteria obtained from most probable number (MPN) assays for 21 patients in the atypical cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the CFU is represented as log-transformed data. On the y-axis, the MPN is represented as log-transformed data. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green).

5.5.3.5 Correlation analysis of MPN with CFU throughout treatment

Previous observations showed that the relationship between MGIT TTP and bacterial load (measured by either MPN or CFU assays) was only significant during the first two weeks of TB treatment. To determine whether the same trend was true for MPN and CFU, Spearman's correlation analysis was carried out on all 75 patients at individual time points, Table 5.9. The analysis showed a significant relationship between the CF^+ MPN and CFU up until 56 days of treatment.

Table 5.9 Correlation between MPN and CFU at treatment time-points

Culture day	No of paired samples	Correlation	P-value
0	73	0.7308	**<0.0001**
3	71	0.6303	**<0.0001**
7	70	0.5673	**<0.0001**
14	73	0.5358	**<0.0001**
35	69	0.3315	**0.0054**
56	65	0.2502	**0.0444**
180	53	0.1417	0.2930

5.6. DISCUSSION

Since the introduction of anti-TB chemotherapeutics more than 70 years ago, the enumeration of *M. tuberculosis* from patient sputa has been used to evaluate the response to treatment against numerous anti-TB agents. Traditionally, this quantification was done by measuring the decline in AFB by smear microscopy or by EBA assays measuring the fall in viable CFU in patient sputum samples during early treatment (Donald et al., 2003, Jindani et al., 1980, Donald and Diacon, 2008, Jindani et al., 2003). While EBA studies are able to show that a potential new drug under development has a detectable anti-TB effect, this method is limited in its approach as it is unable to detect the response to treatment of DCTB that are unable to grow on solid media and require special culture conditions. The development of an assay to detect the killing of these non-replicating tubercle bacilli would allow for the assessment of the sterilizing effect of new agents and significantly improve the value of EBA studies. Recently, attempts to replace CFUs with liquid-based assays such as the MGIT have been carried out with the aim of enhancing sensitivity and obtaining more rapid results (Diacon et al., 2010a, Bowness et al., 2014, Bark et al., 2011, Bark et al., 2013). These studies have demonstrated a direct correlation between MGIT TTP and CFU; however, the estimation of bacterial quantities in MGIT cultures based on the MPN assay is undetermined. Methods of bacterial quantification using 48-well micro-titre plates have been described in the literature for the culture and to estimate the MPN of tubercle bacilli in sputum. The MPN assay in this 48-well format resembles that of the MGIT in that it is liquid based; however, it is miniaturized, carrying less than a tenth of the total volume of the MGIT. In this chapter, this unique format of the MPN assay was correlated with both CFU and MGIT TTP in axenic *M. tuberculosis* H37Rv cultures as well as in clinical samples taken throughout first-line treatment.

In axenic cultures, linear regression analysis revealed that the MPN (no CF) correlated highly with both CFU and MGIT (R^2 = 0.93 and 0.94, respectively). These results indicate that the number of bacteria obtained using the MPN assay can strongly predict MGIT TTP or vice versa. The analysis also revealed that the MPN is more sensitive than the CFU as it was possible to quantitate less than 1 log of bacterial organisms which the CFU could not quantitate. This is in agreement with previous reports that liquid culture is more sensitive than solid culture (Chien et al., 2000, Chihota et al., 2010, Srisuwanvilai et al., 2008, Dhillon et al., 2013, Mukamolova et al., 2010, Chengalroyen et al., 2016).

Following the results obtained in axenic *M. tuberculosis* cultures, retrospective analysis was carried out on longitudinal data obtained from 75 patients that were recruited for the study described in chapter 4. MPN (with and without CF-supplementation) and CFU data were correlated with MGIT TTP throughout first-line treatment. In addition, MPN was correlated with CFU. Spearman's rank sum correlation analysis was performed on the four DCTB decline sub-categories described in chapter 4 (i.e. resist, accumulate, decline and atypical). In three of these groups, namely the resist, accumulate and decline groups, a significant negative correlation was obtained between the CF-supplemented MPN assays and MGIT TTP. These correlations were similar, or slightly stronger than that observed between the CFU and MGIT. As previously described, the results also revealed a strong negative correlation between CFU and MGIT TTP. This negative correlation describes a relationship where an increase in bacterial burden (i.e. obtained in either MPN or CFU assays) is associated with a decrease in MGIT TTP. It is interesting to note that a stronger correlation was observed between the MGIT and the CF-supplemented assays compared to the MPN no CF assays. This is possibly due to the resuscitation of non-culturable/damaged organisms present in sputum (Mukamolova et al., 2010, Chengalroyen et al., 2016). Alternatively, the addition of CF may be able to rescue the growth of bacterial populations that are affected by cell-associated inhibitory activity in sputum. Indeed, previous reports have shown that the addition of Rpf-containing supernatant was able to overcome the growth inhibition observed in liquid media (i.e. MPN no CF) (Mukamolova et al., 2010).

In the atypical cohort, no correlation was observed MGIT TTP and MPN/CFU when all paired samples were analysed. This is most likely due to the low bacillary load observed in these patients throughout treatment. To further investigate the relationship between MGIT TTP and MPN/CFU with regards to bacillary load, correlation analysis was carried out at specified treatment time points for all 75 patients. The results showed that the association was only

significant during the first two weeks of anti-TB treatment when sputum bacillary load is at its highest. In a previous study, the relationship between MGIT TTP and CFU was assessed in 107 TB patients at selected time points during the first two months of anti-TB treatment (i.e. days 0, 2, 4, 7, 14, 21, 28 and 60). The investigators reported that the association was strongest during early treatment, although the association was still significant at all time points assessed (Bark et al., 2011). The patients in this study had baseline sputum smear grades of 3+ and 4+ (Strong and Kubica, 1981), the median baseline CFU was log 6.2 and median TTP was 3.0 days (IQR:2.0 to 4.0) (Bark et al., 2011, Johnson et al., 2003). In contrast, the smear grades in the present study varied from smear negative to 4+, the median baseline CFU was log 3.8 (IQR:1.2 to 5.4) and the median TTP was 6.5 days (IQR: 4.0 to 13.0). The high baseline bacterial loads in patients from the Bark et al. (2011) study may explain why correlation was significant throughout treatment, whereas in the present study was only significant up to day 14. Indeed, Bark et al. (2011) reported that MGIT baseline TTP was predictive of sputum culture conversion at one to two months and that patients with significantly longer median baseline TTP were more likely to have negative cultures after one to two months (Bark et al., 2011). The results from the present study suggest that the MGIT is least variable during early treatment. Furthermore, the MPN cannot accurately predict MGIT TTP (or vice versa) at low bacterial loads.

When CFU (the current gold standard for EBA studies) was correlated with the MPN assay, a strong positive correlation was observed in all four patient sub-categories, although the association was not as strong in the atypical patient category. Furthermore, the MPN correlated with the CFU during the first two months of anti-TB treatment compared to the MGIT that only correlated with the CFU during the first two weeks of TB treatment. These results suggest that the CF^+ MPN assay may be the preferred liquid culture assay (as opposed to MGIT TTP) to monitor the efficacy of new TB drugs, particularly with regards to DCTB.

5.7 CHAPTER CONCLUSION AND FUTURE RESEARCH

In conclusion, these results indicate a strong correlation between the MPN, CFU and MGIT assays when bacteria are cultured in the lab. The MPN assay in the 48-well format proves to be a highly sensitive method to determine the number of organisms in axenic culture. Furthermore, the generation of standard curves from this study has allowed for the prediction of bacterial load in MGIT culture based on the TTP or vice versa. In patient samples, the CF^+ MPN was comparable to the MGIT TTP during the first two weeks of TB treatment. The same

was observed between CFU and MGIT TTP. These results suggest a potential use for the MGIT assay in phase 1 EBA studies. The association between CFU and MPN was stronger than that observed in the MGIT at later treatment time points and in patients with a lower sputum bacillary burden. The ability to measure response of DCTB to anti-TB treatment using the CF^{+} MPN assay will be important in future studies to determine the effect of new TB chemotherapeutics on different *M. tuberculosis* populations.

APPENDICES

APPENDIX 5A: THE RELATIONSHIP BETWEEN MGIT TPP AND BACTERIAL LOAD IN ALL PATIENT SAMPLES

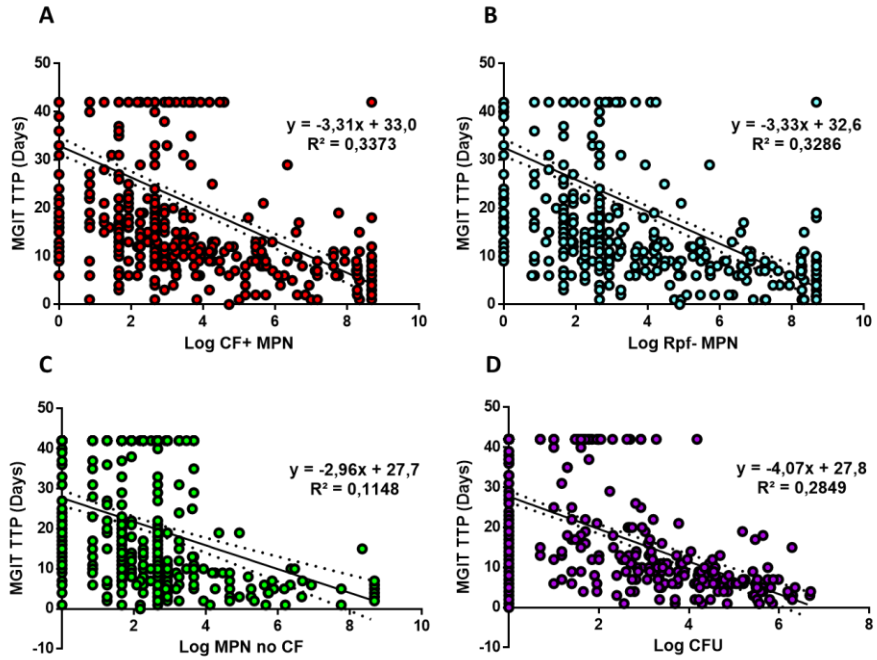

Figure 5A. The linear relationship between the number of bacteria obtained in most probable number and colony forming unit assays with mycobacterial growth indicator tube time to positivity in all patients. The number of bacteria obtained by most probable number (MPN) or colony forming unit (CFU) assays was correlated with the mycobacterial growth indicator tube (MGIT) time to positivity (TTP) obtained for all 75 patients (i.e. all DCTB categories comined). The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the MPN/CFU is represented as log- transformed data. On the y-axis, the MGIT TTP is represented as the number of days to culture TTP. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green) and (D) CFU assays (purple).

APPENDIX 5B: THE RELATIONSHIP BETWEEN MGIT TPP AND BACTERIAL LOAD IN THE ACCUMULATE DCTB COHORT

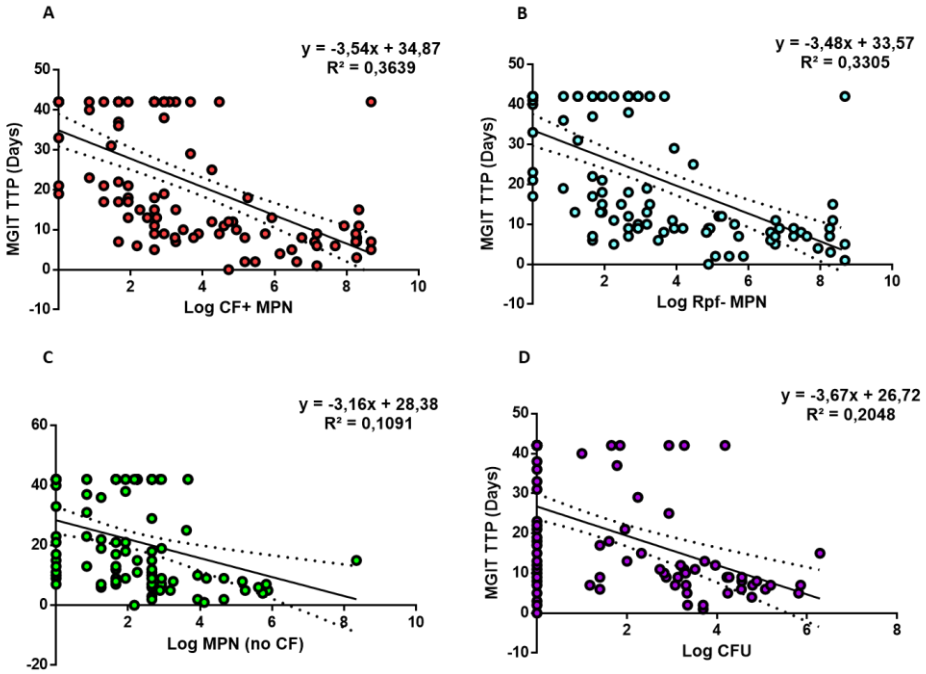

Figure 5B. Correlation between the number of bacteria obtained in most probable number and colony forming unit assays with mycobacterial growth indicator tube time to positivity in patients from the accumulate DCTB cohort. The number of bacteria obtained by most probable number (MPN) or colony forming unit (CFU) assays was correlated with the mycobacterial growth indicator tube (MGIT) time to positivity (TTP) obtained for 17 patients in accumulate DCTB cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the MPN/CFU is represented as log-transformed data. On the y-axis, the MGIT TTP is represented as the number of days to culture TTP. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green) and (D) CFU assays (purple).

APPENDIX 5C: THE RELATIONSHIP BETWEEN MGIT TPP AND BACTERIAL LOAD IN THE DECLINE DCTB COHORT

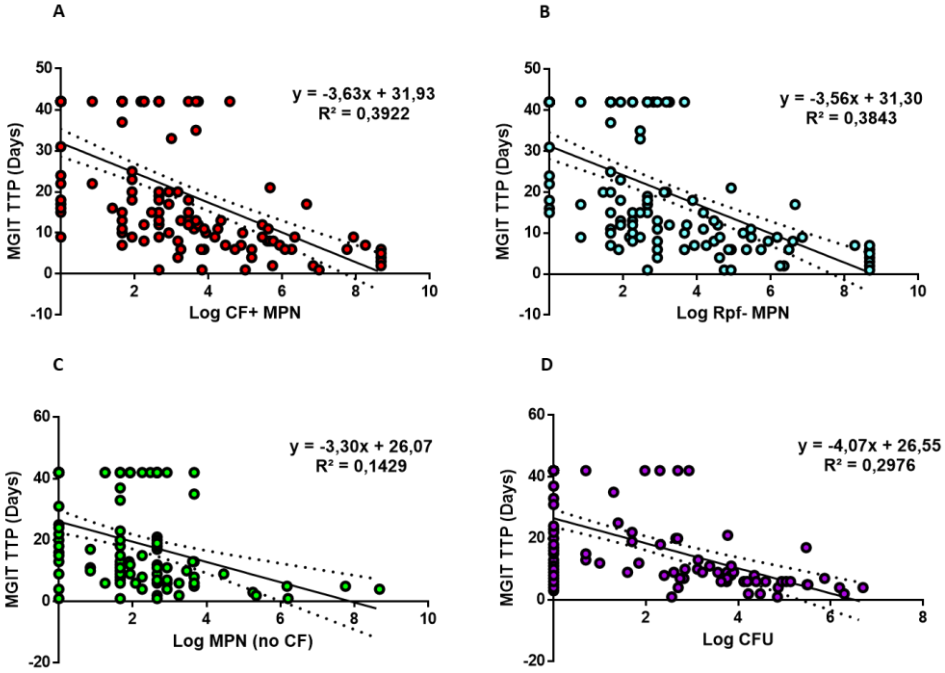

Figure 5C. Correlation between the number of bacteria obtained in most probable number and colony forming unit assays with mycobacterial growth indicator tube time to positivity in patients assigned to the decline DCTB cohort. The number of bacteria obtained by most probable number (MPN) or colony forming unit (CFU) assays was correlated with the mycobacterial growth indicator tube (MGIT) time to positivity (TTP) obtained for 20 patients in decline DCTB cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the MPN/CFU is represented as log-transformed data. On the y-axis, the MGIT TTP is represented as the number of days to culture TTP. (A) CF+ MPN assay (red), (B) Rpf- MPN assay (blue), (C) MPN no CF (green) and (D) CFU assays (purple).

APPENDIX 5D: THE RELATIONSHIP BETWEEN MPN AND CFU IN ALL PATIENT SAMPLES

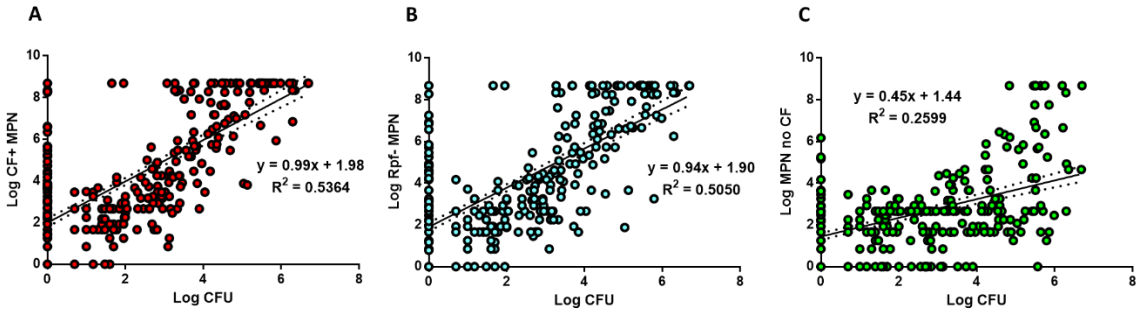

Figure 5D. Correlation between the number of bacteria obtained in colony forming unit assays with most probable number assays in all patients. The number of bacteria obtained by colony forming unit (CFU) assays was correlated with the number of bacteria obtained from most probable number (MPN) assays for 75 patients analysed in the longitudinal cohort from Chapter 4 (i.e. n = 75). The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the CFU is represented

as log- transformed data. On the y-axis, the MPN is represented as log-transformed data. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green).

APPENDIX 5E: THE RELATIONSHIP BETWEEN MPN AND CFU IN THE ACCUMULATE DCTB COHORT

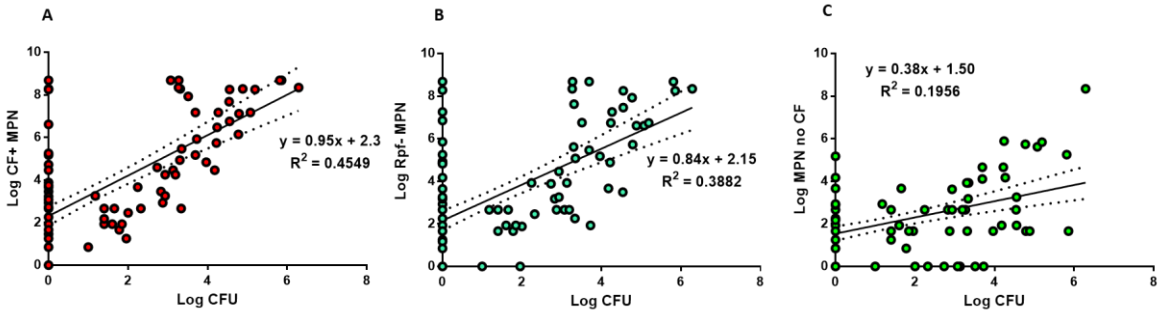

Figure 5E. Correlation between the number of bacteria obtained in colony forming unit assays with most probable number assays in patients from the accumulate cohort. The number of bacteria obtained by colony forming unit (CFU) assays was correlated with the number of bacteria obtained from most probable number (MPN) assays for 17 patients in the accumulate cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the CFU is represented as log-transformed data. On the y-axis, the MPN is represented as log-transformed data. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green).

APPENDIX 5F: THE RELATIONSHIP BETWEEN MPN AND CFU IN THE DECLINE DCTB COHORT

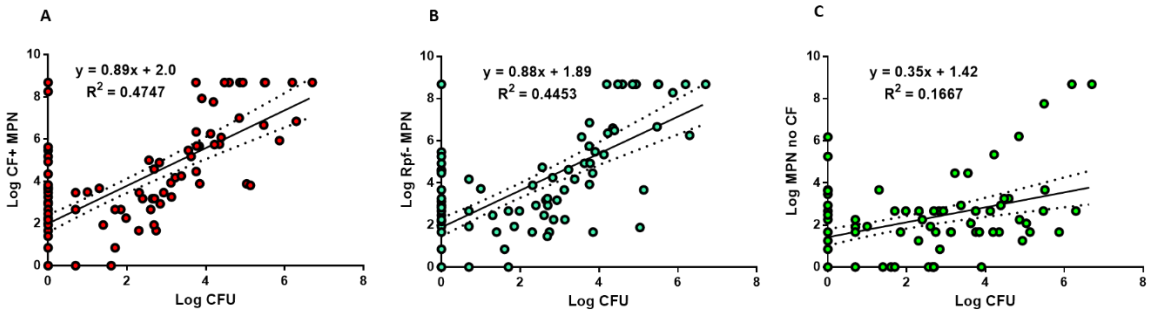

Figure 5F. Correlation between the number of bacteria obtained in colony forming unit assays with most probable number assays in patients from the decline cohort. The number of bacteria obtained by colony forming unit (CFU) assays was correlated with the number of bacteria obtained from most probable number (MPN) assays for 20 patients in the decline cohort. The data featured contains baseline data as well as data from all subsequent time points (i.e. days 3, 7, 14, 35, 56 and 180). On the x-axis, the CFU is represented as log-transformed data. On the y-axis, the MPN is represented as log-transformed data. (A) CF^+ MPN assay (red), (B) Rpf^- MPN assay (blue), (C) MPN no CF (green).

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Detailed components of this PhD

Background and literature review

CHAPER ONE	CHAPTER TWO
TUBERCULOSIS	MICROBIAL DORMANCY

Original Research Chapters

KEY QUESTIONS?	KEY CONCLUSIONS?
CHAPTER 3 Do all TB patients harbor DCTB? What are the various DCTB populations? Are Rpfs solely responsible for unmasking DCTB? Does host immunity affect the quantum of DCTB?	**CHAPTER 3** Four distinct sub-populations of DCTB observed amongst patients Rpf effect was marginal in unmasking DCTB HIV-negative patients harbored a higher quantum of DCTB at baseline CD4 T-cell count not associated with DCTB
CHAPTER 4 How do DCTB respond to first-line TB treatment compared to conventionally culturable bacteria? Are there potential biomarkers to predict patient response to treatment? Do DCTB remain at the end of treatment?	**CHAPTER 4** Four patterns of decline were described An atypical DCTB presentation was associated with HIV-positive patients DCTB were detected in most patients following treatment completion and DMN-Tre staining confirmed the presence of DCTB
CHAPTER 5 What is the relationship between the MPN assay and currently employed *M. tuberculosis* culture methods? Can the MPN possibly be used in EBA studies? Does CF improve the relationship between the MPN and MGIT TTP in clinical samples?	**CHAPTER 5** Strong correlation between MPN and MGIT TTP as well as CFU in axenic cultures Strong correlation between MGIT TTP and CF-supplemented MPN in clinical samples Poor correlation between MPN and MGIT TTP in 'atypical DCTB' patient group
CHAPTER 6 Does the addition of CF to the MGIT enhance diagnostic pick-up? Can modification of the MGIT by the addition of CF improve the time to culture positivity? Does a reduction in total MGIT volume improve TTP by enhanced quorum sensing?	**CHAPTER SIX** ?

CHAPTER 6

ENHANCED DETECTION OF *M. TUBERCULOSIS* IN SMEAR-NEGATIVE/HIV POSITIVE TB PATIENTS WITH REDUCED BACTERIAL LOAD BY SUPPLEMENTATION OF THE BACTEC MGIT 960 CULTURE SYSTEM WITH CULTURE FILTRATE

6.1 INTRODUCTION

Although the introduction of anti-retroviral therapy (ART) programs has saved the lives of millions of HIV-positive individuals in resource-limited settings (UNAIDS, 2003, Haberer et al., 2017, Volberding and Deeks, 2010), mortality amongst patients with advanced disease during the first year of ART initiation is common (Gupta et al., 2011, Lawn et al., 2008). South Africa remains the epicentre for HIV/TB co-infection, with TB being the leading cause of death in patients co-infected with HIV (WHO report 2016). This has been verified in autopsy cases where a systematic review and meta-analysis of 36 eligible studies reported a pooled TB prevalence of 39.7% [95% confidence interval (CI) 32.4 – 47%] in HIV-infected adults (Gupta et al., 2015). The post-mortem TB prevalence varied by region with pooled estimates in adults of 43.2% in sub-Saharan Africa (20 studies), 27.1% in the Americas (eight studies) and 63.2% in South Asia (eight studies) which correlated with the estimates of the National TB prevalence. Furthermore, approximately 50% of these cases remained undiagnosed at death, highlighting current limitations in the approach to TB prevention, diagnosis and treatment in HIV-infected individuals (Gupta et al., 2015).

6.1.1 How does HIV- infection affect TB diagnosis?

TB patients co-infected with HIV have an altered, atypical clinical presentation of disease with reduced prevalence of cavities due to impaired granuloma formation and higher rates of EPTB disease (Palmieri et al., 2002). As a result, these patients have reduced numbers of organisms in their sputum samples when compared to their HIV-uninfected counterparts (Hargreaves et al., 2001, Getahun et al., 2007). In resource-limited settings, screening for TB is predominantly done by sputum staining for AFB which has a low sensitivity that is further reduced with declining CD4 T-cell counts (Singhal et al., 2011). As TB diagnosis and management is predominantly based on sputum, there is a reduced sensitivity in TB tests amongst HIV/TB co-infected patients who are often smear negative (Getahun et al., 2007).

