

Studies on the Phenotypes of *Mycobacterium tuberculosis* in Sputum

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Abstract

Transcriptional, cytological and culture-based analyses of *Mycobacterium tuberculosis* (Mtb) in sputum have revealed multiple traits indicating the presence of a persister-like or dormant mycobacterial population. Between patients, variable proportions of bacilli in sputum appear to be slow or non-growing, contain lipid bodies (LBs) and depend on exogenous Resuscitation promoting factors for growth. More recently by using Auramine O/Nile-red staining the presence of non-acid-fast (NAF) Mtb-like bacilli containing abundant LBs has been noted. Based on these findings, Mtb in sputum may present in multiple populations and express distinctive transmission adapted phenotypes. Identifying these phenotypes and replicating them in *in vitro* settings may lead to important new understanding of Mtb *in vivo*.

To study the suspected NAF Mtb cells in sputum, immunofluorescence (IF), peptide nucleic acid (PNA) probe and fluorescence Kinyoun methods were developed and studied. The IF and PNA methods detected only minor components of sputum and variable proportions of *in vitro* grown cells. Various conditions such as freeze thawing, growth phase and biofilm cultures were shown to alter Auramine NAF proportions. In contrast the fluorescence Kinyoun method labelled the majority of Mtb cells in the preparations studied and provides a promising method for future studies when combined with a suitable LB stain.

The capacity of biofilm cultures to replicate the Mtb bacillary populations in sputum was studied. Three phases of biofilm cultures (pellicle, planktonic and attached layers) were studied for gene expression, cytological, growth, antibiotic tolerance and ³H-uracil labelling properties comparable to the Mtb phenotypes seen in sputum. The three layers replicated to differing degrees the sputum phenotypes including LB and NAF content, and modest Rpf-dependancy. Attached and planktonic cells gave well correlated transcriptional patterns. Overall, it appears plausible that biofilm grown cells in patient's lungs could contribute to the populations seen in sputum.

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List of Abbreviations

AFB	Acid-fast bacilli
AF	Acid-fast
ATP	Adenosine Triphosphate
BCG	Bacille Calmette-Guerin
ADC	Albumin Dextrose Catalase
Att	Attached
BD	BODIPY
CD	Cyclodextrin
cDNA	Complementary Deoxyribonucleic Acid
CFU	Colony Forming Unit
CI	Confidence Interval
CoA	Coenzyme A
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
DosR	Dormancy survival regulator
ECM	Extra Cellulat Matrix
FA	Fatty acid
FAS	Fatty acid synthase
FAS I	Type 1 Fatty Acid Synthetase
FAS II	Type 2 Fatty Acid Synthetase
FI	Fluorescence intensity
GLx	LipidTOX red neutral lipid stain
HIV	Human Immunodeficiency Virus
ICL	Isocitrate lyase
IF	Immunofluorescence
IIF	Indirect Immunofluorescence
II	Inhibition Index
IgG	Immunoglobulin G
IFN γ	Interferon γ
KDa	KDa
LB	Lipid body
LCFA	Long Chain Fatty Acid
LB(s)	Lipid Body (Bodies)
M	Molar
MA	Mycolic acid
mAGP	Mycolyl Arabinogalactan-Peptidoglycan complex
MDR-TB	Multi-drug Resistant tuberculosis
Mg	Microgram
MDR	Multi Drug Resistance
MPN	Most Probable Number

Mtb	Mycobacterium Tuberculosis
NRP	Non-Replicating Persistence
ml	Millilitre
KDa	Kilodalton
OADC	Oleic Acid Albumin Dextrose Catalase
OD	Optical Density
PCR	Polymerase Chain Reaction
PBS	Phosphate-Buffered Saline
Pk	Planktonic
RI	Resuscitation Index
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
Rpf(s)	Resuscitation Promoting Factor(s)
rRNA	Ribosomal Ribonucleic Acid
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RLx	LipidTOX red neutral lipid stain
SD	Standard Deviation
TDM	Trehalose 6'-Dimycolate
Tgs	Triacylglycerol Synthase
UV	Ultra Violet
µl	Microlitre
µg	Milligram
WE	Wax Ester
WHO	World Health Organization
XDR	Extremely Drug Resistant

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Chapter 1

Introduction

1.1. General introduction

Although the worldwide uses of BCG vaccine and antimicrobial therapies have made their mark, tuberculosis (TB), one of the oldest recorded human afflictions, is still amongst the biggest killers within the known infectious diseases (Smith, 2003). TB is caused by infection with *Mycobacterium tuberculosis* (Mtb), from the genus *Mycobacterium*, a group of bacteria characterised by their high lipid content and 'Acid-fastness' which is attributed to a number of mycobacterial cell envelope components (Wayne and Kubica, 1986, Seiler *et al.*, 2003).

Pulmonary TB infection is established when the TB bacillus enters the lung, is phagocytosed by alveolar macrophages, ending with formation of granulomas which contain tubercle bacilli (Saunders *et al.*, 2000). The mechanisms by which the tubercle bacillus stays alive inside the granuloma are still unclear (Peyron *et al.*, 2008). Recently, it has been revealed that Mtb accumulates triacylglycerol (TAG) when exposed to multiple stresses and these may assist its long-term survival (Deb *et al.*, 2009). In this laboratory, analysis of cultures and tuberculous sputum samples demonstrated that the accumulated TAGs appear as intracellular droplets or lipid bodies (LBs) in the cell cytoplasm and that the LBs were associated with non-replicating bacilli (Garton *et al.*, 2002, Garton *et al.*, 2008). LB positive Mtb cells *in vitro* trend to loose acid fastness (Deb *et al.*, 2009) and LB positive non-acid fast bacilli are regularly observed in sputum (Solan, 2008). Further cytological, transcriptional and growth-based sputum studies demonstrated multiple traits indicating the presence of a persister-like or dormant Mtb population. This population was present in varying proportions in samples from different patients and included cells dependent on exogenous resuscitation promoting factors (Rpfs) for growth (Garton *et al.*, 2008, Mukamolova *et al.*, 2010).

The environmental conditions that produced these Mtb phenotypes in sputum remain unclear. In a previous project some potential stimuli have been explored with partial success in replicating *in vitro* the transcriptional signatures seen in sputum (Lee, 2012). This work is extended in the present study, particularly focussing on the suggestion that extracellular growth in TB infected lungs may resemble the biofilm pattern (Lenaerts *et al.*; 2007).

This project is concerned with further exploration of the environmental conditions (particularly biofilm growth) that may have provoked Mtb cells with the phenotypes (non-acid fastness, LB positivity, transcriptional pattern and Rpf-dependency) introduced above. Accordingly, these topics are reviewed in more detail in the following sections.

1.2. The genus *Mycobacterium*

The related genera *Mycobacterium*, *Nocaria* and *Corynebacterium* are, structurally Gram-positive bacteria containing guanine and cytosine (G+C)-rich deoxyribonucleic acid (DNA), lack a true outer membrane, and possess a thick layer of peptidoglycan (PG) (e.g., the PG layer of Mtb ~10 - 20 nm thick) (Wyne & Kubica, 1986, Hett & Rubin, 2008).

Mycobacteria are slender rods, which grow from 0.3 to 0.5 µm in diameter under oxygenated conditions. They are acid-fast (AF), non-motile, and non-spore forming bacilli (Wyne & Kubica, 1986). The rationale behind classifying mycobacteria as acid-fast bacilli (AFB) lies in their ability to resist decolourisation from mineral acids, such as acid alcohol, after staining with cationic dyes, such as carbol-fuchsin (Allen, 1992). This characteristic is associated with the high lipid content in the cell envelope, where mycolic acids (MAs) and waxes are the major components (Watanabe *et al.*, 2001).

The number of recognised *Mycobacterium* species increased from approximately 40 species in 1980 (Skerman *et al.*, 1980) to approximately 120 species in 2009 judged by comparison of their 16S rRNA sequences (Neonakis *et al.*, 2009). Mycobacterial species are classified into slow or fast growers. From dilute inocula, the fast growers demonstrate visible growth within 7 days, whereas the slow growers require more than 7 days for visible growth (Wayne & Sramek, 1992).

The genome size of mycobacterial species ranges from 1,268,203 base pairs (Pb) for *M. leprae* to 6,988,209 bp for *M. smegmatis* mc² 155 all obtained by DNA sequencing (Cook *et al.*, 2009). Classical slow growers such as Mtb possess only one copy of the *rrn* operon, whereas typical fast growers, such as *M. smegmatis* and *M. phlei* have two (Cook *et al.*, 2009).

1.2.1. *Mycobacterium tuberculosis* complex

The *Mycobacterium tuberculosis* complex (MtbC) make up a group with more than 99% identity at the nucleotide level and all have the same 16S rRNA sequence. However, they differ in their phenotypes, pathogenicity, and host tropism (Brosch *et al.*, 2002; Comas & Gagneux, 2009, Dharmadhikari & Nardell, 2009; Cole, 2002). The group comprises Mtb, *M. africanum*, *M. canetti*, *M. bovis*, *M. caprae*, *M. pinnipedii* and *M. microti* (Comas & Gagneux, 2009).

Members of MtbC are straight or curved rods occurring singly or in clumps. The size of MtbC ranges from 0.3-0.6 x 1-4µm. They are strongly acid-fast (AF) under optimal growth conditions (37°C, pH 6.4-7.0) and have a generation time of ~24h. In liquid medium, growth tends to be in serpentine cords, with bacteria showing a parallel orientation. On solid media, Mtb is characterised by formation into rough, raised and thick colonies, with a nodular or wrinkled surface with irregular margins. These colonies may become pigmented with white, buff or yellow colouration (Wayne and Kubica, 1986).

1.2.2. *Mycobacterium tuberculosis*

The characteristic features of Mtb comprise its slow growing, acid-fastness, dormancy, complex cell envelope, intracellular pathogenicity and genetic heterogeneity. The replication time of Mtb is almost 24h in synthetic medium or infected animal models. All of these features contribute to the chronic nature of the disease and elongate the treatment period (Cole *et al.*, 1998).

The Mtb H37Rv (laboratory strain) genome consists of 4.4×10^6 bp and contains about 4,000 genes (Cole *et al.*, 1998). Further, more than 200 Mtb genes are annotated as encoding enzymes for fatty acids (FAs) metabolism, amounting to 6% of the total. Interestingly, among these genes about 100 are predicted to function in the β -oxidation of FAs, whereas in *E.coli*, there are only 50 enzymes involved in FA metabolism (Smith, 2005). This large number of genes involved in FA metabolism may be due to the ability of Mtb to grow in host's tissue where FAs suggested to be the major carbon source (Smith, 2005). Lipid and FA metabolisms are discussed in Section 1.3.

1.2.3. The mycobacterial cell envelope

Mycobacteria have a unique cell envelope structure that differs significantly from most other bacterial species and shares some characteristics with both Gram-positive and negative bacteria. It consists of three major layers: a plasma membrane, a complex of PG and arabinogalactan (AG) covalently linked to MA and a polysaccharide rich capsule (Crick *et al.*, 2001). This complex forms the mycobacterium cell envelope core and is known as the mycolyl AG-PG (mAGP) complex (Brennan, 2003). Figure 1 shows the mycobacterial cell envelope components.

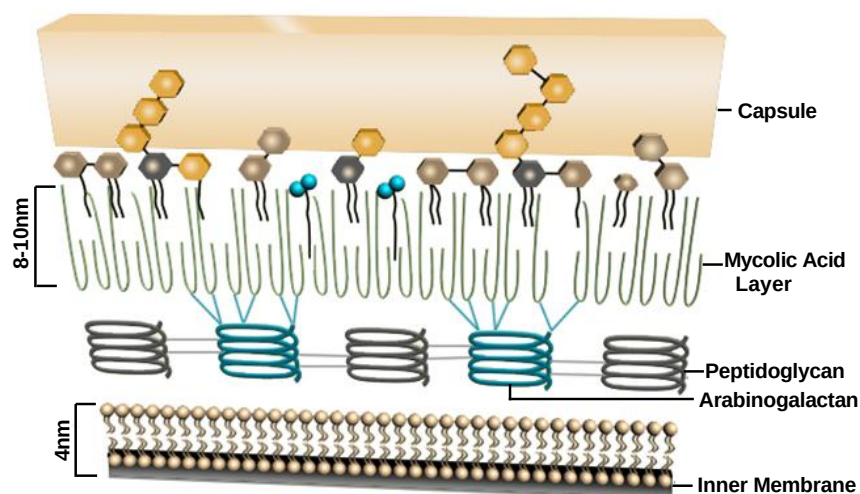


Figure 1: The mycobacterial cell envelope.

The mycobacterial cell wall consists of a large cell wall complex which comprises three major and different covalently linked constructions: arabinogalactan (blue), peptidoglycan (grey) and mycolic acid (green). The outer layer is the capsule (brown), which contains polysaccharides such as glucan and arabinomannan. This 3D schematic diagram is modified from (Abdallah *et al.*, 2007).

In more detail, the outer lipid layer consists of two major components, proteins and free lipids which are non-covalently linked with the MA layer with long and short chain fatty acids complementing the long and short chains of MAs (Hett & Rubin, 2008). The cell envelope also contains glycolipids (lipoarabinomannan (LAM), lipomannan, and phenolic glycolipid (PGL)), phthiocerol dimycocerosates (PDIMs), dimycolyl trehalose (cord factor), sulfolipids, and phosphatidylinositol mannosides (Hett & Rubin, 2008).

Comparing slow and fast growing mycobacteria, in slow growers such as *Mtb* and *M. leprae* the LAMs are capped at the terminal arabinose residue with mannose (Man) residues and are called ManLAMs (Chatterjee *et al.*, 1992, Nigou *et al.*, 2003, Hett & Rubin, 2008). On the other hand, in fast growers such as *M. smegmatis* and *M.*

fortuitum, there are AraLAMs which recently have been called phosphoinositol-capped LAMs and are referred to as PILAMs (Khoo *et al.*, 1995, Vercellone *et al.*, 1998). All the free lipids and proteins are potentially function as signalling and effector molecules (Brennan, 2003) and have a significant role in interacting with the host's immune system (Hett & Rubin, 2008).

Mycolic acids (MAs) in *Mycobacterium*, *Norcardia*, *Rhodococcus*, and *Corynebacterium* are long chain β -hydroxyl α -branched fatty-acids (FAs). The carbon chain extending from C-3 to the methyl-terminal carbon is termed the meromycolate branch. Mycobacterial MAs are distinct from those of related bacteria for two reasons: (i) they contain 70-90 carbon atoms; and (ii) in the meromycolate chain there are only two sites occupied by double-bond cyclopropane rings, or other functional groups (Liu *et al.*, 1996). The high amount and organisation of lipid creates a hydrophilic permeability barrier that surrounds the bacterium and comprises approximately 60% dry weight of the cell (Brennan & Nikaido, 1995; Daffe & Reyrat, 2008).

The permeability of the mycobacterial cell wall is 10 to 100 times lower than that of the notably impermeable *Pseudomonas aeruginosa* (*P. aeruginosa*) (Jarlier & Nikaido, 1990). Therefore, the low permeability of the Mtb cell wall gives it the ability to be sensitive only to aminoglycosides, such as streptomycin and rifamycins among antibiotics, and to fluoroquinolones among general chemotherapeutic agents. Furthermore, it is difficult to prevent the transmission of Mtb in the general environment because mycobacteria are comparatively resistant to alkali, drying, and many chemical disinfectants (Brennan & Nikaido, 1995). Because MAs are acid labile, it has been shown that prior treatment with acid may permeabilise the bacterial cell wall, which allows the fluorescently labelled oligonucleotide probes to access the cell wall (McNaughton *et al.*, 1994).

MAs are present as tetramycolylpentaarabinofuranosyl clusters on the site of arabinan of mycolylarabinogalactan. MAs also occur inside the outer layer in the form of cord-factor which is the trehalose dimycolate (TDM) and trehalose monomycolate (TMM) (Chatterjee, 1997). Cord-factor is significant as a toxic lipid (Hunter *et al.*, 2006) that is associated with Mtb cells growing in cord-like forms *in vitro* (Takayama *et al.*, 2005). Slow growing mycobacteria such as Mtb are able to modify their MAs by cyclopropanation, whilst fast-growing mycobacteria such as *M. smegmatis* do not (Chatterjee, 1997).

MAs give the mycobacterial species unique characteristics including resistance to chemical injury and dehydration, low access to hydrophilic chemotherapies and virulence (Dubnau *et al.*, 2000, Glickman *et al.*, 2000, Glickman & Jacobs, 2001). They also give the mycobacterial species their AF characteristic (Bhatt *et al.*, 2007), which will be discussed in detail in Section 1.4. MAs also play an important role in mycobacterial biofilm formation (Ojha *et al.*, 2005, Ojha *et al.*, 2008, Sambandan *et al.*, 2013), which may help Mtb to persist within the host (Daffe & Draper, 1998, Bhatt *et al.*, 2007). Additionally, enzymes that are involved in mycolate biosynthesis are considered as an ideal target for anti-mycobacterial therapies (Bhatt *et al.*, 2007).

1.3. Lipid bodies in mycobacteria

Lipid bodies (LBs) in mycobacteria were first observed by using Sudan black to stain the lipid droplets in a study done by Burdon, (1946). LBs are intracellular, spherical, neutral lipid-filled inclusions containing triacylglycerol (TAG) and wax ester (WE). They were recently recognised in AFB in sputum from a TB patient in Gambia (Garton *et al.*, 2002). Garton *et al.*, (2008) showed that LB positive Mtb in differing proportions are a universal feature in tuberculous sputum. Figure 2 shows the LB appearance in AFB⁺ sputum sample.

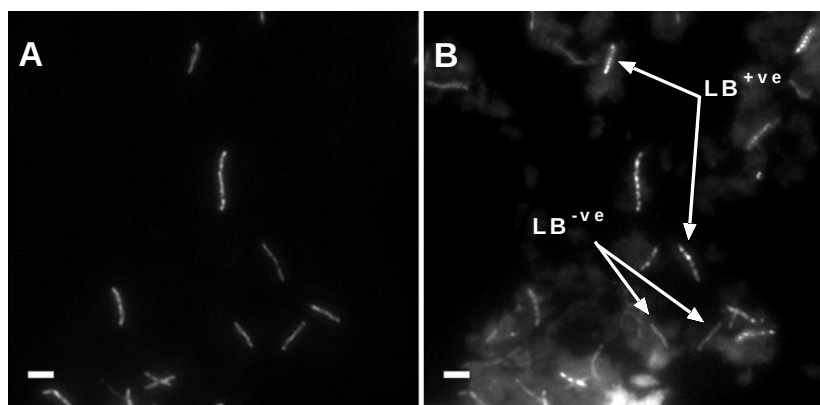
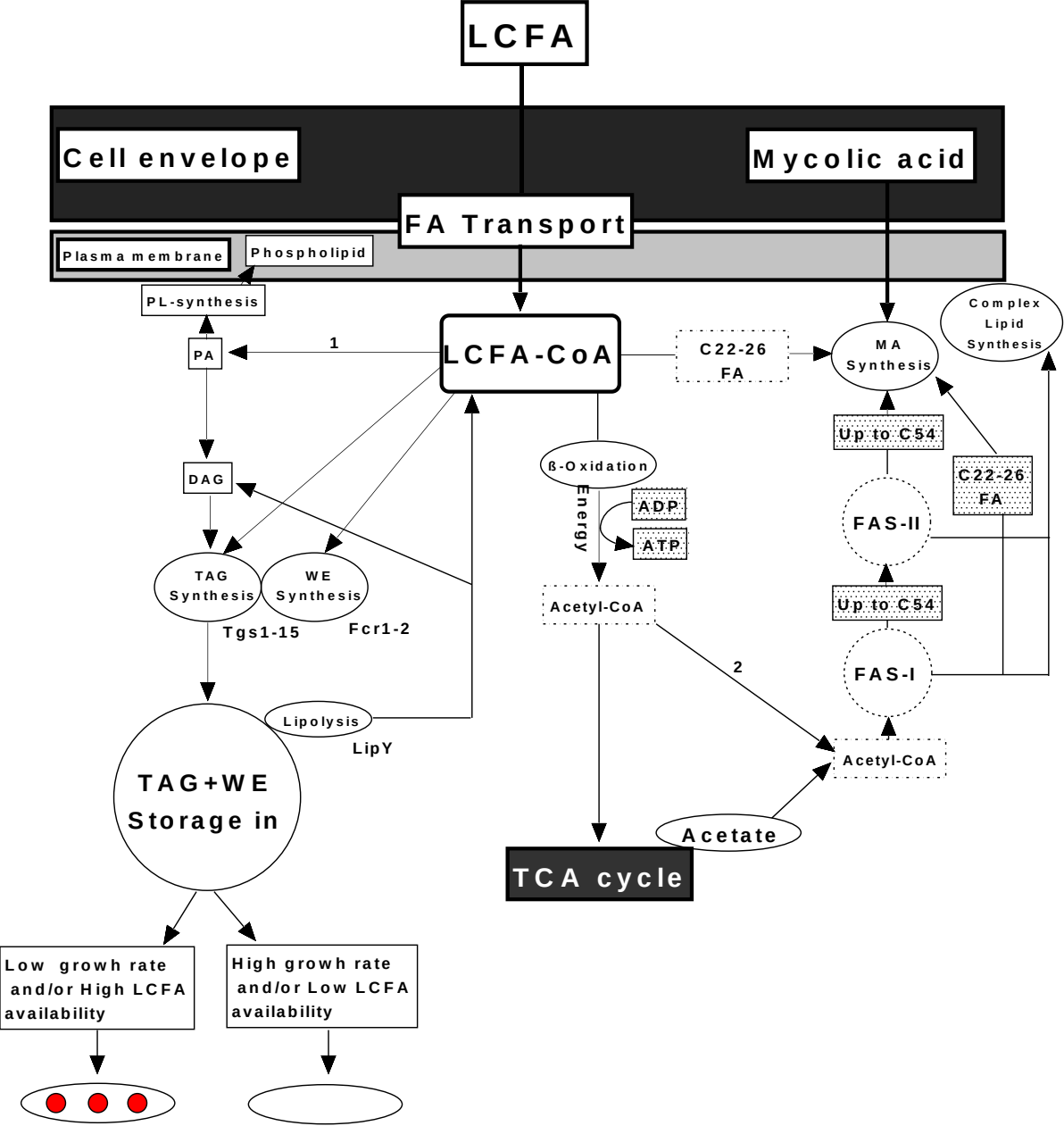


Figure 2: Lipid bodies in tubercle sputum sample.