High rates of smear negative TB disease and attempts to reduce the number of TB-related deaths in HIV-infected individuals has led to the expanded use of empiric treatment (defined as treatment in the absence of microbiological confirmation of TB disease) (Scott et al., 2017). The WHO recommends that HIV-positive patients who screen negative for TB are placed on INH preventative therapy (IPT) and empiric treatment is only used in seriously ill patients in peripheral settings (WHO, 2013). However, whilst IPT has proved to be a successful intervention (Group, 2015, Comstock et al., 1979, Comstock et al., 1967), concerns regarding the tolerability of INH and the treatment of sub-clinical TB with a single antibiotic, has resulted in more clinicians rejecting this method of treatment (Balcells et al., 2006). The Thibela TB study was carried out to investigate the direct protective effect of IPT on gold miners with LTBI in South Africa (Churchyard et al., 2014, Fielding et al., 2011). A high prevalence of HIV-infection along with exposure to silica dust and crowded working and living condition put miners at high risk for TB disease (Corbett et al., 2000). Analysis of the direct effect of INH in 10,909 miners illustrated a reduction in TB incidence during treatment (adjusted rate ratio, 0.42; 95% CI); however, this protection was short-lived. It was recommended that continuous IPT is considered for individuals at highest risk for TB (i.e. HIV-positive or with silicosis). Overall, the investigators concluded that a 9-month course of community-wide IPT did not improve TB control in South Africa (Churchyard et al., 2014). Another large scale trial, named 'REMEMBER' (Reducing Early Mortality and Early Morbidity By Empirical TB treatment Regimens) sought to investigate whether empiric treatment would reduce ART early mortality compared to IPT in high burden settings (Hosseinipour et al., 2016). Results from this multi-centre clinical trial showed that empiric treatment did not reduce mortality in adult outpatients at 24 weeks post-ART initiation compared to IPT.

6.1.2 Current TB diagnostics and the gold standard

Culture remains the gold standard tool for the diagnosis of TB, but is limited in its application in that it can take up to six weeks to yield a positive result, a process that is further protracted in HIV-infected patients (Johnson et al., 1998, Brindle et al., 1993). In recent years, there has been substantive advances in molecular diagnostics with several NAATs to rapidly detect *M. tuberculosis* in patient samples. Among these tests is the recently endorsed Xpert MTB/RIF assay (Cepheid) that detects *M. tuberculosis* and resistance to RIF (which serves as a marker for multi-drug resistant TB) within two hours. In a prospective clinical study conducted in South Africa, this assay was demonstrated to have superior sensitivity when compared to other

NAATs, including the Hain MTBDR*plus* (Hain Lifescience, Nehren, Germany) and LightCycler Mycobacterium Detection (LCTB) (Roche), as well as sputum smear microscopy in an HIV and TB endemic region (Scott et al., 2011). Whilst suggestions to replace smear microscopy with the GeneXpert MTB/RIF assay for the initial diagnosis of TB have been put forward, the availability of this test remains scarce and is most likely hampered due to cost (Fenner et al., 2013). While the GeneXpert has excellent sensitivity in smear-positive sputum samples, sensitivity is decreased in smear-negative samples, usually associated with HIV-infection (Steingart et al., 2013, Lawn et al., 2011, Sohn et al., 2014, Zeka et al., 2011). The new Ultra assay, which is currently being rolled-out in various provinces across South Africa, was shown to have enhanced sensitivity compared to the GeneXpert and was able to detect more cases of smear-negative, culture positive TB (Chakravorty et al., 2017). The overall sensitivity in 277 clinical sputum samples (Ultra versus Xpert) increased from 81% (95% CI, 74.9, 86.2) to 87.5% (95% CI, 82.1, 91,7); in sputum smear-negative samples the sensitivity improved from 66.1% (95% CI, 56.4, 74.9) to 78.9% (95% CI, 70.0, 86.1) (Chakravorty et al., 2017). These results suggest that the GeneXpert Ultra is expected to perform better in HIV-infected individuals.

Smear microscopy and NAATs yield rapid results; however, a limitation of these assays is that they do not give any indication as to whether the organisms are alive or dead. Furthermore, the sensitivity of these assays is reduced compared to culture (Bicmen et al., 2011). Whist these assays may serve a purpose in the initial diagnosis of TB, they cannot be used to monitor response to treatment (WHO, 2014). In addition, MGIT culture is used for down the line DST for many first and second-line anti-TB drugs for detection of drug-resistance. This allows for the prescription of appropriate treatment and limits the emergence of further resistance that may stem from inappropriate treatment. The above highlights the urgent need to develop methods to decrease the rates of culture TTP for TB diagnosis and treatment management in HIV-positive individuals, who often present with low numbers of bacteria in the sputum and are difficult to diagnose. The emergence of drug-resistant *M. tuberculosis* strains has further exacerbated the need for rapid and sensitive TB tests that can provide a diagnosis and guide treatment in this group of individuals. Indeed a systematic review of the published literature reported that HIV/AIDS was significantly associated with an increase in MDR-TB [estimated pooled odds ratio (OR) 1.24; 95%] (Mesfin et al., 2014). Further analysis revealed that these estimates were higher for primary MDR-TB (pooled OR 2.28; 95%), a finding consistent with previous work (Suchindran et al., 2009). Possible explanations for this association may include

the rapid disease progression in immune-compromised individuals in areas where MDR-TB is prevalent. HIV-positive individuals may also have increased contact with MDR-TB infected patients due to increased hospitalization or in social settings with poor infection control (Mesfin et al., 2014). While a common concept with respect to drug resistance suggests that the acquisition of resistance mutations is associated with a fitness cost, more recent evidence suggests that these strains are equally as transmissible as their drug-susceptible counterparts (Gagneux et al., 2006, Dheda et al., 2017, Van Rie and Warren, 2015). This is evident in a number of studies that have linked MDR- and XDR-TB with primary transmission (Ghandi et al., 2006, Klopper et al., 2013, Gilpin et al., 2008, Cox et al., 2010, Andrews et al., 2008). In settings where the transmission of drug-resistant TB occurs due to primary transmission (i.e. infection with a drug-resistant strain as opposed to drug-susceptible strain that acquires resistance through the course of treatment), the rapid diagnosis and treatment of individuals harbouring these strains should be a high priority.

6.1.3 Efforts to enhance culture performance

In light of the above, new methods to improve culture performance are urgently required and enhanced recovery of DCTB may prove beneficial in this regard. In previous work, the addition of Rpf (as recombinant proteins or presumed to be the growth stimulatory molecules in CF) to standard culture medium increased the yield of sputum culture (Mukamolova et al., 2010) and allowed for the culture of *M. tuberculosis* that would ordinarily be negative by conventional methods (O'Connor et al., 2015, Chengalroyen et al., 2016). A few studies have embarked on investigating the effect of recombinant Rpf or CF on reducing culture TTP in conventional and routine diagnostic assays. The results and findings of these studies are summarized in Table 6.1 and are subsequently discussed.

6.1.3.1 The effect of recombinant RpfB on the growth of axenic *M. tuberculosis* cultures

Wu et al. (2008) performed various experiments investigating the relationship between recombinant RpfB supplementation and mycobacterial growth using Middlebrook 7H9, BACTEC 960 and Lowenstein Jensen medium (Wu et al., 2008). In the Middebrook 7H9, *M. bovis* BCG strains were inoculated into media at high (6 X 10^4 CFU per mL) and low (2 X 10^4 per mL) inoculum levels. Growth curves were conducted by measuring the OD_{600nm} at specified time intervals.

Table 6.1 Summary of studies using recombinant Rpf or CF to enhance culture performance

Year	1999	2008	2014	2014	2015
Author	Sun and Zhang	Wu	Huang	Kolwijck	O'Connor
Methods					
Culture system	Liquid incubation followed by plating on solid media	Middlebrook 7H9, BACTEC 960, Lowenstein-Jensen	BacT/Alert MP	BACTEC 960	MPN assay
Recombinant (r) Rpf or CF?	CF	rRpfB	rRpfB and rRpfE	CF	CF (prepared from: H37Rv and ΔABCDE)
Samples					
Axenic cultures or patient samples (n)	Axenic: *M. tuberculosis* H37Rv (8 month aged cultures)	Axenic: *M. bovis* BCG and *M. tuberculosis* H37Rv	Patients (12)	Patients (15)	Patient (1)
If patient samples:					
# of sputum samples collected	NA	NA	23	79	1 lymph node aspirate
# Pre-treatment samples	NA	NA	NS	19	0
# Treatment samples	NA	NA	NS	60	1
Key study conclusion/s:	CF ↑ viability of aged cultures and ↑ growth of small inocula in liquid media	rRPF promoted proliferation of *M. tuberculosis* in 7H9 media, BACTEC 960 and LJ	↑ sensitivity in samples that require longer culture times by standard procedures and ↓ TTP in heat treated samples	TTP reduced in sputum samples collected during the first eight weeks of TB treatment.	*M. tuberculosis* cultured from otherwise undetectable extra-pulmonary TB

Abbreviations: rRpf = recombinant resuscitation-promoting factor, CF = culture filtrate, n = number, NA = not applicable, NS = not specified, LJ = Lowenstein-Jensen, TTP = time to positivity, ↑ = increase, ↓ = decrease.

Results from the high inoculum experiments showed that supplementation with RpfB had no effect on mycobacterial growth compared to the control. In contrast, supplementation with RpfB was shown to have a marked effect on bacterial growth at low starting inoculum levels where the final cell density was three to five times greater than the un-supplemented 7H9 media. These results were in agreement with a previous study where the addition of *M. tuberculosis* H37Rv derived CF increased the viability of aged cultures and promoted the

growth of low-level inocula (Sun and Zhang, 1999). In the BACTEC MGIT 960 experiments, four MGITs were included in the experiments: i) BACTEC 960 (control), ii) BACTEC 960 + OADC, iii) BACTEC 960 + RpfB and iv) BACTEC 960 + OADC + RpfB. Supplementation of BACTEC MGIT 960 culture media with RpfB reduced the TTP of higher-level inoculum densities (10^3 to 10^5; starting bacterial solution contained 10^6 CFU) similar to that achieved by supplementation with OADC (Wu et al., 2008). The biological activity was most prominent at the 10^6 inoculum density where the addition of RpfB in combination with OADC yielded positive growth at 17 days post-inoculum, compared to the control tube, which flagged positive after 37 days (TTP was 33 and 37 days post-inoculation following supplementation with either OADC or RpfB, respectively). These results suggest that stimulation of non-replicating bacteria could shorten the duration of culture period required for TB diagnosis. In the final set of experiments, the addition of RpfB to solid Löwenstein–Jensen media resulted in large, high-density *M. bovis* BCG colonies compared to un-supplemented plates that contained smaller and fewer colonies (Wu et al., 2008). These experiments highlighted a potential use for Rpf in detection of TB infection in clinical samples where the bacterial load is low. However, this study had several limitations: firstly, the authors reported that the recombinant RpfB protein was insoluble, but the solubilisation of this protein, and whether function was restored, was not reported. Similarly, it was reported that this protein was purified under denaturing conditions, but details regarding its renaturation were not given. Therefore, the observations observed may have been due to a non-physiologic action of a denatured protein and not a direct result of recombinant RpfB. Lastly, no quantitative results of CFUs were given. This is problematic as there is no additional confirmation of bacterial load in the experiments.

6.1.3.2 The effect of recombinant Rpf on reducing time to positivity in patient samples

The addition of recombinant RpfB and RpfE to the BacT/Alert MP culture system was investigated (with respect to shortening time to culture positivity) in twelve smear positive patients from Hefei infectious Disease Hospital (Huang et al., 2014). The addition of 20 nm RpfB or RpfE reduced the TTP in samples where the control TTP, performed under standard laboratory procedures, was greater than 20 days. As the ZN staining score did not correlate with liquid culture TTP ($R^2 = 0.36$), the investigators speculated that sputum samples may harbour varying proportions of lipid body positive (LBP) *M. tuberculosis*, and a high proportion of these non-replicating organisms may account for the reduction in TTP. To investigate this, an additional eleven sputum samples (designated A to K) were collected to

determine the effect of LBP-bacilli on TTP. Auramine-Nile Red staining illustrated the presence of LBP-positive *M. tuberculosis* in all sputum samples; however, the proportions varied from 20 to 70%. In agreement with their first conclusion, the TTP was shortened in samples where the control TTP was greater than 20 days (the TTP was reduced in 3 out of 11 samples). Collective data from the two sample groups (first 12 sputum samples, 1-12, and additional 11 samples, A-K) illustrated that the addition of recombinant Rpf non-significantly shortened the TTP (28.8, 21.6 and 22.2 days for the control, the RpfB and RpfE supplemented cultures, respectively; $P < 0.05$). It is unclear whether the reported reduction in TTP was calculated from all 23 samples or only in the samples were TTP was reduced in the two sample groups. The investigators did however report that the effect (i.e. a reduction in TTP compared to the control) was not observed in samples with a shorter culture TTP using standard procedures. To further investigate whether non-replicating *M. tuberculosis* were responsible for the observed decrease in TTP, sputum samples were heat treated for 60 ºC for ten minutes to kill the replicating bacteria. It was assumed in this case that non-replicating bacteria were heat tolerant. Indeed, previous work on *in vitro* cultures illustrated that non-replicating *M. tuberculosis* induced in an anaerobic environment can tolerate heat treatment of 70 ºC for ten minutes (Shleeva et al., 2011). Following heat treatment, the proportion of lipid body-negative samples was reduced compared to untreated sputum samples (55% versus 5%; $P < 0.001$). Furthermore, heat treated samples were undetected by standard culture methods, but could be detected following RpfB supplementation, and in some cases, RpfE supplementation (Huang et al., 2014).

6.1.3.3 The effect of early stationary phase culture supernatant on the growth of *M. tuberculosis* from patients on treatment

Kolwijck et al. (2014) investigated whether supplementation of MGIT tubes with early stationary phase culture supernatant (ESPSN) derived from *M. tuberculosis* H37Rv could aid in the detection of a population of non-culturable *M. tuberculosis* cells during TB chemotherapy (Kolwijck et al., 2014). For this, 500 µL of sputum (n = 79) from 15 patients were inoculated into a total of four MGIT tubes: two with ESPSN (containing 4 mL original medium and 4 mL ESPN) and two with 8 mL of original medium (control). Both pre-treatment (n = 19) and treatment samples from the first two months of treatment were analysed [day 1 to 7 (n=27), day 14 (n = 13), day 21 (n = 2), day 28 (n = 6), day 35 (n = 2), day 42 (n = 4), day 49 (n = 3) and day 56 (n = 3)]. One control MGIT and one ESPN-supplemented MGIT was

harvested following 7 days incubation for CFU analysis and the remaining two tubes were incubated in the BACTEC MGIT 960 to obtain a TTP. Overall, ESPN was shown to non-significantly accelerate the TTP (18.2 ± 128 hours, mean ± SD, P = 0.572, Mann-Whitney U-test) and in contrast, decrease the number of platable bacteria obtained by CFU analysis (0.54 ± 0.35 log CFU, mean ± SD, P = 0.572, Mann-Whitney U-test). The authors note that when categorized by length of treatment (i.e. less than 7 days versus greater than 14 days), there was a significant trend towards increased ΔTTP with length of treatment duration (7.2 ± 32 hours versus 37.8 ± 212 hours, P = 0.01, Kruskal-Wallis test).

6.1.3.4. The effect of Rpfs in diagnosing extra-pulmonary TB disease

The effect of Rpfs on improving diagnosis in EPTB was first reported in a case study involving a 32 year old Asian male (O'Connor et al., 2015). Prior to referral, three sputum samples from this patient were smear- and culture-negative for *M. tuberculosis;* however, pathological intrathoracic lymphadenopathy was seen using computerised tomography. Various clinical samples (including an endobronchial ultra-sound-guided transbronchial needle aspiration of the enlarged paratracheal node) were collected for histopathological analysis and mycobacterial culture, with an additional lymph node aspirate collected to research the effect of Rpfs on culture yield. For this MPN assays were carried out with and without Rpf-containing culture supernatant as previously described (Turapov et al., 2014). After 50 days incubation, no growth was detected in standard liquid culture medium (i.e. MPN no CF) or on agar plates; however, a positive culture was obtained following 25 days incubation in the MPN assay Rpf-containing culture supernatant (i.e. CF^{+} MPN), where the estimated bacterial count was 4.5 cells per/mL. *M. tuberculosis* was confirmed by AFB smear microscopy and PCR using *M. tuberculosis* specific primers. Furthermore, mycobacterial interspersed repetitive-unit-variable-number of tandem repeats strain typing identified the isolate as Dehli/Central Asian (O'Connor et al., 2015). This study illustrates that in some instances CF may be required to stimulate the growth of *M. tuberculosis* from EP sites that would otherwise remain undetected using standard culture methods. Indeed, in a recent study, CF-dependent DCTB [termed culture supernatant (CS) -dependent DCTB) were identified in 10/19 (52.6%) EPTB samples from 18 culture positive *M. tuberculosis* patients, demonstrating that CS-dependent DCTB are not exclusive to sputum and pulmonary TB disease (Rosser et al., 2017a).

Considering the preliminary findings of the aforementioned studies, the addition of Rpf/CF to standard culture media appears to be an attractive avenue to improve TTP and the sensitivity of currently employed TB diagnostics. However; the use of recombinant Rpfs in resuscitating non-culturable organisms is limited and requires further investigation due to the unstable nature of these proteins. In addition to improving culture methods to detect *M. tuberculosis*, the use of CF (or recombinant Rpf) could be further explored for DST, thereby shortening the time to start appropriate treatment for patients with drug resistant TB. Improving culture TTP and yield will also be beneficial for molecular epidemiological studies (i.e. IS*6110* restriction fragment length polymorphism genotyping requires culture material) and advanced 'omic' studies, since the methodology employed for these studies still require positive culture material (Asmar and Drancourt, 2015, Hayer et al., 2013, Ryu, 2015, Rosser et al., 2017b).

6.2 HYPOTHESIS

1. Modification of the BACTEC MGIT 960 culture assay through supplementation of the media with CF derived from *M. tuberculosis* H37Rv (containing Rpfs) will decrease the time to detection of *M. tuberculosis* in sputum samples collected from HIV- positive individuals, with smear negative/paucibacillary disease.
2. Modification of the BACTEC MGIT 960 culture assay by reduction of total volume of culture will decrease the TTP of *M. tuberculosis* in sputum samples collected form HIV-positive individuals, with smear negative/paucibacillary disease.

6.3 AIM OF THE STUDY

The aim of this study was to determine whether modification of the BACTEC 960 MGIT culture assay through supplementation of the media with CF would improve detection in sputum samples collected from HIV-1 positive patients infected with active, drug-susceptible TB disease. This will enhance early diagnosis of TB and timely initiation of drug treatment thereby reducing further transmission within the community.

6.3.1 Specific Objectives

1. To determine whether modification of the MGIT by the addition of CF will reduce the TTP in axenic *M. tuberculosis* cultures
2. To determine whether a reduction in MGIT volume will reduce the TTP in axenic cultures
3. To determine whether the modification of the MGIT by the addition of CF will increase the sensitivity of the assay by decreasing the number of false negative cultures associated with HIV/TB co-infection in patient sputum samples.
4. To determine the proportional decrease in TTP in the MGIT supplemented with CF from *M. tuberculosis* H37Rv in comparison to the original format.
5. To assess whether a reduction in the volume of MGIT media (with or without CF-supplementation) results in a change in TTP.
6. To quantitate the number of DCTB and by performing MPN assays containing CF and standard media, respectively.
7. To determine the number of platable (culturable) bacteria by performing CFU assays.

6.4 METHODS

6.4.1. Preparation of culture filtrate

CF was prepared from *M. tuberculosis* H37Rv cultures as previously described (see section 3.4.3.1.1, page 94) with and without the addition of Tween 80 (polysorbate 80, polyoxyethylene sorbitan monooleate). Briefly, Freezer stocks containing 1 mL aliquots of cells were grown in standard media, sub-cultured and grown to an OD_{600nm} of 0.6 – 0.9 after which the cells were harvested by centrifugation. The supernatant was filtered through a sterile 0.22 µm syringe filter to obtain the CF.

6.4.2 Quality controls

Quality controls were performed to ensure the sterility of the CF as previously described (see section 3.4.3.1.2, page 98). In addition, a MGIT control was included to ensure that no bacterial contamination was present following preparation of the CF. For this, 3.5 mL of MGIT media was removed from a MGIT and replaced with 3.5 mL CF. The control was also inoculated with 800 mL PANTA reconstituted in OADC. The MGIT was labelled ‘CF control’ with the

appropriate date and scanned into the BACTEC MGIT 960 instrument and incubated for 42 days to ensure the absence of *M. tuberculosis* as well as other contaminants. For an example of the quality controls, see Appendix 5A.

6.4.3 Detection of differentially culturable and conventionally culturable tubercle bacilli

MPN assays were performed as previously described in section 3.4.3.1 (page 94). CF-supplemented (CF^+ only) and –un-supplemented 7H9 media was used for MPN assays. For axenic experiments, CFU assays were performed on all serial dilutions as described in section 3.4.3.2 (page 104). For patient samples, approximately 500 µL of decontaminated sputum sediment was used to perform MPN and CFU assays. CFU assays were performed from the decontaminated sputum to determine the number of platable bacteria as described in section 3.4.3.2. Neat to 10^3 serial dilutions were performed. GeneXpert and smear microscopy was done at CLS.

6.4.4 Methods specific to axenic experiments

6.4.4.1 Preparation of bacterial strains

Three H37Rv cultures were grown to OD_{600nm} = 0.6. These cultures were subsequently diluted to an OD600nm = 0.05 in standard 7H9 media. Serial dilutions were subsequently performed from the diluted cultures (10^3 to 10^6). Five hundred µL of each serial dilution was inoculated into five different MGIT tubes (see section 6.4.4.2). The experiment was repeated in triplicate from three separate cultures. A total of 62 MGIT tubes (including two control tubes) were required for this experiment. The experiment was repeated as above, where 100 µL (as opposed to 500 µL) of each serial dilution was inoculated into each of the five MGIT tubes. This was done to assess how reductions in the numbers of bacteria inoculated affected any possible TTP enhancement.

6.4.4.2 Preparation of modified MGITs

Five MGIT tubes were required for each experiment. The preparation of each tube is described below:

The first MGIT tube (A) served as the control and was not modified (7 mL of original MGIT media). For the second MGIT tube (B), 3.5 mL of MGIT medium was removed and replaced with 3.5 mL of freshly prepared CF (7 mL volume with 1:1 ratio of media and CF). MGIT tube C was prepared in the same manner as MGIT tube B, however, the CF added was prepared from bacterial cultures grown in media containing Tween 80. To investigate whether a reduced MGIT volume would result in a faster TTP, an additional two MGITs were prepared. For the reduced volume control, five mL of media was removed from MGIT tube D (2 mL of original MGIT media). Six mL of MGIT medium was removed from the last MGIT tube (E) and replaced with 1 mL of freshly prepared CF (2 mL volume with 1:1 ratio of media and CF). Following preparation of the five MGIT tubes, 800 µL and 230 µL of PANTA reconstituted in OADC was added to MGIT tubes containing 7 mL and 2 mL volumes of media, respectively.

Table 6.2 MGITs prepared for axenic culture experiments

Experimental conditions	**MGIT A**	**MGIT B**	**MGIT C**	**MGIT D**	**MGIT E**
Modified?		✓	✓	✓	✓
CF-supplemented?		✓	✓		✓
Reduced volume?				✓	✓
CF prepared from cultures grown in Tween 80?			✓		
Designated name and colour	Control green	CF^+ red	CF^+ (Tween 80) blue	2 mL (no CF) teal	2 mL CF^+ orange

Abbreviations: MGIT = mycobacterial growth indicator tube, CF = culture filtrate, Tween 80 = polysorbate 80, polyoxyethylene sorbitan monooleate.

6.4.4.3 Processing and inoculation of MGITs

Following preparation of the MGIT tubes, 500 µL of each serial dilution factor from cultures one, two and three were inoculated into MGIT tubes A, B, C, D and E. Each tube was labelled with the appropriate culture number (i.e. 1, 2 or 3) and dilution factor (i.e. 10^3, 10^4, 10^5 or 10^6). A control MGIT containing a 1:1 ratio of CF and original media was prepared.

6.4.5 Methods specific to clinical samples

This component of the study was conducted to determine whether the addition of CF to the MGIT in a ratio of 1:1 with the original MGIT media (consisting of 110 µL fluorescent indicator and 7 mL of modified 7H9 broth base) would both, increase the sensitivity of the assay by allowing for the further identification of *M. tuberculosis* in isolates that are negative after 42 days incubation in the control MGIT or decrease the time to positivity when compared to control samples containing no CF. A ratio of 1:1 CF to 7H9 broth in the MGIT (as opposed to broth only) allowed for the replenishment of nutrients utilized by the bacteria during culture. This approach has been used successfully in other published work (Mukamolova et al. 2008).

6.4.5.1 Patients/Sources of sputum

One hundred and twenty newly diagnosed HIV-positive patients were recruited for the study at a rate of one to three patients per week through the clinical platforms of the PHRU on the basis of a positive GeneXpert result (Ethics clearance certificate number: M161058, see Appendix 3A, page 133). HIV-positive patients were selected based on previous observations that these individuals present with low numbers of bacteria in the sputum and are difficult to diagnose with culture. Hence, the greatest benefit in CF-supplementation of MGIT culture is expected in this group. Once informed consent was obtained, a spot sample was collected at baseline after which the patient was placed onto a standard first-line TB regimen. The enrolment and exclusion criteria are given below:

Enrolment criteria: 18 years and older, able to produce a sputum sample > 3 ml, HIV sero-positive, no previous history of TB/TB treatment.
Exclusion criteria: Unable to produce a sputum sample > 3ml, HIV sero-negative, drug resistant TB.

Sputum collection was performed in a designated area away from staff and other patients. In order to minimize contamination, the patients were asked to rinse their mouths with water and take several deep breaths before coughing into a pre-labelled sputum jar. A blood sample was also taken from patients who did not have a recent CD4 count (more than 3 months) report. After collection, sputum samples were placed in a plastic bag and immediately transported to CLS where decontamination and diagnostic tests were performed. Samples were kept at 4 ºC (preferably in the dark) during transportation and whilst awaiting decontamination. Samples

were batched and processed once a week for decontamination and MGIT assays. The diagnostic tests performed included auramine smear microscopy (to differentiate patients into smear-negative and smear-positive groups), GeneXpert and conventional MGIT culture (designated MGIT 'A'). A smear grading was performed on all smear-positive samples.

6.4.5.2 Processing at of routine diagnostic data at Clinical Laboratory Services

Sputum decontamination, smear microscopy, GeneXpert, CD4 counts and MGIT culture (for 35 patients only) were performed at CLS as described in Appendix 3B, chapter 3.

6.4.5.3 Experimental design and set-up of protocols one, two and three

The 120 patients recruited for this study were divided and processed according to three different protocols which are detailed out below. Results from the first two protocols yielded unexpected results, leading to subsequent modification of the protocols. A methods overview is given in Figure 6.1.

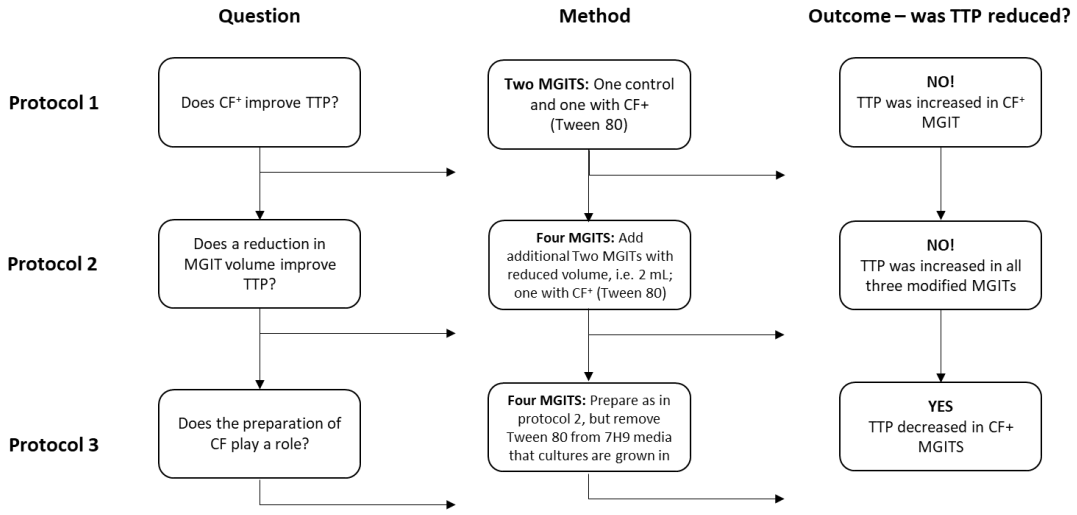

Figure 6.1 Methods map for patient samples. Patient samples were processed by protocols one, two and three as the study progressed. These protocols represent variations that were introduced as results were obtained and the hypothesis modified. In protocol one, the addition of culture filtrate [CF^+ (Tween 80)] prepared from *M. tuberculosis* H37Rv cultures to the MGIT did not decrease time to positivity (TTP). Since most probable number (MPN) assays revealed the presence of differentially culturable tubercle bacilli (DCTB), the effect of culture volume on MGIT TTP was investigated. In protocol two, four MGITs were included. Two had a reduced overall total volume of 2 mL of which one was supplemented with a 1:1 ratio of MGIT media to CF. Results showed that a reduction in overall culture volume (with or without CF) did not decrease TTP. In protocol three, Tween 80 was removed from the *M. tuberculosis* cultures from which the CF was derived. The MGIT tubes for protocol three were prepared in the same manner as protocol two but Tween 80 deficient CF was used. This method resulted in a reduction in overall TTP in CF-supplemented MGITs.

6.4.5.3.1 Protocol one – does the addition of CF enhance TTP?

The BACTEC MGIT 960 mycobacterial detection system was used to ascertain whether the addition of CF to the MGIT could enhance the sensitivity of this assay. For the first 35 patients, two MGIT tubes were prepared for each patient. The first MGIT tube served as the control, was not modified and was labelled 'A'. The second MGIT tube, contained 50% of H37Rv CF and was labelled 'B'. For the preparation of tube B, 3.5 mL of MGIT media was removed with a sterile pipette and substituted with 3.5 mL of freshly prepared CF. This was followed by the addition of 800 µL of PANTA reconstituted in OADC supplement. Once prepared, each MGIT tube was inoculated with 500 µL of decontaminated sputum sample after which they were scanned into the BD BACTEC MGIT 960 instrument for incubation and monitoring of growth. The preparation and inoculation of MGIT tubes A and B are shown in Figure 6.2. Positive cultures were confirmed as MTBC by ZN staining and the MGIT TBc identification test (TBc ID) (BD diagnostics, Sparks, MD). The TBc ID test is a highly sensitive and specific lateral-flow immune-chromatographic assay that is able to detect the MPB64 antigen for the confirmation of MTBC strains (Yu et al., 2011, Martin et al., 2011). Positive MGITs were also streaked out onto blood agar to rule out the presence of a contaminant. In the indication of any contaminant, the pair of MGITs were excluded from analysis, even if smear microscopy detected the presence of *M. tuberculosis*.

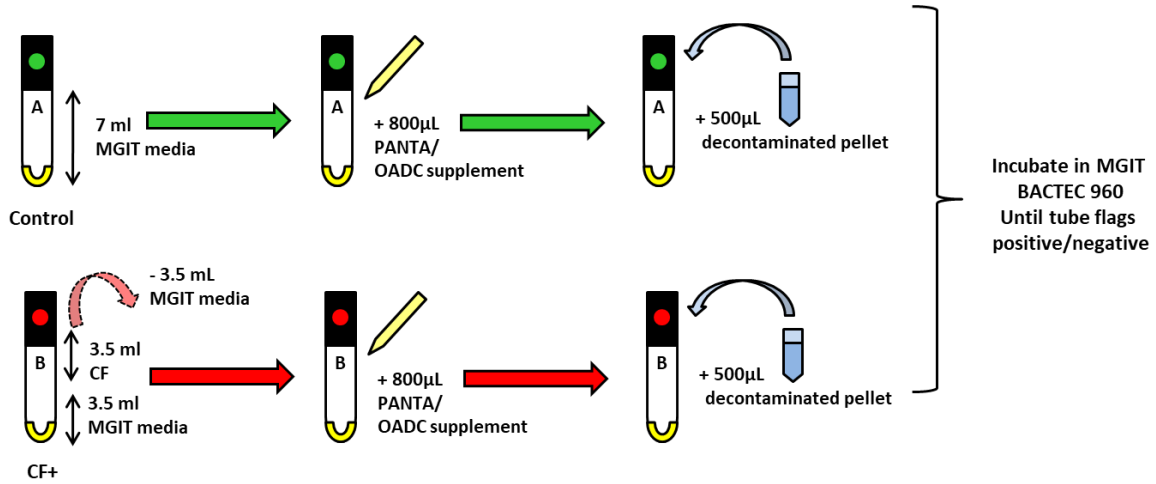

Figure 6.2 Set-up of mycobacterial growth indicator tubes A and B for protocol one. Two mycobacterial growth indicator tubes (MGITs) were prepared for the first 35 patients recruited into the study. Three and a half mL of MGIT media was removed from MGIT tube B, and replaced with 3.5 mL of freshly prepared culture filtrate (CF) derived from *M. tuberculosis* H37Rv. Both MGIT tubes A and B were subsequently inoculated with 800 µL of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA) reconstituted in Oleic acid, Albumin, Dextrose, Catalase (OADC). Following preparation of MGIT tubes A and B, both were inoculated with 500 µL of decontaminated patient sputum. Each tube was incubated in the BACTEC MGIT 960 instrument until flagging positive or negative after which time to positivity was recorded.

6.4.5.3.2 Protocol two – does reduction in total MGIT volume reduce TTP?

Preliminary analysis of the initial 35 patients recruited indicated a slight increase in TTP following the addition of CF. The MPN assays on the other hand, showed the presence of a population of bacteria that could only be cultured in the presence of CF. As the MPN assay is performed in a smaller culture volume (total volume 500 µL) compared to the MGIT (total volume approximately 8.3 mL), the effect of culture volume on TTP was investigated in the MGIT in the second protocol. Here it was hypothesized that a smaller culture volume would enhance any growth stimulatory effects by molecular crowding. For this an additional two MGITs with a reduced volume of original medium (C) and original medium supplemented with CF (D) were prepared, Figure 6.3. MGIT tubes A and B were prepared as described in section *6.4.5.3.1*. Sputum samples from 15 patients were processed according to protocol two.

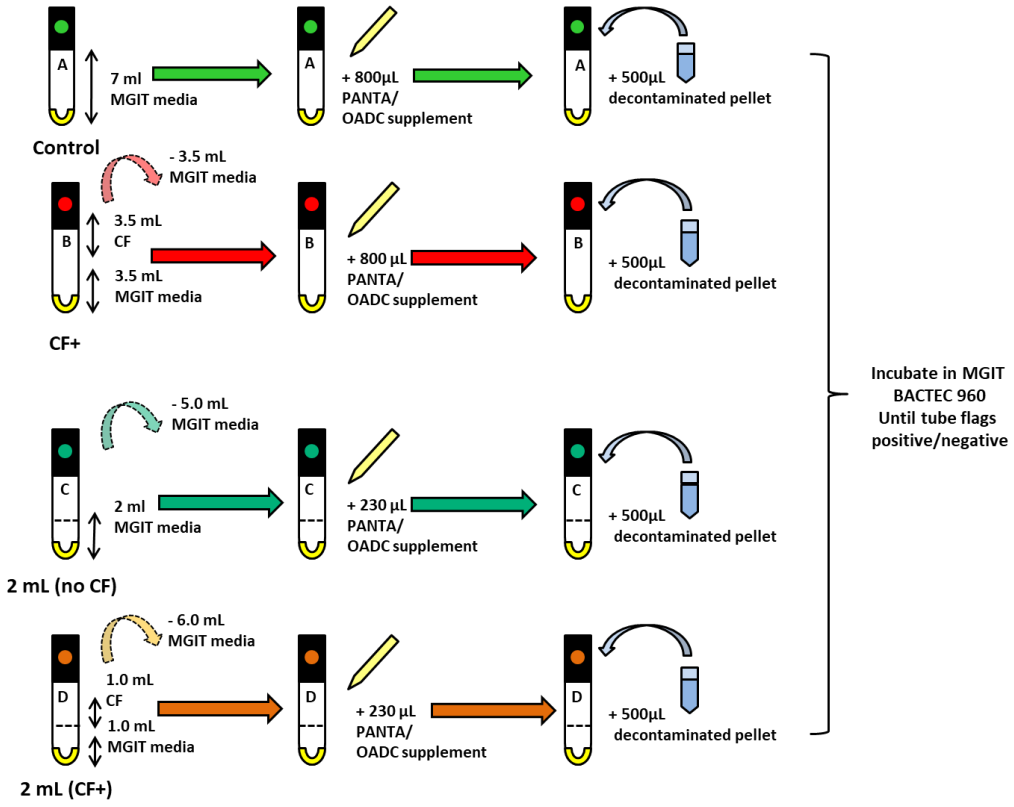

Figure 6.3 Set-up of mycobacterial growth indicator tubes A, B, C and D for protocol two. Four mycobacterial growth indicator tubes (MGITs) were prepared for fifteen patients. The control MGIT, A, was not modified and processed using standard procedures. MGIT tube B was prepared by removing 3.5 mL of MGIT media and replacing it with 3.5 mL of freshly prepared culture filtrate (CF) derived from *M. tuberculosis* H37Rv. MGIT tube C was prepared by removing 5 mL of MGIT media. MGIT D was prepared by substituting 6 mL of MGIT media with 1 mL of CF. Following MGIT preparation, 800 µL and 230 µL of Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin (PANTA) reconstituted in Oleic acid, Albumin, Dextrose, Catalase (OADC) was added to MGITs A/B and C/D, respectively. All four MGITs were inoculated with 500 µL of decontaminated patient sputum. Each tube was incubated in the BACTEC MGIT 960 instrument until flagging positive or negative after which time to positivity was recorded.

6.4.5.3.3 Protocol three – does removal of Tween 80 from CF enhance MGIT TTP?

The third protocol was used for the remaining 69 patients recruited into the study. To assess possible reasons for the observed increase in MGIT TTP following CF supplementation, (see Results, section 6.5.2.1 and 6.5.2.2 for protocols one and two, respectively) Tween 80 was removed from the 7H9 media for the growth of *M. tuberculosis* H37Rv strains used in the preparation of CF.

6.4.6 Data analysis

***Axenic cultures*:** To determine statistical significance between groups, the students paired t-test was used. Analysis was two-sided and a 95% level of statistical significance was employed.

Patient samples: For patient demographics, differences between patients processed by protocols one, two and three were determined using the chi-squared test for proportions and analysis of variance (ANOVA). To determine statistical significance between MGIT TTP (in hours) in modified and control MGITs, the Wilcoxon signed-ranks tests was used. Correlations between MGIT time to positivity and smear status was assessed by the Pearson's and Spearman's correlation coefficients and linear regression analysis. Patients were further stratified according to smear status (positive versus negative). All statistical analyses were two-sided and performed at a 95% level of significant using GraphPad Prism, version 6.01 for Windows (GraphPad Software, San Diego, California, USA).

6.5 RESULTS

6.5.1 Modification of the MGIT by CF-supplementation and reduction of the total volume, decreases time to positivity in axenic culture

The association between culture volume and CF-supplementation on culture TTP in the MGIT system was investigated in axenic cultures with the hypothesis that a reduction in culture volume will reduce TTP due to molecular crowding of bacteria and growth stimulatory factors. Figure 6.4 and Table 6.3 illustrates the decrease in culture TTP in modified MGIT formats.

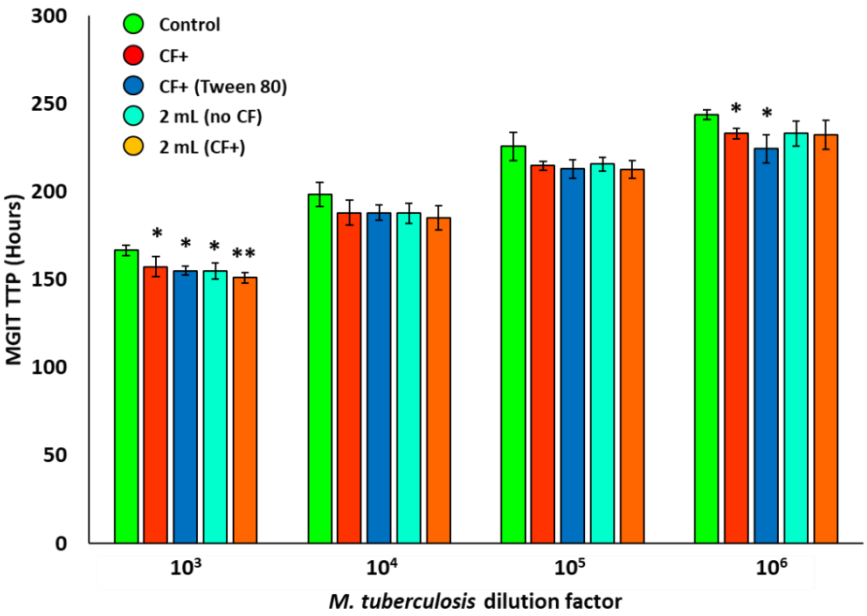

Figure 6.4 Modification of the BACTEC mycobacterial growth indicator tube (MGIT) enhances time to detection in *Mycobacterium tuberculosis* H37Rv, Experiment 1. The culture time to positivity (TTP) in the control MGIT (green) was compared to four experimental MGITs using axenic *M. tuberculosis* H37Rv cultures prepared at different inocula. The control MGIT was unmodified. Modified MGITs consisted of a culture filtrate $(CF)^+$ MGIT (containing 3.5 mL standard media and 3.5 mL CF) (red), CF^+ MGIT containing CF derived from *M. tuberculosis* H37Rv grown in standard 7H9 media supplemented with the detergent Tween 80 (blue), 2 mL (no CF) MGIT (teal) and 2 mL (CF^+) MGIT (containing 1mL standard media and 1 mL CF) (orange). All MGITs were inoculated with 500 µL of prepared bacterial inoculums. The experiment was repeated in triplicate. Error bars represent the standard deviation. Statistical significance was determined using the students paired t-test with a 95% confidence interval (CI). * /** Significant with a 95% and 99% CI, respectively (i.e. $P<0.05$ and $P<0.01$).

Table 6.3 MGIT modification by culture filtrate supplementation and volume reduction reduces time to detection in axenic *M. tuberculosis* cultures, Experiment 1.

	Days of culture (Mean ± SD)					
***M. tuberculosis* dilution factor**	**Control**	**CF+ (-Tween 80)**	**CF+ (+Tween 80)**	**2 mL (no CF)**	**2 ml CF+ (- Tween 80)**	**CFU (mean ± SD)**
10^3	6.94 ± 0.12	6.56 ± 0.24	6.46 ± 0.11	6.46 ± 0.19	6.29 ± 0.13	TNTC
10^4	8.26 ± 0.28	7.83 ± 0.29	7.83 ± 0.18	7.82 ± 1,24	7.71 ± 0.29	83.50 ± 6.36
10^5	9.40 ± 0.34	8.94 ± 0.10	8.88 ± 0.22	8.99 ± 0,16	8.86 ± 0.21	35.67 ± 9.79
10^6	10.15 ± 0.12	9.71 ± 0.13	9.35 ± 0.34	9.71 ± 0,29	9.68 ± 0.34	11.0 ± 2.94

Abbreviations: CF^+ = culture filtrate derived from *M. tuberculosis* H37Rv; + Tween 80 = CF was prepared in 7H9 media supplemented with Tween 80; – Tween 80 = CF was prepared from media deficient in Tween 80; CFU = colony forming units; TNTC = too numerous to count. Data represent the average of three independent experiments.

Preliminary analysis in axenic cultures illustrated that the greatest improvement in MGIT TTP was obtained following supplementation of the MGIT with CF prepared from bacterial strains grown in the presence of Tween 80. Figure 6.4 and Table 6.3 indicate that at a 10^3 inoculum density a significant decrease in time to positivity (TTP) was observed in all four modified MGITs when compared to the control ($P < 0.05$ and 0.01, students paired t-test). A non-significant decrease was observed it 10^4 and 10^5 inoculum densities. A significant decrease in TTP was observed in the two 7 mL CF-supplemented MGITs at the 10^6 inoculum density [$P = 0.0472$ and 0.0260 for CF^+ and CF^+ (Tween 80), respectively]. Two negative control MGITs (with and without CF-supplementation) were included in each experiment to rule out contamination and CF^+ sterility. Plating of positive MGIT on blood agar incubated for 48 hours confirmed that positive cultures were not contaminated.

Since previous studies found that the greatest change in culture TTP occurred in samples with very low bacterial loads, the experiment was repeated and only 100 μL (as opposed to 500 μL) of axenic culture from each respective serial dilution was inoculated into each MGIT, Figure 6.5 and Table 6.4. Again, the greatest improvement in MGIT TTP (when compared to the control) was observed in the CF^+ (Tween 80) MGIT. Significant differences were observed in the 10^3, 10^4 and 10^5 dilution factors ($P = 0.0091$, 0.0029 and 0.0019, respectively). A significant decrease in MGIT TTP was also observed between the control and CF^+ MGIT at 10^4 and 10^5 inoculum densities ($P = 0.0017$ and 0.0064, respectively). In contrast to experiment 1, reduction in total culture volume (2 mL no CF) did not result in a decrease in MGIT TTP.

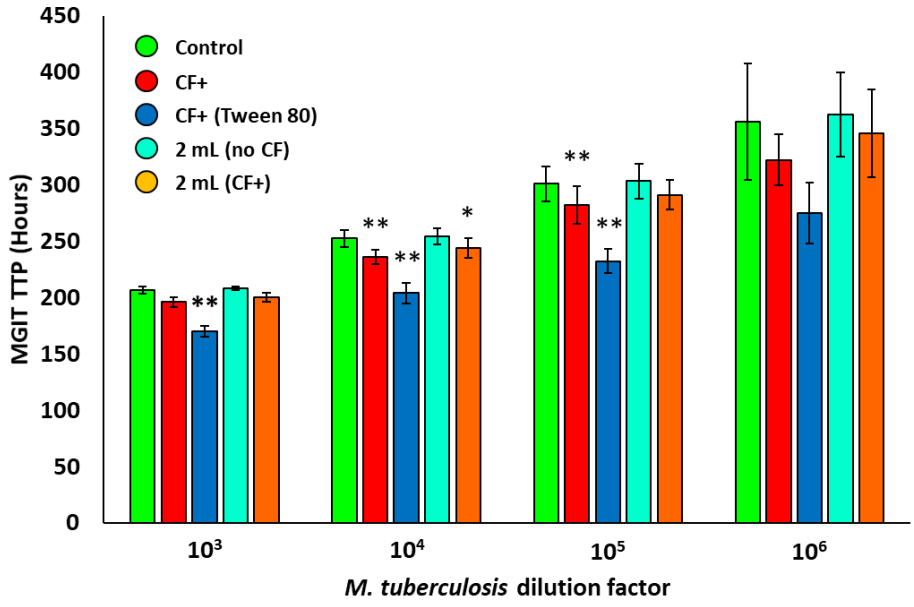

Figure 6.5 Modification of the BACTEC mycobacterial growth indicator tube (MGIT) enhances time to detection in *Mycobacterium tuberculosis* H37Rv, Experiment 2. The culture time to positivity (TTP) in the control MGIT (green) was compared to four experimental MGITs using axenic *M. tuberculosis* H37Rv cultures prepared at different inocula. The control MGIT was unmodified. Modified MGITs consisted of a culture filtrate $(CF)^+$ MGIT (containing 3.5 mL standard media and 3.5 mL CF) (red), CF^+ MGIT containing CF derived from *M. tuberculosis* H37Rv grown in standard 7H9 media supplemented with the detergent Tween 80 (blue), 2 mL (no CF) MGIT (teal) and 2 mL (CF^+) MGIT (containing 1mL standard media and 1 mL CF) (orange). All MGITs were inoculated with 100 µL of prepared bacterial inoculums. The experiment was repeated in triplicate. Error bars represent standard deviations. Statistical significance was determined using the students paired t-test with a 95% confidence interval (CI). * /** Significant with a 95% and 99% CI, respectively (i.e. P<0.05 and P<0.01).

Table 6.4 MGIT modification by culture filtrate supplementation and volume reduction reduces time to detection in axenic *Mycobacterium tuberculosis* cultures, Experiment 2.

	Days of culture (mean ± SD)					
***M. tuberculosis* dilution factor**	**Control**	**CF+ (-Tween 80)**	**CF+ (+Tween 80)**	**2 mL (no CF)**	**2 ml CF+ (- Tween 80)**	**CFU (mean ± SD)**
10^3	8,60 ± 0,13	8,17 ± 0,18	7,07 ± 0,20	8,67 ± 0,07	8,35± 0,17	TNTC
10^4	10,51 ± 0,31	9,83 ± 0,26	8,50 ± 0,38	10,60 ± 0,31	10,17 ± 0,36	253 ± ND
10^5	12,54 ± 0,65	11,75 ± 0,70	9,68 ± 0,46	12,64 ± 0,64	12,13 ± 0,54	84 ± 50.3
10^6	14,83 ± 2,15	13,42 ± 0,94	11,44 ± 1,13	15,10 ± 1,56	14,42 ± 1,62	18.7 ± 13.0

Abbreviations: CF^+ = culture filtrate derived from *M. tuberculosis* H37Rv; + Tween 80 = CF was prepared in 7H9 media supplemented with Tween 80; – Tween 80 = CF was prepared from media deficient in Tween 80; CFU = colony forming units; TNTC = too numerous to count. Data represent the average of three independent experiments.

6.5.2 Patient samples

The participant disposition flow chart is given in Figure 6.6. One hundred and twenty patients were recruited into the study from which, 119 samples were processed. One patient was a screen failure. Thirty five, 15 and 69 sputum samples from these patients were processed according to protocols one, two and three respectively. As mentioned previously, these protocols were iterative attempts to improve the study outcome by modification of the methods. The patient demographics are shown in Table 6.5.