This figure shows images from a 4+ smear-positive sputum sample of (A) Auramine O stained Mtb bacilli from a sputum sample and (B) the same cells stained with LipidTox Red Neutral Lipid stain (RLx). Arrows represent the different LB populations in the same sputum sample as they vary in having LB, as well as in the number of lipid droplets. Scale bar is 2 μm.

The lipid contents on the mycobacterial cell wall cause an important burden on cell synthesis. There are about 250 identified genes in Mtb implemented in FA metabolism

compared with 50 genes in *E. coli* (Cole *et al.*, 1998). The mycobacterial cell wall, phospholipids and acylglycerols are derived from the long chain FA (LCFA). Although the cell is actively growing, great amounts of LCFAs are necessary to synthesise new cell wall components; nevertheless, exogenous FA sources, whereas beneficial are also potentially bactericidal, because of their detergent like activities (Kondo and Kanai, 1972). In contrast, additional LCFA is concentrated into TAG and LB synthesis under conditions of stress, which leads to intervals of slow growth. This TAG can be hydrolysed back to LCFA to refill cell reserves (Russell, 2011). Figure 3 shows the principle Mtb lipid biosynthetic and catabolic pathways relevant to the present study.



Caption overleaf

Figure 3: Schematic diagram of the interaction among lipid metabolism pathways and fatality of LCFA in *M. tuberculosis*

This figure shows that long chain fatty acid (LCFA) may be transported into the mycobacterial cell by the action of FA transport proteins or alternatively synthesised by fatty acid synthase I (FAS-I); both are coenzyme A (CoA) dependent. Complex lipids such as TDMs, TMMs and PDIMs, phospholipids in addition to MA, are produced from LCFA. The action of lipases leads to release of LCFA from stored in triacylglycerols (TAGs) or wax esters (WEs). Due to degradation of LCFA via β -oxidation, acetyl-CoA is generated and generates ATP via the tricarboxylic (TCA) cycle. Acetyl-CoA can also be used for elongation of FA. There are additional enzymes implicated in this process such as Tgs1-15, Fcr1-2 and LipY. However, Tgs1-15 have either WE synthase or diacylglycerol acyltransferase activity. Rv0308 is a putative phosphatidate phosphatase membrane-bound protein that yields diacylglycerol (DAG) from phosphatidic acid (PA); 1, PA is synthesised through two cycles of sequential concentration of FA-CoA to glycerol-3-phosphate to produce 1 acyl-glycerol-3-phosphate followed by 1,2 diacyl-glycerol-3-phosphate (PA). PA is used to create complex PLs; 2, LCFA-CoA cannot be directly combined into MA precursors in fatty acid synthase II (FAS-II) as a only FA carried by AcpM can be synthesised by FAS-II. Transformation from CoA to AcpM is done by mtFabH, which forms the link between FAS-I and FAS-II. It is suggested that LBs are formed according to an environmental equilibrium of available LCFA and cell growth conditions. When the cell is actively growing, LCFA flux is also active as a result of cell wall lipid and phospholipid turnover. On the other hand, when the cell is growing slowly or stressed the syntheses of cell wall lipids and phospholipids are repressed and LCFA can be transported into TAG synthesis for storage. (Figure modified from (Sherratt, 2008, Lee, 2012).

The FAS-I system of Mtb is encoded by *fab* (Rv2524c) whose substrates are a FA. The FAS-I domain of Mtb is organised in this order: acyltransferase, enoyl reductase, dehydratase, malonyl/palmi- toyl transferase, acyl carrier protein, β -ketoacyl reductase, and β -ketoacyl synthase which supports the short chain acyl-coA substrates for further elongation by FAS-II system (Takayama *et al.*, 2005). FAS-I is thought to be essential for Mtb viability (Sasseti *et al.*, 2003). Although FAS-I and FAS-II have different mechanisms, the FAS-II mechanism is dependent on substrates made by FAS-I (Takayama *et al.*, 2005). The link between FAS-I and FAS-II systems is initiated by the mtFabH condensing enzyme. The role of mtFabH enzyme was shown to be as an initiator of MA elongation (Bhatt *et al.*, 2007b).

Following the condensation process catalysed by mtFabH, the precursor FA-AcpM is exposed to keto-reduction, dehydration and enoyl-reduction catalysed by MabA, FabG (β -ketoacyl-AcpM) and InhA (enoyl-AcpM) reductases. As a consequence, the FA is extended and β -acyl-AcpM initiates further rounds of extension by β -ketoacyl-AcpM, KasA and KasB synthases (Bhatt *et al.*, 2007b).

KasA and KasB encode a distinct FAS-II β -ketoacyl-ACP synthases (Cole *et al.*, 1998); the former is essential in Mtb but the latter is not. In *M. marinum* and *M. smegmatis* KasB appears to be an accessory gene that is not essential for MA synthesis (Bhatt *et al.*, 2007b). KasB is implicated in the carbon chain extension to full length mero-MAs

where the first step is initiated by KasA (Gao *et al.*, 2003, Bhatt *et al.*, 2007a). A significant observation was reported by Bhatt *et al.*, (2007a) where a *KasB* mutant resulted in a change in colony morphology and the loss of Mtb acid-fastness.

During dormancy, Mtb accumulates triacylglycerol (TAG). Mtb triacylglycerol synthase 1 (*tgs1*) is the major source of TAG synthesis and deletion of *tgs1* leads to an almost complete loss of TAG (Daniel *et al.*, 2010). LB formation is likely due to activation of the dormancy associated DosR regulon (see 1.5.7) as *tgs1* is highly responsive to DosR (Boshoff and Barry, 2005). Although LB formation and DosR expression are clearly related, LB formation may be influenced by many other factors including expression of the other 14 *tgs* homologues in Mtb, expression of lipases and the likely LB accessory proteins recently identified in *M. bovis* BCG.

1.4. Mycobacterial acid-fastness

Acid-fastness is a key property differentiating mycobacteria from other bacterial species (Murohashi *et al.*, 1968). Mycobacterial acid-fastness is routinely identified as the ability of the cell to retain fuchsin, crystal violet, or Auramine O when presented in a phenol-water mixture (as carbol-fuchsin, carbol crystal violet, or carbol Auramine O) and resists decolourisation by acidic alcohol (Barksdale *et al.*, 1977; Nyka *et al.*, 1970). The resultant acid-resistant mycobacterial cells appear red (carbol-fuchsin retained), purple (crystal-violet retained) or yellow-green by fluorescence microscopy (Auramine O retained) (Barksdale *et al.*, 1977).

1.4.1. The mechanism of acid-fastness

The exact mechanism of acid-fastness is not fully understood (Fukunaga *et al.*, 2002). Berg (1955) stated that when mycobacterial cells are broken they are only weakly AF, with a poor brightness of colour because of the obscuring methylene-blue counter stain. When mycobacteria are exposed to carbol-fuchsin, the whole mycobacterial cell preserves it into its interior and also binds fuchsin to the MA residues of the PG of the outer cell wall. Free MAs of the mycobacterial cells bind fuchsin via an acid-stable bonding. The cell surface then became highly hydrophobic once the mycobacterial MA complexed with an arylmethane dye (Berg, 1955).

Following this mechanism when a carbol-fuchsin stained mycobacterial cell was subjected to decolourisation with acid-alcohol (EtoH-HCl), the carbol-fuchsin taken inside the internal mycobacterial cell creates leftovers there and also enhances the lightly stained MAs complex of the external layer (Barksdale *et al.*, 1977). In an experiment to determine which part of the mycobacterial cell wall preserves the carbol-fuchsin after decolourisation, it was observed that from isolated MA cell wall fractions, which contain a high density of molecules other than MAs, a weak pink colour is retained following decolourisation (Cho *et al.*, 1966). Consequently, the mycobacterial acid-fastness brilliance is dependent on trapped carbol-fuchsin, which is assured by the fuchsin-mycolate of the external peptidoglycolipids (Barksdale *et al.*, 1977).

There are some bacterial species that resist decolourisation by dilute (1-10%) mineral acids such as *Nocardia* and *Corynebacterium*, which when stained by AF stain and grown in specific media contain glycerol (Harrington, 1966). Although, *Nocardia* and *Corynebacterium* resist decolorization by dilute mineral acids (Beaman *et al.*, 1973; Cho *et al.*, 1966), they do not resist decolourisation by EtoH-HCl (Barksdale *et al.*, 1977).

Knowledge about the target of AF staining on the mycobacterial cell is incomplete. Z-N and Kinyoun staining have been held to target the lipid complex of the mycobacterial cell wall (Richards, 1941; Harada, 1976; Shapiro *et al.*, 2008) while Auramine O staining has been thought to target both MA (Richards, 1941) and nucleic acid (Oster, 1951; Hanscheid *et al.*, 2007) content of the mycobacterial cell.

1.4.2. Non-acid-fast alternates of mycobacteria

Although the AF characteristic is the most important property that distinguishes mycobacteria from other bacterial species (Murohashi *et al.*, 1968), it has been reported that *Mtb* bacilli become non-acid-fast (non-AF) with Z-N staining when caseous lesions begin to liquefy and form tuberculous cavities in the lung (Takahashi, 1979). *In vitro* studies also noted that some growth conditions resulted in the formation of non-AF mycobacterial cells with Z-N staining (Mudd & Mudd, 1927, Yegian & Porter, 1944, Murohashi & Yoshida, 1965, Nyka, 1974, Gangadharam & Stager, 1975, Yuan *et al.*, 1998, Bhatt *et al.*, 2007a, Bhatt *et al.*, 2007b). Furthermore, staining a tissue section from the lung with Z-N staining revealed that *Mtb* bacilli

appear to be non-AF (Seiler *et al.*, 2003). The reason behind the loss of acid-fastness is still unclear. It may be due to mycobacterial cell envelope alteration due to stress.

Acid-fastness can be affected by many conditions. For example, a multiple-stress model for Mtb was incubated under several conditions including low oxygen, high carbon dioxide, starvation and acidic pH and this resulted in loss of acid-fastness (Deb *et al.*, 2009). Several other procedures result in the formation of non-AF Mtb and these include, cell wall disruption via physical or chemical changes (Yegian & Porter, 1944), exposure to UV irradiation (Murohashi & Yoshida, 1965), lack of growth nutrients or starvation (Nyka, 1974), mycobacteriophage infection (Gangadharam & Stager, 1975), cell wall alteration resulting in changes in MA contents (Yuan *et al.*, 1998, Bhatt *et al.*, 2007a) and isoniazid antibiotic exposure (Bhatt *et al.*, 2007b). Sputum decontamination with N-Acetyl-L-Cysteine (NALC) and NaOH also reduces the AF proportion. It was also shown that dry heating the sputum and culture smears in a heat block might alter the cell wall and as a result reduce acid-fastness (Gokhale *et al.*, 1990).

1.4.3. Carbol-fuchsin based staining

1.4.3.1. Zeihl-Neelsen stain

Z-N is a staining method described by Zeihl and Neelsen followed by Robert Koch's identification of Mtb 130 years ago. Subsequently, Z-N was developed by Neelsen, Ehrlich and Rindfleisch (Bishop *et al.*, 1970). A carbol-fuchsin-based stain with Z-N requires heating and is the standard staining method for processing sputum smears to detect Mtb (Pandey *et al.*, 2009).

Tubercle bacilli appear under transmitted-light microscopy as pink rods against a blue background (methylene blue countersatin. Shapiro and colleagues (2008) developed detection of Z-N staining in sputum smears using fluorescence microscopy. They showed that some Mtb bacilli were detected by fluorescence but not by transmitted light (Shapiro *et al.*, 2008).

1.4.3.2. Kinyoun stain

One of the alternative techniques to classical Z-N that is widely used in clinical laboratories the method developed by Kinyoun (1915). Cold Z-N staining or Kinyoun staining is carbol fuchsin-based and uses a higher phenol concentration, presumably to increase the permeation of fuchsin into the mycobacterial cell without heating. Kinyoun staining was developed to shorten the staining time and improve laboratory efficiency (Pandey *et al.*, 2009).

As with the Z-N method, tubercle bacilli appear under transmitted-light microscopy as pink rods against a blue background. It has been shown that the Z-N staining technique is more sensitive than Kinyoun (Somoskovi *et al.*, 2001).

1.4.3.3. Auramine O stain

Another alternative method to Z-N that does not require heating is Auramine O staining (Truant *et al.*, 1962). Auramine O is a cationic dye that was first introduced by Hagemann in 1937 (Hagemann, 1937); it is excited by blue light (450–480 nm) and emits in the green-yellow or golden range (500–600 nm) (Marais *et al.*, 2008).

Many studies have compared conventional Z-N and fluorescence Auramine O staining, reporting that Auramine O has the same specificity as Z-N and a higher sensitivity (about 10% higher) than Z-N staining for detection of Mtb (Selvakumar *et al.*, 2002, Steingart *et al.*, 2006, Marais *et al.*, 2008). However, there are no comparative studies comparing fluorescence Z-N and Kinyoun with fluorescence Auramine O staining. Generally, fluorescence microscopy has a better minimal screening time (Marais *et al.*, 2008) and test slides are examined under a lower magnification (Habeenzu *et al.*, 1998).

1.5. Tuberculosis

Robert Koch was the first to describe Mtb bacilli arranged in cords of cells by light microscopy of sputum samples. Despite many advances in diagnosis and treatment the disease still kills 1.4 million individuals worldwide annually and about 9 million people became sick from TB according to CDC records (Centres for Disease Control and Prevention, 2013). Even though the incidence of TB has been declining in

developed countries, recent years have seen an increase in the number of cases (Dutt, 2011) in the UK. This partly reflects the high numbers of immigrants from high-incidence areas such as Sub-Saharan Africa. Although, many antibiotics have been discovered, treatment remains a problem due to the rise in multi-drug resistance (MDR) and extensively drug-resistant (XDR) Mtb strains (Dutt, 2011, Williams, 2012).

1.5.1. Clinical disease

The majority of TB cases present as pulmonary disease, resulting from reactivation of latent TB infection, reinfection, or initial development from a primary infection see 1.5.4 (Lin and Flynn, 2010). However, any organ can be involved as extra-pulmonary disease as TB can display varied disease expression (Hopewell and Jamser, 2005). Symptoms of pulmonary TB begin with a persisting cough as a common symptom, which might not be productive early on, but as the disease develops and results in inflammation and necrosis of the lung tissue, mucoid or purulent sputum is frequently coughed up (Hopewell and Gamser, 2005). Expectoration of blood-stained sputum (haemoptysis) may be present with pulmonary TB, but does not necessarily indicate active TB; haemoptysis may be a consequence of ruptured blood vessels in an old cavity and other bacterial or fungal infections (Hopewell and Gamser, 2005). Chest pain and dyspnea may also occur as a result of spontaneous pneumothorax. TB may also end up with respiratory failure in advanced disease stages (Huseby and Hudson, 1979, Murray *et al.*, 1978).

1.5.2. Treatment of tuberculosis

Streptomycin (SM) was introduced as a first TB drug, however, monotherapy with this drug led to resistance and treatment failure in many cases. In 1952, Isoniazid (INH) provided the first modern anti-TB therapy (Mitchison, 2005). The use of INH monotherapy then provoked a search for other anti-TB therapies that could be used in combination in order to prevent INH resistance. In 1961, pyrazinamide (PZA) was introduced followed by ethambutol (EMB) and rifampicin (RIF) in 1967. INH, RIF, EMB and PZA are used today as the first-line TB drug regimen (Mitchison, 2005, Alcalá *et al.*, 2003). All four drugs are given for the first 2 months and INH and RIF continued for the remaining months of the six month standard treatment. This extended treatment poses major problems for patient compliance. The target of each drug is shown in Figure 4. Only INH and RIF have been used in this study.

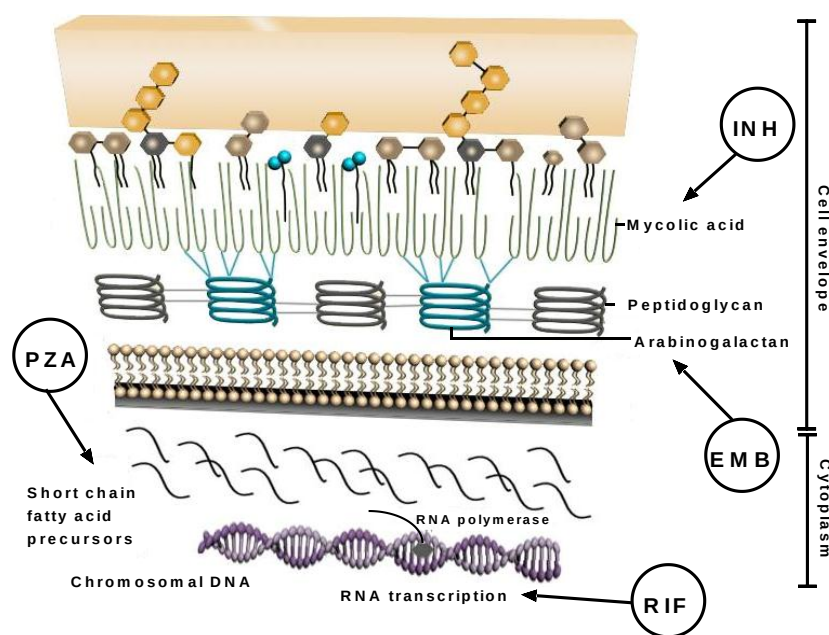


Figure 4: Antimycobacterial chemotherapy sites of action.

This figure shows the different sites of action of isoniazid (INH), pyrazinamide (PZA) and rifampicin (RIF) in an Mtb cell envelope and cytoplasm. As shown, INH inhibits mycolic acid synthesis; PZA inhibits short-chain FA synthesis, whereas RIF inhibits the transcription process by binding to the β -subunit region of RNA polymerase Figure adapted from (Somoskovi *et al.*, 2001).

INH was introduced in 1952 as the first modern TB drug and is more useful than SM because there is a large therapeutic margin (Bloch *et al.*, 1994). INH is bactericidal and is both the oldest synthetic anti-TB drug and most extensively prescribed medication against active TB infection and prophylaxis (Blanchard, 1996). INH acts by inhibiting the synthesis of cell envelope MA (Winder and Collins, 1970). As a consequence, INH-treated Mtb bacilli lose their AF staining characteristic (Zhang and Young, 1993).

INH enters the bacterial cell by passive diffusion (Bardou *et al.*, 1998). It is a pro-drug activated by the KatG enzyme (Zhang and Young, 1993), a multifunctional catalase peroxidase that has other activities such as peroxynitritase and NADH oxidase. KatG activates INH by producing intracellular INH derived damaging species. However, KatG can act as an effective producer of a range of INH-derived radicals as a result of the presence of INH, which can inhibit some KatG catalytic activities (Timmins and Deretic, 2006). As a result of this, it has been hypothesised that these INH-derived

damaging species (oxygen and carbon centred free radicals) are directly important in mycobacterial cell lethality (Shoeb *et al.*, 1985) because INH damages a range of mycobacterial cell components, mainly the lipids, by inhibiting cell-wall lipid synthesis (Timmins and Deretic, 2006).

RIF is a fermentation product extracted from *Nocardia mediterranei* as a semisynthetic derivative of rifamycins (Sensi *et al.*, 1959). Since the discovery of RIF in 1967 (Mitchison, 2005), it has been a valuable antibacterial chemotherapy effective at low concentrations against mycobacteria and Gram-positive species (Wehrli, 1983). RIF provides an early bactericidal effect on metabolically active Mtb. In addition, it displays late sterilizing action on semi-dormant bacteria with low metabolic activity. The introduction of RIF and PZA shortened TB treatment from 1 year to 6 months. Monoresistance with INH is quite common and uncommon for RIF resistance, which usually occurs with INH resistance. Hence, RIF resistance is used as a marker for MDR TB (Somoskovi *et al.*, 2001).

RIF was first shown to inhibit the RNA polymerase of *E. coli* (Hartmann *et al.*, 1967). More than 96% of RIF resistance (Ramaswamy and Musser, 1998) relates to mutations in a well-defined, 81 base-pair central region of the gene that encodes the β -subunit of the RNA polymerase (Telenti *et al.*, 1993).

The need for long-term treatment of TB is not due to conventional drug resistance but is generally associated with physiological states of bacteria that play a major role in drug tolerance (McKinney, 2000). It has been proposed that there are four Mtb populations during infection with distinct physiological states. These states are: fast growing bacilli, slow growing bacilli, dormant or non-replicating persistent (NRP), and bacilli tolerant to the acidic environment (Mitchison, 1979). Although INH is effective against slow growing bacilli, fast growing bacilli can be killed effectively by INH within the first two wks (McKinney, 2000, Zhang and Amzel, 2002). The remaining populations can be eliminated by RIF, and PZA has been thought to also kill bacteria in an acidic environment and in NRP (McKinney, 2000).

The challenge of TB patient compliance has been addressed by the World Health Organisation (WHO) with the introduction of a Directly Observed Therapy – Short Course (DOTS) treatment program. A DOTS program establishes a treatment course for each TB patient that is supervised by a health or social worker (McKinney, 2000). DOTs program are expensive and difficult to apply, and as a result only 1 of 5 TB

patients is treated this way. DOTS is extremely effective in treating TB cases with high cure results (McKinney, 2000, Zhang and Amzel, 2002).

1.5.3. Transmission of tuberculosis

TB is spread through airborne droplet nuclei of sizes about 1 – 5 µm in diameter that contain *Mtb* bacilli when an individual with pulmonary or laryngeal TB coughs, sneezes, sings or even talks (Leung, 1999). An individual with active pulmonary TB who coughs up frequently is able to introduce thousands of these droplets into the air. A study conducted by Fennelly *et al.*, (2004) using a cough aerosol sampling system showed that aerosol particles from TB patients can reach 7 or more µm in diameter. These particles containing *Mtb* bacilli settle very slowly and can remain airborne from minutes to several hr after coughing (Beggs *et al.*, 2003, Frieden *et al.*, 2003). Thus, TB is transmitted most easily in overcrowded and poorly ventilated residences that typically reflect low-income level accommodation (Beggs *et al.*, 2003).

When infectious droplet nuclei are inhaled, they may lodge in the alveoli (Frieden *et al.*, 2003) where they meet the initial defence, alveolar macrophages. The level of infectiousness varies between TB patients. There are many factors that may control the probability for the transmission of TB and the establishment of infection, these include: host immune status, the force of the cough and properties of the bacillus such as virulence factors and bacterial load (Ahmad, 2011, Dharmadhikari and Nardell, 2009). Variation in TB infectiousness has been measured by subjecting Guinea pigs to the air generated from a TB hospital ward. Guinea pigs are used in these type of studies because they are extremely vulnerable to low numbers of bacilli (a single infectious droplet can cause an infection) (Riley, 1961). Results of this study revealed that 4% of infected patients in a particular ward were responsible for developing the TB in 73% of the Guinea pigs (Sultan *et al.*, 1960).

Another risk factor that increases the probability of transmitting infection is prolonged and close contact to a TB patient, hence, co-habiting family members are more likely be infected than the wider community (Dutt, 2011). Risk factors are summarised in Figure 5.

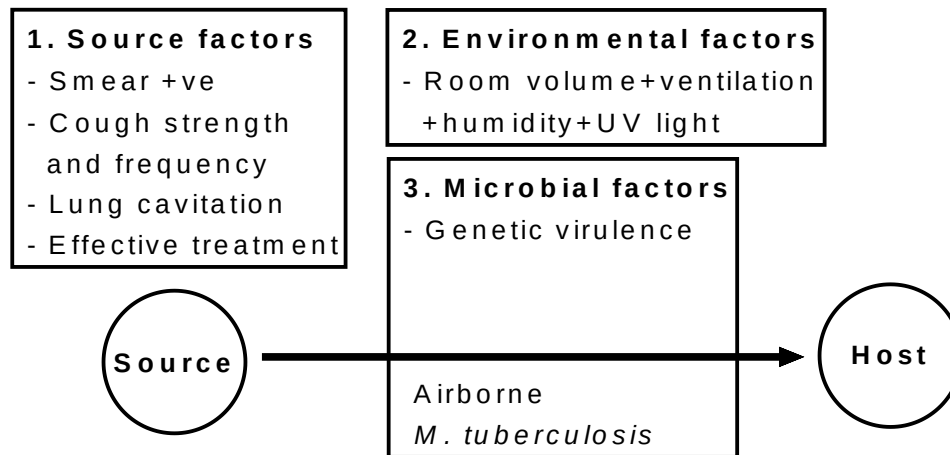


Figure 5. Factors affecting tuberculosis transmission.

This schematic figure displays the different factors affecting the transmission of TB. These factors containing source factors, environmental factors and microbial factors (Figure modified from Dharmadhikari and Nardell, 2009).

1.5.4. Pathogenesis

As a first event, Mtb is believed to enter the alveolar space of the exposed person in an aerosol droplet, where they are ingested by alveolar macrophages or by alveolar epithelial type II pneumocytes (Smith, 2003). A key cell that plays an important role in early stage infection and activation of T-cells by presenting Mtb antigens (Ags) is the dendritic cell (DC) which is migratory (Smith, 2003). This feature gives DCs an important role in dissemination (Lipscomb and Masten, 2002). However, most research has concentrated on the Mtb-macrophage interaction, and as such is the focus in this section.

Following macrophage phagocytosis, infection is arrested by the bactericidal activities of activated macrophages such as reactive oxygen intermediates (ROIs), reactive nitrogen intermediates (RNIs), lysosomal enzymes, acidic pH and toxic peptides (Smith, 2003, Ehrt and Schnappinger, 2009). Inside macrophages, Mtb possesses a number of survival mechanisms to avoid death from these bactericidal processes (Smith, 2003). These mechanisms include inhibition of phagosomal maturation, inhibition of apoptosis, inhibition of macrophage response to interferon- γ (INF- γ) through the 19-KDa protein (Fortune *et al.*, 2004), and polymerisation of actin (actin is required for the scaffolding of endosomes throughout phagosome-endosome connections and a correlation between the polymerisation of actin by Mtb and a delay

in phagosomal maturation) (Hestvik *et al.*, 2005). Following the primary infection of the macrophage in a naïve host's lung, in the absence of an immune response, Mtb undertakes a period of rapid growth. Once the host's immune system gains awareness, infected macrophages pass through the lymphatic system to the hilar lymph nodes (Honer zu Bentrop and Russell, 2001, Frieden *et al.*, 2003). The macrophage plays an important role as a primary host cell in TB infection, by supporting a significant environment for bacterial multiplication and distribution, and as a possible niche for persistent infection (Cosma *et al.*, 2003).

The state of TB disease during the course of infection reflects the interaction between the host and microbial factors (Hopewell and Gamser, 2005). The possibility of developing active disease becomes higher when the immune system cannot effectively contain the replication and proliferation of tubercle bacilli, such as in children under five-years-old and in immunocompromised adults, such as those with HIV (Frieden *et al.*, 2003).

Cell-mediated immunity in most individuals develops after infection of 2-8 wks (Frieden *et al.*, 2003). *In vitro* studies in measuring the response of murine and human macrophages to Mtb infection reveal that cells produce a strong proinflammatory response via the activity of Toll-like receptor (TLR) agonists, which are rich on the surface of the bacteria (Russell, 2007). The growth of Mtb in alveolar macrophages leads to stimulation of this proinflammatory response via the release of Tumour Necrosis Factor- α (TNF- α) and chemokines, attracting mononuclear cells such as monocytes, lymphocytes and neutrophils, from blood vessels towards the site of infection (Cosma *et al.*, 2003, Russell, 2007, Smith, 2003). Natural killer (NK) T cells are first recruited by chemokines to the site of infection, following by recruitment of CD4⁺ T cells, CD8⁺ T cells and $\gamma\delta$ T cells and B cells (Russell, 2007). Granule-mediated lysis of Mtb-infected macrophages is achieved mostly by CD8⁺ T cells and NK T cells expressing perforin and granulysin proteins (Stenger *et al.*, 1998, Gansert *et al.*, 2003). Granulysin protein level is decreased in children after therapy which suggests that granulysin can be used as a marker of disease activity in childhood TB (Di Liberto *et al.*, 2007). However, CD4⁺ T cells produce interleukin-2 (IL-2) and TNF- α which are important to control the infection and also produce IFN- γ to activate macrophages. IFN- γ controls and amplifies the cellular enrolment of both cytokines and chemokines which are released from CD4⁺ and CD8⁺ T cells (Russell, 2007). All these immune cells are building blocks for the classic lesions of TB, the granuloma (Russell, 2007).

1.5.5. Granuloma formation

The granuloma is considered a hallmark of TB (Silva Miranda *et al.*, 2007). It can be defined as a central, compact collection of inflammatory cells in which mononuclear cells predominate (Zumla and James, 1996), and where the infection can be controlled and limited from dissemination by the immune system (Russell, 2007). However granulomas could be beneficial for Mtb by providing the bacilli with a niche in which it can stay alive, modulating the immune response to allow its persistence without lysis over long periods of time.

Granulomas consist of foamy macrophages which are highly lipid-laden and other mononuclear phagocytes surrounding the core infected macrophages (Russell, 2007, Gonzalez-Juarrero *et al.*, 2001, Silva Miranda *et al.*, 2007). These macrophages are surrounded by a layer of lymphocytes forming a fibrous layer of collagen and other extracellular matrices, which outline the structure from normal lung tissue (Honer zu Bentrup and Russell, 2001, Russell, 2007).

The Mtb granuloma is characterised by the formation of central caseation (Zumla and James, 1998). In advanced stages, the granuloma forms a fibrous sheath and the number of blood vessels that go to the structure diminishes. The central zone or the caseation involves a cheese-like semi solid-structure that is rich in lipids and proteins from live bacteria as well as dead cells placed in a hypoxic environment (Honer zu Bentrup and Russell, 2001, Russell, 2007). Necrotic granulomas are produced by a cellular necrotic section in the centre of the granuloma with a rim of lymphocytes of the T- and B cell forms surrounded by epithelioid macrophages (Flynn *et al.*, 2011, Huynh *et al.*, 2011). Bacteria in the centre of the necrotic granuloma are believed to use the cheese-like matter as a source of nutrients (Wayne and Sohaskey, 2001). Within this environment, hypoxia is one of the conditions that are speculated to shift Mtb bacilli to a NRP state (Wayne and Hayes, 1996). The formation and maturation of tuberculous pulmonary granuloma is shown in Figure 6.

Although Mtb is thought to be in the centre of the necrotic lesion, several studies speculated that a substantial portion of bacteria or bacterial antigens were associated with macrophages in the peripheral leukocytic infiltrate (Russell, 2007). These bacteria, which were found to be in the peripheral macrophages, express highly upregulated *icl1* (isocitrate lyase-1) (McKinney *et al.*, 2000). It was assumed through

histological investigations on infected human lung that the margins of liquefied lesions in lung cavities are the location of tubercle bacilli showing rapid bacterial growth (Canetti, 1955b). This view is currently supported by Lenaerts *et al.*, (2007), who studied the location of persisting mycobacteria in a Guinea pig model. These worker also demonstrated a large number of AF-stained Mtb bacilli in the acellular rim of the primary granuloma in extracellular microcolonies or clusters. This appearance has been thought to resemble biofilm growth within the necrotic granuloma.

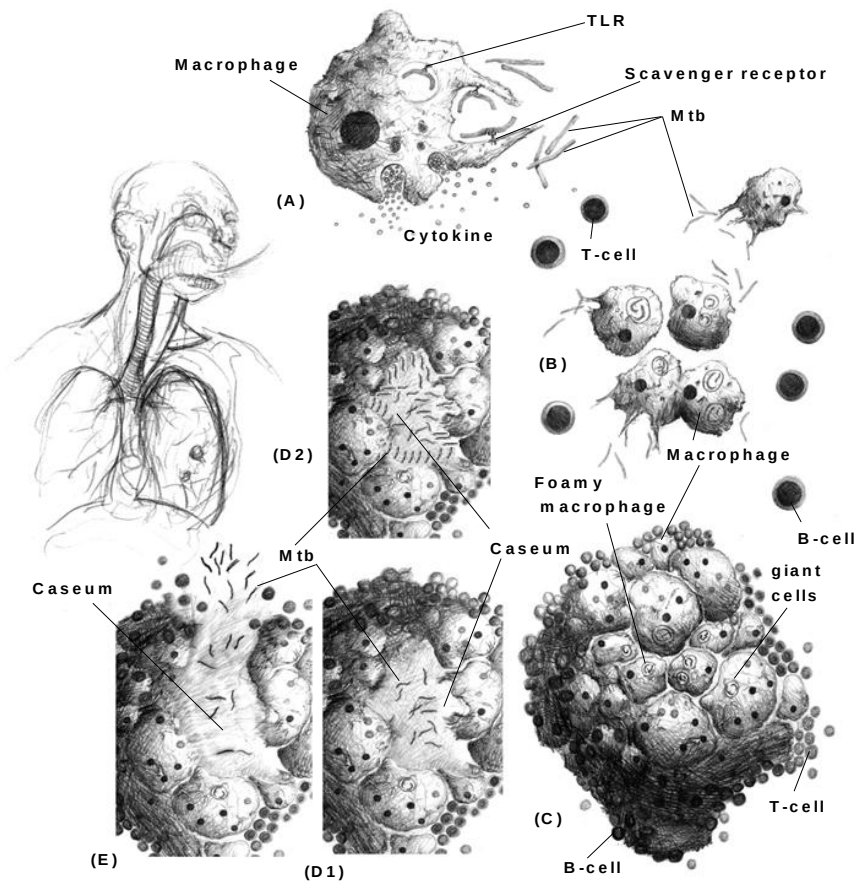


Figure 6: Formation and maturation of the tuberculous pulmonary granuloma.

Once Mtb cells are inhaled, surface receptors or toll-like receptors (TLRs) on alveolar macrophages enable uptake of bacteria and trigger innate immune signalling pathways resulting in production of many cytokines and chemokines (A) This attracts other immune cells such as DCs, lymphocytes and more macrophages to the site of infection (B) Immune cells organise in a spherical structure with mycobacterial exposed macrophages in the central zone surrounded by lymphocytes, mostly CD4+, CD8+ and $\gamma\delta$ T cells. Macrophages often fuse to form multinucleated giant cells or separate into foamy macrophages (C) Mtb have the ability to survive for many decades inside the granuloma. Due to some factors (e.g., HIV and malnutrition) Mtb reactivate and provoke the death of the infected macrophages and a caseating necrotic region develops (D1) It is also suggested that there are a large number of AF-stained Mtb bacilli in the acellular rim of the primary granuloma (D2) Ultimately, the structure breaks down, letting the bacteria spread to other regions of the lungs to form more lesions. At this stage the infection can be transmitted to other individuals (E) Figure modified from (Miranda *et al.*, 2007).

1.5.6. Latent infection

After infection is established there are no symptoms in most individuals. This is termed latent infection and may reflect Mtb dormancy (no multiplication) or suppression by the immune system and this condition may last a few months, decades or be lifelong (Tufariello *et al.*, 2003, Wayne and Sohaskey, 2001). Latency is associated with a positive tuberculin skin test (Boshoff and Barry, 2005).

Individuals with latent TB have a 2 to 25% chance of developing active disease (Parrish *et al.*, 1998). Latency refers to the clinical disease condition, whereas dormancy refers to the condition of the surviving Mtb bacilli in infected host. Bacterial dormancy is defined as 'a reversible state of low metabolic activity in a level that maintains viability' (Kaprelyants and Kell, 1993). It is widely believed that Mtb bacilli maintain a dormant or "Non-Replicating Persistent (NRP) state during latency, as a result of the inhibitory growth conditions in the macrophage and granuloma (Boshoff and Barry, 2005, Parrish *et al.*, 1998, Wayne and Sohaskey, 2001).

1.5.7. Gene expression NRP *M. tuberculosis*

As noted above (1.1) a study on Mtb transcription in sputum (see 1.7) led to the recognition of a pattern similar to that observed in NRP bacilli (Garton *et al.*, 2008). One of the regulons that may play an important role in Mtb survival during latency is the "Dormancy Survival Regulator" or DosR regulon (Wayne and Sohaskey, 2001, Leistikow *et al.*, 2010). The DosR regulon provides genetic programming upregulated by environments that inhibit aerobic respiration and as limit bacillary replication (Leistikow *et al.*, 2010). There appears to be a strong association between oxygen tension and the formation and maintenance of latency (Wayne and Sohaskey, 2001). According to this link between oxygen depletion and latency Sherman *et al.*, (2001) identified 48-genes that were shown to be induced in H37Rv with response to 0.2% oxygen for 2h in a culture flask. During this study, within the 48-upregulated genes, an obvious operon that contains the putative two-component response regulator pair Rv3133c/Rv3132c was identified. The deletion of a Rv3133c regulator resulted in a repressed expression of the α -crystallin hypoxic response gene, whereas deletion of the Rv3132c sensor kinase, resulted in no particular phenotype being detected (Sherman *et al.*, 2001). In addition to the DosR, there is a two-component regulatory

system that essentially contains two sensor histidine kinases, where *DosS* acts as a redox sensor and *DosT* acts as a hypoxia sensor (Kumar *et al.*, 2007).

The 48-genes shown to be induced during hypoxia have been associated with dormancy. Muttucumaru *et al.*, (2004) examined H37Rv NRP gene expression using the model devised by Wayne and colleagues and showed that 36 out of 49 predicted members of the DosR regulon were induced in the resultant NRP state. Another study done by Voskuil *et al.*, (2004) using the same model, demonstrated that 42 predicted members of the DosR regulon were induced during NRP state whereas 27 DosR predicted members of the DosR regulon were also induced in a stationary phase culture. Other studies on this topic showed that 35 DosR regulon genes were upregulated in a stable state chemostat culture under low oxygen tension (Bacon *et al.*, 2004). In a static culture that was allowed to settle for 30 minutes, there were 31 DosR regulon genes up regulated (Kendall *et al.*, 2004). All these studies support hypoxia as a major factor in DosR regulon induction.

Another factor that stimulates the DosR regulon in Mtb is a non-lethal nitric oxide (NO) concentration that reversibly reduces respiration and growth and as a result induces the dormancy program (Voskuil *et al.*, 2003). Exposure to a bacteriostatic concentration of NO on Mtb *in vitro* reveals transcriptional changes such as upregulation of about a 20-gene set regulated by the DosR/DosS/DosT system (Shi *et al.*, 2005). The DosR regulon was shown to be induced at a high level when the Mtb H37Rv strain exposed to 1 mM of s-nitrosoglutathione (GNSO, a NO releasing agent), 5% ethanol and to a lesser extent 10 mM hydrogen monoxide (H₂O₂), but not heat and cold shock conditions (Kendall *et al.*, 2004). Another study using a DosR mutated Mtb strain revealed that deletion of DosR resulted in a loss of Mtb cell culturability in solid culture and that growth could be recovered in liquid culture. Furthermore, the DosR mutant showed 10,000-fold defect in an anaerobic dormancy survival condition. This finding indicates DosR as significant in the continuation of growth from a hypoxic or NO repressed non-respiring state (Leistikow *et al.*, 2010). In addition to the *in vitro* studies, *in vivo* work was done by Parish *et al.*, (2003) showing that DosR mutants in mice infection became more virulent than wild type. In contrast, DosR in Guinea pig was shown to be essential for infection and formation of granuloma, whereas disruption of DosR attenuates Mtb (Malhotra *et al.*, 2004). However, it should be taken into account that there is a difference between the mouse and Guinea pig models; in mice there is no caseous necrotic centre (Rhoades *et al.*, 1997).

Late-stage persistence in a murine model has revealed that a FA enzyme, isocitrate lyase-1 *icl1*, originally recognised under NRP conditions by Wayne and Sohaskey (2001), is essential at this stage (McKinney *et al.*, 2000). The *icl1* gene of Mtb was shown to be upregulated in THP-1 macrophage infection (Schnappinger *et al.*, 2003). This enzyme is a significant component of the glyoxylate cycle that permits Mtb to achieve a source of energy from FAs broken down to acetyl coenzyme A. The *icl1* enzyme is also essential for Mtb pathogenesis and intracellular survival in mice infection (Munoz-Elias and McKinney, 2005). Furthermore, *in vivo* studies on mice confirmed that deletion of *icl1* resulted in almost a ten-fold decline in Mtb titres in lungs during the chronic phase of infection (Ehrt and Schnappinger, 2009). Although there is noted upregulation of *icl1* gene during dormancy, it is not part of the DosR regulon. Hence, DosR regulon does not control all genes required for NRP Mtb (Zhang *et al.*, 2012).

1.6. Resuscitation Promoting Factors

Sputum contains MTB cells that depend on Resuscitation Promoting Factors (Rpfs) to grow (mukamolova 2010). Rpfs are muralytic enzymes that induce the culturability of dormant or non-culturable bacteria (Kana and Mizrahi, 2010). Mukamolova and colleagues (1998) were the first to identify and characterise the single Rpf in *Micrococcus luteus* as a ~16 KDa secreted protein that, when added to extended stationary phase cells incapable of growth in liquid or solid media, rendered them culturable in a most probable number (MPN) liquid culture assay. Homologues of the *M. luteus* protein are found in many Gram positive organisms (actinobacteria and firmicutes) and, in particular mycobacteria including Mtb (Kana and Mizrahi, 2010; Gupta and Srivastava, 2012). Rpf proteins are known to be PG-glycosidases, but the mechanism of action and role of Rpf in mediating reactivation is still unknown.