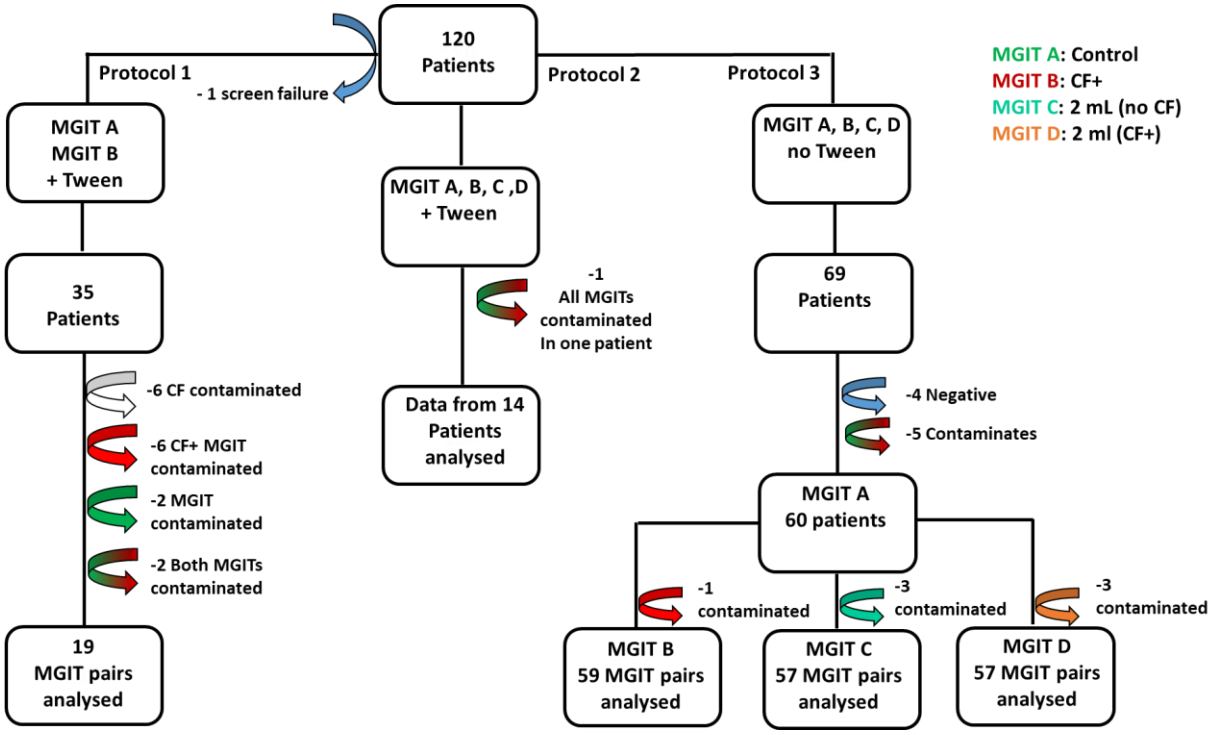

Figure 6.6 Patient disposition flow chart. A total of 120 patients were recruited into the study, of which one was classified as a screen failure. These patients were treatment naïve, HIV-positive individuals with a strong indication of TB disease through a positive GeneXpert result. Sputum samples from 35, 15 and 69 patients were processed according to protocols one, two and three, respectively. In the first protocol, culture was performed in two mycobacterial growth indicator tubes (MGIT) [one control and one supplemented with culture filtrate (CF)] for each patient. Arrows indicate MGIT pairs that were excluded due to CF or MGIT contamination. A total of 19 pairs were available for analysis. In protocol two, culture was performed in one control MGIT and three modified MGITs, designated B, C and D. MGITs C and D had a reduced total volume of 2 mL, with MGIT tube D containing a 1:1 ratio of MGIT media to CF. CF prepared for protocols one and two was derived from *M. tuberculosis* H37Rv grown in 7H9 media supplemented with 0.05% Tween 80. Protocol 3 was performed in the same manner as protocol two with the exception that CF was derived from *M. tuberculosis* H37Rv grown in 7H9 media that was not supplemented with 0.05% Tween 80.

The majority of the patients recruited were male (63.4%) and the median age of study participants was 38 years (IQR: 32 – 44 years old). Most patients (67.7%) had a normal BMI and the median CD4 T-cell count was 130.5 cells/mm^3. Predominately, spot sputum samples

were collected from patients (83.9%). In some cases, overnight sputum samples were collected (16.1%). In terms of pathology, the majority of patients did not present with lymphadenopathy (92.5%). Furthermore, in the majority of patients (approximately 40%), no cavities were seen on the chest X-ray. No significant differences in clinical characteristics were observed between patients processed according to protocols one, two and three (Table 6.5).

Table 6.5 Patient demographics

Variable	Overall (n = 93)	Protocol 1 (n = 19)	Protocol 2 (n = 14)	Protocol 3 (n = 60)	P-value
Demographics					
Male, n (%)	59 (63.4)	13 (68.4)	10 (71.4)	36 (60.0)	0.6394 *
Female, n (%)	34 (36.6)	6 (31.6)	4 (28.6)	24 (40.0)	
Age, yr, median (IQR)	38.0 (32.0 – 44.0)	36.0 (33.5 – 43.5)	41.5 (31.0 – 46.8)	38.0 (31.8 – 44.0)	0.4395 †
BMI					
Underweight, n (%)	21 (22.6)	2 (10.5)	3 (21.4)	16 (26.7)	0.1766 *
Normal, n (%)	63 (67.7)	17 (89.5)	10 (71.4)	36 (60.0)	
Overweight, n (%)	9 (9.7)	0 (0.0)	1 (7.1)	8 (13.3)	
Median (IQR), kg/m^2	20.4 (18.5 – 22.7)	19.8 (18.5 – 21.1)	19.5 (18.7-20.8)	21.0 (18.4 – 23.3)	0.1894 †
Immunology cells/mm3, median (IQR)	130.5 (61.5 – 274.3)	104.0 (51.5 – 181.0)	271.0 (158.0 – 281.0)	131.0 (61.5 – 310.5)	0.2320 †
Sample					
Spot	78 (83.9)	16 (84.2)	12 (85.7)	50 (83.3)	0.9755 *
Overnight	15 (16.1)	3 (15.8)	2 (14.3)	10 (16.7)	
Lung Pathology					
Lymphadenopathy Yes	7 (7.5)	1 (5.3)	1 (7.1)	5 (8.3)	0.8989 *
No	86 (92.5)	18 (94.7)	13 (92.9)	55 (91.7)	
Cavitation 1	37 (39.8)	8 (42.1)	7 (50.0)	22 (36.7)	0.1574 *
2	24 (25.8)	8 (42.1)	4 (28.6)	12 (20.0)	
3	17 (18.3)	3 (15.8)	2 (14.3)	12 (20.0)	
ND	15 (16.1)	0 (0.0)	1 (7.1)	14 (23.3)	

Definition of abbreviations: n = number; yr = year; BMI = body mass index; IQR = interquartile range; ND = note done. For cavitation, 1: absent, 2: single or multiple cavities with diameter of <4cm in aggregate and 3: single or multiple cavities with diameter ≥4cm in aggregate. * P-values were determined using the chi-squared test for proportions with a 95% confidence interval; † P-values were determined using analysis of variance (ANOVA) with a 95% confidence interval.

6.5.2.1 Change in MGIT TTP in patient samples processed by protocol one

6.5.2.1.1 Contamination

Out of the initial 35 samples processed, data from 19 MGIT pairs were available for analysis. Reasons for the exclusion of 16 samples included a high contamination rate that was observed in the initial 22 samples processed, which suggests a possible non-specific resuscitative effect of the CF that may have stimulated the growth of other organisms present in the sputum. These organisms would ordinarily be suppressed by the addition of the antibiotic mixture, PANTA. Whilst AFB were observed in the ZN stain, the presence of a contaminant as detected by blood agar and the ZN stain made it impossible to include the MGIT TTP in the analysis as these tubes most likely flagged positive due to the presence of a contaminant as opposed to the *M. tuberculosis*. A contamination rate of 27.3% was observed in CF-supplemented MGIT tubes versus 9.1% in un-supplemented MGIT tubes. During each batch of processing, a PANTA control and CF control tube were included to ensure the sterility of the media (see section 6.4.2. and Appendix 5A). These quality controls were negative, suggesting that the contamination was most likely due to other micro-organisms in the sputum samples (ordinarily suppressed by PANTA) and not introduced during the preparation of the MGIT test and control tubes.

To address the high rates of contamination, the concentration of PANTA added to both the test and the control MGIT tube was increased. The contamination rates decreased from 27.3% to 11.6% in the CF- supplemented MGIT tube and from 9.1% to 3.8% in the un-supplemented MGIT tube. The improved contamination rates were just above and within the acceptable range that is given at up to 8% for liquid media, respectively (Siddiqi and Rüsch-Gerdes, 2006).

6.5.2.1.2 Changes in MGIT time to positivity following CF supplementation

To investigate the changes in TTP between the control and the CF- supplemented MGIT, the number of days and hours to positivity was converted to the total number of hours taken to flag positive for each tube (Total hours = χ number of days x 24 + χ number of hours). These results are given in Figure 6.7 for each individual patient and overall data is summarized in Table 6.6. Next, the change in TTP was calculated using the following equation: ΔTTP = TTP control – TTP CF^{+}.

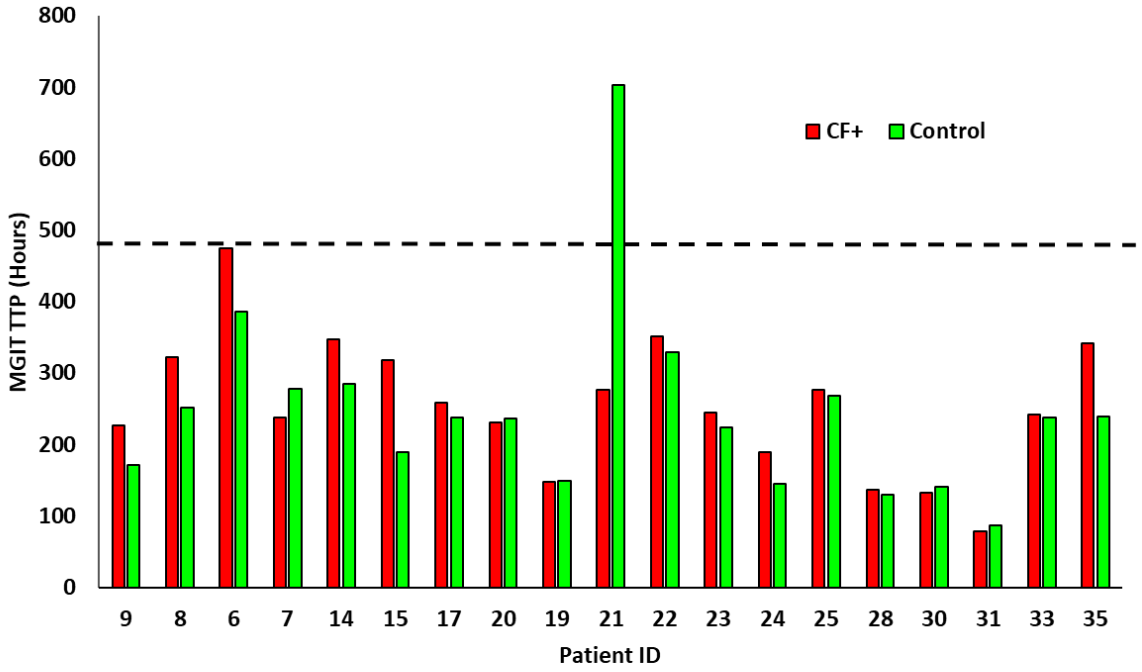

Figure 6.7 The effect of culture filtrate on the time to positivity in clinical samples analysed in protocol 1. In the experimental group (red bars), mycobacterial growth indicator tubes (MGIT) were prepared to yield a 1:1 ratio of culture filtrate (CF) to standard MGIT media (total volume 7 ml before the addition of PANTA reconstituted in OADC). The control MGIT (green bars) was cultured using standard laboratory procedures. The time to positivity (TTP) of the cultures are depicted in days. The dashed line indicates 20 days.

Figure 6.7 illustrates the hours to positivity in control and CF^+ supplemented MGIT tubes. For the most part, a marginal increase in TTP was observed following the addition of CF^+ to the MGIT (observed in 13 out of 19 patients). The median increase in TTP in these 13 patients was 40.0 hours (IQR: 20.0; 70.0). The remaining six patients showed a median decrease of 8.5 (IQR: 5.8; 32.3) hours in TTP following supplementation with CF^+. Interestingly, the patient who showed the most dramatic decrease in TTP (Patient 21) had a TTP of greater than 20 days in the control MGIT. The dotted horizontal line in Figure 6.7, denotes a TTP of greater than 20 days. These results are in agreement with that reported in previous studies where a significant decrease in TTP can only be seen in a low starting inoculum load (Huang et al., 2014, Wu et al., 2008, Sun and Zhang, 1999)

Table 6.6 Culture growth in conventional and CF-supplemented MGITs

Variable	Control MGIT	CF+ MGIT	P- value
Mean (SD), hours	246.6 (132.9)	254.1 (94.6)	**0.0457**
Median (IQR), hours	238.0 (149.0 – 278.0)	244.0 (189.0 – 322.0)	
Minimum, hours	86.0	78.0	
Maximum, hours	703.0	474.0	

Definition of abbreviations: CF = culture filtrate; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation and IQR = interquartile range. To determine statistical significance between the two groups, the Wilcoxon signed-ranks test was used with a 95% confidence interval.

The overall TTP in the control versus CF^+ supplemented MGIT tubes is given in Table 6.6. In the control MGIT, a median TTP of 238.0 hours (IQR: 149.0 – 278.0), equivalent to 9.9 days was obtained versus 244.0 hours (IQR: 189.0 – 322.0) in the CF^+ MGIT, equivalent to 10.2 days. This result was found to be significant (P = 0.0457; Wilcoxon signed-ranks test). The maximum number of hours was reduced in the CF- supplemented test, due to a decrease in TTP in MGIT cultures with low starting bacterial load. The result was in contrast to the predicted hypothesis and to further investigate it, DCTB were assessed in the corresponding samples.

6.5.2.1.3 Are the changes in TTP associated with the presence of CF-dependent bacteria?

To assess whether the increase/decrease in TTP following CF-supplementation was associated with CF-dependency, the resuscitation index (RI – MPN/CFU) for each patient was calculated using the following equation: log (CF^+ MPN/CFU). Figure 6.8 indicates that the majority of patients analysed (17/19) harboured CF-dependent DCTB. Only two patients had no DCTB, where the CFU obtained was greater than the MPN. These results suggest that the observed changes in TTP are not associated with the stimulation of non-culturable organisms, but rather accelerate the growth where few *M. tuberculosis* bacilli are present. It is however noteworthy, that the sputum sample that benefitted the most from CF-supplementation in reducing TTP in MGIT, from patient 21, displayed the highest quantum of DCTB.

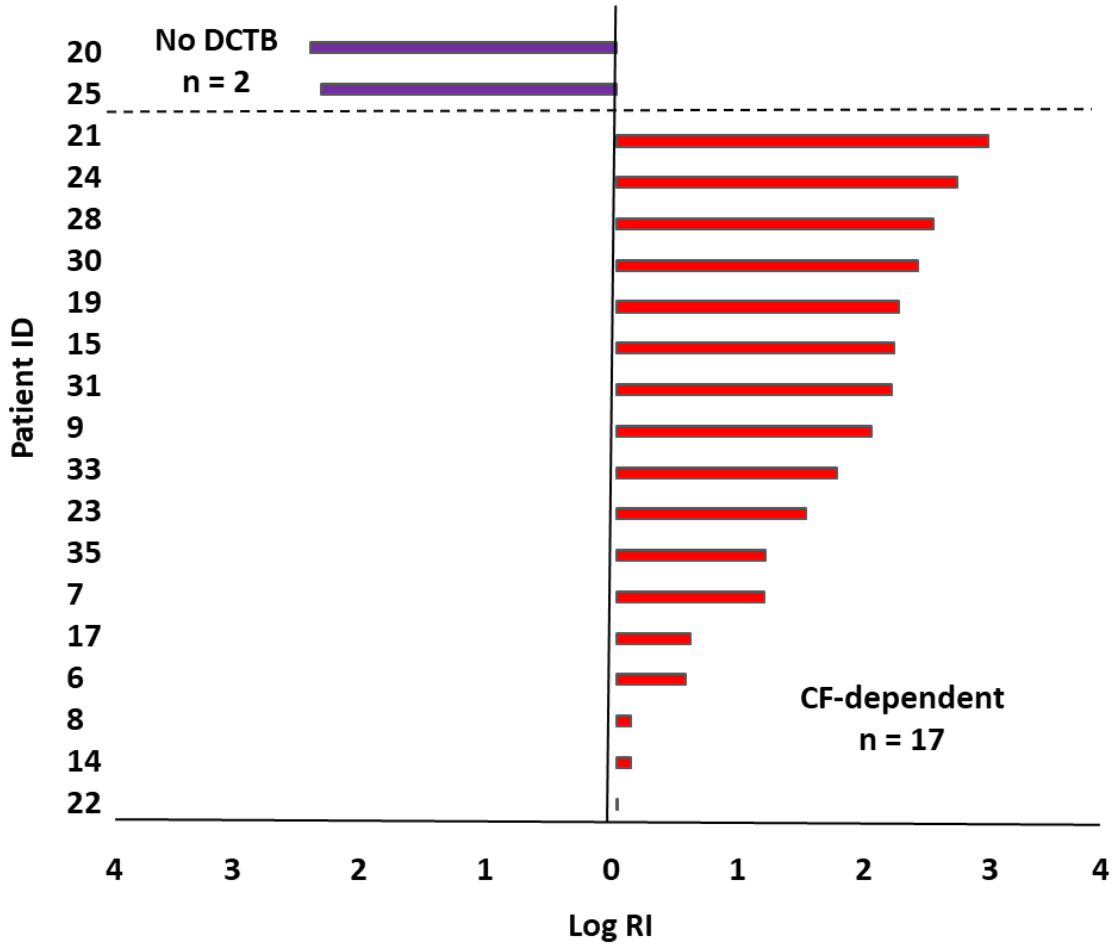

Figure 6.8 Distribution of CF-dependent bacteria in a cross sectional group of patients with tuberculosis. Individual patients with their relative proportions of CF-dependent bacteria are represented on the *y-axis*. CF-dependency is reported using the resuscitation index (RI) using the following equation: RI = Log (CF^+ MPN/CFU), reflected in red. In instances where no colony forming units (CFU) were obtained, a value of 1 was used as the denominator. In two patients (25 and 20), no CF-dependent bacteria were cultured in the most probable number assay (MPN), but CFUs were isolated on solid media.

6.5.2.2. Change in MGIT TTP in patient samples processed by protocol two

In contrast to the hypothesis, the addition of CF^+ to the MGIT using the first protocol resulted in an overall increase in culture TTP. Conversely, bacterial load measured by the MPN and CFU assays illustrated that the majority of clinical samples tested contained a population of CF-dependent DCTB. To investigate these discrepancies two additional MGITs with a lower culture volume were included in the second protocol. In this case, the hypothesis was that a reduction in culture volume will enhance growth stimulatory effects of CF.

Overall, the analysis of results from protocol two indicated an increase in TTP in all three of the modified MGITs when compared to the control which was unexpected, Figure 6.9 and Table 6.7. These changes in TTP were significant between the control and the CF^+ and 2 mL

(CF^+) MGITs, where the P-values obtained were 0.0125 and 0.0059, respectively. Hence, a reduction in total MGIT volume also resulted in an increase in TTP and a P-value of 0.0604 was obtained.

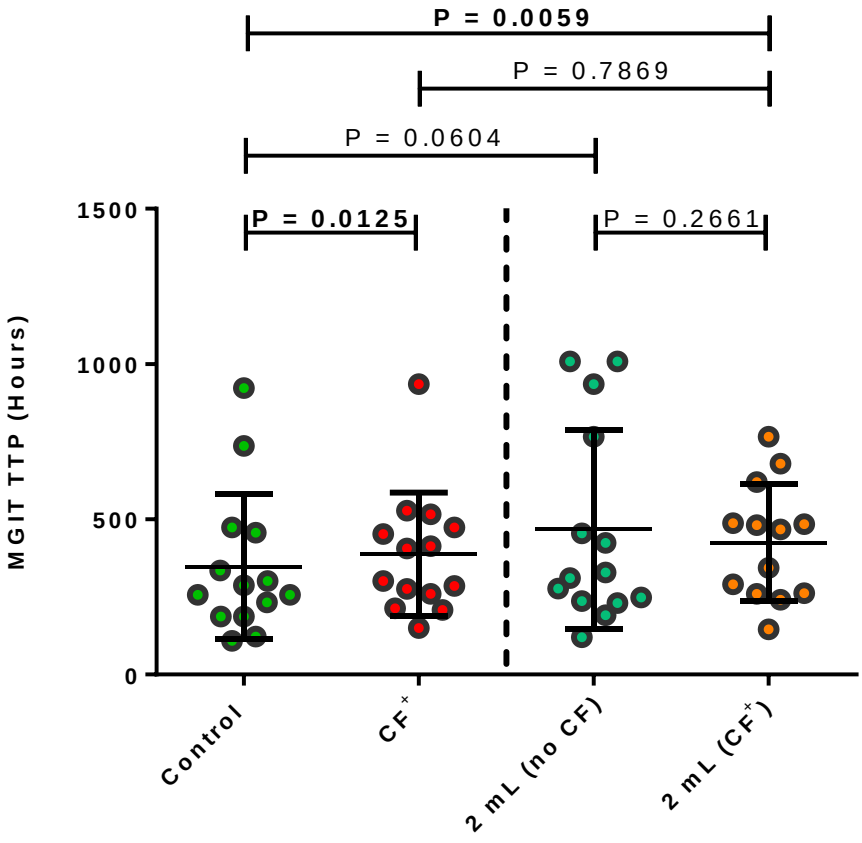

Figure 6.9 Overall culture time to positivity in control and modified mycobacterial growth indicator tubes. The time to positivity (TTP) obtained in the control mycobacterial growth indicator tube (MGIT), performed under standard laboratory conditions was compared to three modified MGITs in 14 patients. One MGIT TTP in the 2 ml (CF^+) experiment was removed as this culture was contaminated (n = 13). Error bars represent medians and interquartile ranges. To determine statistical significance, the Wilcoxon signed-ranks test was used with a 95% confidence interval. The control MGIT (green) was conducted using standard laboratory procedures. Modified MGITs consisted of the 2 ml [no culture filtrate (CF)] MGIT (teal), CF^+ MGIT (containing 3.5 mL standard media and 3.5 mL CF) (red) and 2 mL (CF^+) MGIT (containing 1mL standard media and 1 mL CF). An overall increase in MGIT TTP was obtained in all three experimental conditions, but the increases observed where not significant (P>0.5).

Table 6.7 Culture growth in control, CF-supplemented and reduced volume MGITs (protocol 2)

Variable	Control n = 14	CF^+ n = 14	2 ml (no CF) n = 14	2 ml (CF^+) n = 13
Mean TTP (SD), hours	347.4 (233.0)	387.0 (198.9)	467.1 (319.7)	425.2 (187.4)
Median TTP (IQR), hours	272.0 (187.0 – 461.3)	353.5 (248.3 – 484.5)	320.0 (253.3 – 808.3)	467.0 (261.0 – 553.5)
Minimum TTP, hours	108.0	150.0	120.0	146.0
Maximum TTP, hours	923.0	935.0	1008.0	766.0

Definition of abbreviations: CF = culture filtrate; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation and IQR = interquartile range

In the case of protocol two, two patients had a control MGIT with recorded TTPs that were greater than 20 days. *M. tuberculosis* was not detected in either of these patients by the GeneXpert assay, both were smear negative and no bacteria were seen in the MPN or CFU assays. The TTPs were 38 days and 30 days for patient 38 and 47, respectively. In patient 38, the TTP was reduced to 11 days in the 2 mL (CF^+) tube; however, no AFB was observed on the ZN slide, TBc Id test was negative and growth was present on the blood agar, showing that the reduction in TTP was likely due to a contaminating organism. The TTP obtained from this MGIT was removed from analysis and only 13 samples were analysed in the 2 mL (CF^+) experiment. Results from patient 47 showed that the TTP was reduced in both CF-supplemented MGITs. A reduced TTP of 18 and 28 days was obtained in the 7 mL and 2 mL MGITs, respectively. The TBc ID test was positive, blood agar was negative and AFB was observed on the ZN slide. These data suggested again that the largest benefit in CF-supplementation of MGIT culture should occur in samples with low bacterial loads.