Five Rpf proteins in Mtb (Rpf A-E) were shown to be expressed and to have resuscitating activity on *M. luteus* (Mukamolova *et al.*, 2002). Dormant Mtb cells produced in some in vitro systems require an exogenous source of Rpf to grow (Sheelva *et al.*, 2002). The *Rpf* gene homologues in Mtb (Rv0867c (*rpfA*), Rv1009 (*rpfB*), Rv1884c (*rpfC*), Rv2389c (*rpfD*), and Rv2450c (*rpfE*)) were shown to stimulate growth of Mtb and *M. bovis* BCG from stationary-phase cultures (Mukamolova *et al.*, 2002). Tufariello *et al.*, (2006) have shown that the deletion of *rpfB* gene from the Mtb chromosome led to a delayed resuscitation in a murine mouse infection model, but the growth and persistence of Mtb in mice were not affected. Another study done by Kana

et al., (2008) demonstrated that combinations of single and combined deletion mutants of the five Rpf proteins results in reduced Mtb recovery from dormancy. Individual *Rpf*-like genes are unnecessary for Mtb growth *in vitro* as the Rpf mutants showed no significant growth curve difference than wild type, but Rpf deletion led to small colony phenotype (Tufariello *et al.*, 2004). Through comparison between exponential-phase and stationary-phase Mtb cells, expression of the *Rpf*-like genes is found to be growth phase-dependent (Tufariello *et al.*, 2004).

Because Rpf proteins are expressed by active cells, the mycobacterial NRP-like population in TB needs actively dividing cells to be able to be resuscitated (Kana *et al.*, 2008, Mukamolova *et al.*, 2010). In smear-positive sputum samples, it was observed that between 80 to 99.9% of Mtb cells present are Rpf-dependant (Mukamolova *et al.*, 2010). Rpf-dependant populations may signify the population of dormant cells in a patient.

1.7. Sputum transcriptome

Expectoration or production of sputum is a major feature of pulmonary TB. The medical definition of production or expectoration is 'spitting out or coughing material produced in the respiratory tract (Farzan, 1990). Sputum expectorated from pulmonary TB patients contains Mtb populations expressing different properties that may be connected with transmission (Garton *et al.*, 2008). The physiology of Mtb in sputum has been largely unknown but the transcriptional pattern determined in this study revealed expression of 182 genes to be significantly upregulated and 334 genes were significantly downregulated when compared with log-phase aerobic growth (Garton *et al.*, 2008). Further, when Mtb cells were exposed to the Wayne model of dormancy, they accumulated LBs at a similar level to those present in sputum, thus the sputum transcriptome was compared to two NRP stages (NRP1: hypoxic condition for 70 h and NRP2: hypoxic condition for 200 h) (Garton *et al.*, 2008).

This comparison between sputum transcriptome and the two NRP stages showed that *in vitro* grown cells do not match with the transcriptional signature from sputum. However, there were number of similarities in the regulated genes between sputum, slow growth, NRP2 and those identified in murine macrophage infection (Schnappinger *et al.*, 2003). Further analysis revealed that 11 out of 48 DosR regulon genes were identified to be induced greater than 2.5-fold in sputum including *hspX*,

tgs1 and *narK2*. In addition the *icl1* was upregulated greater than 3.5-fold. Moreover, sputum downregulated genes also suggested presence of NRP Mtb bacilli and included repression of genes essential for aerobic respiration, ATP synthesis and ribosomal proteins.

Three genes involved in cholesterol utilisation were shown to be upregulated in sputum; all three genes are from the putative *kstR* regulon and induced greater than 2.5-fold upregulation. In Mtb, the *kstR* regulon involves 74 genes including *mce4* and *igr*, which control the expression of genes used for the utilisation of various lipids including cholesterol as energy sources (Kendall *et al.*, 2007, Kendall *et al.*, 2010). There are around 64 genes in Mtb that may be involved in cholesterol utilisation. All these genes were significantly induced in sputum in comparison with log-phase aerobic growth.

All 21 genes identified as involved in aerobic respiration in Mtb were repressed in both sputum and NRP2 compared with log-phase aerobic growth. Of these genes, *atpA* (adenosine triphosphate (ATP) synthase α -chain), *nuoB* (probable NADH dehydrogenase type-I), *ctaD* (*aa3*-type-I cytochrome *c* oxidase), *qcrC* (cytochrome *bc1* complex) (Shi *et al.*, 2005), and *atpD* (β -chain ATP synthase) corresponded with the downregulated genes earlier observed in bacillary stasis in the chronic murine infection model (Shi *et al.*, 2005). In addition to aerobic respiration genes, 45 ribosomal genes were repressed in comparison with log-phase aerobic growth. These genes include *rpsL*, which was repressed greater than 5-fold in sputum.

Overall, the main features of the sputum Mtb transcriptome indicated induction of the DosR regulon and genes required for lipid and cholesterol utilisation together and downregulation of aerobic respiration and ribosomal genes. These features were used in a previous study to select transcripts to monitor when trying to find conditions inducing the sputum Mtb phenotypes *in vitro* (Lee, 2012 and see chapter 5).

1.8. Bacterial biofilms

Mtb has the ability to evade a host's immune response and persists for decades, with possible reactivation that results in an active disease (Flynn and Chan, 2001). The physiology of Mtb bacilli within this microenvironment is still unclear (Miranda *et al.*, 2012). However, there has been some debate concerning whether Mtb in human lung

is present as a biofilm. Canetti, (1955b) noted that when TB becomes active, expectoration of tubercle bacilli originates from rapidly and extensively growing cells in the margins of liquefied lung caseum. Recently, Lenaerts *et al.*, (2007), studied the location of persisting Mtb in a Guinea pig model. This work showed a large number of AF-stained Mtb bacilli in the acellular rim of the primary granuloma, which live in microcolonies or clusters. There is also a large body of evidence that Mtb form cords *in vivo* in Guinea pig and mice infection (Middlebrook *et al.*, 1947, Bloch and Noll, 1955). Cords have also been shown in Zebrafish *M. marinum* infection (Tobin *et al.*, 2010). A recent discovery is that Mtb produces a pilin-like protein during human infection. This protein is produced in the extracellular matrices of many bacteria during biofilm formation when bacteria are engaged in surface attachment (Alteri *et al.*, 2007).

1.8.1. What is bacterial biofilm?

Bacterial biofilm formation has not been extensively investigated until Costerton *et al.*, (1978) put forth the theory that 99.9% of bacteria in natural environments grow in biofilms on a wide range of surfaces. Most bacteria do not live in dispersed single-cell pure cultures, but are often at interfaces in polymicrobial aggregates, for instance, as sludge, mats, flocs and “biofilms” (Flemming and Wingender, 2010).

Bacterial biofilm was defined by Donlan *et al.*, (2002) as a community of bacterial-derived sessile characterised by populations that are irreversibly stuck to a substratum or interface or to both, that are fixed in an extracellular matrix of polymeric substances that they have formed, and show a modified growth rate and gene transcription phenotypes.

1.8.2. Bacterial biofilm mechanisms

There is large body of evidence of that bacterial communities which grow in self-organised biofilm are present in a NRP state (Hall-Stoodley *et al.*, 2004, Donlan *et al.*, 2002). Several Gram positive and negative bacteria live as long-term colonizers of humans, including *P. aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Candida albicans*, *Haemophilus influenzae* and *E. coli*. These grow as extracellular or intracellular biofilm, on tissues, or even on medical devices (Ojha and Hatfull, 2012).

The mechanism of bacterial biofilm development has been studied extensively over the last 20 years (O'Toole *et al.*, 1999, Hall-Stoodley and Stoodley, 2002). Regardless of differences in specific genetic requirements and structural constituents, biofilms of all bacterial species are produced via common development mechanisms. This mechanism is initiated by attachment on a surface, cell-to-cell communication, extracellular matrix (ECM) formation and synthesis that encloses constituent cells and ends by cellular detachment (Hall-Stoodley and Stoodley, 2002, Ojha and Hatfull, 2012).

Bacterial biofilms are produced in many steps involving intercellular signalling and they exhibit a profile of gene transcription that is distinct from that of planktonic cells or cells in ordinary culture (Watnick and Kolter, 2000). These bacterial biofilms communicate through the production and sensing of autoinducer (AI) to be able to control the expression of specific genes in response to population density. This bacterial phenomenon is known to be produced only when bacterial species form biofilms (Costerton *et al.*, 2003). Development has been proposed to have multiple stages according to proteomic studies on *P. aeruginosa* biofilm (Stoodley *et al.*, 2002). The first stage involves forming a transient association with the surface allowing the bacterium to find an appropriate attachment location. Once the bacterium establishes a stable association with the surface a group or microcolony is formed. In the next stage an extracellular matrix is produced and establishes a three-dimensional biofilm (Watnick and Kolter, 2000). When matured, the biofilm structure becomes flat or mushroom shaped depending on the nutrient source (Stoodley *et al.*, 2002). These stages of biofilm development were also shown in motile bacterial species such as *E.coli* and *Vibrio cholera* as well as non-motile species such as *S. aureus* and *Mycobacterium spp* (Kaiser, 2003, Hall-Stoodley and Lappin-Scott, 1998).

The biofilm matrix comprises different types of biopolymers known as Extracellular Polymeric Substances (EPS). EPS are responsible for the cell adhesion to the surface as well as cohesion within the biofilm. Furthermore, EPS immobilise the biofilm cells and facilitate cell-to-cell communication. Another function of EPS is that they provide a nutrient source and also serve like a recycling centre of lysed cell components such as nucleic acids. One of the most important functions of EPS is in protection against dehydration, oxidation and against some anti-microbial agents and UV light (Flemming and Wingender, 2010).

1.8.3. The mycobacterial biofilm

A mycobacterial biofilm studies report a slightly different structure including cells that are attached to hydrophobic solid surfaces (Attached) and floating mats (pellicles) on the surface of a liquid culture media in addition to the (planktonic) cells in the liquid (Figure 7). Several biofilm studies have been carried on *M. smegmatis* (Recht *et al.*, 2000, Ojha *et al.*, 2005, Ojha *et al.*, 2010). The glycopeptidolipids of *M. smegmatis* were shown to be significant for initial surface attachment during biofilm formation (Recht *et al.*, 2000). Another two components required for *M. smegmatis* biofilm development apart from glycopeptidolipids are free-MAs and mycolyl-diacylglycerol (MDAG); this indicates that lipids have a key role in biofilm formation.

In addition to lipids, iron is another component significant for *M. smegmatis* biofilm development (Ojha *et al.*, 2008, Ojha *et al.*, 2010). It has been shown that FAs through the *GroEL1* gene, the member of Hsp60 family of chaperon proteins found in mycobacteria, play a crucial role in *M. smegmatis* biofilm architecture and development (Ojha *et al.*, 2005). Glycopeptidolipids were also observed to be essential during the multicellular growth of *M. avium* suggesting that both mycobacterial species share mechanisms of biofilm formation (Yamazaki *et al.*, 2006).

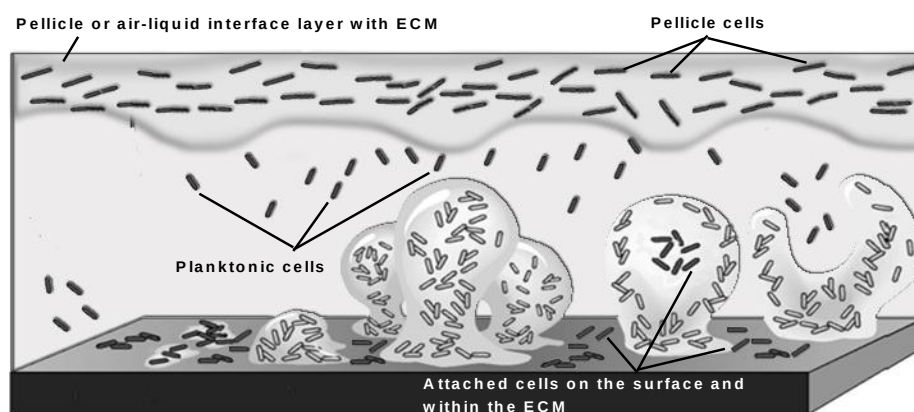


Figure 7: Mycobacterial biofilm layers.

Schematic figure of mycobacterial biofilm Attached, Planktonic and Pellicle layers during the development cycle. Figure adapted from (Sauer, 2003).

In *Mtb* biofilms, the pellicle layer requires keto-MAs, as shown by mutants of MAs species that are unable to produce pellicle (Sambandan *et al.*, 2013). The last stage of *in vitro* *Mtb* biofilm development there is maturation free-MA rich at the air-media interface achieved via genetically controlled developmental stages (Ojha *et al.*, 2008).

Three key genes are implicated in Mtb the biofilm formation, *pks16*, *heliY* and *pks1*, as mutants of these genes failed to form biofilm (Ojha *et al.*, 2008, Pang *et al.*, 2012). The Mtb biofilm formation is also sensitive to gaseous conditions on the air-media interface. This finding is consistent with the hypothesis that a different gaseous composition such as raised CO₂ might stimulate cell surface interactions in slow growing mycobacteria (Ojha *et al.*, 2008).

The ECM of mycobacterial Pellicle consists of polysaccharides (exopolysaccharides), proteins, and nucleic acids (Zambrano and Kolter, 2005). The main composition of the mycobacterial extracellular matrix consists of lipid-rich contents that are secreted from cells as shown by Ojha *et al.*, (2008). This content gives the mycobacterial biofilm cells extensive tensile integrity and a highly drug-tolerant phenotypic feature (Pang *et al.*, 2012).

Another characteristic of mycobacterial biofilm that is shared with other bacterial biofilms is drug tolerance. Teng and Dick, (2003) revealed the link between drug tolerance and detergent-free biofilm formation on fast growing *M. smegmatis* as it shows resistance to higher RIF and INH concentrations compared with the planktonic aerated grown cells (Ojha *et al.*, 2005, Ojha *et al.*, 2010). Another significant *in vitro* study on Mtb detergent-free biofilm showed that Mtb cells within the biofilm are tolerant to high RIF and INH concentrations (Ojha *et al.*, 2008). A more recent study showed that keto-MAs are responsible for drug tolerance in the pellicle. This was shown by comparing the wild type, which is highly tolerant to RIF, with the keto-MA mutant which was hypersensitive to RIF (Sambandan *et al.*, 2013).

The biofilm mechanism of resistance to antimicrobial agents could come from many factors. One of these factors is reduced penetration of antimicrobial agents (Donlan and Costerton, 2002). The ECM forms a potential diffusion barrier for drugs and may in some cases react directly with specific agents (Donlan and Costerton, 2002).

The modified growth rate of biofilm associated cells may also contribute to drug resistance/tolerance. Organised growth in clusters or cell communities can stimulate phenotypic persistence of essential bacilli through physical protection from the antimicrobial threats (Kapoor *et al.*, 2013). Mukamolova *et al.*, (2010) have shown that Mtb bacilli from sputum can be tolerant to high RIF concentrations. This might reflect either Mtb cells in a NRP or that the tolerant cells are derived from a biofilm-like

structure *in vivo*. However, the precise mechanism of persistence and the drug tolerance remains unclear (Islam *et al.*, 2012).

The question that should be addressed is whether the *in vitro* Mtb biofilm reflects sputum phenotypes. This speculation was raised by Lenaerts and his colleagues, as in early lung infection in a Guinea pig model profiles, which show some of the Mtb bacilli deprived of their favoured intracellular niche due to cellular necrosis, were found in the extracellular liquid in biofilm-like formation (Lenaerts *et al.*, 2008). This suggests that the *in vitro* Mtb biofilm may correlate with some or all phenotypes already observed sputum. These phenotypes comprise gene expression profile, which shows slow or no growth signals, high LB percentage (Garton *et al.*, 2008), variable Auramine O acid-fastness (Solan, 2008) and Rpf-dependant populations with high tolerance to RIF (Mukamolova *et al.*, 2010). The Mtb biofilm development and the correlation with sputum phenotypes will be discussed in details in Chapter 5.

1.9. Aims and objectives of this study

The main hypothesis of this study is that Mtb forms biofilm-like growth at the lung cavity air-liquid interface. The overall aims of this study were to achieve further understanding of the Mtb observed populations in sputum first by improving the staining/labelling techniques by which they may be described and then by attempting to replicate the phenotypes *in vitro* settings under various conditions including biofilm growth.

The specific objectives were to:

- 1. Detect, identify and characterise the majority of Mtb bacilli in sputum using:**
 - a. Auramine O**
 - b. Immunofluorescence**
 - c. Peptide nucleic acid probes**
 - d. A Fluorescence-based Kinyoun method**
- 2. Apply selected methods to quantify Mtb sub-populations in sputum.**
- 3. Explore use of an *in vitro* biofilm system to replicate the sputum phenotypes by monitoring:**
 - I. Gene expression profiles**
 - II. LB and AF proportions**
 - III. Drug tolerance**
 - IV. Rpf-dependancy**

Chapter 2

Materials and methods

2.1. Mycobacterial strains

Table 1: Bacterial strains.

Strain	Description	Source
<i>M. smegmatis</i> mc ² 155	Strain with high transformation efficiency	Laboratory stocks
<i>M. bovis</i> BCG Glaxo	Attenuated category II TB vaccine strain	Laboratory stocks
<i>M. tuberculosis</i> H37Rv	Virulent laboratory strain	Laboratory stocks (Bill Jacobs)
<i>M. tuberculosis</i> Beijing 65	Clinical strain	Laboratory stocks (Mark Nicholls)
<i>Pseudomonas aeruginosa</i>	Wild type strain	Laboratory stocks
<i>Escherichia Coli</i> DH5α	Wild type strain	Laboratory stocks

Table 2: DNA used in this study.

Genomic DNA	Description	Source
<i>M. tuberculosis</i> H37Rv	Virulent laboratory strain	BEI Resources
<i>M. tuberculosis</i> CDC 1551	Virulent and highly infectious strain	BEI Resources

Table 3: Sputum samples used in this study

Smear #	Origin	Amount (μl)	Sample status	Classification	Comments
22	Ethiopia	300	Decontaminated (NALC/NaOH)	3+	Freeze-thaw at least once
23	Ethiopia	400	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
41	Ethiopia	500	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
42	Ethiopia	900	Decontaminated (NALC/NaOH)	2+	Freeze-thaw at least once
43	Ethiopia	500	Decontaminated (NALC/NaOH)	3+	Freeze-thaw at least once
44	Ethiopia	500	Decontaminated (NALC/NaOH)	3+	Freeze-thaw at least once
58	Ethiopia	300	Decontaminated (NALC/NaOH)	2+	Freeze-thaw at least once
85	Ethiopia	500	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
88	Ethiopia	200	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
90	Ethiopia	500	Decontaminated (NALC/NaOH)	3+	Freeze-thaw at least once
93	Ethiopia	500	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
96	Ethiopia	400	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
103	Ethiopia	500	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
104	Ethiopia	900	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once
115	Ethiopia	300	Decontaminated (NALC/NaOH)	4+	Freeze-thaw at least once

2.2. Laboratory reagents and culture media

2.2.1. Chemicals and media

All chemicals were obtained from Thermo Fisher Scientific (Loughborough, Leicestershire, UK) or Sigma-Aldrich Company Limited (Poole, Dorset, UK), unless otherwise stated. Middlebrook media were obtained from Difco Laboratories (Detroit, USA). Other growth media were obtained from Becton Dickinson Biosciences (Oxford, UK) or Sigma-Aldrich Company Limited, unless otherwise stated.

2.2.2. Growth media

Middlebrook 7H9 broth

Middlebrook broth was prepared by dissolving 4.7g of broth powder in 900ml distilled water containing 2.5g glycerol. The solution was sterilised by autoclaving at 15lbs in-2 pressure (121°C) for 15 min. Prior to use, the broth was supplemented with Albumin-dextrose-catalase (ADC) supplement at a concentration of 10% (v/v) and 10% (w/v) Tween-80 at a concentration of 0.05% (v/v).

Middlebrook 7H10 agar

Middlebrook 7H10 agar was prepared by dissolving 19g of agar powder in 900 ml distilled water containing 6.25g glycerol. The agar was boiled for 30 min to fully dissolve the powder and turns to yellowish colour and then sterilised by autocleaving at 15lbs in-2 pressure (121°C) for 17 min. The agar media was supplemented with Oleic acid-albumin-dextrose-catalase (OADC) at a concentration of 10% (v/v) prior to use.

Albumin-Dextrose-Catalase (ADC) Supplement

The ADC was prepared by dissolving the following substances in 150 ml distilled water:

Bovine Serum Albumin (BSA) fraction V	7.50g
D-Glucose	3.00g
Sodium Chloride	1.28g
Catalase	6.0mg

The solution was centrifuged at $6371 \times g$ for 30 min to remove undissolved particles. The supplement then was filter-sterilised (0.2 μ m filter, Nalgene, Hereford, UK) and stored at 4°C.

Oleic Acid-Albumin-Dextrose-Catalase (OADC) Supplement

Oleic acid is a supplement for mycobacterial growth (Winn *et al.*, 2006). OADC supplement was prepared as per ADC supplement above, with the addition of 8.63 ml of Oleic Acid solution (1% w/v) in 0.2 M NaOH prewarmed to 50°C). The OADC solution was filter sterilised through a 0.2 μ m filter.

Tween 80

Tween 80 is used to minimise clumping of the mycobacteria species during growth in liquid culture. Tween 80 was prepared by dissolving 10g of stock solution in distilled water to a final volume of 100 ml and final concentration of 10% (w/v). The solution was sterilised by filtration through a 0.2 μ l filter unit and stored at 4°C.

Oleic acid in BSA

The oleic acid solution was prepared by dissolving 100 μ l of oleic acid (Sigma-Aldrich) in 100ml 5% (w/v) bovine serum albumin fraction V (BSA), to give a stock concentration of 3.35mM. The solution was sonicated for 1 hr to allow the oleic acid to emulsify before being filter sterilised through 0.2 μ m filter. The solution was stored at 4°C. The solution was melted at 50°C prior to use.

Sauton`s Broth

Potassium dihydrogen orthophosphate	0.5g
Magnesium sulphate	0.5g
L-Asparagine	4.0g
Glycerol	75.0g
Ferric ammonium citrate	50.0mg
Citric acid	2.0g
1% (w/v) zinc sulphate	0.1ml
Tween 80	0.5g

The solution was made up to final volume of 1 litre and the pH was adjusted to 7.0 with NaOH. The solution was autoclaved at 15lbs in-2 pressure (121°C) for 20 min and stored in room temperature away from direct light.

Luria-Bertani (LB) agar

Bacto-tryptone	4 g
Bacto-yeast extract	2 g
NaCl	2 g
Agar-powder	1.5% (w/v)

The solution was made up to 400 ml with distilled water and the pH was adjusted to 7.4. LB agar was autoclaved at 121°C for 15 min.

Phosphate-buffered Saline (PBS)

Phosphate-buffered saline (PBS) was prepared by dissolving one PBS tablet (Sigma-Aldrich, cat no: P3813) in 200ml double-distilled water to give a solution with final concentration of 0.01M phosphate buffer, 0.002M potassium chloride and 0.137M sodium chloride at pH7.4. The solution was autoclaved at 15lbs in-2 pressure (121°C) for 20 min.

10% (w/v) glycerol solution

Glycerol (10g) was made up to 100ml in distilled water and sterilised by autoclaving at 121°C for 15 min then stored at 4°C.

1% (w/v) BSA solution

This solution was prepared by dissolving 1 g of BSA fraction V in PBS to a final volume of 100 ml and final concentration of 1% (w/v). The solution was filter sterilised through 0.2 µl filter and stored at 4°C. This solution was used to block un-specific binding of primary and secondary antibodies during immunofluorescence technique.

10% (v/v) normal goat serum in BSA

This solution was prepared by dissolving 1 g BSA fraction V and 10 ml of normal goat serum (Invitrogen, USA) in PBS to a final volume of 100 ml to reach the final concentration of 10% (v/v) normal goat serum in 1% (w/v) BSA in PBS. The solution was filter sterilised through 0.2 µl filter and stored at 4°C. This solution was used for block nonspecific binding of primary and secondary antibodies during sputum labelling.

N-acetyl-L-cysteine (NALC)

NALC solution was used to digest and decontaminate sputum samples. NALC solution was prepared by combining the following reagents:

Trisodium citrate	50 ml	50mM
NaOH	50 ml	4% (w/v)
NALC	0.5 g	0.5% (w/v)

Phosphate buffer (67mM, pH 6.8) was prepared by combining the following reagents:

NaH₂PO₄	51 ml	134mM
Na₂HPO₄	49 ml	134mM
d. H₂O	100 ml	-

Preparation of Guanidine Thiocyanate (GTC) Solution

Guanidine Thiocyanate (GTC) was prepared by combining the following reagents:

Guanidium thiocyanate	295.4g
N-lauryl sarcosine	2.5g
Trisodium citrate	12.5ml
10% (w/v)Tween 80	25ml

Reagents were dissolved in 200ml of distilled water incubated at 37°C with shaking (100 rpm) for 6 hr. When fully dissolved, solution was made up to a final volume of 500ml with distilled water. GTC then was separated into 20ml aliquots in 50ml polypropylene centrifuge tubes and stored away from heat and light. Immediately prior to use, 140 µl of β-mercaptoethanol was added to 20 ml of the GTC solution to reach the final concentration of 7µl/ml.

Preparation of Mtb H37Rv Culture Supernatant (SN)

A freeze dried pellet (kindly supplied by Dr. Galina Mukamolova) from 32ml of *M. tuberculosis* H37Rv culture supernatant prepared as described by Mukamolova *et al.* (2010) and stored at -20°C was used for Rpf-dependency assays. Prior to use, H37Rv SN was dissolved in 16ml of sterile distilled water and 16ml of 7H9-ADC-Tween 80 and then was kept on ice for 30-60 min before use.

Preparations of Rpf-dependacy assessment for *M. tuberculosis* cells

Control 7H9 plate

In a sterile falcon tube 45 ml Middlebrook 7H9 autoclaved fresh broth, 5 ml ADC and 0.125 ml of 10% (w/v) Tween-80 were combined and mixed carefully.

Supernatant plate

A 32 ml of dried powdered sterile H37Rv SN was dissolved in 16 ml of sterile d.H₂O and kept on ice for an hr to dissolve all solid particles. Once ready, the SN was diluted with the same control preparation of 50 % (v/v) of Middlebrook 7H9 broth, ADC and Tween-80 then used directly.

Preparation of media with cholesterol

The preparation of media supplemented with cholesterol (Sigma-Aldrich) was adapted from Klein *et al.*, (1995). Methyl- β -cyclodextrin (Sigma-Aldrich) 1g was dissolved in 11 ml of PBS in a sterile glass test tube at 80°C in plate stirrer (Grant Instruments SUB6, Cambridge, England) with continuous stirring. Cholesterol (30mg) was dissolved in 400 μ l isopropanol/chloroform (2:1 v/v) in a small glass tube. Cholesterol solution was added to the warmed methyl- β -cyclodextrin solution in 50 μ l aliquots, stirring was continued until all the cholesterol went into solution before adding additional material. The final concentration of cholesterol in solution is 6.8 mM cholesterol and 70 mM cyclodextrin (~9% w/v cyclodextrin). The combined mixture was cooled down and kept at room temperature. Methyl- β -cyclodextrin (70mM) solution was used as a control solution.

The cholesterol/Cyclodextrin mixture and Methyl- β -cyclodextrin control solution were added to Sauton`s broth warmed at 80°C in water bath. The final concentration of cholesterol and Cyclodextrin mixture is 0.01% (v/v).

Addition of antibiotics

Antimycobacterial drugs were used according to Inderlied, (1991). Antibiotics were ordered from SIGMA and stock solutions sterilised by filtration though 0.22 μ m pore membrane then stored at -80°C for up to 12 months. Concentrations used and diluents are mentioned in the table 4 below:

Table 4: Antibiotics used in the study

Drug	Solvent	Diluent	Stock Conc ($\mu\text{g/ml}^{-1}$)	Final Conc ($\mu\text{g/ml}^{-1}$)
Isoniazid (INH)	SDW	SDW	1000	1
Rifampicin (RIF)	DMSO	SDW	1000	5

2.3. Cultivation of bacteria

2.3.1. Measuring the optical density

The optical density (OD) was measured at wavelength of 580nm (OD_{580nm}). The OD was measured by transferring 1 ml of culture into a 1.5 ml cuvette (Fisher Scientific), which was then sealed with autoclave tape and Nescofilm (Bando Chemical, Kobe, Japan). The OD of *M. smegmatis* and *M. bovis* was measured using a Sanyo SP75 UV/Vis spectrophotometer (Watford) in Category 2 laboratory whereas for *Mtb* a Jenway 6300 spectrophotometer (Stone) was used in the Category 3 laboratory. To insure accuracy, thick cultures (OD > 1.0) were diluted 1:10 prior to measurement. The OD_{580nm} of all cultures were measured against blank of relevant medium (7H9 or Sauton's).

2.3.2. Stock cultures for long term storage

Frozen stocks of *M. bovis* and *M. smegmatis* were prepared by storing 1 ml of exponentially growing culture (whole culture) in 1.5 ml microfuge tubes which were stored at -80°C. The 1 ml *Mtb* stocks (whole cultures) were stored in 1.5ml cryovials (NUNC, Thermo Fisher) at -80°C. *M. bovis* and *Mtb* doubling every 24 h, *M. smegmatis* doubling every 4 h, assessed through OD readings.

2.3.3. Cultivation of *M. smegmatis*

M. smegmatis was prepared by thawing -80°C (whole culture) stock and then used to inoculate 5 ml of Middlebrook 7H9-ADC-Tween-80 broth in a 30 ml Universal plastic tube (Sterilin, Bargoed). The culture was incubated at 37°C with shaking at 200 rpm overnight. Then, the starter culture was used to inoculate 25 ml of 7H9-ADC-Tween 80 broth in a 125 ml conical glass flask to OD₅₈₀ of 0.02, and grown at 37°C with shaking at 200 rpm overnight to reach OD₅₈₀ of 1.

2.3.4. Cultivation of *M. bovis* BCG

The *M. bovis* BCG was prepared by thawing -80°C 1ml stock BCG culture aliquot and then used to inoculate 5 ml of Middlebrook 7H9-ADC-Tween-80 broth in a 30 ml Universal plastic tube (Sterilin, Bargoed). The culture was incubated at 37°C statically for 10 days to reach OD₅₈₀ of 1.0. Then, the starter culture was used to inoculate 25 ml of 7H9-ADC-Tween 80 broth in a 125 ml conical glass flask to OD₅₈₀ of 0.05, and grown at 37°C statically for 5-6 days to reach OD₅₈₀ of 1.

2.3.5. Cultivation of *M. tuberculosis*

Mtb is classified by the Advisory Committee on Dangerous Pathogens (ACDP) as Category 3 hazardous pathogen requiring biohazard containment at level 3. Therefore, all Mtb work was carried out in Class I or Class II microbiological safety cabinets within the Containment laboratory suite, in accordance with the suite code of practice.

Mtb starter cultures were prepared as described for *M. bovis* BCG (Section 2.3.4). Mtb starter culture then was sub-cultured into 25ml of Middlebrook 7H9-ADC-Tween-80 in a 125ml polycarbonate conical flask (Corning Life Science, Massachusetta, USA) and incubated with shaking at 100 rpm for H37Rv and 80 rpm for Beijing 65 at 37°C. All *Mtb* solid and liquid cultures were double bagged during incubation.

2.3.6. Enumeration of colony-forming units (CFU)

Colony counting was carried out according to the modified version of the Miles and Misra (surface viable count) method. Ten-fold serial dilutions of cell suspension were performed in 450 µl of 7H9-ADC-Tween 80 medium in 1.5 ml microfuge tubes (Axygen, USA). Three 20 µl drops from each dilution were plated out onto Middlebrook 7H10 agar in duplicate plates. Each agar plate was separated into 6 sectors. Agar plates were sealed with Nescofilm to avoid drying and incubated at 37°C until isolated colonies became visible. The incubation time for *M. smegmatis* plates about 2-3 days while generally *M. bovis* and *Mtb* required 14-30 days.

The dilution produces 10-100 colonies (averaged over the six replicate spots) were used for the final calculation of CFUs, using the equation:

$$CFU/ml = A \times 50 \times D$$

A = Average colony count per 20µl spot

D = Dilution factor

2.3.7. Enumeration of most probable number (MPN)

MPN assays were performed in quadruple replicates in 48-well microtitre plates (Greiner Bio-One, Frickenhausen, Germany), by diluting 50 µl of cell suspension into 450 µl of 7H9-ADC-Tween 80. Each microtitre plate was separated into 6 or 7 dilution sectors according to the growth nature of the strain. Plates were then sealed with PetriSEAL™ (Diversified Biotech, Dedham, UK) tape, to avoid drying and incubated statically at 37°C. The incubation time for Mtb MPN plates about 4-6 wks.

2.3.8. Nitric Oxide Exposure

Solutions of Nitric Oxide donor, Spermine NONOate (SPER/NO, ENZO, UK) and its control compound, Spermine tetrahydrochloride (SPER.4HCl, Sigma-Aldrich, UK) were both prepared to a concentration of 10 mM in sterile PBS as stock solutions. SPER/NO is a fine powder stock, SPER/NO working solution was prepared immediately prior to use under flow of nitrogen gas in the fume hood due to its oxygen sensitive nature and discarded after 30 min. When SPER/NO powder dissolved in PBS, it was filter sterilised through 0.2 µm filter and used immediately. Both SPER/NO and its control SPER.4HCl were added to the Mtb culture at the final concentration of 100 µM according to Daniel *et al.* (2004) and Sherratt (2008) protocols. All cultures were exposed to the nitric oxide or the SPER.HCl control for 4 h.

2.4. Immobilising of bacteria on glass slides for microscopy

2.4.1. Immobilising of *M. bovis* BCG and *M. smegmatis* onto slides for microscopy

M. bovis BCG and *M. smegmatis* cells were immobilised onto 76 x 26 mm glass slides using rectangular Belco slide chamber system (Figure 8). A 50 µl cell suspension was dispensed into each well of the silicone block followed by centrifugation at 1000 xg for 10 min in an IEC Centra-4X centrifuge (International Equipment Company, Dunstable, Bedfordshire, UK). The supernatant then was removed and the slide was air dried and was finally fixed with formaldehyde vapour overnight (see below 2.4.3).

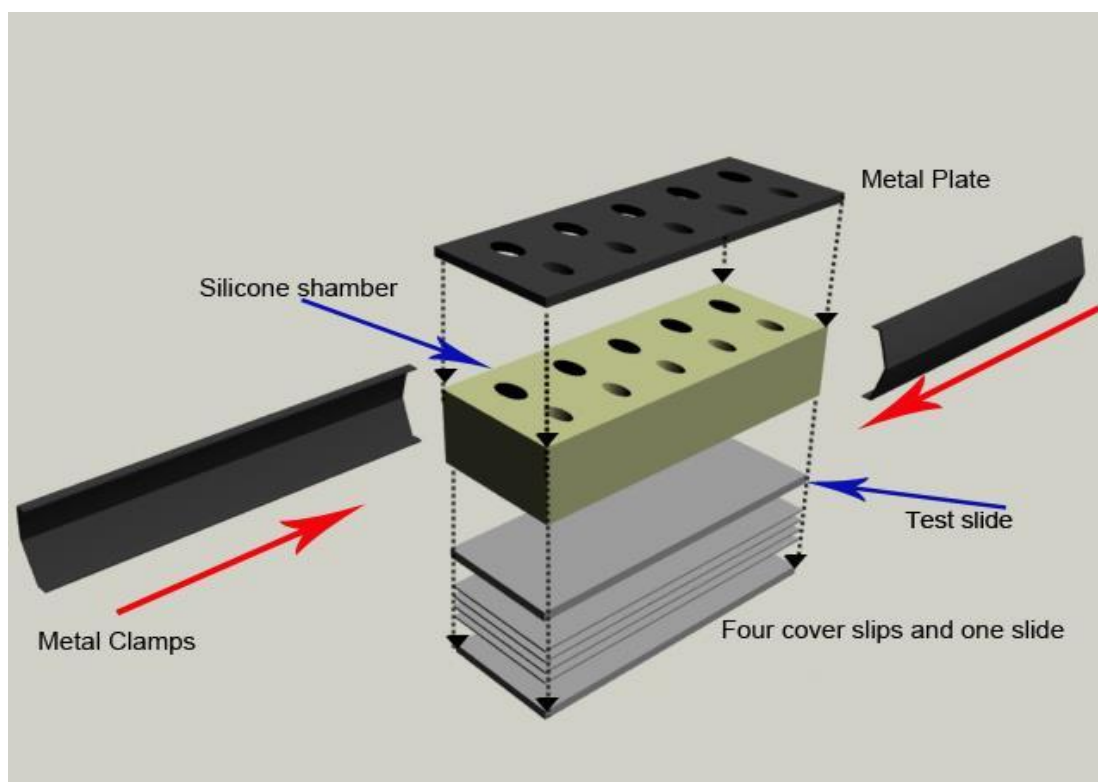


Figure 8: Assembly of the rectangular Bellco slide silicon chamber system for the immobilisation of mycobacteria on slide.

The tube silicone chamber system was congregated as shown by short dashed arrows. 50µl of mycobacterial cells was dispensed into each well placed on the silicon block. Image modified from (Sherratt, 2008).

2.4.2. Immobilising of *M. tuberculosis* onto slides for microscopy

From 20-30 µl of *Mtb* cell suspension was spotted on 76 x 26 mm glass slides and then spread carefully to cover 1cm x1cm of the slide area. Smears were then dried at 65°C on a using heat block (Fisher Scientific, Loughborough, Leicestershire, UK). Slides were then fixed with formaldehyde vapour overnight prior to remove from Category 3 suite.

2.4.3. Formaldehyde fixation of mycobacteria immobilised on glass slides

Heat-fixed slides of *Mtb* bacilli were fixed with formaldehyde vapour to allow for their safe removal from the containment suite as well as cross linking the surface antigens. Slides or coverslips were placed in a plastic slide rack. The open slide rack was placed within a plastic food box (15cmx8cm), alongside a tissue soaked with 10ml of 23% (w/v) formaldehyde. The plastic box was sealed, and the slides or coverslips left overnight with formaldehyde vapour before being removed from the microbiological safety cabinet.

2.4.4. Syringe treatment to break cultural clumps

Where necessary, clumps were broken by passage of the cell suspension through a blunt 25G needle (Becton Dickinson Biosciences, Oxford, UK) 5-7 times.

2.5. Staining and sample preparation for fluorescence microscopy

2.5.1. Acid-fast staining techniques

Auramine O

Auramine O stain was prepared by mixing the following solutions:

Solution 1: 0.2 g Auramine O powder (Sigma Aldrich) was dissolved in 20 ml 95% (v/v) absolute ethanol in distilled water.

Solution 2: 6 g phenol crystals (Sigma Aldrich catalogue number) were dissolved in 174 ml distilled water.

Solutions 1 and 2 were mixed in a glass bottle then sealed tightly and stored away from the heat and light at room temperature.

Acid-alcohol decolourisation solution

Acid alcohol decolourisation solution was prepared by adding 1 ml of concentrated Hydrochloric acid (HCl) to 200 ml of 70% (v/v) ethanol.

Potassium permanganate solution

Potassium permanganate solution was prepared by dissolving 5 g of potassium permanganate particles into 1L of distilled water to give a concentration of 0.5% w/v of Potassium permanganate aqueous solution.

<http://www.sahealthinfo.org/tb/microacid.htm>

Modified Kinyoun staining

Modified Kinyoun staining was prepared by combining the following reagents:

Solution 1: 4 g Basic-Fuchsin (Sigma-Aldrich) was dissolved in 10 ml absolute ethanol.

Solution 2: 8 g phenol crystals were dissolved in 100 ml distilled water.

Solutions 1 and 2 were mixed properly and the mixture was filtered using filter paper size 150mm (Whatman, UK) prior to use (Van Deun A *et al.*, 2005). The glass bottle was closed tightly and stored away from the heat and light at room temperature.

Kinyoun decolourisation solution

Decolourisation solution (acid-alcohol) was prepared by adding 3ml of concentrated hydrochloric acid (HCl) to 97ml of 95% (v/v) ethanol (Van Deun A *et al.*, 2005).

Counter staining (transmitted light)

Methylene blue counter stain was prepared by dissolving 0.3 g of methylene blue crystals (Fisher Scientific, UK) in 100 ml of distilled water to reach the final concentration of 0.3% (w/v).

Counter staining (fluorescence light)

Methylene blue counter stain was prepared by dissolving 0.1 g of methylene blue crystals (Fisher Scientific, UK) in 100 ml of distilled water to reach the final concentration of 0.1% (w/v).

2.5.2. Lipid labelling techniques

HCS LipidTOX™ Red Neutral Lipid Stain

LipidTOX™ Red Neutral Lipid (Invitrogen, UK) Stain (RLx) working solution was prepared by making a 1:50 dilution in PBS. The working solution was separated into 100 µl aliquots and stored at -20°C away from light until use.

HCS LipidTOX™ Green Neutral Lipid Stain

LipidTOX™ Green Neutral Lipid (Invitrogen, UK) Stain (GLx) working solution was prepared as detailed above for LipidTOX™ Red Neutral Lipid staining.

4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY® 493/503)

BODIPY 493/503 (Invitrogen, UK) powder was dissolved in absolute ethanol to give a stock of final concentration 1 mg/ml. A working solution (50µg/ml) was prepared at ratio of 1:50 dilution in ethanol. The working solution was separated into 100 µl aliquots and stored at -20°C in dark until use.

2.5.3. Immunostaining techniques

Purified Protein Derivative anti-M. tuberculosis complex primary antibody (PAb)

Purified Protein Derivative (PPD) anti Mtb complex is an IgG polyclonal un-labelled primary antibody (PAb) raised in rabbit against Mtb H37Rv PPD (Abcam, UK). PPD anti-Mtb antibody stock solution was prepared at ratio 1:25 in PBS. The stock solution was stored at -20°C whereas the working solution was stored at 4°C.