6.5.2.3 Change in MGIT TTP in patient samples processed by protocol three

The results obtained from protocols one and two were unexpected, as the CF^+ appeared to have an inhibitory effect on the growth of *M. tuberculosis* in the MGIT format. This was in contrast to observation in the MPN assay, where growth was stimulated. It was hypothesized that the addition of Tween 80 to the 7H9 media from which the CF^+ was derived may have contributed to the increase in MGIT TTP (although this was not the case in axenic samples). To assess the overall changes in TTP between the control and three modified MGIT tubes in protocols one

and two, Tween 80 was removed from the 7H9 media that the *M. tuberculosis* H37Rv bacterial strains were grown in. The remaining 69 patients recruited were processed following protocol three. Here, the hypothesis was that removal of Tween 80 will result in clumping of bacteria, which would then settle to the bottom of the MGIT tube. The fluorescent dye in MGIT tubes is retained at the bottom of the tube in a silica matrix. If bacteria settle to the bottom quickly, this may enhance TTP in CF-supplemented tubes. Figure 6.10 and Table 6.8 illustrate that the addition of CF^+ to the MGIT, in this format, decreases the overall TTP. In the control MGIT, a median TTP of 274.5 hours (IQR: 163.3 – 392.3), equivalent to 11.4 days was obtained versus 250.0 hours (IQR: 177.0 – 370.0), equivalent to 10.4 days in the CF^+ MGIT. This result was found to not be significant (P = 0.1558, Wilcoxon signed-ranks test). The result obtained using protocol three were in contrast to that obtained using protocols one and two, where the addition of CF^+ to the MGIT resulted in an overall increase in TTP. It is speculated that while Tween 80 may have had a beneficial effect in reducing the overall TTP in experiments conducted in axenic cultures, it a chaotropic agent and may have an inhibitory effect in *M. tuberculosis* populations derived from sputum samples. Indeed, studies have shown Tween 80 to be toxic to mycobacterial growth in the absence of bovine serum albumin (Meyers et al., 1998, Wayne, 1994, Lyon et al., 1963, Masaki et al., 1990). Alternatively, since Tween 80 is a detergent, this may have prevented bacterial clumping leading to a distribution of bacterial organisms throughout the MGIT tube. This dispersal of organisms may have affected the gradient of oxygen depletion, causing reduced fluorescence and thus an increase in TTP. Further work is required to prove this hypothesis. A significant decrease in TTP was also obtained between the 2 mL (no CF) and 2 mL (CF^+) MGITs where the median TTP decreased from 273.3 hours (11.4 days) to 263.0 hours (11.0 days) (P = 0.0105, Wilcoxon signed-ranks test). While the reduced volumes resulted in an overall increase in TTPs compared to the control, the benefit of CF^+ in reducing TTP is observed between these two MGITs.

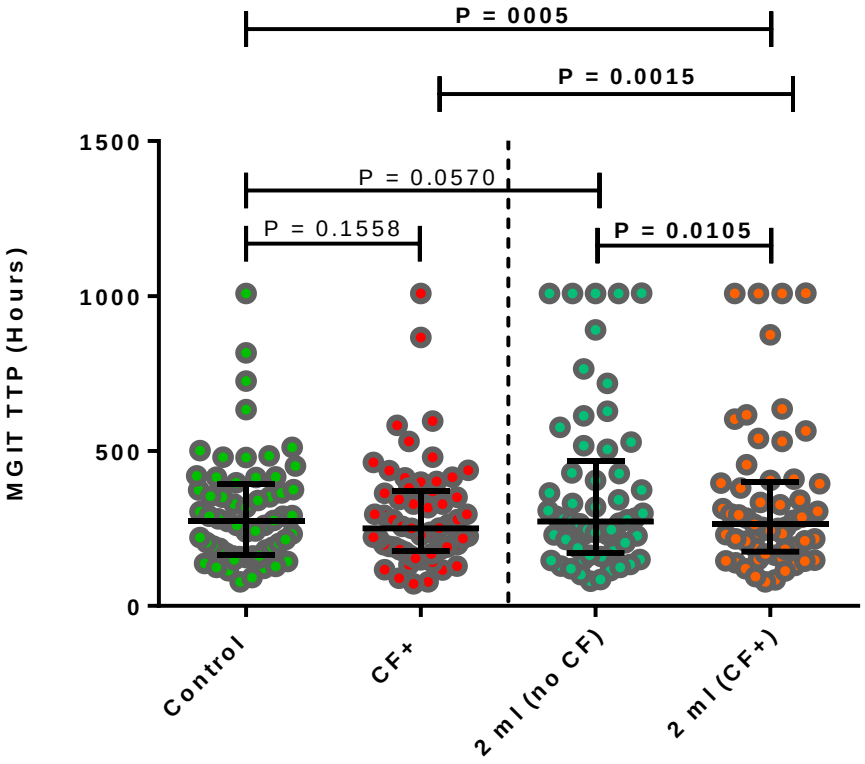

Figure 6.10 Overall time to positivity (hours) in the mycobacterial growth indicator tube (MGIT) with reduced volume and with and without culture filtrate supplementation. Scatterplots depicting mycobacterial growth indicator tube **(**MGIT) time to positivity (TTP) in the overall patient cohort was determined using three different experimental conditions in the MGIT. The control MGIT (green) was conducted using standard laboratory procedures. Modified MGITs consisted of the CF^+ MGIT (red: containing 3.5 mL standard media and 3.5 mL CF), 2 mL [no culture filtrate (CF)] MGIT (teal), and 2 mL (CF^+) MGIT (orange: containing 1mL standard media and 1 mL CF). Error bars represent medians and interquartile ranges. To determine statistical significance, the Wilcoxon signed-ranks was used with a 95% confidence interval. Overall, a significant increase in mean TTP was observed between the control and 2 mL (CF^+). A non-significant decrease in mean TTP was observed between the control and CF^+ MGIT.

Table 6.8 Culture growth in control, CF-supplemented and reduced volume MGIT tubes for all patients (protocol 3)

Variable	Control n = 60	CF^+ n = 59	2 mL (no CF) n = 57	2 ml (CF^+) n = 57
Mean (SD), hours	305.9 (178.9)	291.0 (174.7)	364.8 (266.9)	340.0 (243.2)
Median (IQR), hours	274.5 (163.3 – 392.3)	250.0 (177.0 – 370.0)	273.3 (170.5 – 467.0)	263.0 (175.5– 400.5)
Minimum, hours	78.0	71.0	80.0	78.0
Maximum, hours	1008.0	1008.0	1008.0	1008.0

Definition of abbreviations: CF = culture filtrate; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation and IQR = interquartile range

The association between culture volume and TTP was also investigated. In contrast to what was observed in axenic cultures (experiment 1), a reduction in the total MGIT volume (7 mL to 2 mL prior to the addition of PANTA/OADC) resulted in an overall increase in TTP, i.e. average 364.8 hours versus 305.9 hours. It was initially hypothesized that a reduction in MGIT volume may have resulted in a reduction in TTP due to increased quorum sensing. Indeed, this reduction in TTP was observed in axenic cultures, albeit marginal. It is suspected that this increase in TTP may be due to a reduction in the amount of nutrients available in reduced volume MGITs, particularly the 2mL (CF^+) MGIT. Alternatively the increase in TTP may result from the increase in concentration of inhibitory factors present in sputum. The inhibitory activity of sputum against *M. tuberculosis* growth in liquid media has been previously described (Mukamolova et al., 2010). The observation that un-supplemented MPN counts (i.e. MPN no CF) were lower than CFUs led to hypothesis that cell-associated inhibitory activity is present in sputum. When *M. tuberculosis* H37Rv cultures were inoculated into low-positive patient sputum samples (rendered negative by freezing) and incubated at 4ºC for 24 hours, substantial growth inhibition was observed in the un-supplemented MPN assay (Mukamolova et al., 2010). Currently it is not known what factors are responsible for the observed growth inhibition; however, secreted leukocyte protease inhibitors on the surface of sputum derived *M. tuberculosis* cells have been suggested as a possible explanation (Gomez et al., 2009, Mukamolova et al., 2010).

As the preliminary findings indicated that the greatest change in TTP was observed in patients with a low bacterial load, auramine smear grade was correlated with MGIT TTP obtained in both the control MGIT and the CF^+ MGIT, Figure 6.11. Pearson's and Spearman's correlation analysis revealed a significant negative correlation between auramine staining score and control and CF^+ MGIT TTP (r_p = -0.6968 and -0.5342, P>0.0001; r_s = -0.7926 and -0.6363, P>0.0001). Linear regression analysis revealed a poorer linear relationship between auramine staining score and CF^+ MGIT TTP compared to the control MGIT TTP (R^2 = 0.28 versus 0.48). Following CF-supplementation, regression analysis revealed a decreased in both the slope and the y-axis intercept. The average TTP across staining scores was reduced in smear negative patients only (19.38 days versus 16.59 days). Following this observation, patients were stratified into auramine smear-positive and smear–negative. A total of 22/69 patients processed by protocol three were smear negative. Four of these patients had negative cultures in all four MGITs and were removed from analysis. In addition, contaminated cultures were removed from analysis.

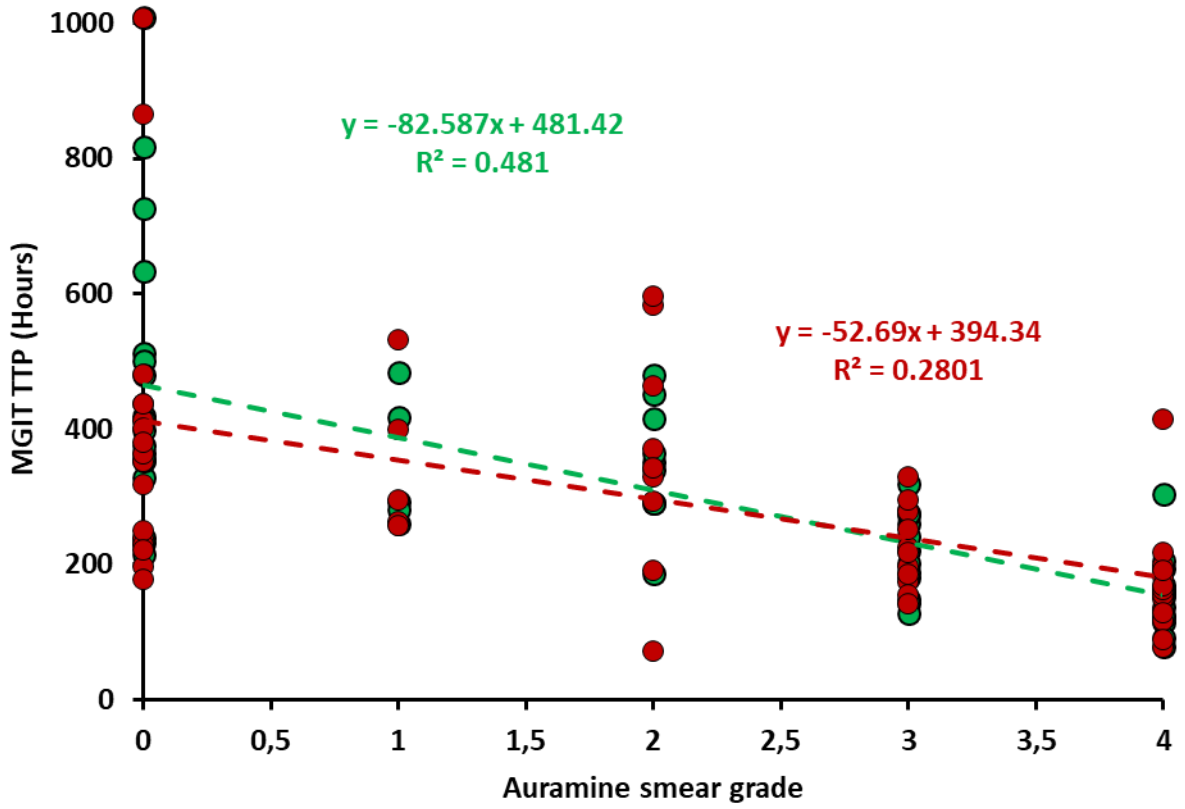

Figure 6.11 Increase in culture positivity is correlated with decreasing smear grade. Culture positivity obtained using the Mycobacterial growth indicator tube (MGIT) was correlated with auramine smear grade. Time to positivity was plotted for both the control (green) and culture filtrate (CF) supplemented (red) MGIT. P+++, P++, P+, scanty and negative smear grades were quantified as 4,3,2,1 and 0 respectively. The average TTP of P+++, P++, P+, scanty and negative auramine staining scores was 6.48, 8.96, 14.66, 14.45 and 19.38 days in the control MGITs, respectively. The average TTP of P+++, P++, P+, scanty and negative auramine staining scores was 7.00, 9,47, 16.49, 14.56 and 16.59 days in the CF+ MGITs, respectively A significant negative linear relationship was obtained between auramine smear grade and time to positivity in the control and CF^+ MGIT systems (R^2 = 0.48 and 0.28, respectively. Pearson's and Spearman's correlation, P<0.0001, 95% confidence interval).

In smear negative patients, the TTP was reduced in the CF^+ MGIT when compared to the control with median TTPs of 364.0 and 406.0 hours, respectively (P = 0.2788, Wilcoxon signed-ranks test). In terms of the reduced volume MGIT culture, TTP was non-significantly reduced in the 2 mL (CF^+) MGIT when compared to the 2 mL (no CF) MGIT, with median TTPs of 396 versus 529 hours, respectively (P = 0.7869, Wilcoxon signed-ranks test).

Table 6.9 Culture growth in control, culture filtrate-supplemented and reduced volume MGITs in smear negative patients

Variable	Control n = 18	CF^+ MGIT n = 17	2 mL (no CF) n = 16	2 mL (CF^+) n = 15
Mean (SD), hours	465.0 (211.2)	398.2 (224.7)	581.7 (294.2)	491.6 (276.7)
Median (IQR), hours	406.0 (346.3 – 542.5)	364.0 (233.0 – 542.5)	529.0 (325.0 – 949.0)	396.0 (263.0 – 617.0)
Minimum, hours	214.0	177.0	226.0	154.0
Maximum, hours	1008.0	1008.0	1008.0	1008.0

Definition of abbreviations: CF = culture filtrate; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation and IQR = interquartile range

To further investigate the effect CF^+ in reducing MGIT TTP in samples with a low bacterial load, five patients that qualitatively had a 'low' Gene Xpert report (Ct value range: 22 to 28) were subsequently removed from analysis. Therefore, additional analysis was conducted on smear negative individuals who had 'very low' GeneXpert CT (i.e. cycle threshold >28) values and on samples where *M. tuberculosis* was not detected using the GeneXert. A non-significant decrease in TTP was observed in the 7 mL and 2 mL supplemented MGITs (median TTP: 407.5 and 468.0 hours) when compared to the control and 2 mL (no CF) MGITs (median TTP: 480.0 and 613.0 hours) (P = 0.1968 and 0.1309, Wilcoxon signed-ranks test), Table 6.10 and Figure 6.12A. In one patient, the addition of CF^+ to both the 7 mL and 2 ml MGITs showed an increase in TTP compared to the respective controls (Figure 6.13 and Table 6.11). As this effect was in contrast to what was observed in the other samples, it was suspected that a laboratory error may have occurred and these tubes may have been mislabelled. When this patient was removed from analysis, the decrease in TTP between the control and CF^+ MGIT in both 7 mL and 2 mL MGITs approached significance (Figure 6.12 B; P = 0.0635 and 0.0742, Wilcoxon signed-ranks test).

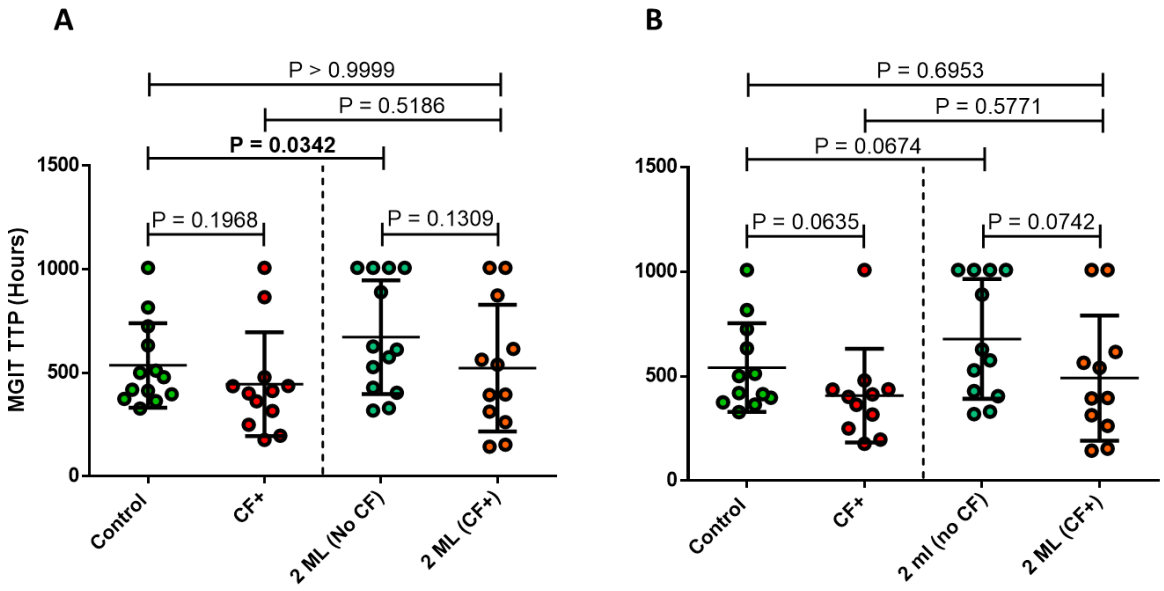

Figure 6.12 Time to positivity in control and modified mycobacterial growth indicator tubes in smear negative and GeneXpert very low/negative patients. Scatterplots depicting MGIT time to positivity (TTP) in patients that were both smear negative and had a very low or negative Gene Xpert test was determined using four different experimental conditions in the Mycobacterial growth indicator tube (MGIT). (A) Data includes patient 83. (B) Patient 83 was removed from analysis. Error bars represent medians and interquartile ranges. To determine statistical significance, the Wilcoxon signed-ranks test was used with a 95% confidence interval. The control MGIT (green) was conducted using standard laboratory procedures. For the modified MGITs, one standard volume MGIT (7 mL) containing a 1:1 ratio of MGIT media and culture filtrate (CF) was prepared (red). The volumes of two MGITs were reduced to 2 mL, one with no CF (teal) and the other containing a 1:1 ratio of MGIT media and CF (orange). The dotted line separates MGITs with and without CF-supplementation. Significance with a 95% confidence interval is depicted in bold ($P<0.05$).

Table 6.10 Culture growth in control, culture filtrate-supplemented and reduced volume MGIT in smear negative patients with GeneXpert very low/*M. tuberculosis* not detected.

Variable	Control n = 13	CF^+ MGIT n = 12	2 mL (no CF) n = 13	2 ml (CF^+) n = 11
Mean (SD), hours	536.8 (203.7)	445.8 (250.9)	673.3 (274.7)	523.3 (305.8)
Median (IQR), hours	480.0 (386.5– 680.0)	407.5 (266.8 – 469.5)	613.0 (417.0 – 1008.0)	468.0 (275.8 – 810.5)
Minimum, hours	329.0	177.0	319.0	145.0
Maximum, hours	1008.0	1008.0	1008.0	1008.0

Definition of abbreviations: CF = culture filtrate; MGIT = mycobacterial growth indicator tube; TTP = time to positivity; SD = standard deviation and IQR = interquartile range. Data includes patient MP83.

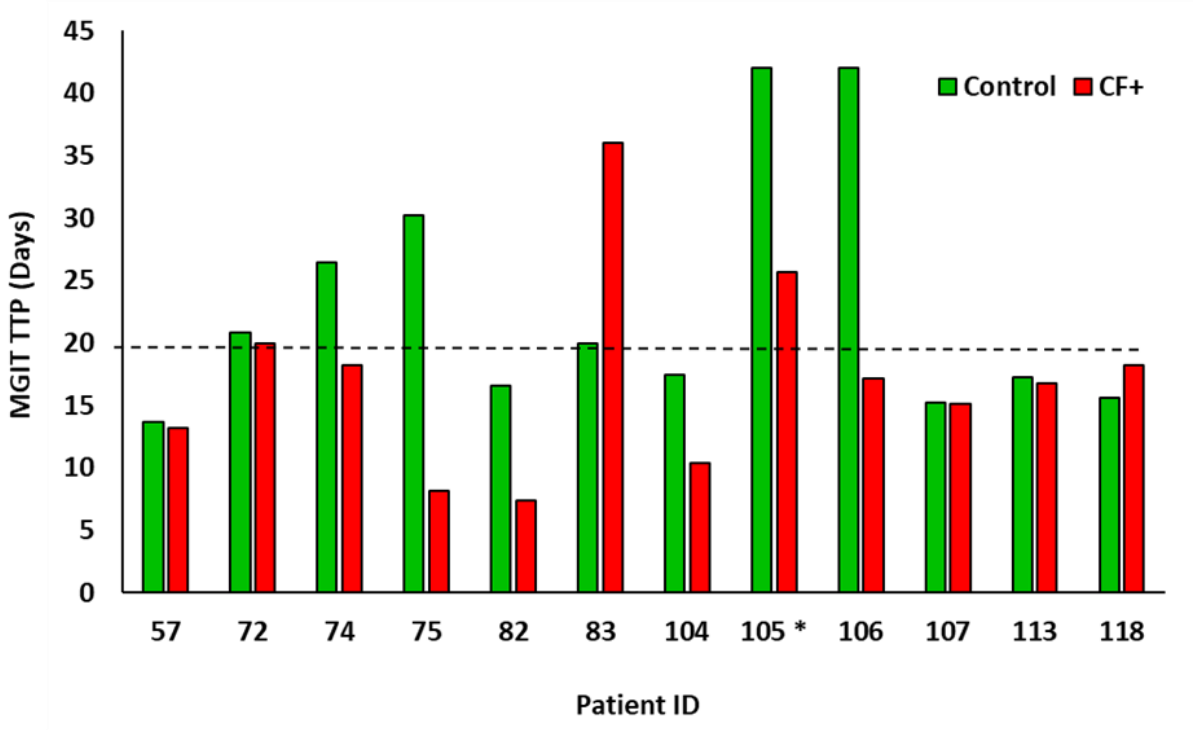

Figure 6.13 The effect of the addition of culture filtrate on the time to positivity in smear negative and Gene Xpert very low/negative clinical samples. In the experimental group, MGIT tubes were prepared to yield a 1:1 ratio of culture filtrate (CF) to standard MGIT media (total volume 7 mL before the addition of PANTA reconstituted in OADC). The control MGIT (green bars) was cultured using standard laboratory procedures. The time to positivity (TTP) of the cultures are depicted in days. The dashed line indicates 20 days. * Patient 105: the TTP from the 2 mL CF+ MGIT (red) is represented relative to the control. All other samples show the control and CF^+ MGIT pairs.

Figure 6.13 illustrate the TTP obtained in the BACTEC 960 culture system in control and CF^+ MGITs (both 7 mL MGITs, with the exception of 105 where the result from the 2 mL CF^+ MGIT is used) in 12 smear negative patients with GeneXpert results that were qualitatively very low or *M. tuberculosis* negative. For patient 105, the 2 mL CF^+ TTP was used as the CF^+ MGIT was negative after 42 days incubation. This MGIT was positive for MTBC using the TBc ID test and no contamination was present on the blood agar plate. Patients showing the greatest decrease in TTP following CF-supplementation included patients 74, 75, 82, 104, 105 and 106. As previously discussed, the addition of CF^+ increased the TTP in patient 83 which may have possibly occurred due to a laboratory error.

Next the association between change in TTP and the presence of DCTB was investigated (Table 6.11, page 276). Patients showing the greatest change in TTP (i.e. 74, 75, 82, 83, 104, 105 and 106) all had very low Gene Xpert results (i.e. CT value >28) or *M. tuberculosis* was not detected using this assay. In addition, the majority of these patients had no CFUs, and the

three that did had less than 10 platable bacilli per mL. Furthermore, the RI was calculated to determine the quantum of resuscitatable bacteria in the sputum sample, calculated as log (CF^+ MPN/CFU). In most cases, the patients showing the greatest culture acceleration following supplementation of the MGIT with CF^+ were negative for both the MPN and CFU assay. In three patients, however, the calculated RI was below 1, indicating the presence of very few CF^+ dependent bacilli. In one patient (106) the addition of CF to the MGIT was able to stimulate the growth of *M. tuberculosis* and yield a positive MGIT culture where the control sample was negative. The GeneXpert and MPN for this patient was negative, but 1 single *M. tuberculosis* colony emerged on one of the two neat solid media plates, predicting approximately 5 bacilli per mL of sputum.

In contrast, smear negative patients that did not show a drastic change in TTP (i.e. 55, 56, 72, 88, 107, 110, 113, 115 and 118) generally harboured more than 35 bacteria per mL as determined by CFU (with the exception of patient 113). DCTB were also present in patients where an increase in TTP was obtained following CF-supplementation (110, 115 and 118). These findings suggest that growth factors present in CF^+ are more likely to have an effect in the BACTEC MGIT 960 system by accelerating the growth of few viable organisms present in sputum as opposed to resuscitating non-culturable organisms.

To further assess whether the reduced TTP observed in the CF^+ MGIT was due to accelerating the growth of few viable organisms and not from resuscitation of non-culturable bacilli, weekly growth in CF-supplemented and un-supplemented MPN assays from 31 patients was assessed over six weeks. After one week's incubation at 37 °C, the average log bacterial growth in the CF^+ assay was 3.2 versus log 1.3 in the un-supplemented MPN assay. After two weeks, the quantum of organisms in the CF^+ and un-supplemented MPN assays was log 7.7 and log 3.5, respectively. By the third week, on average growth in the CF^+ MPN had reached log 8.2 versus log 7.7 in the un-supplemented assays. At the end of the six week incubation period, the growth in the un-supplemented MPN assay was similar to that obtained in the CF^+ MPN assay (log 8.2 versus log 8.1). It is however important to acknowledge that interpretation of these results are limited due to the upper limit of log 8.7 in the MPN assay; Nevertheless, these results show an accelerated growth in the CF-supplemented MPN compared to the un-supplement MPN assay during the first few weeks of growth.

Table 6.11 Change in MGIT TTP is associated with low sputum bacterial load rather than the presence of differentially culturable tubercle bacteria in smear negative patients

Patient No.	ΔTTP (hours)	GeneXpert		Log MPN		Log CFU	Log RI
		Qualitative	Ct value (Average)	CF^+	Media (No CF)		
106	595	MTB ND	NA	0	0	0.70	No DCTB
75	529	Very low	31.5	0	0	0	0
105*	391	MTB ND	NA	0.85	0.85	0	0.85
82	221	Very low	30.98	0	0	0	0
74	196	MTB ND	NA	0	0	0	0
104	170	MTB ND	NA	1.25	0	1.00	0.25
72	21	MTB ND	NA	0	0	1.54	No DCTB
88	21	low	23.58	3.26	0	3.02	0.23
57	12	Very low	29.98	0	0	C	NA
113	12	Very low	31.46	0	0	1.00	No DCTB
56	11	low	27.20	0	0	4.06	No DCTB
107	1	MTB ND	NA	1.93	1.46	1.78	0.15
55	-6	low	25.58	0	0	3.78	No DCTB
110	-8	low	24.24	5.49	4.34	4.29	1.20
115	-27	low	23.86	2.66	0	2.06	0.60
118	-62	Very low	29.78	2,26	0	2,06	0.30
83	-386	MTB ND	NA	0.85	0	0.70	0.15

Definition of abbreviations: MPN = most probable number; CFU = colony forming units; CT = cycle threshold; CF = culture filtrate; MTB ND = *M. tuberculosis* not detected; NA = not applicable; DCTB = differentially culturable tubercle bacilli. Negative values indicate an increase in MGIT TTP following CF-supplementation of the MGIT. Data are arranged according to ΔTTP. * The change in TTP for patient 105 was calculated using the 2 mL CF^+ result.

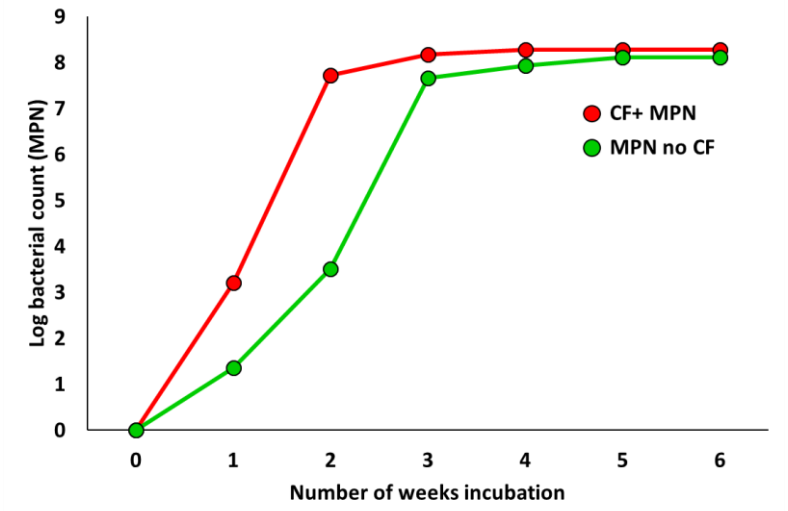

Figure 6.14 Average weekly bacterial growth in culture filtrate-supplemented and un-supplemented most probable number assays in patient samples. The average weekly growth in culture filtrate (CF)-supplemented (red) and un-supplemented MPN assays (green) from 31 patients was recorded. MPN plates were incubated at 37 ºC and scored every seven days for up to six weeks. More bacteria were isolated in the CF-supplemented MPN assays during the first three weeks of incubation.

6.6 DISCUSSION

In resource poor countries where TB is endemic, AFB smear microscopy is widely utilised because it is affordable, easy to perform and has a fast turnaround. While AFB smear-microscopy can be specific, its sensitivity is variable (Ryu, 2015). Furthermore, the high burden of HIV/AIDS has led to an increase in the number of smear-negative pulmonary TB cases, with frequencies of 24 to 61% reported (Getahun et al., 2007). These figures illustrate that smear-negative patients represent a significant caseload of the overall global TB burden (Siddiqi et al., 2003). It is generally accepted that individuals who are smear-positive are more infectious than those that are smear-negative (Hernandez-Garduno et al., 2004). For this reason, the diagnosis and treatment of smear-positive individuals is commonly seen as priority. However patients who are smear-negative are also capable of transmitting TB disease and are associated with at least one-sixth of culture-positive TB episodes (Bicmen et al., 2011, Behr et al., 1999, Hernandez-Garduno et al., 2004, Tostmann et al., 2008, Campos et al., 2016). When compared to smear-positive individuals, patients who are smear-negative or have extra-pulmonary TB disease are more likely to experience treatment delays, hospitalisation and poorer treatment outcomes (Whitehorn et al., 2010, Hargreaves et al., 2001).

Sputum culture remains the gold standard for the diagnosis of TB disease and is 100-fold more sensitive than smear-microscopy (Caulfield and Wengenack, 2016). In addition, the material obtained from positive culture can be used for downstream DST. As liquid culture is faster than culture on solid media, it is generally preferred as it improves patient management and clinical outcomes (Moreira et al., 2015). With regards to growth of clinical *M. tuberculosis* strains, on average 10 days are required for culture positivity in liquid culture compared to 20 to 25 days on solid media (Caulfield and Wengenack, 2016, Pfyffer et al., 1997, Cruciani et al., 2004, Tortoli et al., 1999, Hanna et al., 1999). For these reasons, automated liquid culture systems such as the BACTEC MGIT 960 are widely employed for TB culture and DST (Cruciani et al., 2004, Dheda et al., 2016). MGIT TTP has been shown to correlate directly with bacterial load (as determined by CFUs on solid media) (Bark et al., 2011) as well as smear grade (Olaru et al., 2014). Whilst the BACTEC MGIT 960 instrument has a high sensitivity for the recovery of mycobacteria (Hanna et al., 1999), recent reports have shown that in few cases this system fails to detect mycobacteria at the end of the 42 day incubation protocol (Mahomed et al., 2017, Pang et al., 2016). These false negatives have been attributed to the granular growth pattern of the organism which may result in oxygen consumption remaining below the detection threshold (Mahomed et al., 2017). The sputum decontamination procedure has also been proposed as a

stress that may damage certain organisms resulting in a false negative result in the MGIT (Pang et al., 2016). The above factors highlight the urgent need to develop more timeous and sensitive culture methods to diagnose *M. tuberculosis*. In this study, the addition of CF^+ to the BACTEC MGIT 960 on accelerating the TTP and increasing the sensitivity in HIV-positive individuals was investigated.

The most noteworthy finding of this study was that the addition of CF^+ (derived from *M. tuberculosis* cultures grown in the absence of the detergent, Tween 80), to the MGIT BACTEC 960 system decreased the TTP in smear-negative patients with pulmonary TB. The effect was most prominent in patients that had very low bacterial loads as detected by GeneXpert, or where *M. tuberculosis* was below the limit of detection. Indeed, the majority of patients that benefited from the addition of CF^+, had less than 10 bacteria per mL sputum (as determined by CFU assays) and were negative in CF-supplemented and un-supplemented MPN assays. As, the limit of detection of widely used nucleic acid amplification tests, such as the Gene Xpert is 131 bacteria per mL (Helb et al., 2010), our findings suggest that this method of culture may be particularly useful in identifying patients with disseminated TB disease where bacterial loads in sputum are extremely low and cannot be detected using current molecular methods. As the decrease in TTP did not correlate with the presence of DCTB (as detected by the MPN assay), our data further suggest that we have created an acceleration growth model as opposed to a resuscitative model, or perhaps a combination of both.

In one patient, the sputum was negative for all routine diagnostic tests performed (i.e. smear negative, culture negative and GeneXpert negative); however, *M. tuberculosis* was cultured in the CF^+ MGIT after 17 days incubation. In addition, 1 colony emerged on solid 7H11 media. In terms of quality control, the blood agar was negative showing an absence of bacterial contamination and the MGIT TBc ID test was positive. This phenomenon has been previously described in a case study where an EP specimen was negative by standard laboratory methods, but positive following supplementation of the MPN assay with CF^+ (O'Connor et al., 2015).

Another noteworthy finding of this study was that a reduction in culture volume resulted in a significant decrease in bacterial growth as evident by an increase in MGIT TTP. It was hypothesized that the decrease in culture volume would result in an increase in the concentration of growth inhibitory factors present in sputum, possibly explaining this. Alternatively, the concentration of residual sodium hydroxide from the decontamination

procedure may have affected bacterial growth. A decrease in TTP between the 2 mL no CF and 2 mL (CF^+) tube illustrates that factors present in CF^+ may be able to promote the growth of bacterial organisms in the presence of these inhibitory substances or rescue damaged organisms. These findings are in agreement with previous work (Mukamolova et al., 2010). It was also found that the addition of Tween 80 had a detrimental effect on TTP and removal of this detergent from bacterial cultures from which CF^+ was derived improved bacterial growth. This is an important methodological finding for future work investigating the effect of CF^+ on TTP.

Our study had several limitations. The first was that CF^+ is likely to contain other molecules (other than Rpfs) that may stimulate or inhibit the growth of *M. tuberculosis*. Further work investigating the effect of recombinant Rpf on reducing TTP in a HIV-positive, smear-negative population may prove to have an added benefit in stimulating the growth of *M. tuberculosis*. However, the literature investigating the effect of recombinant Rpf on *M. tuberculosis* growth is limited. The work carried out by Mukamolova et al. (2010) probably provided the strongest evidence, where RpfE was effective at stimulating growth in five out of 25 patients studied. While RpfB was reported to be effective, the data was not provided. Furthermore, the effect of recombinant RpfB and RpfE was not reported in the other 20 patients. The authors also reported that since CF was the most potent, it was used more extensively. Huang et al (2014) added 20 nM recombinant RpfB and RpfE to the BACTEC MGIT 960 to investigate the effect on TTP in 15 patients. Overall, the addition of these proteins was found to improve TTP in only five/12 patient samples. These samples required long culture times to flag positive using standard procedures. For these reasons, the decrease in TTP cannot solely be attributed to Rpfs and further work investigating the responsible growth stimulatory compounds in CF^+ is required

Furthermore, our preliminary analysis indicated an increase in bacterial contamination in the CF-supplemented MGITs most likely due to a non-specific resuscitative effect of Rpfs or other molecules present in CF^+. To address this, we increased the PANTA concentration to twice the original concentration used in standard laboratory practice in both the control (2 mL and 7mL) and CF-supplemented MGITs. A main concern was that an increase in the antibiotic concentration would decrease the recovery and impact the growth of *M. tuberculosis*, although previous reports have shown that the use of MGIT with 2X PANTA reduced overall contamination rates without affecting the culture yield (Peres et al., 2011). In addition, the baseline oxygen concentration may have varied between the control and modified MGITs. As

the BACTEC MGIT 960 measures growth via oxygen fluorescence-quenching technology, these changes may have accounted for the decrease in TTP. For example, a lower baseline oxygen concentration may have been present in the CF-supplemented media due to the growth of the laboratory strain H37Rv in the preparation of CF^+. However, the drastic decrease in TTP in patient samples with low bacterial load in the CF^+ MGIT does not support this hypothesis. The use of recombinant Rpf (or other recombinant identified growth factors) is important in this regard as it would also result in minimal modification of the MGIT and not affect the baseline oxygen concentration.

CONCLUSION/ FUTURE RESEARCH

The results indicate a promising and potential use for CF-supplementation in enhancing the culture detection rate and sensitivity of the currently used culture diagnostic methods such as the BACTEC MGIT 960 assay. Furthermore, the culture-based detection can be enhanced in one of the most vulnerable patient populations (i.e. HIV-positive patients with paucibacillary disease) where TB is difficult to diagnose and subsequently treat. Current TB diagnostic algorithms may therefore benefit by the addition of specialized culture whereby a MGIT containing CF^+ is included for patients that are auramine smear-negative. Research exploring the potential use of CF^+ and recombinant Rpfs is required for a greater cohort of patients with smear negative TB disease where molecular diagnostic tests may not be adequately sensitive to diagnose TB in these patients. Also, the possible application of specialist culture to the timeous diagnosis of TB in patients with sub-clinical disease will have positive outcomes for both the individual patient and reducing the transmission of disease within the community. In terms of feasibility, methods to simplify the addition of CF^+ or recombinant Rpfs to the MGIT is required so that it can be performed routinely in most laboratories. This may include working with biomedical/pharmaceutical companies to commercialise the product, i.e. lyophilized CF that can be added to the MGIT during set-up or producing MGITs that already contain growth stimulatory molecules.

APPENDIX 6A: CULTURE FILTRATE AND MEDIA QUALITY CONTROL

Date Processed	Liquid		7H11			MGIT		PCR
	RV	Media	H37Rv (CF)	Media	H37Rv (strain)	Control	CF+	H37Rv
17.06.2016	Contam	Contam	Contam	Contam	H37Rv	ND	ND	yes
23.06.2016	NG	NG	NG	NG	H37Rv	ND	ND	yes
30.06.2016	NG	NG	NG	NG	H37Rv	ND	ND	yes
07.07.2016	Contam	Contam	Pink Cols	Pink Cols	H37Rv	Negative (CLS)	Negative (CLS)	yes
14.07.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
28.07.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
06.08.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
11.08.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
18.08.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
25.08.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
31.08.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
08.09.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	yes
15.09.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	ND
22.09.2016	NG	NG	NG	NG	H37Rv	Negative (CLS)	Negative (CLS)	ND
06.10.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
13.10.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
21.10.2016	NG	NG	NG	NG	Bacterial Contam	Negative	Negative	ND
27.10.2016	NG	NG	NG	NG	H37Rv	ND †	ND †	ND
10.11.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
17.11.2016	NG	NG	NG	NG	H37Rv	Negative	Positive *	ND
24.11.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
01.12.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
09.12.2016	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
19.01.2017	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
26.01.2017	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
03.02.2017	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
18.02.2017	NG	NG	NG	NG	H37Rv	Negative	Negative	ND
23.02.2017	NG	NG	NG	NG	H37Rv	Negative	Negative	ND

† Not enough culture filtrate (CF) to set up controls; Aliquots of RV CF and media incubated to ensure sterility

*** Results included as RV CF aliquotes were clean. In addition MGIT TTP did not vary between control MGITs and CF+ supplemented MGITs**

ND: not done. H37Rv strain was confirmed by PCR analysis from 07/06/2016 to 08/09/2016.

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Detailed components of this PhD

Background and literature review

CHAPER ONE	CHAPTER TWO
TUBERCULOSIS	MICROBIAL DORMANCY

Original Research Chapters

KEY QUESTIONS?	KEY CONCLUSIONS?
CHAPTER 3 Do all TB patients harbor DCTB? What are the various DCTB populations? Are Rpfs solely responsible for unmasking DCTB? Does host immunity affect the quantum of DCTB?	**CHAPTER 3** Four distinct sub-populations of DCTB observed amongst patients Rpf effect was marginal in unmasking DCTB HIV-negative patients harbored a higher quantum of DCTB at baseline CD4 T-cell count not associated with DCTB
CHAPTER 4 How do DCTB respond to first-line TB treatment compared to conventionally culturable bacteria? Are there potential biomarkers to predict patient response to treatment? Do DCTB remain at the end of treatment?	**CHAPTER 4** Four patterns of decline were described An atypical DCTB presentation was associated with HIV-positive patients DCTB were detected in most patients following treatment completion and DMN-Tre staining confirmed the presence of DCTB
CHAPTER 5 What is the relationship between the MPN assay and currently employed *M. tuberculosis* culture methods? Can the MPN possibly be used in EBA studies? Does CF improve the relationship between the MPN and MGIT TTP in clinical samples?	**CHAPTER 5** Strong correlation between MPN and MGIT TTP as well as CFU in axenic cultures Strong correlation between MGIT TTP and CFU with CF-supplemented MPN in clinical samples Poor correlation between MPN and MGIT TTP in 'atypical DCTB' patient group
CHAPTER 6 Does the addition of CF to the MGIT enhance diagnostic pick-up? Can modification of the MGIT by the addition of CF improve the time to culture positivity? Does a reduction in total MGIT volume improve TTP by enhanced quorum sensing?	**CHAPTER 6** The addition of CF and reduction of total MGIT volume enhances TTP in axenic cultures CF non-significantly enhanced TTP in HIV positive patients – greater effect in smear-negative patients Reduction in total MGIT volume was inhibitory

FINAL SUMMARY

Key findings:

In this thesis, DCTB were characterized in sputum samples from patients with active, drug-susceptible TB disease. In line with previous observations, four distinct populations of DCTB were observed in treatment naïve patients (chapter 3). In most patients, mixed populations of both CF-dependent and Rpf-independent bacteria emerged suggesting that stimulatory molecules, other than Rpfs, are involved in the resuscitative or growth stimulatory process. Furthermore, sputum samples obtained from HIV-negative individuals had a higher quantum of DCTB than HIV-positive patients, suggesting a possible role for host immunity in generating DCTB. In contrast to previous reports, CD4 T-cell count was not correlated with DCTB. To assess how DCTB respond to chemotherapy, patients were followed-up throughout their treatment. Four patterns of DCTB decline were described, namely (i) drug-tolerant populations that resists decline during early treatment, (ii) populations that accumulate during early treatment, (iii) populations that decline rapidly following the onset of treatment and (iv) populations exhibiting an atypical DCTB presentation. Following treatment completion, DCTB were cultured in two thirds of the patient population, suggesting that complete bacteriological sterilization of the lung was not achieved. Whether or not these residual organisms can emerge later to cause relapse disease requires further investigation.

The presence of DCTB (cultured in the MPN assay) was correlated with existing TB culture diagnostic tests, including CFU and MGIT. The MPN was found to directly correlate with both these assays in axenic cultures and in patient samples (when supplemented with CF). Furthermore, in patient samples the MPN assay correlated better with CFU when compared to the MGIT in samples with a low bacterial burden and at later treatment time-points. These observations suggest that the MPN assay may be a useful tool to monitor the sterilizing effect of potential new drugs on DCTB populations. In a separate patient cohort, the addition of CF to the MGIT was found to have a beneficial effect on reducing the turn-around-time in HIV-positive, smear-negative patient samples. In contrast to the hypothesis, a reduction in MGIT TTP was not associated with the presence of DCTB. As dramatic changes in TTP were observed in patients with a low bacillary burden, it is postulated that the addition of CF to the MGIT may accelerate the growth of few viable organisms rather than resuscitate non-culturable organisms.

Implications:

The quantification and monitoring of how DCTB respond to treatment has numerous clinical implications. For instance, DCTB may serve as a novel biomarker for treatment response and possible relapse disease. In addition, patients that are 'DCTB positive' at the end of treatment could possibly be candidates for isoniazid preventative therapy to prevent subsequent relapse. The use of CF to unmask DCTB populations in the MPN assay has potential implications in EBA studies to monitor the sterilising effect of new anti-TB compounds on DCTB populations. The addition of CF to routinely used diagnostic culture assays, such as the BACTEC MGIT 960, has implications in TB diagnosis, treatment monitoring and DST. The results indicated that the addition of CF was particularly effective in patient sputum samples with a low bacillary load. Studies to optimise the use of CF, or recombinant stimulatory molecules, in routine culture assays would be an important technical advancement in reducing the time required to yield a positive culture. The addition of CF to the MGIT would also be beneficial in terms of shortening the time required for DST. This would result in patients receiving prompt and appropriate TB treatment, thus reducing transmission within the community.

Quo Vadis:

Mechanistic studies are required to identify the relevant stimulatory molecules in CF as well as to provide a more in-depth understanding of bacterial culturability. In terms of clinical work the following questions still need to be answered:

1. What is the link between residual DCTB at the end of treatment and relapse disease?
2. Are DCTB more likely to present in patients with a long history of disease (long standing latent infection) compared to patients who rapidly progress to active TB disease?
3. Are DCTB associated with certain *M. tuberculosis* strain types?
4. Which host factors are responsible for the formation of DCTB?
5. Are DCTB influenced by blood and sputum drug concentration levels? To investigate this pharmokinetic studies using HPLC or mass spectrophotometry are required to analyse drug concentration levels.

APPENDICES

Ethics clearance is provided in Appendix 3A, page 134.

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Minireview

MINIREVIEW

Relapse, re-infection and mixed infections in tuberculosis disease

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One sentence summary: This review provides a synthesis on the prevalence of mixed tuberculosis infection, which can provide insight on transmission rates, strain diversity and drug tolerance and resistance within select high and low burden settings.

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ABSTRACT

Tuberculosis (TB) disease can be characterized by genotypic and phenotypic complexity in *Mycobacterium tuberculosis* bacilli within a single patient. This microbiological heterogeneity has become an area of intense study due its perceived importance in drug tolerance, drug resistance and as a surrogate measure of transmission rates. This review presents a descriptive analysis of research describing the prevalence of mixed-strain TB infections in geographically distinct locations. Despite significant variation in disease burden and a rampant human immunodeficiency virus (HIV)-TB co-epidemic, there was no difference in the prevalence range of mixed infections reported in African countries when compared to the rest of the world. The occurrence of recurrent TB was associated with a higher prevalence of mixed-strain infections, but this difference was not reported as statistically significant. These interpretations were limited by differences in the design and overall size of the studies assessed. Factors such as sputum quality, culture media, number of repeated culture steps, molecular typing methods and HIV-infection status can affect the detection of mixed-strain infection. It is recommended that future clinical studies should focus on settings with varying TB burdens, with a common sample processing protocol to gain further insight into these phenomena and develop novel transmission blocking strategies.

Keywords: tuberculosis; recurrent disease; relapse; re-infection; mixed infections; heteroresistance

INTRODUCTION

Efforts to contain tuberculosis (TB) with combination chemotherapy for the past 50 years have met with mixed success as this disease remains a global public health concern and is now the leading cause of death due to an infectious bacterial agent (WHO 2016a). Current TB treatment is characterized by a variety of disease outcomes including clinical cure, treatment failure and the development of latent infection, with the latter two carrying the risk of subsequent relapse disease. Chemotherapy of TB is complex and while ultimately aimed at eliminating tubercle bacilli, treatment can also result in the selection of various forms of antimicrobial resistance. This can lead to emergence of one dominant resistant bacterial genotype or the appearance of heteroresistance, comprising mixtures of drug-susceptible and drug-resistant organisms.

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This complexity in bacterial populations, which can occur in a single patient, presents unique challenges and hampers the rapid clearance of organisms during chemotherapy. In addition, differences in bacterial growth states can further contribute to the establishment of drug-tolerant populations as chemotherapeutic agents are generally targeted at metabolic pathways that are a hallmark of active bacterial replication (Zumla, Nahid and Cole 2013; Kana *et al.* 2014). This is evidenced by the demonstration that sputum from TB patients contains a mixture of actively replicating and differentially culturable tubercle bacteria (DCTB), the latter displaying tolerance to first-line TB chemotherapeutics (Mukamolova *et al.* 2010; Chengalroyen *et al.* 2016; Loraine *et al.* 2016). It is proposed that these drug-tolerant populations provide the framework for the selection and emergence of genetically stable drug-resistant strains (Loraine *et al.* 2016). In view of this, the genomic plasticity and resulting strain complexity of *Mycobacterium tuberculosis* populations during infection is a key feature of the success of this deadly pathogen and merits further consideration. As a result, the complexity in *M. tuberculosis* population dynamics in human populations has become the focus of intense study. The advent of molecular typing methods and next-generation sequencing technologies has allowed for the genetic characterization of *M. tuberculosis* strains at a resolution that was previously not possible (Gan *et al.* 2016; Dheda *et al.* 2017).

DNA fingerprinting technologies, developed over the past two decades, have been widely used to study the diversity of *M. tuberculosis* strains and to investigate episodes of exogenous re-infection and relapse (van Rie *et al.* 1999; Bandera *et al.* 2001; Caminero *et al.* 2001), as well as the presence of heteroresistance in sputum isolates (Kaplan *et al.* 2003; Post *et al.* 2004; Shamputa *et al.* 2004), and the occurrence of mixed *M. tuberculosis* strain infections (Warren *et al.* 2004; van Rie *et al.* 2005; Shamputa *et al.* 2006; Cohen *et al.* 2011). Prior to these advances, TB disease was assumed to occur as a consequence of infection with a single *M. tuberculosis* strain, this event was thought to confer a measure of protection against infection with a secondary strain (Stead 1967). Recurrence of the disease was understood to be the result of endogenous re-activation of a non-replicating, immune-subversive variant of the strain that was responsible for the original disease episode (Stead 1967). In this context, the importance of re-infection remained relatively unexplored. However, molecular-based genotyping has subsequently demonstrated that exogenous re-infection with different *M. tuberculosis* strains does indeed occur in both high- and low-incidence settings, thus confirming that previous infection does not provide protection against subsequent infection (Small *et al.* 1993; van Rie *et al.* 1999; Sonnenberg *et al.* 2001; Kruuner *et al.* 2002; Andrews *et al.* 2008; Charalambous *et al.* 2008). Furthermore, studies have shown that many TB cases occur as a result of recent transmission and depending on setting; these transmission events are likely to take place outside of the household within the community (Verver *et al.* 2004; Dheda *et al.* 2017). High rates of re-infection have important implications for TB control strategies and highlight the need to reduce TB transmission in the community, together with the importance of early diagnosis, coupled with treatment initiation. TB disease due to recent transmission and re-infection has become an increasingly relevant topic of study in the era of human immunodeficiency virus (HIV) infection, as the resulting reduced immunity may result in vulnerable populations becoming increasingly susceptible to infection or re-infection with *M. tuberculosis*. Moreover, increased transmission in communities with a diversity of circulating *M. tuberculosis* strains can lead to mixed-strain infections, which have been documented in many distinct geographical locations, emphasizing the importance of interventions that limit or interrupt transmission. The complex environment of the lung as a site for TB disease, combined with sustained exposure to tubercle bacilli in high-TB-endemic settings, enhances the occurrence of mixed-strain infections, which have been associated with unfavorable treatment outcomes (Theisen *et al.* 1995; Niemann *et al.* 2000; Baldeviano-Vidalon *et al.* 2005; van Rie *et al.* 2005; Kamakoli *et al.* 2017). Given the emerging importance of phenotypic diversity in bacterial populations during TB pathogenesis, this review takes a retrospective view of published studies that report the prevalence of mixed-strain infections within select populations. The prevalence of these phenomena was interrogated amongst studies conducted within and outside the African continent to identify patient and population-level risk factors associated with mixed-strain TB infections, as well as possible avenues for future research. Recurrent TB and factors associated with relapse versus re-infection are discussed, together with any association with mixed-strain infections. For clarity, the terminology and methods used to describe and study these clinically complex presentations of TB disease are detailed below.