Whole cell lysate anti M. tuberculosis polyclonal primary antibody

Whole cell lysate (WCL) anti-Mtb PAb is un-labelled PAb rose in rabbits against Mtb H37Rv (BEI resources) whole cell lysate. WCL anti-Mtb antibody stock solution was prepared at ratio 1:25 in PBS. The stock solution was stored at -20°C whereas the working solution was stored at 4°C.

Anti M. tuberculosis Rv polyclonal primary antibody

Rv anti-Mtb antibody (BEI resources) is an unlabelled PAb raised in Guinea pig against live Mtb H37Rv infection. Anti-Rv antibody working solution was prepared at a ratio of 1:25 in PBS. The stock solution was stored at -20°C whereas the working solution was stored at 4°C.

Anti M. tuberculosis CDC1551 polyclonal primary antibody

CDC1551 anti-Mtb antibody (BEI resources) is an unlabelled PAb raised in Guinea pig against live Mtb CDC1551 infection. Anti-CDC1551 antibody working solution was prepared at a ratio of 1:25 in PBS. The stock solution was stored at -20°C whereas the working solution was stored at 4°C.

Goat anti-rabbit IgG secondary antibody

Goat anti-rabbit IgG secondary antibody FITC labelled (Invitrogen, UK) working solution was prepared at ratio 1:500 in PBS. The secondary antibody stock and working solution were stored at 4°C away from the light.

Goat anti-guinea pig IgG secondary antibody

Goat anti-Guinea pig IgG secondary antibody FITC labelled (Invitrogen, UK) working solution was prepared at ratio 1:500 in PBS. The secondary antibody stock and working solutions were stored at 4°C away from the light.

2.5.4. Peptide Nucleic Acid Probes (PNA)

The PNA probe (Cambridge Research Biochemicals) used was that of Stender *et al.*, 1999 and were as shown in table 5.

Table 5: OK682 probe sequence.

Reference	Description	Target	Sequence
Stender <i>et al.</i> , 1999	PNA OK682	23S rRNA	5'-UGAUCGUAUUGGGCAUCUAACCUCGAACCCU-3'

2.5.5. (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) INT

A fresh stock solution was prepared by dissolving 10mM INT (Sigma) in SDW. Because the INT was difficult to dissolve in distilled water, heating in water bath at 55°C for 30 min was performed, then the solution was briefly sonicated (Decon, ultrasonic, LTD, England) for 30 min. From the stock solution a 2mM working solution was prepared in PBS and filter-sterilised. The stock solution and working solution were stored at 4°C until used (Cooney, 2000).

2.5.6. SYTO9 nucleic acid and propidium iodide staining of *M. tuberculosis*

SYTO9/Propidium iodide (PI) (Invitrogen, UK) working solution was prepared by combining 5 µl of SYTO 9 green nucleic acid stain and 5 µl red Propidium iodide stain at ratio (1:1) and mixed thoroughly.

2.6. Labeling and staining protocols

1.6.1. Labelling *M. tuberculosis* and *M. bovis* with Auramine O staining

All staining were done by spreading cells on the slide and fixed with 23% (v/v) formaldehyde prior to staining. Smears were flooded with Auramine O staining for 15 min. Smears were washed with water for few seconds then decolourised by flooding with acid-alcohol for 15 min followed by washing with water for few seconds. Slides were then covered with 0.5% (w/v) potassium permanganate background followed by washing with water thoroughly. The slides were air dried away from the light as Auramine O is light sensitive. Smears then were mounted in 10% (w/v) glycerol in PBS and sealed with transparent nail varnish.

1.6.2. Labelling of mycobacteria with Red LipidTOX neutral lipid stain

Cells then were placed on rectangular Bellco slide silicon camber system as shown in Figure 2. In each well 40µl of 1:50 dilution in PBS of red LipidTox stain was spotted and incubated at 37°C for 20 min by placing the chamber system in the incubator. After incubation, staining solution was discarded and cells washed once with PBS by pipetting off. Blocks were disassembled, slides air dried and mounted with 10% (w/v) glycerol in PBS.

1.6.3. Labelling mycobacteria with (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride) INT

1ml of culture was collected in sterile microfuge tube and spun down at 3,300 xg for 2 min. Cells were then washed twice with 1 ml PBS pelleting in between at 3,300 xg for 2 min. The pellet was then re-suspended in 300 µl PBS. An equal volume of INT working solution was added to the bacterial suspension, mixed gently, and incubated at 37°C for 1 hr. Cells were then harvested by centrifugation as above, washed twice in PBS and spotted on slides as described Section 2.4.2, smears were fixed with 23% (v/v) formaldehyde prior to be mounting with the mounting medium. Formazan deposits were observed by bright field microscopy.

1.6.4. Labelling *M. tuberculosis* with SYTO9 nucleic acid and propidium iodide staining

Mtb cells were harvested, washed 3X with PBS and then resuspended in 1 ml of PBS. To each 1ml of *Mtb* cell suspension, 3µl of SYTO9/PI working solution was added and mixed thoroughly by inverting the tube then incubated for 15min at room temperature away from the light. Following incubation, cells were spotted on the slide as described in Section 2.4.2.

2.7. Recording fluorescence images

All stained slides were mounted with 10% (w/v) glycerol in PBS. For visualisation a Nikon Ti-E eclipse inverted microscope with a Intensilight C-HGFIE pre-centred fiber optic light source (motorised type). Bacterial cells were visualised using a 100X magnification oil immersion lens using Citifluor immersion oil. Images were recorded with a 12/10bit, high speed DS-U3 CCD camera Build 831 (Nikon Corporation, Japan) using Nikon NIS Elements Imaging Software. The filter sets which were used for epifluorescence microscopy are shown in table 6. The exposure time of each staining is shown in Table 7.

Table 6: Filter sets which were used for epifluorescence microscopy.

Filter block (Chroma)	excitation	dichroic mirror	emission
ET-DAPI (49000)	350 ± 25nm	400LP	460 ± 25nm
Auramine (31015 bespoke)	460 ± 25nm	500DCLP	550 ± 25nm
ET-GFP (49002)	470 ± 20nm	495LP	525 ± 25nm
ET-Texas Red /mCherry (49008)	560 ±20nm	585LP	630 ± 37.5nm

Table 7: Exposure time of each staining.

Stain	Exposure time
Auramine O	60ms
Fluorescence Kinyoun	80ms
RLx	10ms
GLx	60ms
BD	40ms
SYTO9 and PI	300ms
IF and IIF	300ms
PNA	1.5sec

2.7.1. Image analysis: measuring cell Fluorescence Intensity and the relative proportions of cells with various phenotypes

Fluorescence intensity data and cell sub-population sizes were acquired from fluorescence images using bespoke image analysis algorithm developed at the University of Leicester by Andrew Bell (Bell, 2013). This process uses ImageJ-based software (National Institutes of Health, Bethesda, Maryland) to count and analyse fluorescence intensities and staining proportions. For each phase image, ImageJ identifies the region of interest through thresholding. The area of interest was applied to the consistent fluorescent image and then the fluorescence intensity was measured (Bell, 2013). The acid-fastness and proportions of lipid body positive cells were assessed by measuring the fluorescence intensity of each cell per area and comparing the cell intensity with the cut off value that determined automatically by the ImageJ software (Bell, 2013).

To filter out non-cellular fluorescence signals, the R Project Software Environment for Statistical Computing (R Development Core Team, GNU General Public License and The University of Auckland, New Zealand) was developed and applied (Bell, 2013).

2.7.2. Displaying images

In this project, the images displayed are either phase contrast and fluorescence images. Any additional format is mentioned in the figure legend. Some of images are displayed with a Lookup table (LUT). The Green-Fire-Blue format (Figure 9) was used. TOP-HAT filtering was also used in some cases through Image-Pro-Plus 5.0 software.



Figure 9: LUT Green-Fire-Blue.

The Green-Fire-Blue LUT imaging format application to help in differentiating fluorescence intensities. Colour ranges from the lowest fluorescence value in (black) to the highest in (white).

2.8. Sputum smear classification results

Table 8: Sputum smear classification results according to the centres for disease control (CDC) guidelines

Smear classification	Number of AFB observed at 1000X magnification
4+	(>9/field)
3+	(1-9/field)
2+	(1-9/10 fields)
1+	(1-9/100 fields)
+/-	(1-2/300 fields)*
Negative	No acid-fast bacilli seen

(*) Doubtful

2.9. Statistical analyses and graphical representation of significance

Calculations and confidence intervals were calculated using Excel 2010 (Microsoft Corp.), whereas complex statistical analyses and significance tests were performed using Prism 6 (GraphPad Software, Inc.) statistical software. The significance between results is displayed graphically according to Table 9.

Table 9: Display of significance used in this thesis.

Significance	Denoted by
$P \leq 0.05$	*
$P \leq 0.01$	**
$P \leq 0.001$	***

Chapter 3

Development of staining
techniques to detect
M. tuberculosis from sputum and
pure culture

3.1. Introduction

Sputum is considered the key source of TB infection (Leung, 1999). When it is expectorated from pulmonary TB patients, it contains different Mtb populations expressing properties that are thought to be required for transmission and initiation of the disease (Garton *et al.*, 2008). These tubercle bacilli originate from the margins of liquefied lesions in the lung that are characterized by extensive and rapid bacterial growth (Young & Duncan, 1995 and Canetti, 1955). In an attempt to stay alive, Mtb faces dominant selection pressures to preserve and express these un-defined transmission properties (Garton *et al.*, 2008). Although the identification of Mtb in sputum came more than a century ago, these expectorated phenotypes in sputum remain unclear (Barksdale & Kim, 1977). Therefore, the identification of any of these bacillary phenotypes in sputum might give clues for recognising these undefined properties and ultimately offering improved treatment and prevention of TB (Garton *et al.*, 2008, Mitchison, 2004).

One of these phenotypes which are thought to be feature of Mtb in sputum is LBs (Garton *et al.*, 2008). It has been shown that the fast-growing *M. smegmatis* mc² 155 strain accumulates intracellular triacylglycerol (TAG) LB *in vitro* (Garton *et al.*, 2002). Although these LBs have been observed in sputum, Mtb did not produce them in the same culture conditions as used to observe them in *M. smegmatis* (Garton *et al.*, 2002). In addition, it has been shown that when Mtb is conditioned, under various stresses it accumulates TAG (Daniel *et al.*, 2004), which might later be consumed for long-term survival (Garton *et al.*, 2008). The transcriptome signatures and presence of LB suggest that lipid body positive cells populations in sputum may exist in a NRP condition (Garton *et al.*, 2008). Therefore, it is considered a significant challenge to detect and identify mycobacterial phenotypes and their expressed properties in sputum and in culture media.

It has been hypothesised that under multiple-stresses including hypoxia, Mtb enter a NRP state. Deb *et al.*, (2009) showed that the proportion of acid-fastness decreases and the LB proportion increases. This change in Mtb characteristics might be due to the alteration of the mycobacterial cell envelope composition, thus leading to a loss of acid-fastness (Deb *et al.*, 2009). Therefore, conventional acid-fast (AF) staining methods, such as Z-N, Kinyoun and Auramine O staining, will not detect the non-AF bacilli.

Examination of some Auramine O/Nile-red stained sputum samples has revealed populations of non-AFB that contain abundant LBs (Figure 10). These bacilli are of similar size and morphology to Mtb cells and this led us to hypothesise that non-AFB Mtb are

present in sputum. This population has been thought to be non-AF Mtb bacilli that might be in the NRP state. Identifying any of these populations may lead us to a better understanding of Mtb physiology during transmission. Therefore, in order to identify these phenotypes, it would be desirable to develop a staining technique that detects all Mtb bacilli in sputum.

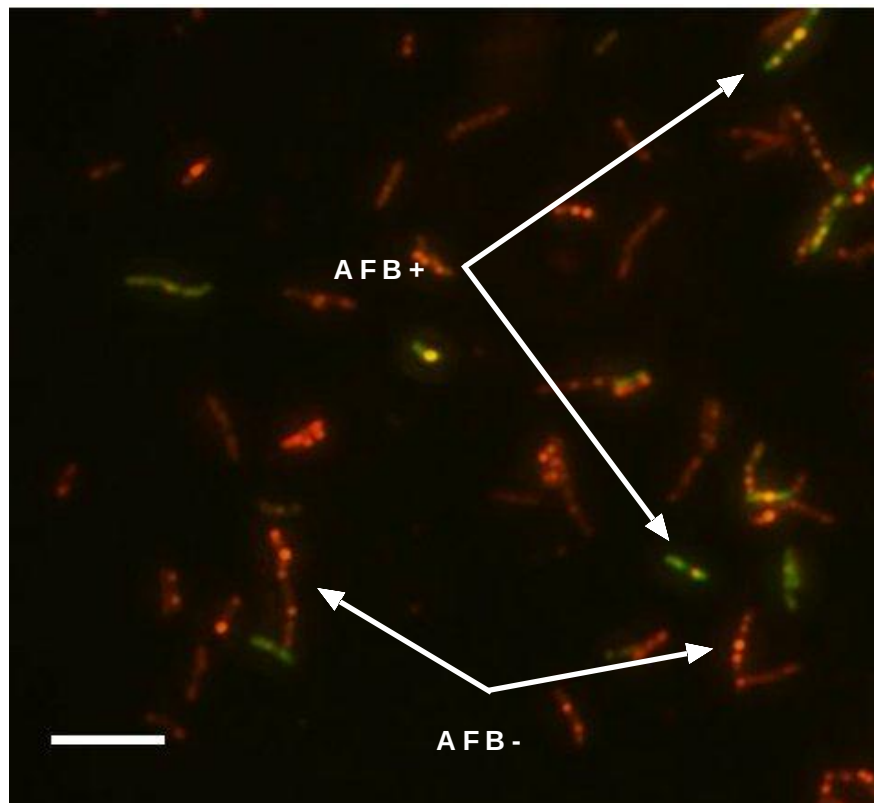


Figure 10: Tubercle sputum sample.

This image shows Mtb heterogeneity in sputum. A (4+) tubercle sputum sample stained with Auramine O AFB staining (green pseudocolour) and Nile-Red neutral lipid staining (red pseudocolour). The image suggests that there are multiple populations of Mtb in sputum: AFB⁺ population stained with Auramine O and the other population show the same Mtb morphology and decent LB, however, it did not stain with Auramine O. Scale bar 4 μ m. Image modified from (Sloan, 2008).

The main aim of this work was to find an effective way to quantify the Mtb populations in culture and ultimately in sputum samples. To accomplish this objective, techniques were used in this work to initially assess their ability to detect all Mtb sub-populations *in-vitro*. Then, the techniques were applied on sputum samples to detect all Mtb sub-populations *ex-vivo*.

3.2. Aims and objectives

The aim of this research was to develop cytological staining techniques that detect and classify the majority of Mtb cells in a growth culture and sputum samples with particular reference to their LB content.

The specific objectives were:

1. To investigate the staining of Mtb populations *in vitro* and in sputum taking into account:
 - a. Growth conditions
 - b. The effects of freezing for storage
 - c. Use of different reagents including:
 - i. Auramine O
 - ii. Antibodies for immunofluorescence
 - iii. rRNA-directed Peptide Nucleic Acid (PNA) probes
 - iv. Carbol-Fuchsin detected by fluorescence
 - v. LipidTox Red and Green Neutral Lipid staining

3.3. Methods

3.3.1. Immunofluorescence staining

3.3.1.1. Direct immunofluorescence (DIF)

Smears from cultures were treated with 1% (w/v) BSA, and sputum slides were treated with 10% (v/v) of Normal Goat serum (NGS) in BSA for 2h to block the non-specific binding. Smears were then washed 3 times with sterile PBS for 5 min. Immediately after washing, a 1:25 dilution of IgG polyclonal primary Ab (Pab) raised against Mtb H37Rv in an *in vivo* infection in 1% (w/v) BSA was added for 1h at 37°C. Subsequently, solutions were removed and slides were washed 3 times with PBS for 5 min. Blocks were then disassembled and slides were air dried. Finally, slides were mounted by 10% (w/v) glycerol in PBS and covered with a glass coverslip and then sealed with nail varnish for microscopy.

3.3.1.2. Indirect immunofluorescence (IIF)

For the secondary Ab, after washing with PBS, 1:500 of an appropriate secondary Ab (SAb) was added and incubated for 1h at RT. Slides were then washed with PBS 3 times for 5 min. Blocks were then disassembled and slides were air dried. Finally, slides were mounted by 10% (w/v) glycerol in PBS and covered with a glass coverslip and then sealed with nail varnish for microscopy.

3.3.2. FISH–PNA staining technique

Mtb bacilli from sputum or pure culture were fixed on slides as described above in Section 2.4. The protocol was carried out by immersing the smears in 80% (v/v) ethanol for 15 min followed by air drying. Subsequently, 50 µl of the hybridization buffer (Table 8) was added to the slide and a glass cover slip was carefully lowered onto the slide, taking care not to trap air bubbles. The slide and coverslip were placed inside a Petri dish (Sterillin, Bargoed) with wet tissue to ensure a humidified environment. Slides were then incubated in a hybridization oven in the dark (Appligene, UK) at 55°C for 90 min. Afterwards, slides were carefully taken from the oven and immersed in a pre-warmed washing buffer (5 mM Tris.HCl, 15 mM NaCl, 0.1% (v/v) Triton X-100, pH 10) for 30 min at 55°C in a water bath. Slides were then immersed in sterile distilled water, air dried, and finally mounted with mounting media and sealed with nail varnish (Stender *et al.*, 1999). The hybridization buffer was prepared by combining the solutions shown in Table 10.

Table 10: Hybridization buffer reagents used in the PNA-FISH technique.

Component	Stock conc.	Final conc.	Hybrid. Buffer (µl)
Formamide	100%	30% (v/v)	300
Dextran sulphate	25% (w/v)	10% (w/v)	400
EDTA	0.5M	5mM	10
Polyvinylpyrrolidone	10% (w/v)	0.2% (w/v)	20
Ficoll	10% (w/v)	0.2% (w/v)	20
Triton X-100	10% (v/v)	0.1% (v/v)	10
Sodium pyrophosphate	20% (w/v)	0.1% (w/v)	5
Tris/ NaCl	2M/400mM pH7.5	50mM/10mM	25
OK682 hybridisation probe (Stender <i>et al.</i> , 1999)	6.25µM	25nM	5
H ₂ O	-	-	105

3.3.3. Modified Kinyoun staining protocol

Mtb bacilli were spread and fixed on glass slides as described in section 2.4. The protocol was carried out by flooding the smears with Carbol-Fuchsin stain for 5 min. Slides were then washed with tap water for a few seconds followed by flooding with a decolourising agent (Kinyoun's acid-alcohol) for 3 min followed by washing with tap water. The decolourisation step was performed again for 2 min to ensure that there is no red colour remains on the smear (which might give high background). Finally, Slides were covered with the modified counterstain (0.1% w/v methylene blue) for 2 min followed by a thorough washing with water. Slides were air dried, mounted with 10% (w/v) glycerol in PBS and sealed with transparent nail varnish.

3.3.4. Labeling of mycobacteria with Green LipidTOX neutral lipid stain

In each well, 40 µl of 1:50 dilution in PBS of LipidTox Green neutral lipid stain was spotted and incubated at RT in the dark for 20 min. After incubation, the staining solution was discarded and the cells were washed once with PBS. Blocks were then disassembled, slides air dried and mounted with 10% (w/v) glycerol in PBS.

3.3.5. Labeling of mycobacteria with BODIPY® 493/503 neutral lipid stain

BODIPY staining was supplied in powder, thus, the stock solution was prepared in ethanol to give a stock of 1mg/ml as mentioned in Section 2.5.2. In each well, 40 µl of a 1:50 dilution in ethanol of BODIPY 493/503 stain was spotted and incubated at 37°C for 20 min. After incubation, the staining solution was discarded and the cells washed once with PBS. The

blocks were then disassembled, slides air dried and mounted with 10% (w/v) glycerol in PBS.

3.3.6. Spiking a sputum sample with *M. tuberculosis* from culture

Mtb H37Rv cells from a mid-exponential phase with an OD of 1 were spiked with a 3+ AFB decontaminated frozen sputum sample. The spiking step was done by separating the sputum sample into two portions, with each portion containing 60 µl. Then, 10 µl of H37Rv culture was added to the first portion and vortexed for 5 sec. H37Rv was incubated for 30 min at RT to let the H37Rv cells merge with the sputum. The two portions were then spotted onto glass slides.

3.3.7. Bacterial strains used in the study

This study used Mtb H37Rv laboratory strain as the LB percentage of H37Rv strain is known to be low at mid-exponential phase under aerated growth condition compared with other clinical strains, such as Beijing and CH Mtb strains. In contrast, *M. bovis* BCG is known to produce LBs even in mid-exponential phase, which is useful for comparing the LB counting staining techniques. To assess the cross reactivity of IIF and PNA staining techniques, three bacterial species were used. *M. smegmatis* was used as it is one of the mycobacterial species that shares many features with Mtb, such as some surface Ags (He & De Buck, 2010). The other Gram negative bacteria *E. coli* DH5α and *P. aeruginosa* were used because they might be found in sputum in some TB patients.