DEFINITIONS AND TERMINOLOGY

Definitions of terms and descriptions of features relating to TB disease, pathogenesis and epidemiology that are commonly encountered in the literature and feature in this review are given below. These are further clarified in Fig. 1.

Primary TB disease

Primary TB, Fig. 1, is defined as disease in a patient who has never been treated for TB or has taken anti-TB medication for less than a month (WHO 2014).

Endogenous re-activation

Endogenous reactivation refers to incident cases of TB (both new and recurrent) that occur as a result of re-activation/re-emergence of a previous infection that was contained by the host immune response, in the case of individuals with latent TB infection, and/or the application of chemotherapy in patients with active TB disease, Fig. 1. This is in contrast to TB disease (both new and recurrent) that is a result of a recent transmission event (Mathema *et al.* 2006).

Recurrent TB disease

Recurrent TB refers to a repeat occurrence (second, third or subsequent episode) of TB disease in a patient that occurs as a result of either relapse or re-infection. Recurrent TB occurs after the previous/initial episode has been classified as clinically cured according to WHO guidelines [smear or culture-negative sputum specimens in the last month of treatment and on at least one previous occasion, (WHO 2016a)].

Re-infection

Recurrent TB disease also occurs as a result of re-infection, whereby a patient is exogenously infected with a *Mycobacterium tuberculosis* strain that is distinct from the organism that caused the original infection, Fig. 1B (Mathema *et al.* 2006). A caveat here is that in high-incidence settings, patients, on rare occasions,

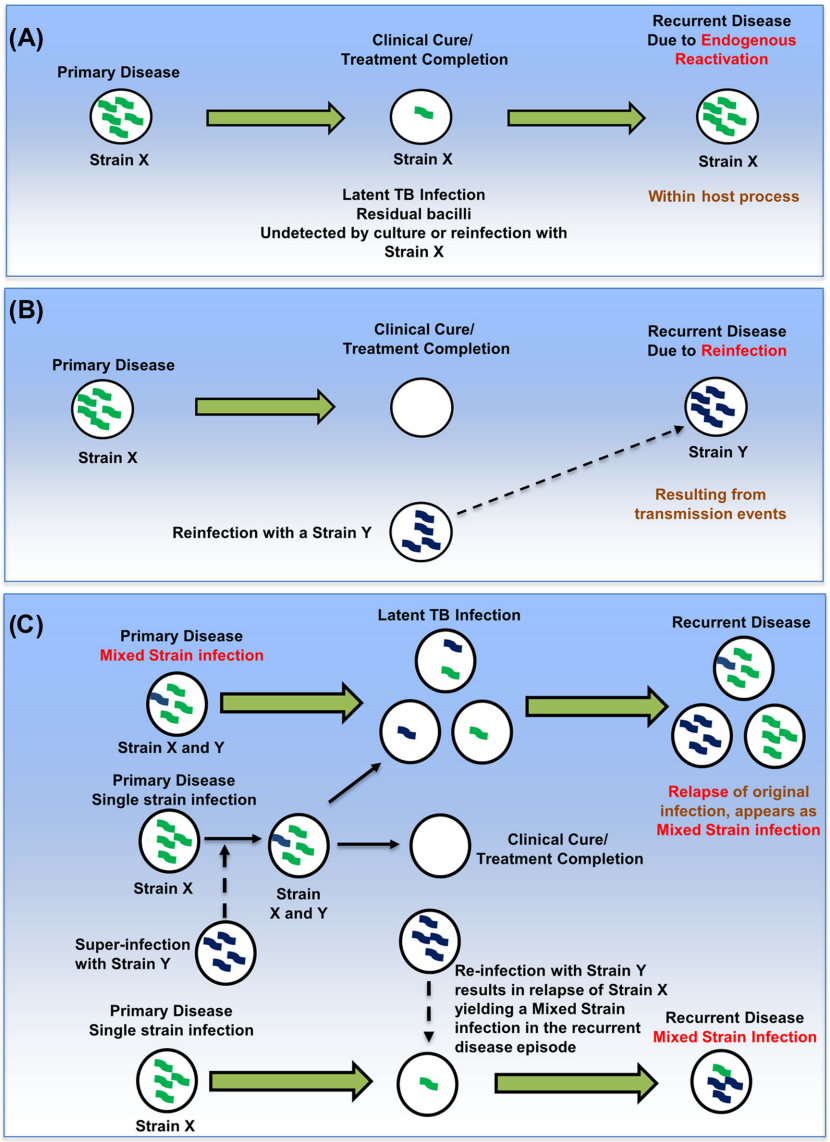

Figure 1. Recurrent disease and mixed-strain *M. tuberculosis* infections. Shown are the various scenarios that give rise to recurrent TB disease and mixed infections. (**A**) Relapse disease occurs due to the re-emergence of an *M. tuberculosis* strain that caused the original infection, suggesting that complete eradication of tubercle bacteria was not achieved during the primary disease episode. In this case, the second disease episode results from the presence of persisting organisms that emerge when treatment is stopped and environmental factors are favorable for bacillary survival. (**B**) Re-infection occurs when a patient is infected with an *M. tuberculosis* strain that is distinct from the strain that caused the original infection. High rates of re-infection can be attributed to both environmental and host factors. Alternatively, an individual can be functionally cured, with no surviving bacteria but can then be re-infected with the same strain that caused the primary disease episode. In the absence of molecular tools to distinguish these events, this would result in the incorrect classification of re-activation, see (**A**). (**C**) Mixed infections can occur in both primary TB disease and recurrent TB disease. During primary disease, mixed infections can occur during a single infectious episode, whereby two genetically distinct strains are present at the same time during the initial infectious episode. Alternatively, infection with a second distinct strain (super infection) can occur during latent infection or active disease. Re-infection with a second strain may also result in re-activation of an underlying strain.

may be exposed to or be infected by a very similar or the same strain as in the primary infection, which makes differentiation between relapse and re-infection in these particular cases difficult.

Relapse

Relapse disease is defined as a second (or third) episode of active TB disease due to re-emergence of the original infection, as determined by genotypic analysis of the prevailing tubercle bacilli (Mathema *et al.* 2006). As indicated for re-infection, substantive homogeneity in *M. tuberculosis* strains in any given setting, combined with high transmission rates, will make the classification relapse versus re-infection difficult. In these cases, whole-genome sequencing (WGS) to identify minor differences will provide the greatest insight (Gan *et al.* 2016).

Mixed-strain TB infection

This refers to TB disease caused by more than one clonally distinct *M. tuberculosis* strain, either through a single transmission event involving more than one distinct strain or through multiple transmission events (super-infection) during a single-

disease episode, Fig. 1C. The simultaneous transmission of multiple strains resulting in mixed infection may occur in populations of vulnerable individuals, whereby both strains are able to bypass the host's defense system and resist killing (Warren *et al.* 2004). Super-infection can also occur when the severity of a current disease episode is such that it compromises the host innate immune response to a point that leads to increased susceptibility to infection with a secondary strain (Warren *et al.* 2004). Alternatively, mixed-strain infections can arise if a subsequent infectious episode, which was caused by a distinct strain, results in relapse of the original infection, yielding disease with two unique *M. tuberculosis* strains that may have the same or different drug susceptibility profiles.

Heteroresistance

The term heteroresistance refers to the occurrence of populations of both drug-susceptible and drug-resistant isolates within the same clinical sample (Rinder 2001). This phenomenon may arise in a single infection as tubercle bacilli undergo genetic change through mutation of genes associated with drug resistance. It may also arise in mixed-strain infections, whereby one strain is resistant to a particular TB drug, while the other strain is susceptible. When heteroresistance occurs within the same strain type, it involves sub-populations of *M. tuberculosis*, for example resistant mutants co-existing with susceptible wild-type genotypes of specific resistance-related genes such as *katG or rpoB* in the same sputum specimen. This phenomenon is known as clonal heteroresistance, which is dependent solely on within-host processes affecting a single causative strain, in comparison to a mixed infection that is reliant on the presence of more than one strain.

METHODOLOGY USED TO STUDY RECURRENT TB AND STRAIN HETEROGENEITY

Numerous approaches have been employed to describe the microbiological complexity associated with TB disease, further detailed below. While some methods rely on variations in the biochemical properties of tubercle bacilli, the most popular methods used for the identification of mixed-strain infections include IS*6110*-based restriction fragment length polymorphism (IS*6110* RFLP) analysis, spacer oligonucleotide genotyping (spoligotyping) and the mycobacterial interspersed repetitive-unit-variable-number of tandem repeats (MIRU-VNTR) typing. In more recent work, metagenomics (Kay *et al.* 2015) and WGS approaches have been used (Ford *et al.* 2012; Gan *et al.* 2016).

Phage typing of *Mycobacterium tuberculosis* strains

Mixed-strain infections were first described in the 1970s using phage typing of *M. tuberculosis* cultures (Mankiewicz and Liivak 1975; Bates, Stead and Rado 1976). This method demonstrated that distinct strains of *M. tuberculosis* could be isolated from a single sputum sample by the typing of multiple colonies on solid culture media (Mankiewicz and Liivak 1975).

IS6110 RFLP typing

IS*6110* RFLP typing is an attractive tool to study transmission dynamics and relatedness of *M. tuberculosis* isolates (Cave *et al.* 1994; Warren *et al.* 2004). Strain identification using this method relies on the number and position of the transposable insertion sequence element, IS*6110*, in *M. tuberculosis* strains after restriction digestion, yielding a unique fingerprint pattern (van Embden *et al.* 1993). In studies investigating recurrent TB disease, strains exhibiting an identical banding pattern to the primary infectious episode point to relapse of TB disease, whereas re-infection and recent transmission is implied when distinct banding patterns are obtained. IS*6110* RFLP technology has also been utilized to identify mixed infections either by the identification of low-intensity banding patterns (LIBs) present in the same sputum sample (de Boer *et al.* 2000) or by the identification of unique fingerprints from the selection of multiple single colonies from the original sample (de Viedma *et al.* 2003; Das *et al.* 2004; Shamputa *et al.* 2004). One limitation of this method is that the identification of LIBs requires that at least 10% of the extracted DNA originates from the minority strain in order to be detected (Cohen *et al.* 2012). An additional limitation is that it fails to reliably discriminate between strains with less than six IS*6110* copies i.e. low-copy-number isolates (Gutacker *et al.* 2006). Some of these strains, displaying identical banding patterns on IS*6110* typing, have been shown to have unique genetic identities when using a secondary genotyping method (Bauer *et al.* 1999; Rhee *et al.* 2000).

Spoligotyping

Spoligotyping is the most commonly used PCR-based method for genotyping and differentiating between *M. tuberculosis* complex (MTBC) strains and is based on the visualization of 43 interspersed spacer sequences in a genomic direct repeat locus of MTBC strains (Kamerbeek *et al.* 1997). Although spoligotyping is a popular and simple molecular tool used in the study of molecular epidemiology, it has lower discriminatory power when compared to IS*6110* RFLP typing and is generally not recommended for use on its own in the study of mixed infections or transmission patterns (Kremer *et al.* 1999).

MIRU-VNTR typing

This method uses PCR to categorize the number and sizes of repeats in a minimum of 12 independent loci, each of which have a unique repeat sequence (Barnes and Cave 2003). As this method examines heterogeneity within a limited set of loci, its discriminatory power is linked to the number of loci that are evaluated. An advantage of MIRU-VNTR typing is that it can identify strains that are under-represented in a mixed-strain infection. Thus, a minority strain representing as little as 1% of the entire bacterial population can be identified using this technique (de Viedma *et al.* 2005). One limitation of this method is that it may present difficultly in differentiating between mixed infection and microevolution of strains (Cohen *et al.* 2012).

Whole-genome sequencing

WGS has become an established method in the field of molecular epidemiology. The application of this technology provides the capability of in-depth analysis into the diversity of *M. tuberculosis* strains that cannot be achieved by other currently used molecular methods. However, its use has been hampered by high cost and the complexity of data analysis but it is likely that the application of WGS will increase as new-generation sequencing becomes more affordable and simpler data analysis tools become available.

APPROACH FOR RETROSPECTIVE ANALYSIS

To retrospectively assess the prevalence of mixed-strain infections, the published literature was interrogated for reports that described these and related occurrences. Publications selected for analysis were obtained through the Pubmed database (www.ncbi.nlm.gov/pubmed) using the following keywords and Boolean operators: (mixed OR multiple) AND tuberculosis AND (strain OR strains), which yielded 1749 hits. Relevant English articles published from the beginning of records till December 2016 that reported the occurrence of mixed-strain infections using molecular genotyping techniques in adult TB patients were chosen. These articles were selected using several criteria which included, geographic location, patient population, recurrent TB and where possible, congruent processing protocols. We also endeavored to differentiate our analysis to a previous review published 4 years ago (Cohen *et al.* 2012), which affected our inclusion/exclusion of studies. Studies with a starting sample size of less than 10 patients were excluded from analysis. In addition, relevant articles that were referenced by papers obtained through the original search were also included for analysis. The analysis of mixed infections was further stratified by primary and recurrent disease. In some instances, where not reported, the proportion of mixed infections was calculated in each cohort based on the information available. The resulting synthesis of research on mixed *Mycobacterium tuberculosis* infections worldwide, spanning over the last 15–17 years is provided in this review.

MIXED *MYCOBACTERIUM TUBERCULOSIS* INFECTIONS

Studies of TB cases in deceased patients from the 18th century, while limited, revealed that in five out of eight bodies investigated using metagenomics, more than one *Mycobacterium tuberculosis* genotype was identified, suggesting that mixed-strain infection may have been a common occurrence during a time of rampant TB in Europe (Kay *et al.* 2015). Current studies investigating the frequency of present day mixed *M. tuberculosis* infections confirm that they can occur regularly but the frequency of detection varies according to the design of the studies, the sample size and methodology used.

African versus non-African studies

For the greatest coverage possible, geographically distinct regions covering Africa and countries outside Africa, respectively, were included in this review and the findings summarized in Tables 1 and 2. The rationale for this stratification was based on the observation that countries in Africa (especially southern Africa) carry a high burden of HIV-associated TB (WHO 2016a,b). We hypothesized that the compromised immune response in individuals associated with these dual epidemics, together with a high incidence of TB and elevated transmission rates, would give rise to a greater prevalence of mixed infections on the African continent. The reported TB incidence figures per 100 000 population in the select countries used for this analysis were South Africa, 834; Rwanda, 63; Zambia, 406; Malawi, 164; Uganda, 161. In similar studies from outside of Africa, the TB incidence figures per 100 000 population were Spain, 12; India, 167; Bangladesh, 227; Georgia, 106; China, 68; Kyrgyzstan, 142; Vietnam, 140; Guyana, 93; Surinam, 33 and Pakistan, 270 (WHO 2016b). Amongst studies from African countries, 15 that investigated the occurrence of mixed infections in patients with pulmonary TB were included for analysis. These studies were selected on the basis of the methodology employed as well as the number of patient samples analyzed. Of the selected African studies, nine were conducted in South Africa, and the remaining six were carried out in Rwanda, Zambia, Malawi (one study from each country) and Uganda (three studies). The frequency of mixed infections observed in sputum samples from African studies varied between 2.3% and 19% (Table 1). Frequencies of mixed infections reported in 19 studies performed in countries outside of Africa were similar to those in Africa and varied from <0.4% to 15.7% (Table 2). Given the substantive variation in overall study design, it is unclear whether the difference in the upper limit of mixed infection prevalence, 14%–19%, can be considered as significant. Possible reasons for the large variation in the occurrence of mixed infection in any particular setting or between the select studies are outlined below.

Effect of quality, volume and processing of specimens

A major contribution to variations between studies in similar settings is likely to be sputum collection and processing. The collection of a single, low-volume sputum sample may greatly decrease the odds of finding mixed infection episodes. For example, a poor-quality sputum sample containing a significant amount of upper airway secretions is unlikely to be representative of the entire *M. tuberculosis* population in the bronchial tree, while the collection of multiple sputum samples may increase the odds of sampling from different cavities within the lung. Several studies reviewed here, relied on the collection of sputum cultures from multiple pre-treatment specimens to increase the sensitivity of their assay (Richardson *et al.* 2002a; Shamputa *et al.* 2006; Peng *et al.* 2013). Similarly, for the demonstration of mixed infections, typing of cultures from multiple sputum samples from smear-positive individuals, collected serially over time, was used (Braden *et al.* 2001; Baldeviano-Vidalon *et al.* 2005; van Rie *et al.* 2005; Dickman *et al.* 2010; Mallard *et al.* 2010; Mulenga *et al.* 2010; Cohen *et al.* 2011). Considering this, an in-depth comparison of findings between these studies was not possible due to differences in methodology and study design. However, intra-study comparisons of the frequency of mixed infections have concluded that the analysis of a single sputum sample, as opposed to multiple samples, decreases the likelihood of detecting mixed infections (Shamputa *et al.* 2006; Peng *et al.* 2013).

Effect of specimen processing

We hypothesize that additional factors that could affect the detection of mixed-strain infections include delays in transportation, breaks in cold-chain transport and delays in sputum processing. Furthermore, it is not known whether the decontamination procedure may result in a loss of bacterial populations. To our knowledge, no research has focused on the impact of these procedures on detecting mixed strains, and further research is required to corroborate these hypotheses. Findings of studies on mixed infections are also not directly comparable as the population structure of mixed infection is strain genotype and culture medium dependent (Hanekom *et al.* 2013). Moreover, the clonal composition of cultures from clinical specimens could be modified by the culture techniques used (Martín *et al.* 2010). Hanekom *et al.* (2013) used a PCR-based molecular typing approach to identify strain genotypes in both Löwenstein-Jensen (LJ) and mycobacterial growth indicator tube (MGIT) cultures. The investigators noted that Beijing and Haarlem strain

Table 1. Studies conducted on mixed tuberculosis infection in Africa.

Year	2001	2002a	2004	2005	2007	2008	2009	2010	2010	2010	2011	2013	2013	2014	2015
Author	du Plessis *et al.*	Richardson *et al.*	Warren *et al.*	Van Rie *et al.*	Umubyeyi *et al.*	Andrews *et al.*	Stavrum *et al.*	Dickman *et al.*	Mallard *et al.*	Mulenga *et al.*	Cohen *et al.*	Hanekom *et al.*	Muwonge *et al.*	Shin *et al.*	Ssengooba *et al.*
Location	Western Cape, RSA	Cape Town, RSA	Cape Town, RSA	Cape Town, RSA	Rwanda	KwaZulu-Natal, RSA	Eight provinces in RSA	Kampala, Uganda	Karonga District, Malawi	Ndola, Zambia	KwaZulu-Natal, RSA	Khayelitsha, RSA	Uganda	Botswana	Kampala, Uganda
Strain typing method used	IS*6110* RFLP DR Sequence	IS*6110* RFLP DR Sequence	PCR (Beijing and non-Beijing) Spoligo	IS*6110* RFLP PCR	Spoligo MIRU-VNTR	Spoligo	Spoligo MIRU-VNTR IS*6110* RFLP	MIRU-VNTR Spoligo	PCR (LAM and Beijing)	Spoligo MIRU-VNTR	MIRU-VNTR Spoligo	PCR (LAM, Haarlem, S, Beijing, LCC)	MIRU-VNTR Spoligo	MIRU-VNTR	Spoligo MIRU-VNTR
Number of enrolled	17	210	407	768	710 (69 MDR)	23[c]	252 isolates	113	72	361	240	535	74	370	66
Number of patients/ samples analyzed	13[a]	131	186	48 MDR-TB	22 MDR 1 Non-MDR	17	54 isolates	113	72	273	56	206 cultures[f]	72	370	51
First incidence of TB	No	210	200	23	10	14	141	NS	62	Yes	NA	NA	49	142	47
Retreatment records	Yes	NA	207	25	12	3	102	3	10	Yes	NA	NA	23	228	4
HIV status	All negative	All negative	10% HIV+	2% HIV+, 47% HIV– 51% NA	54.5% HIV +	15 + 2 NA	44% HIV + 22% HIV– 34% NA	14.4% HIV+	62.7% HIV+	NA	96% HIV+	NA	24.7% HIV+ 17% HIV– 59% NA	75.4% HIV+	All positive
Samples															
Single	No	No	Yes	No	47	No	NS	No	25	Yes	No	Yes	Yes	Yes	No
Multiple	Yes[b]	Yes	No	Yes	22	Yes	NS	Yes	47	Yes	Pooled[e]	No	No	No	Yes[g]
AFB +ve	Yes	Yes	Yes	NA	Yes	NA	NS	Yes	Yes	Yes	No	NS	NS	NA	Yes/No
Sputum	Yes/No	Yes	Yes	Yes	Yes	Yes	NS	Yes	Yes	Yes	No	Yes	Yes/No	Yes	Yes/No
Culture step	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
Collected before treatment	Yes (4); No (9)	Yes	Yes	Yes/No	Yes/No	Yes/No	Yes/No	Yes/No	NS	Yes/No	Yes (42%) No (58%)	NA	Yes/No	Yes/No	Yes/No

Table 1. (*Continued*).

Year	2001	2002a	2004	2005	2007	2008	2009	2010	2010	2010	2011	2013	2013	2014	2015
Percentage of mixed infections in:															
Primary TB disease	NA	2.3	17	NS	NS	NS	NS	NS	2.8	NS	NA	NA	12	5.6	53
Recurrent TB disease	15.4	NA	23	NS	NS	NS	NS	NS	0	NS	NA	NA	4.3	12.7	25
Total	IS*6110*: **15.4** DR: **7.7**	**2.3**	PCR:**19** Spoligo: **4.8**	PCR: **10.4** IS*6110*: **2**	**4.3**	**11.7 (29.4)**[d]	**18.5**	**7.1** (HIV +: **37.5**)	**2.8**	**3.2**	**9**	**15**	**11.1**	**10**	**4** (blood and sputum: **51**)

[a]Twelve autopsied cases and one pneumonectomy, both lung and extra-pulmonary samples were collected.
[b]Sampled from 6 to 15 lesions from anatomically distinct sites.
[c]Patients who developed multi drug-resistant (MDR) or extremely drug-resistant (XDR-TB) after treated for less resistant form.
[d]Two patients infected with multiple genotypes at initiation (11.7%). During follow-up, an additional three patients had two strains with different DST patterns in a single episode (29.4%).
[e]Pooled biopsy specimens from lung, liver and spleen.
[f]Convenience sample consisting of paired MGIT and LJ cultures.
[g]Two sputum and one blood sample collected at baseline for culture.
AFB: acid fast bacilli
DR: direct repeat sequence
IS*6110* restriction fragment length polymorphism analysis
LCC: low copy clade
MDR: multidrug-resistant tuberculosis
MIRU-VNTR: mycobacterial interspersed repetitive-unit-variable-number of tandem repeats
NA: not available
NS: not specified
RSA: Republic of South Africa
Spoligo: spacer oligonucleotide genotyping
The boldface is the total percentage of mixed-strain infections reported in each study.

Table 2. Large studies conducted on mixed tuberculosis infections in countries outside of Africa.

Year	1999	1999	2003	2004	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2015	2015	2015	2015	2016
Author	Chaves *et al.*	Yeh, Hopewell and Daley	de Viedma *et al.*	Das *et al.*	Shamputa *et al.*	de Viedma *et al.*	Sham-puta *et al.*	Cox *et al.*	Fang *et al.*	Mokrousov *et al.*	Huang *et al.*	Wang *et al.*	Huyen *et al.*	Peng *et al.*	Wang *et al.*	Pang *et al.*	Streit, Millet and Rastogi	Zheng *et al.*	Mustafa *et al.*
Location	Madrid, Spain (TB prison hospital)	United States	Madrid, Spain	Thirivallur, India	Bangladesh (Hospitals in Mymensingh)	Madrid, Spain	Georgia (TB prison hospital)	Central Asia	Shanghai, China	Kyrgyzstan	Eastern Taiwan	Taiwan	Southern Vietnam	Heilon-gjiang, China	Inner Mongolia, China	China	Guyana and Suriname	Sichuan Province, China	Punjab District Pakistan
Typing method	IS6110 RFLP	IS6110 RFLP	Spoligo DRE-PCR IS6110 RFLP	IS6110 RFLP DR-RFLP	IS6110 RFLP Spoligo MIRU-VNTR	IS6110 RFLP Spoligo MIRU-VNTR	IS6110 RFLP MIRU-VNTR	IS6110 RFLP Spoligo	MIRU-VNTR	IS6110 inverse PCR MIRU-VNTR, spoligo	PCR (Beijing and non-Beijing)	PCR (Beijing and non-Beijing)	IS6110 RFLP Spoligo MIRU-VNTR	MIRU-VNTR	MIRU-VNTR	MIRU-VNTR	Spoligo MIRU-VNTR	Spoligo MIRU-VNTR	PCR (Beijing and non-Beijing)
Number of patients enrolled	226	49	123	543	132	NS	385	416 strains	561	56	185	868	1890	174	384	3929 strains	161 isolate[c]	5090	102[d]
Number of patients/ samples analyzed	37	49	50	543	97	115 Cultures	199	397 isolates	249	56	185	466	1248	89	384	3248 strains	154	499 isolates	95 MDR-TB
First incidence of TB	25	NS	NA	NS	1	NA	134	198	217	56	144	436	1107	NA	325	2519	NA	NA	44
Retreat-ment records	12	NS	NA	NS	131	NA	65	184	249	NA	41	30	139	NA	59	729	NA	NA	51
HIV status	All positive	NS	84% positive	NA	NA	NA	NA	NA	NA	All negative	1 HIV + patient identified	NA	NA	NA	NA	NA	26.6% positive (not all tested)	NA	NA
Samples																			
Single	No	No	No	No	Yes	NS	No	NS	Yes	Yes	Yes	Yes	Yes	No	Yes	No	NS	Yes	Yes
Multiple	Yes	Yes	Yes[a]	Yes	No	NS	Yes	NS	Yes	No	No	No	No	Yes	No	Yes	NS	No	No
AFB +ve	NS	NS	NA	NA	Yes	Yes	Yes	NS	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	NS	NA	Yes
Sputum	Yes/No	Yes	Yes/No	Yes	Yes	Yes	Yes	Yes	isolates	Yes	Yes	Yes (292)	Yes	Yes	NS	Yes	NS	NS	Yes
Culture step	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No	Yes (174)	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Table 2. (*Continued*).

Year	1999	1999	2003	2004	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2015	2015	2015	2015	2016
Collected before TX	NS	Yes/No	NS	Yes/No	Yes	NS	Yes	Yes/No	Yes (217)	NS	Yes (144)	Yes	NS	NS	NS	Yes	NS	NS	NS
DST	S,I,R,E	NA	S,I,R,E	NA	Yes	NA	S,I,R,E	S,I,R,E, PZA	Yes	I, R	S,I,R,E	Yes	S,I,R,E	Yes	S,I,R,E,O,K	NA	Yes	S,I,R,E	S,I,R,E
# Colonies selected	0	0	10	15–20[b]	10	Yes	0	0	30	0	0	0	0	0	30[b]	0	0	0	0
Percentage of mixed infections in:																			
Primary disease	NA	NA	NA	NA	NA	NA	15.7	NA	4.1	13.7	10.4	2.8	3.4	NA	2.8	3.3	NA	NA	27.2
Recurrent disease	NA	NA	NA	NA	2.1	NA	7.7	NA	15.6	NA	14.6	6.6	0.7	NA	5.1	4.4	NA	NA	5.9
Total	**3.6**	**2**	**6**	**<0.4**	**2.1**	VNTR: **2.6**	IS6110: **9** VNTR:**13.1**	**5**	**5.6**	**13.7**	**11.3**	**3%**	RFLP/ spol: **3.1** VNTR: **4.8**	**11.2%**	**3.13**	**3.5**	**0.6**	**2.4**	**15.7**

[a]One respiratory and one extra-respiratory sample analyzed less than 30 days apart.
[b]Monoclones were subsequently isolated and tested after mixed infections were identified using initial typing methods.
[c]Seventy-four isolates from Guyana and 80 from Suriname.
[d]Patients suspected of MDR-TB.
AFB: acid fast bacilli
DST: drug susceptibility testing
E: Ethambutol
IS6110 RFLP: IS6110 restriction fragment length polymorphism analysis
I: isoniazid
K: kanamycin
MDR: multidrug-resistant tuberculosis
MIRU-VNTR: mycobacterial interspersed repetitive-unit-variable-number of tandem repeats
NA: not available or not applicable
NS: not specified
O: Ofloxacin
PZA: Pyrazinamide
R: Rifampicin
Spoligo: spacer oligonucleotide genotyping
S: streptomycin
The boldface is the total percentage of mixed-strain infections reported in each study.

families were more likely to be associated with mixed infections and showed a predilection for some culture media (Beijing MGIT, $P = 0.02$, LJ $P < 0.01$; Haarlem MGIT $P < 0.01$, LJ $P = 0.01$). Other strains investigated included the Latin American Mediterranean (LAM) and S-family genotypes (Hanekom *et al.* 2013). In addition, mixed infections were identified in 23 of 206 MGIT cultures and 28 of 206 LJ cultures, indicating that LJ media may be marginally preferable to liquid media in identifying mixed infections (Hanekom *et al.* 2013). In a separate study, the clonal complexity of *M. tuberculosis* populations from clinical specimens was investigated by mixing strains in different volumetric proportions before culturing was performed. Using MIRU-VNTR typing, marked changes in the clonal composition were observed, including strains with different resistance profiles. The authors concluded that clonal complexity can be lost after culture, therefore genotyping techniques should be optimized in such a way that they can be performed directly on clinical samples (Martín *et al.* 2010). Four studies included herein were performed directly on sputum samples of which three used a PCR amplification method to detect different lineages (Mokrousov *et al.* 2009; Huang *et al.* 2010; Mallard *et al.* 2010; Wang *et al.* 2011). PCR methods to detect Beijing and non-Beijing lineages identified mixed infection frequencies to occur at 3% and 11% (Huang *et al.* 2010; Wang *et al.* 2011) while PCR to detect LAM and Beijing lineages reported mixed infection frequencies of 2.8% (Mallard *et al.* 2010). In the remaining study, 13.7% was identified by 12 locus MIRU-VNTR typing (Mokrousov *et al.* 2009). Another study investigated the proportion of mixed infections in both sputum and liquid culture-positive medium and reported a frequency of 2.1% and 4.6%, respectively (Wang *et al.* 2011). In a subsequent study, Wang *et al.* (2015) further sub-cultured the mixed strains initially observed and selected 30 clones from the sub-culture for each mixed infection. The authors reported that after the addition of a culture step, 8 of 12 (66.7%) strains with mixed infections had converted into a single genotype (Wang *et al.* 2015). Mixed-strain infections involving heteroresistance may also be missed if sputum samples are collected from a patient who has started treatment at a time when resistance is being amplified by first-line therapy (Hingley-Wilson *et al.* 2013). Pre-treatment samples may therefore be preferable for studies trying to identify mixed infections/clonal heteroresistance within an infecting bacterial population.

Techniques used for demonstrating mixed infections

The prevalence of mixed-strain infections varied between <0.4% using IS*6110* genotyping on colonies from 543 patients in India (Das *et al.* 2004), to 15.4% using the same technique on 12 HIV-negative autopsy cases in the Western Cape (du Plessis *et al.* 2001). In studies employing MIRU-VNTR typing, mixed infections were detected at an average frequency of 5.6% in Shanghai, China, with re-treatment cases having a higher rate of mixed infections than new TB cases (15.6% versus 4.1%) (Fang *et al.* 2008). Compared to these figures, mixed infections were encountered in 10% of all TB cases in Botswana (Shin *et al.* 2014). Only one study used spoligotyping as its primary method of genotyping and reported mixed infections to occur in 18% of samples analyzed, which is high in comparison to rates reported in other studies using other molecular methods (Andrews *et al.* 2008).

Frequencies of certain *Mycobacterium tuberculosis* genotypes involved in mixed infections

In the African studies, the incidence of mixed infections varied from 2.3% in diagnostic sputum cultures in patients from Cape Town, South Africa (Richardson *et al.* 2002a) to 19% in sputum cultures obtained from other patients suspected of suffering from TB from the same region (Warren *et al.* 2004). The highest frequency of mixed infections in sputum samples was obtained using a novel PCR technique demonstrating that patients analyzed in the study were simultaneously infected with a Beijing and non-Beijing *M. tuberculosis* strains (Warren *et al.* 2004). These results probably underestimate the frequency of mixed infections in this population as the PCR was conducted with primers specific to the Beijing lineage, and mixed infections may have also been present within the non-Beijing lineages. However, this method was still superior to spoligotyping, which identified only 4.5% of mixed cases in the same population (Warren *et al.* 2004). In another study, a PCR-based approach was used to determine the occurrence of mixed infections in sputum samples taken from patients in Malawi (Mallard *et al.* 2010). The investigators applied two lineage-specific PCR assays to sputum samples, targeting the LAM and non-LAM genotypes, as LAM strains are the most common genotypes present in northern Malawi. This yielded mixed infections in 2.8% of sputum samples, significantly lower than the 19% mixed infection rates observed in the Cape Town study that investigated the occurrence of Beijing and non-Beijing strains (Warren *et al.* 2004; Mallard *et al.* 2010). It was proposed that the differences found between the two studies could be explained by differences in the annual risk of infection [3% in Cape Town (Kritzinger *et al.* 2009) versus 1% in the rural Karongo district in Malawi (Crampin, Glynn and Fine 2009)]. Alternatively, inherent properties such as transmissibility of the Beijing versus LAM genotypes could explain the discrepancy. In addition, there were differences in the methods employed in the two studies.

Middelkoop *et al.* (2014) investigated factors associated with *M. tuberculosis* strain success in a high-TB-burdened community, with high strain diversity. Using IS*6110* RFLP, the authors noted that only 4% of strain types were persistently successful over a 10-year study period. These were strains from the W-Beijing family (W451 W724, W181, W330) and four from the CC-related lineage (CC27, CC61, CC73, CC77), as well as NG24 and AI265 strains. As host factors examined, such as age and gender, were not associated with strain success, it was concluded that pathogen rather than host characteristics appear to play a greater role in strain success (Middelkoop *et al.* 2014).

Effect of disease burden on prevalence of mixed infections

A high number of incident cases should logically influence the frequency of mixed infections, as was the case with the increased prevalence of 19% of mixed infections identified in the Cape Town study by Warren *et al.* (2004). However, Richardson *et al.* (2002a) who conducted their study in the same setting but found the frequency of mixed infections to be markedly lower, at 2.3%. This result was similar to the mixed infection rate seen in Bangladesh (2.1%) and Spain (2.6%). As mentioned previously, detailed comparisons between these studies are not scientifically valid due to differences in study design and methodology; however, the findings do suggest that overall disease burden is not the only arbiter of strain diversity or mixed infections. Alternatively, the differences in methods used in these studies may have resulted in an underestimate of mixed infections, as studies that used IS*6110*-based RFLP as their primary typing technique tended to report a low incidence of mixed infections, Tables 1 and 2. In contrast, when used in a congregate setting,

IS*6110*-based RFLP on multiple pre-treatment sputum samples identified mixed infections at a frequency of 9% in adult inmates in a prison TB hospital in Georgia (Shamputa *et al.* 2006). When MIRU-VNTR was used as a secondary typing tool, mixed infections were observed in 13.1% of the patients. The high mixed infection rates seen in this study occurred in settings of overcrowding coupled with a high incidence of TB.

Mixed infections in extra-pulmonary sites

While the majority of analyses reported herein was focused on sputum, the occurrence of mixed infections has also been demonstrated in extra-pulmonary samples taken from culture-positive biopsy specimens in two studies, both reporting a frequency of 9% (Cohen *et al.* 2011; Lieberman *et al.* 2016). Sampling of different anatomical sites in another study yielded a frequency of 3.6% (Chaves *et al.* 1999) while a frequency of 6% was recorded in another study where both extra-pulmonary and pulmonary specimens were collected (de Viedma *et al.* 2003). In an autopsy study, using lung and extra-pulmonary samples, a mixed infection frequency of 15.4% was reported (du Plessis *et al.* 2001). The highest frequency of mixed infections involving extra-pulmonary samples was reported by Ssengooba *et al.* (2015) who found that 51% of HIV-infected individuals with CD4 counts $>$ 200 cells/mm^3 displayed discordant *M. tuberculosis* genotypes between isolates from blood and sputum samples, respectively, providing evidence of substantive mixed infection frequencies in different body compartments (Ssengooba *et al.* 2015). In agreement with this, genomic analysis of postmortem lung and extra-pulmonary biopsies from 44 HIV-positive patients in KwaZulu-Natal reported that *M. tuberculosis* sub-lineages, including mixed infections, were distributed differentially throughout the lung, pointing towards temporary barriers to pathogen migration in the lung (Lieberman *et al.* 2016). In addition, multiple genotypes were identified between *M. tuberculosis* organisms in the lungs and extra-pulmonary sites, which may have occurred through a single transmission event involving multiple genotypes or by means of super-infection episodes (Lieberman *et al.* 2016). The genomic diversity identified by the occurrence of mixed-strain infections as well diversified sub-lineages provide an interesting record of evolution and dissemination across the body in HIV-infected individuals.

RECURRENT TB DISEASE

A select few studies analyzed here investigated whether mixed infections were more likely to occur during a primary versus a recurrent disease episode. From these, the frequency of mixed infections were reported as being higher in the recurrent disease episode in seven out of 13 studies, although most of these studies found this difference not to be significant (Warren *et al.* 2004; Fang *et al.* 2008; Huang *et al.* 2010; Wang *et al.* 2011, 2015; Shin *et al.* 2014; Pang *et al.* 2015). The six remaining studies reported lower frequencies of mixed infections in the recurrent episode, Tables 1 and 2. However, in three of these studies, less than 20% of the patients analyzed were recurrent cases, making it difficult to draw any definitive conclusions on the likelihood of previous TB exposure being a risk factor for mixed-strain infections.

A review of earlier studies yielded little consensus regarding the proportions of recurrent disease due to relapse and re-infection (Lambert *et al.* 2003). Although re-infection is most likely to occur in geographical locations where the incidence of TB disease is high (Romeyn 1970; Vynnycky and Fine 1997; Godfrey-Faussett *et al.* 2000; Richardson *et al.* 2002b; Middelkoop *et al.* 2015), studies performed in low- to moderate-incidence settings have also reported high rates of re-infection (Caminero *et al.* 2001). A poor association between re-infection and recurrent TB disease in high-burden settings has also been reported, suggesting that a consistent trend in this regard is lacking (Shamputa *et al.* 2007; Luzze *et al.* 2013). Annual rates of relapse versus re-infection in patients with recurrent disease in Cape Town, South Africa, indicate that relapse generally occurred within a year following treatment, while re-infection, responsible for at least half of all cases of recurrent disease in this study, predominated after the first year following treatment (Marx *et al.* 2014; Guerra-Assunção *et al.* 2015). Hence, it is difficult to make direct comparisons between studies where the length of follow-up after treatment differs. Co-infection with HIV and antiretroviral treatment has been shown to be associated with increased risk for recurrent disease due to re-infection as opposed to relapse (Godfrey-Faussett *et al.* 1994; Sonnenberg *et al.* 2001; Middelkoop *et al.* 2012, 2015; Guerra-Assunção *et al.* 2015). It was also demonstrated that there is a 2.4 times higher hazard ratio for recurrent disease in HIV-1-positive individuals, compared to HIV-1-negative counterparts (Sonnenberg *et al.* 2001). The risk of re-infection has also been linked to previous TB infection, where disease attributable to re-infection after successful treatment completion was four times higher than that attributable to new TB disease (Verver *et al.* 2005). A major limitation of this study is that the HIV status of most patients was unknown; however, the authors concluded that re-infections observed could not be explained by HIV status alone, as the HIV prevalence in this study group was relatively low (1.2% to 5.2% in antenatal clinics and 11% amongst newly diagnosed TB patients). These findings suggest that a subset of individuals within a population may be predisposed to TB disease, with previous TB exposure itself being a risk factor for recurrent disease. We hypothesize that mixed infection prevalence will be higher in these individuals.

INTERPRETATION AND RECOMMENDATIONS

Interruption of TB transmission is pivotal to eliminating this disease from human society (Pai *et al.* 2016). The science surrounding transmission of the tubercle bacillus is an evolving discipline and much work is still needed to elucidate the biophysical, environmental, genetic and immunologic determinants in those individuals that transmit disease and those that get infected. We propose that the analysis of mixed infection rates provides an interesting and useful proxy for the level of transmission in communities within TB-endemic regions. However, retrospective analyses of studies conducted in various settings to assess this are difficult due to numerous factors which include differences in study design, sample processing, bacterial culture and molecular typing tool employed. Although molecular approaches for identifying mixed-strain TB infections are limited in their sensitivity, studies relying on sputum samples alone have reported that up to 19% of individuals are infected with more than one *Mycobacterium tuberculosis* strain. Furthermore, studies utilizing a combination of both pulmonary and extra-pulmonary samples have reported mixed infections to occur at a frequency of up to 51%. As these results are likely to be a conservative estimate, this suggests that mixed-strain infections occur at reasonably high frequencies. Whether this is a result of high transmission rates in endemic settings remains unclear as mixed infection rates seems similar in high- and low-

burden settings, based on the studies analyzed herein. Various factors need to be considered when assessing the prevalence or risk of mixed infections. These are detailed below.

What is the role of HIV?

From our collective analysis, factors associated with mixed-strain infection among HIV-infected patients include prior TB treatment [adjusted prevalence ratio, 2.11] and CD4+ T-cell count < 100 cells/μl (adjusted prevalence ratio, 10.18), suggesting that patients with advanced immune suppression are at increased risk of contracting a mixed-strain infection (Shin *et al.* 2014). Consistent with this, a study in Uganda on 113 smear and culture-positive patients established that mixed infections are more likely to occur in HIV-seropositive individuals, 37.5% versus 12.6% in HIV-positive versus HIV-negative patients with TB, respectively (Dickman *et al.* 2010). These results may still underestimate the proportion of mixed infections as TB/HIV co-infection often results in disseminated/extra-pulmonary disease, therefore reducing the chances of detecting mixed infections from pulmonary specimens alone, which may be paucibacillary in nature (de Viedma *et al.* 2003; Dickman *et al.* 2010). Various studies have now shown that multiple genotypes can be detected by sampling from both respiratory and extra-pulmonary sites in HIV-positive individuals, illustrating the presence of migration routes within and between organs. To our knowledge, no studies have investigated this sampling strategy in HIV-negative individuals. Despite the demonstrated increased risk of mixed infections in HIV-infected individuals, we found no substantive differences in mixed infection incidence when collectively comparing studies within Africa, an HIV-endemic region, and the rest of the world. This could reflect the lack of studies in the correct populations or the above-mentioned limitations in our comparative analysis.

How do mixed infections affect disease/treatment outcome?

The presence of mixed-strain infections involving heteroresistance results in poor treatment outcome (Theisen *et al.* 1995; Niemann *et al.* 2000; Baldeviano-Vidalon *et al.* 2005; van Rie *et al.* 2005; Kamakoli *et al.* 2017). For this reason, patients with various forms of drug-resistant TB may require a combination of first- and second-line antibiotics to target both drug-sensitive and -resistant organisms, respectively (Post *et al.* 2004; van Rie *et al.* 2005). Diagnostically, if only the sensitive strain is cultured from a mixed-strain infection, the administration of a drug-sensitive regimen will result in the selection of the resistant strain. This highlights the importance of early detection of mixed-strain infections with different resistance profiles to tailor the treatment regimen accordingly.

Mixed infections and the immune response

It has been demonstrated that different *M. tuberculosis* strains elicit distinct immune responses in the lung, ultimately leading to differences in disease pathology and mortality (Post *et al.* 2004). Consistent with this, immune protection conferred by one *M. tuberculosis* strain may differ when compared to distantly related strains due to differences in antigenic properties. Indeed, studies assessing the effectiveness of Bacille Calmette-Guerin (BCG) have varied worldwide and are possibly complicated by the heterogeneity in *M. tuberculosis* strains circulating within vaccinated communities (Fine 1995). Direct intra-tracheal injection of BALB/c mice indicated that BCG vaccination was less effective against infection with Beijing strains when compared to H37Rv (Lopez *et al.* 2003). Furthermore, infection with Beijing strains was characterized by excessive pneumonia, early but short-lived tumor necrosis factor-α (TNF-α) and nitric oxide synthetase (INOS) expression and an increase in mortality, while infection with *M. canetti* resulted in limited pneumonia, sustained TNF-α and INOS expression in the lung, with 100% survival (Lopez *et al.* 2003). This indicates that different *M. tuberculosis* strains elicit varying immune-pathological events. In this regard, the simultaneous interaction between multiple strains and the host immune system in a mixed infection scenario remains to be explored. In addition, it is not known whether all resident strains in an infected individual are transmitted simultaneously or whether one strain is more transmissible than the other. These remain as critical knowledge gaps that limit the development of novel interventions to reduce transmission.

Phenotypic heterogeneity

An important aspect of sputum microbiology, for which there has been growing appreciation, is the failure to detect non-replicating organisms that could influence the probability of diagnosing mixed-strain infections (Dartois *et al.* 2016). It is predicted that a sub-population of organisms withstands treatment and can enter into a drug-tolerant, quiescent state (Kell and Young 2000). Consistent with this, *M. tuberculosis* grown under hypoxic conditions is able to enter into a drug-tolerant, non-replicating state and accumulate lipid inclusion bodies (Garton *et al.* 2008). Bacteria with a similar physiology have been isolated directly from patient sputum, suggesting that these cells reflect those studied *in vitro* (Garton *et al.* 2008). Subsequent work has also demonstrated that pre-treatment sputum samples are dominated by a DCTB population that can only be grown in liquid media (Mukamolova *et al.* 2010; Chengalroyen *et al.* 2016; Loraine *et al.* 2016). Considering this, we hypothesize that it is possible that specific strains of *M. tuberculosis* adopt this differentially culturable state, and thus are not identifiable by routine culture. Currently, there is no data to corroborate this, but the upregulation of the DosR regulon in W-Beijing strains, and concomitant accumulation of triacylglycerides, suggests that this family of strains may have a greater propensity to adopt these differential growth states (Reed *et al.* 2007). Hence, the association of Beijing lineage 2 strains with relapse (Lan *et al.* 2003; Bryant *et al.* 2013) may be linked to a proportion of this strain assuming a differentially culturable state, thereby providing the organism with an adaptive advantage against immune assault and drug treatment. Furthermore, the occurrence of DCTB could result in an underestimation of mixed infections as some strains may not emerge on solid media.

Limitations in molecular typing tools

Current molecular methods do not provide adequate information on the mechanism of transmission and order in which each individual infection was acquired, for the latter continuous and very expensive molecular surveillance would be required. This information would be invaluable in the interpretation of epidemiological data. For example, in a scenario where re-infection resulted in the re-activation of an underlying strain, IS*6110* may only identify the secondary strain which could be interpreted as the strain causing the original infection and result in inaccurate identification of the source of infection in contact tracing. High

rates of mixed infections in a recurrent disease episode highlight the importance of re-infection in disease transmission, which has previously been questioned (Stead 1967). These limitations call for wider use of high-resolution techniques such as WGS.

Diagnosing mixed infections

In high-burden settings, where the majority of TB disease is likely (but not necessarily) due to re-infection /recent infection, TB control strategies will be highly reliant on the early identification and early treatment of infectious individuals to prevent transmission of both drug-susceptible and -resistant strains within the community. High rates of disease recurrences due to re-infection pose a serious problem to vulnerable populations, such as HIV-infected mining communities where post-treatment prophylaxis was proposed as an important public health strategy (Sonnenberg *et al.* 2001). Exogenous re-infection has also been implicated in the acquisition of drug resistance in patients (Andrews *et al.* 2008). Several studies in South Africa investigating extremely drug-resistant (XDR)-TB have been associated with primary transmission (Ghandi *et al.* 2006; Klopper *et al.* 2013). These cases have been attributed to 'super spreaders'—a small percentage of infected individuals who have the propensity to transmit TB disease as identified by a cough aerosol sampling system (Jones-López *et al.* 2013). The long-term treatment outcome in patients with XDR-TB is very poor, and the growing infectious pool of drug-resistant TB disease is of major concern as it threatens global TB control efforts (Dheda *et al.* 2010). Where drug resistance is often the result of primary transmission of resistant strains, the rapid identification and treatment of patients harboring these strains should be a high priority. Furthermore, if mutations are more likely to emerge from certain strains associated with re-infection, strain differentiation may need to be included in the diagnostic process for early identification and the development of tailored treatment regimens. With regard to implementation, while the addition of strain typing to current diagnostic algorithms in high-income, low-burden nations would seem plausible, the introduction of this technology in developing nations with a high disease burden would be unrealistic due to the high costs and the laboratory expertise required. These challenges highlight the need for the development of simpler and more affordable technologies for the characterization of TB strains (e.g. point-of-care multiplex tool to differentiate strain type). Successful interventions to decrease transmission will reduce the levels of recurrent TB disease (due to re-infection) as well as reduce the opportunities for mixed-strain TB infection.

CONCLUDING REMARKS

Molecular fingerprinting techniques have allowed for the differentiation of recurrent TB disease into relapse versus re-infection, as well as the identification of TB infection with multiple strains. The development of improved techniques for the identification of relapse, re-infection and mixed infections has important implications for the understanding of TB epidemiology and the implementation of related technologies in TB-control programs, patient management and outcome analysis of clinical trials. Ongoing research using WGS to provide more detailed insight into these phenomena is imperative to design effective transmission intervention strategies. However, a limitation with the substantive research effort in these areas over the last two decades is the lack of a common sample collection and processing protocol for specimens to allow for a direct comparison of mixed infections and recurrent disease in geographically distinct settings with varying burdens of disease. Such studies should take high priority on the global TB research agenda.

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