3.4. Results

3.4.1. Studying acid-fastness of *M. tuberculosis* by Auramine O staining

A combination of multiple stresses has been reported to result in loss of acid-fastness including low O₂, high CO₂, low nutrients and acidic pH (Deb *et al.*, 2009). A new observation that is reported as responsible for low AF proportions of Mtb cells is seen when growth is done with poor nutrients and a low O₂ environment where bacteria form a net to share the nutrients in the form of biofilm (Ehlers & Schaible, 2012). This condition will be discussed and studied extensively in Chapter 5 of this thesis.

Mtb planktonic (Pk) cells that were generated and taken from a biofilm Pk phase (See chapter 5) were used in this chapter to assess the detection of acid-fastness with various staining techniques. As the non-AF population is thought to exist in sputum samples (see Figure 10), being able to detect these sub-populations might help to identify the non-AF population in sputum. Figure 11 shows an example of non-AF populations of Mtb H37Rv from the mid-exponential phase.

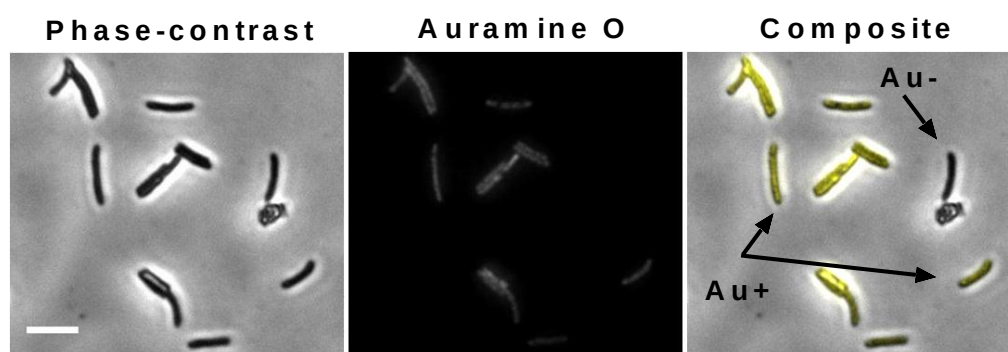


Figure 11: Non-AF populations of H37Rv culture.

Mid-exponentially grown Mtb H37Rv in Sauton's medium. Cells stained with Auramine O and arrows show the Auramine O $\pm$ populations. Scale bar 4 μ m.

Mtb Pk cells and Mtb H37Rv mid-exponential phase cells (both grown in Sauton's) were stained with Auramine O in triplicate. Phase contrast and Auramine O images were taken and the AF proportions were counted. The results are shown in Table 11, indicating that the AF proportions of the Pk cells with Auramine O have are extremely low compared with the mid-exponential phase cells control. Therefore, detecting these populations might lead to their identification in sputum samples.

Table 11: Mtb H37Rv cells with Auramine O AF staining.

Sample (Mtb H37Rv)	(%) Auramine O AF $\pm$ SD	No. cells
Pk cells	7.5 $\pm$ 3.1	275
Mid-exponential phase (control)	96.9 $\pm$ 1.3	432

3.4.1.1. Loss of Auramine O labeling due to several freeze and thaw cycles

Sputum samples which were used in this study are taken from frozen stocks. Hence, prior use of these samples should be taken into account whether frequent freeze and thaw cycles affect the proportion of the acid-fastness. To assess this, the study was done by using mid-exponential phase Mtb strain H37Rv grown in Sauton's medium and a previously decontaminated frozen sputum sample was also studied.

The first set (Time 0) from culture and sputum was prepared followed by storing aliquots of 1 ml of H37Rv and 60 μ l of sputum at -20°C. Every 24h, an aliquot was removed from the freezer. It was then defrosted and refrozen again. This process was done continuously for 4 cycles of 24h, except for the last cycle, which lasted 48 h. Once all cycles were carried out, the microscope slides were prepared and stained. Slides for both H37Rv and sputum were stained in triplicate for each cycle with Auramine O. An independent user assigned a unique code to slides. The images of phase contrast and fluorescence Auramine O were captured. In each replicate of H37Rv, at least 100 cells were counted automatically whereas for sputum, 15 fields were counted. Once all images were captured, the AF proportion of H37Rv was counted. Sputum AF cells were counted as a total AF number in each replicate by eye.

Figure 12 shows that the AF proportion decreased within two freeze-thaw cycles and then steadily decreased afterwards to reach the minimum AF proportion of < 1 after 7 days (5 cycles). This result shows that repeated freezing-thawing reduced the Auramine O AF proportion of H37Rv cells from culture and sputum.

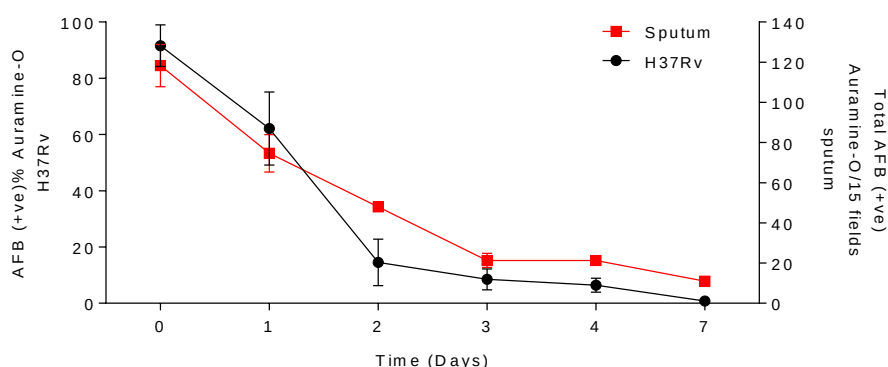


Figure 12: Effect of several freezing and thawing cycles on H37Rv and Mtb from sputum during five time points.

The graph shows the effect of multiple freeze-thaw cycles on Mtb H37Rv and from sputum. There is a decline in trend of Auramine O acid-fastness with an increase of the number of freeze-thaw cycles over several days. Three H37Rv from mid-exponential phase replicates were processed from each time point and almost 100 cells were counted automatically from each replicate. The average number of AF cells of 4+ sputum sample per 15 fields of view was counted by eye in three replicates of each cycle. Error bars represent the SD of the three replicates.

3.4.1.2. LipidTOX™ Red Neutral Lipid staining development to combine with Auramine O AF staining

In attempts to detect and identify the Mtb populations in sputum and pure culture, an Auramine O and LipidTox Red Neutral Lipid (RLx) dual-staining technique was developed. A late-exponential phase culture *M. bovis* BCG Glaxo cells was grown in Sauton's medium. Cells were then harvested and slides were prepared. The dual staining procedure was done by staining slides with Auramine O followed by RLx (a neutral lipid stain has an extremely high affinity for neutral lipid droplets with approximate excitation/emission of 577/609 nm) on top, slides were air-dried and sealed.

This technique was developed by using specific microscope filters that were selected carefully to enable imaging of the individual probes (Table 5). Figure 13 shows the AFB BCG cells with Auramine O (yellow pseudocolour) and the RLx labelling with (red pseudocolour). Auramine O was taken first followed by RLx, and this preserves the fluorescence signal from fading.

These results show that Auramine O could not detect most Mtb populations in sputum and pure culture. Therefore, other staining techniques toned to be developed to detect these populations.

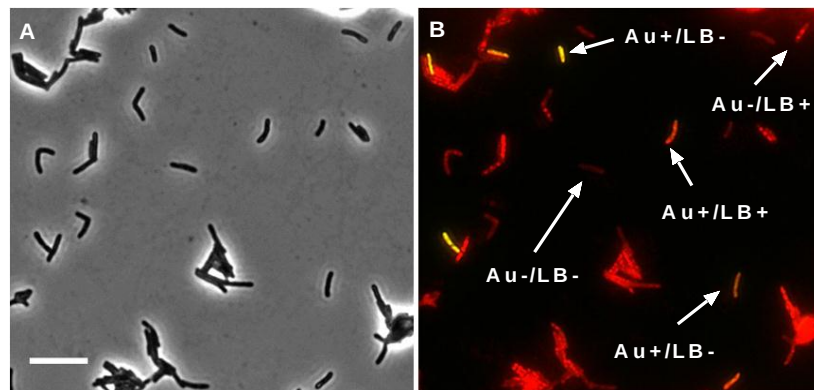


Figure 13: Auramine O combination with LipidTOX™ Red Neutral Lipid Staining labelling.

Representative images show Auramine O with RLx staining of *M. bovis* BCG (Glaxo) late-exponentially grown cells in Sauton's medium. Image (A) shows the phase contrast, while image (B) shows a composite image with Auramine O/RLx dual staining. The arrows indicate Auramine O fluorescence (yellow pseudocolour) and RLx (red pseudocolour) BCG different cell populations. Scale bars 5 μ m.

3.4.2. IF staining Development to detect *M. tuberculosis* from sputum and culture

An alternative technique to detect Mtb is immunofluorescence (IF) staining. Although IF has been used as a diagnostic technique to detect microorganisms in culture and clinical samples, there are no published studies on Mtb in sputum. Thus, considerable effort was required to optimize the procedure and manage the challenge of cross reactivity (Ulrichs *et al.*, 2005). This section shows the developed IF approach to detect Mtb from sputum and pure culture.

3.4.2.1. Antibody detection of *M. tuberculosis* from pure culture

Mtb H37Rv cells were grown to the mid-exponential phase prior to labelling them with Purified Protein Derivative (PPD) Ab. PPD used in this study was boiled, and crude extract of Mtb H37Rv proteins was taken from filtrates of sterilized and concentrated culture medium containing 8-12 week old Mtb bacilli (Landi, 1982). H37Rv cells were treated with 23% formaldehyde vapor. In the current study, a primary rabbit polyclonal PPD IgG antibody was used. It is known that IIF gives a stronger signal compared to IF (Lamvik *et al.*, 2001). Therefore, IIF technique was applied using an unlabelled rabbit polyclonal PPD as a primary Ab (PAb) and Alexa-Fluoro goat anti-rabbit as a secondary antibody (SAb).

Figure 14A shows that when H37Rv cells are labelled directly, heterogeneous antibody labelling patterns are formed around cells with a lower signal than the more uniform pattern obtained with IIF (Figure 14B).

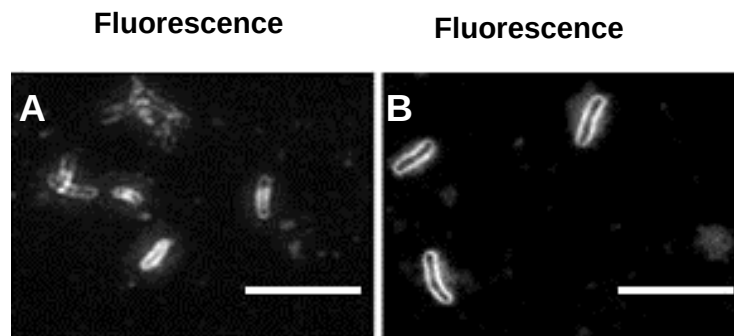


Figure 14: Indirect immunofluorescence staining gives better labelling of *Mtb* *in vitro*.

The displayed images show a comparison between (A) direct PPD antibody detection of exponentially grown *Mtb* H37Rv grown in Middlebrook 7H9, which shows a higher background and lower fluorescence signal (assessed by eye), and (B) the same set of cells labeled indirectly with PPD followed by staining with FITC-labeled goat anti-rabbit Alexa-Fluoro SAb, which shows a lower background with a higher fluorescence signal around the cells. Antibody fluorescence fields are exposed. Scale bars 4 μ m.

In the current study, four anti-*Mtb* Abs were assessed. These polyclonal Abs included: Ab raised in rabbit against H37Rv PPD, Ab raised in rabbit against H37Rv whole cell lysate (WCL) and Abs raised separately against H37Rv live infection (Rv and CDC1551). The same preparations of the mid-exponentially grown *Mtb* H37Rv cells were labelled indirectly with these anti-H37Rv Abs. Cells were labelled and examined by fluorescence microscopy at the same time in triplicates for each sample.

The results in Table 12 show that more than 95% of cells were detected with PPD and Rv Abs, whereas CDC155 and WCL showed a lower detection rate. PPD and Rv Abs were chosen for further assessment, as they had slightly higher detection rate.

Table 12: *M. tuberculosis* H37Rv cells detection proportions with anti-*Mtb* SAb.

Ab (<i>Mtb</i> H37Rv)	(%) SAb (+ve) $\pm$ SD	# cells(total triplicates)
PPD	97.3 $\pm$ 2.5	251
Rv	99.0 $\pm$ 1.0	310
CDC1551	93.6 $\pm$ 6.1	301
WCL	91.3 $\pm$ 3.0	296

This table illustrates the PPD, Rv, CDC1551 and WCL Ab detection proportions indirectly with Mtb H37Rv from mid-exponential phase grown in Sauton's medium. Proportions were taken from the average of approximately 100 cells per slide in three replicates of each. Fluorescently labelled cells were counted manually against the phase contrast.

3.4.2.2. PPD and Rv Antibody detection assessment during *M. tuberculosis* growth cycle

Cocito & Vanlinden, (1988) reported that mycobacterial surface antigen levels vary during the growth cycle. Also, the obvious Mtb characteristic in sputum is the heterogeneity of the mycobacterial bacilli (Garton *et al.*, 2002, Garton *et al.*, 2008). Hence, it would be expected that in sputum, Mtb may be found in different growth phases. For this reason, it would be necessary to assess Ab detection with Mtb from different phases during the growth cycle.

Mtb H37Rv cells in Sauton's medium were grown to early-exponential phase OD of 0.2, mid-exponential phase OD of 1, late-exponential phase OD of 1.5 and stationary phase OD of 1.9. Cells from different growth phases were labelled with PPD and Rv Abs. The labelling was done according to the standard IIF staining protocol in Section 3.3.1.2. Figure 15 reveals that the PPD Ab detects a lower detection proportion of Mtb H37Rv cells in early-exponential phase, while in other growth phases the Ab has > 90% detection. In contrast, the Rv Ab shows almost constant detection proportion during all Mtb growth phases. Therefore, Rv Ab was used for further analysis.

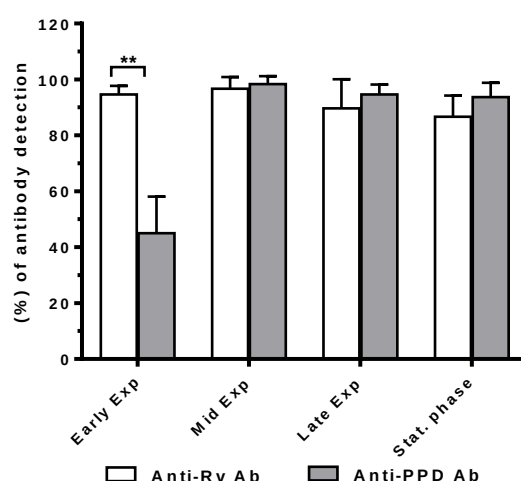


Figure 15: PPD and Rv Ab IF detection proportions in different H37Rv growth phases.

This bar chart shows detection proportions of different growth phases of Mtb H37Rv cells with Rv Ab (open columns) and PPD Ab (grey columns). Each phase was done in triplicate and from each replicate 100 cells were counted. Error bars display standard deviation. Unpaired t-tests were used to compare the antibody proportions. The asterisks designate significant results.

3.4.2.3. Assessment of non-specific binding of indirect IF with different bacterial species

The major difficulty in using Ab-based techniques to detect Mtb is the cross-reactivity with other bacterial strains (Yanez *et al.*, 1986). As such, the final stage of this work is to optimize the IF technique to detect Mtb from sputum. It is known that a sputum sample contains a mixture of bacterial and fungal species (McClellan *et al.*, 2011). Hence, assessing Ab cross-reactivity *in vitro* with a variety of bacterial strains will give an idea of Ab cross-reactivity performance. The Ab showing the highest detection proportion in all Mtb H37Rv growth phases was assessed with *P. aeruginosa*, *E. coli*, and *M. smegmatis* with Rv Ab.

Samples of *P. aeruginosa*, *E. coli*, and *M. smegmatis* were stained indirectly with Rv Ab according to the standard Ab staining protocol in Section 3.3.1.2. Figure 16 shows that there is no cross-reactivity between Rv Ab and the other bacterial strains.

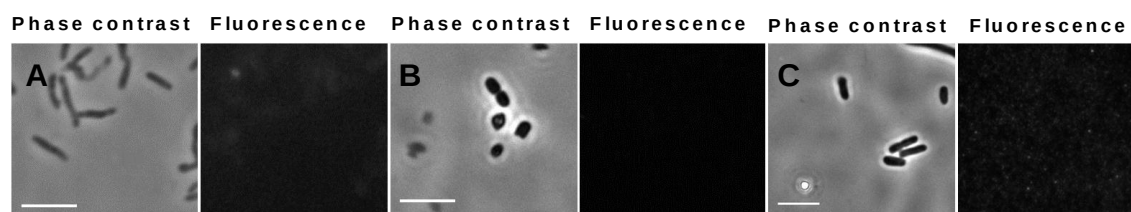


Figure 16: Rv Ab cross reactivity assessment with different bacterial strains.

The images shown here represent IIF reactions with different bacterial species. A, B and C show Rv Ab with *M. smegmatis* mc² 155, *E. coli* DH5α, and *Pseudomonas aeruginosa*. Scale bar 5 μm.

3.4.2.4. Antibody detection of *M. tuberculosis* in sputum

A sputum sample classified as (See Table 8) was stained with the IIF technique. It was observed that when the Mtb from sputum was stained with Rv Ab using the standard IIF protocol, it shows a high background signal (Figure 17A). In comparison, using a normal goat serum (NGS) blocking solution, which is widely used in IF staining and where the secondary antibody was produced in goat, minimises the high background of the sputum (Figure 17B).

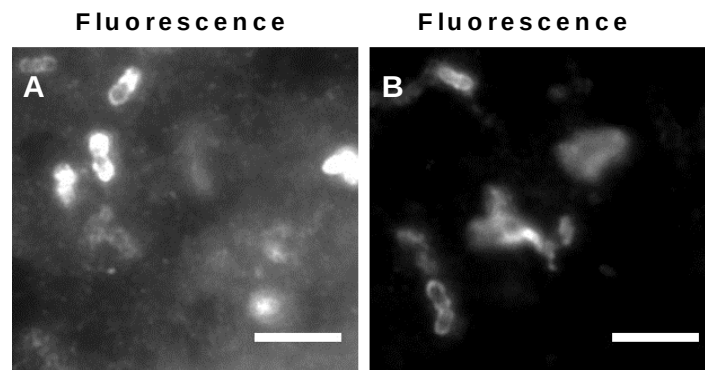


Figure 17: Rv Ab labelling on *M. tuberculosis* from sputum using NGS treatment.

The images represent fluorescence fields of (A) Mtb cells from a (4+) sputum sample labeled indirectly with Rv Ab with no NGS treatment. (B) The same sample with NGS treatment which resulted in minimising the high background of sputum. Scale bar 5 μ m.

3.4.2.5. IIF staining reveals cross reactivity with non-*M. tuberculosis* cells in sputum

Rv Ab was applied on a known AFB^{-ve} sputum sample (from non-TB patient). This sample was stained with Kinyoun AFB staining (an acid-fast staining which may be targeting nucleic acids and MAs) visualised under the bright field, and no AFB detected as shown in Figure 18A. It was also stained with Auramine O AFB staining, which showed no AFB bacilli as depicted in Figure 18B. Nevertheless, Rv Ab shows distinctive labelling patterns of 1-2 μ m in diameter as presented in Figure 18C. These cells do not look like Mtb and this may indicate cross-reactivity.

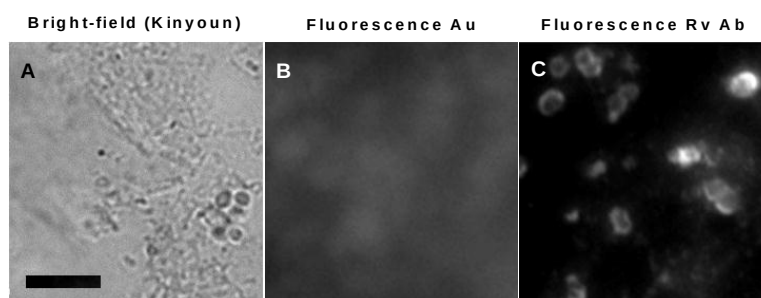


Figure 18: Assessment of Rv Ab cross reactivity in sputum.

The fluorescence images show a known AF negative sputum sample stained with (A) Kinyoun (X1000 magnification), (B) Auramine O AF staining and (C) Rv Ab staining. All three images were taken from the same sample. These images indicate the cross reactivity of Rv antibody with sputum contents. Scale bar 5 μ m.

3.4.2.6. Detection proportion of *M. tuberculosis* from spiked sputum

To assess the detection of Ab, H37Rv from mid-exponential phase culture was spiked into a known AFB⁺ve sputum sample. Unspiked sputum was used as a control sample as described in Section 3.3.6. Both samples were stained with IIF whereas Auramine O was applied on un-spiked sputum sample only. The Mtb counting was done on 10 fields manually by eye.

The results in Figure 19A show a significantly higher detection of Mtb cells with Rv Ab from spiked sputum compared with lower detection of Ab with the unspiked sputum. This result is shown clearly in Figure 19B. In Figure 19B, the bright Ab labelling is may be due to Mtb clumps or non-specific labelling with the sputum contents.

These results show that the total detection number is much lower for IIF compared to Auramine O in sputum sample. Hence, further studies are required to develop another staining technique targeting another property of Mtb cell.

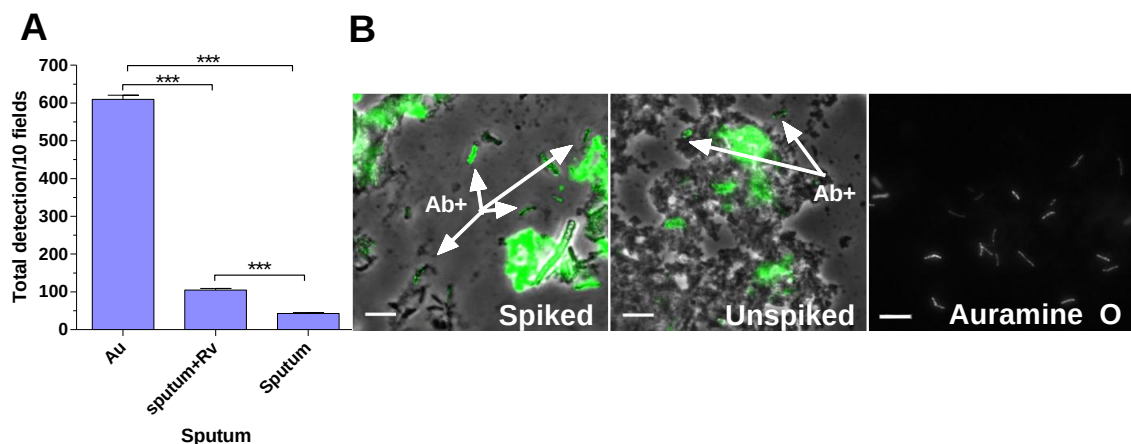


Figure 19: Detection of *M. tuberculosis* in spiked sputum sample using Rv-Ab.

Mtb from spiked and unspiked sputum were detected with Rv-Ab. (A) shows the total cells count of Auramine O and Rv-Ab with both spiked and unspiked sputum all per 10 fields. (B) Shows composite images of phase contrast (grey) and the fluorescence field (green) of unspiked and spiked sputum and Auramine O fluorescence field. Scale bar 5 μ m.

3.4.3. PNA Probe-FISH staining development to detect *M. tuberculosis* from sputum and growth culture

In order to pursue more specific and sensitive detection and differentiation of Mtb cell states from tissue and smears, recent research efforts have looked at peptide nucleic acid (PNA) probes (Stender *et al.*, 1999). PNA are described as pseudo-peptides having the capability

to bind with DNA (Stender *et al.*, 1999). The nucleobases of the PNA are covalently bonded with the polyamide backbone of *N*-(2-aminoethyl) glycine units; this polyamide backbone gives it a relative hydrophobic character compared to that of DNA (Egholm *et al.*, 1993). This characteristic allows the PNA probes to diffuse through the hydrophobic cell envelope of mycobacteria under conditions that do not lead to disturbance of the bacterial structure (Stender *et al.*, 1999). Because of all these characteristics, PNA probes were used in the detection of Mtb from sputum and growth culture.

3.4.3.1. PNA detection of *M. tuberculosis* from culture

In the current study, after extensive work to optimize use of FITC labelled OK682 probe, which targets the 23S rRNA successful hybridization to H37Rv cells was obtained. As shown in Figure 20, mid-exponential phase H37Rv cells in Sauton`s medium were detected by FISH using the OK682 probe. PNA probe labelling of Mtb cells (Figure 20A) shows a characteristic spotted accumulation. However, not all cells are equally labelled, spotted distribution and more intense labelling is obvious in two cells. In contrast, the control sample (Figure 20B), shows the background fluorescence.

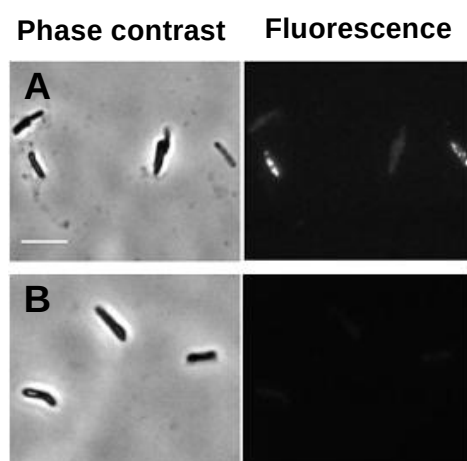


Figure 20: *M. tuberculosis* sample stained with OK682 FISH-PNA.

The images shown represent OK682 PNA-FISH staining of Mtb H37Rv mid-exponential phase cells. (A) Shows H37Rv cells labelled with OK682 targeting 23S rRNA. (B) Shows H37Rv negative control with d.H₂O instead of the probe. Scale bar 5 μ m.

3.4.3.2. Assessment of the PNA cross-reactivity with different bacterial species

As presented above, spot accumulation is the marker of PNA labeling of some Mtb cells. Prior to applying the PNA probes on Mtb from sputum, it was necessary to assess probe specificity. Thus, OK682 was applied to Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*) and *E. coli* DH5 α , and fast-growing *M. smegmatis* mc² 155. The results show a limited (faint) labeling of the OK682 probe in Figure 21A with *M. smegmatis* mc² 155. *P. aeruginosa* and *E. coli* DH5 α in (Figure 21 B&C) showed no labelling with the PNA probe as expected.

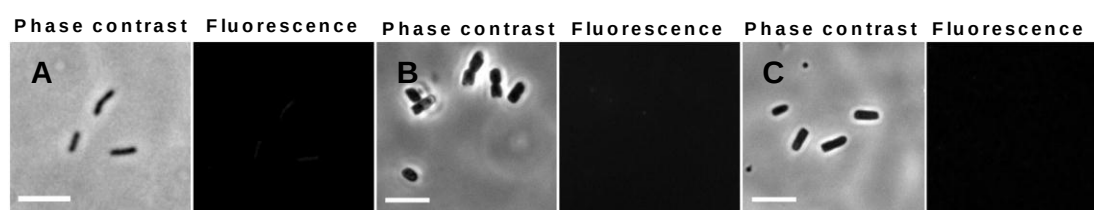


Figure 21: OK682 PNA probe cross reactivity assessment in bacterial pure cultures.

The example images represent an OK682 PNA probe reaction with different bacterial species. A, B, C show OK682 probe with *M. smegmatis* mc² 155, *E. coli* DH5 α , and *P. aeruginosa*, respectively. Phase contrast (where applicable) is illustrated, along with PNA fluorescence images. Scale bars are 5 μ m.

3.4.3.3. PNA detection of *M. tuberculosis* from sputum

The detection of Mtb in sputum requires a highly-specific and sensitive technique in order to pick up the majority of Mtb populations in sputum. Previously, PNA probes were developed to detect Mtb from sputum samples and paraffin-embedded tissues (Stender *et al.*, 1999). As here, OK682 was applied on Mtb cells from 4+ sputum samples. There was much effort put into optimizing the PNA-FISH technique and Stender published a protocol to label Mtb from sputum. Subsequently, however, attempts at labelling using the published protocol were unsuccessful. Later efforts with Stender's protocol applied to sputum and pure culture was done by modifying the PNA probe concentration (Table 8). The Mtb bacilli from sputum have similar to labelling of the *in vitro* culture and spotty appearance when labelled with an OK682 probe as shown in (Figure 22A), compared with no labelling with the d.H₂O control (Figure 22B). To make a comparison with the modified and published protocol, (Figure 22C) shows the results of following Stender's published protocol and (Figure 22D) shows the negative control with d.H₂O instead of the probe, which is similar.

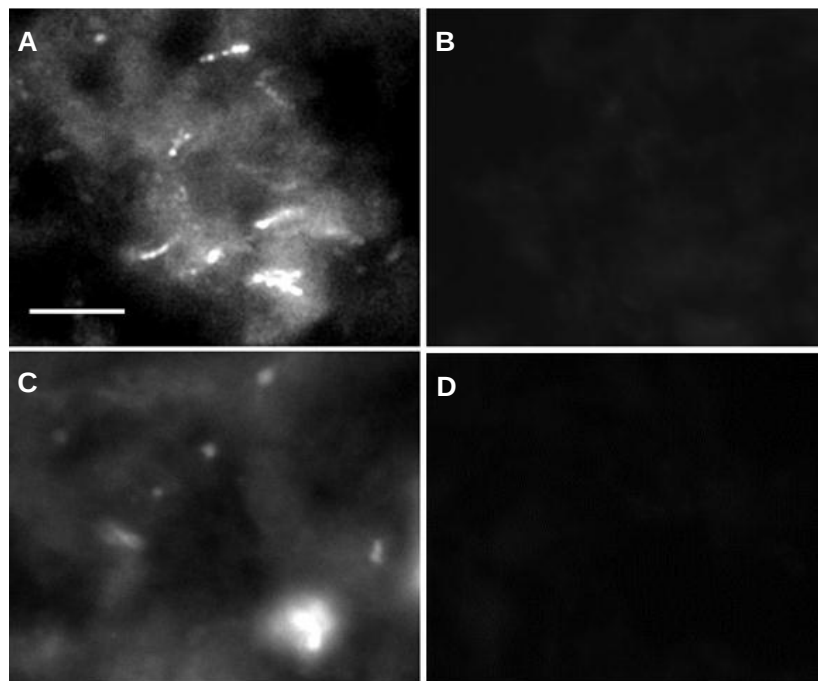


Figure 22: Sputum sample stained with OK682 probe.

The images represent the modification of a PNA-FISH staining protocol in tubercle sputum. (A) The modified protocol shows localisation spots of the OK682 probe targeting 23S rRNA of Mtb cells. (B) Shows the negative control of the modified protocol (d.H₂O instead of the probe) showing no fluorescence localisation of 23S rRNA. (C) Shows OK682 labelling with Stender's published protocol, whereas (D) is the negative control sample from Stender's published protocol (d.H₂O instead of the probe). Scale bar 5 μ m.

3.4.3.4. Lysozyme treatment

Lysozyme (10mg/ml) at 37°C for 1h of incubation was applied on two 4+ decontaminated frozen sputum samples before hybridisation in an attempt to improve the penetration of the OK682 probe. Counting was done manually by eye in 25 fluorescence field for samples with and without lysozyme treatment. The average detection number was then calculated. The results in Figure 23 show that lysozyme treatment was able to significantly enhance the detection proportion of the OK682 probe with a hybridisation time of 90 min at 55°C.

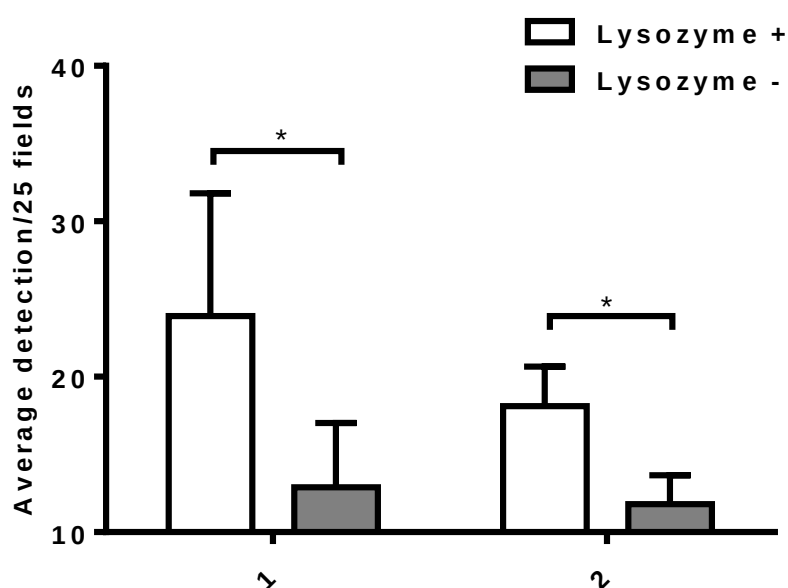


Figure 23: PNA detection averages on *M. tuberculosis* from sputum using lysozyme treatment.

This bar chart shows the average cell detection rate in two (4+) sputum samples stained with an OK682 probe with lysozyme treatment (open columns), and without lysozyme treatment (grey columns). The results show the average of three replicates of each sample with 25 fields of view and were counted in each replicate. PNA proportions were counted by eye in triplicates for each sample. The error bars show standard deviation. Paired t-tests were used to compare lysozyme and no-lysozyme treatment proportions. The asterisks designate significant results.

3.4.3.5. Assessing OK682 for the detection of *M. tuberculosis* in sputum

To assess the detection proportion of the OK682 PNA probe, H37Rv from a mid-exponential phase culture was spiked into a known AFB⁺ sputum sample. Unspiked sputum was used as a control sample as described in Section 3.3.6. The OK682 probe was hybridized to Mtb smears and images were taken from 10 fields of views for both spiked and unspiked sputum.

Figure 24A shows a significant difference in the total detection number per 10 fields between spiked and unspiked sputum. This difference suggests that an OK682 probe detects significantly more cells from the mid-exponential phase growth than Mtb from sputum (Figure 24B).

PNA detection of Mtb from sputum showed higher total detection number compared to IIF. However, in comparison with Auramine O, PNA failed to detect higher number of Mtb in sputum. Thus, the development of another staining technique to detect higher number of Mtb populations in sputum is necessary.

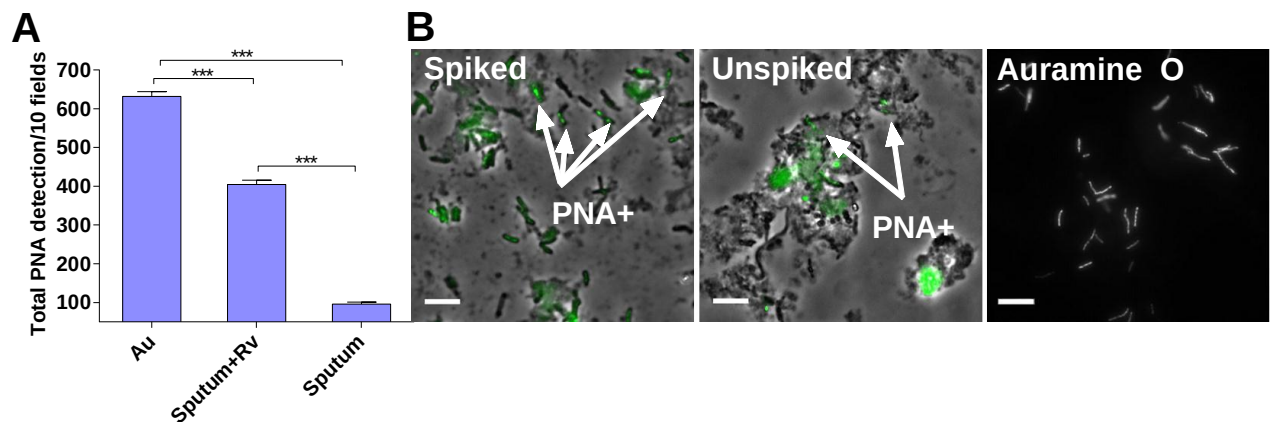


Figure 24: Detection of *M. tuberculosis* in spiked sputum sample using OK682 PNA probe.

Mtb from both spiked and unspiked sputum were detected with OK682. (A) Shows the total cell count of OK682 with both spiked and unspiked sputum per 10 fields. (B) Shows composite images of phase contrast (grey) and a fluorescence field (green) of spiked and unspiked sputum in addition to Auramine O fluorescence field of the same sample. Scale bar 5 μ m.

3.4.4. Kinyoun staining development to detect *M. tuberculosis* populations from sputum and pure culture

A central aim of this work has been to develop a staining technique to detect all or at least the majority of Mtb populations from sputum. By developing this technique, it will be possible to identify Mtb sub-populations. Research has demonstrated that Auramine O has similar sensitivity and specificity to Z-N staining (Ba & Rieder, 1999 and Steingart *et al.*, 2006). As shown earlier in this chapter, however, there are apparent Mtb populations that show distinct LBs and they have a similar Mtb cell morphology, but they are non-Auramine O AF.

3.4.5. *M. tuberculosis* detection with a fluorescence Kinyoun AF staining technique

Because Kinyoun staining does not require heating it is easier and safer to perform than Z-N (Kinyoun, 1915). Thus, Kinyoun AF staining, which uses a higher concentration of phenol than Z-N, increases the penetration of fuchsin; with the result that staining can occur at room temperature (Kinyoun, 1915). Shapiro *et al.*, (2008) modified the Z-N technique for use as a fluorescence staining method to detect Mtb from sputum (Shapiro and H nscheid, 2008). A

modified Kinyoun AF staining technique as a fluorescence staining method to detect Mtb from sputum and pure cultures with approximate excitation/emission of 546/590 nm, was applied here.

The Pk biofilm cells (cultured in Sauton`s medium) are characterised by a low Auramine O AF proportion (3%-14%), thus they were used to measure Kinyoun staining sensitivity. Mid-exponential phase Mtb strain H37Rv cells grown in Sauton`s medium were used as a positive control. Slides were then prepared in triplicates and stained with the modified Kinyoun staining technique (3.3.3) and at least 100 cells were counted in each replicate.

Figure 25A shows the Pk Mtb cells with Kinyoun, which detects almost 99% of Mtb bacilli out of 475 cells compared with phase contrast (Table 13). Figure 25B shows the control cells from a mid-exponential phase with Kinyoun, which detects almost 100% of the Mtb bacilli out of 645 cells compared with phase contrast (Table 13). Additionally, Kinyoun staining was applied on Mtb cells from sputum in Figure 25C. These results show that fluorescent Kinyoun AF staining shows a higher detection proportion with the already known non-Auramine O AF Pk Mtb cells.

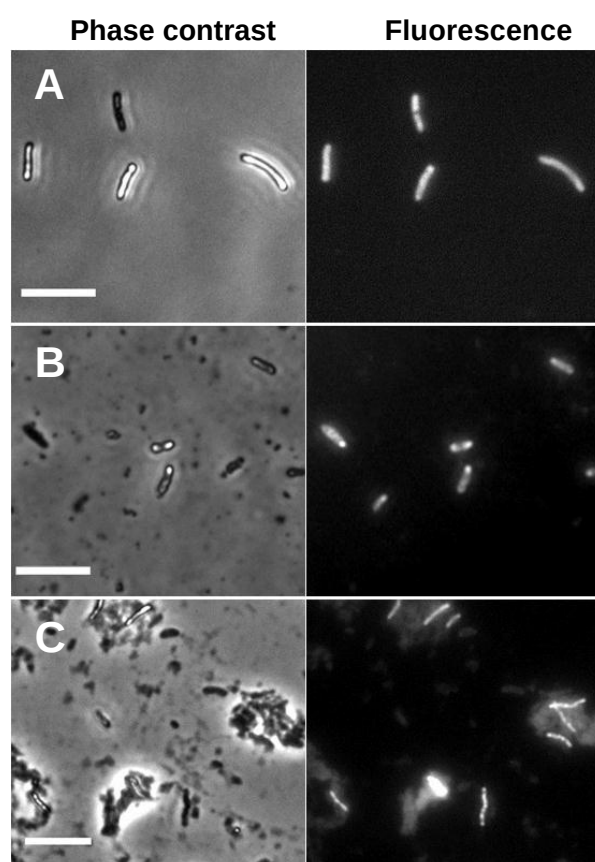


Figure 25: *M. tuberculosis* H37Rv cells with Kinyoun fluorescence AF staining.

The images show Kinyoun stained Mtb H37Rv cells. Image (A) shows mid-exponential H37Rv cells with fluorescence Kinyoun, (B) shows Pk Mtb cells with fluorescence Kinyoun staining whereas (C) shows the fluorescence Kinyoun with Mtb cells from sputum. The microscopic images were captured using phase contrast and fluorescence microscopy (TexasRed filter). Scale bar 5 μ m.

Table 13: Kinyoun AF proportions in Mtb from growth culture.

Sample (Mtb H37Rv)	AF ^{+ve} (%) $\pm$ SD	# cells
Pk cells	98.9 $\pm$ 1.2	475
Mid-exponential phase	99.7 $\pm$ 2.4	645

The table shows the AF proportions of mid-exponential phase and Pk Mtb H37Rv cells with fluorescence Kinyoun staining. AF proportions were counted in triplicate for each sample (almost 100 cells per slide).

3.4.6. BODIPY (493/503) staining shows no localisation of labelling to lipid bodies

BODIPY (BD) is a neutral lipid due to its nonpolar structure and long-wavelength absorption with approximate excitation/emission of 493/503 nm with cell-permeable lipophilic molecule (Listenberger & Brown, 2007). It can emit bright green fluorescent and red fluorescent alternatives. BD fluorophores differ from RLx and GLx in fluorescence stability (Chen *et al.*, 1998).

Late-exponential phase *M. bovis* BCG Glaxo cells were grown in Middlebrook 7H9 medium. The dual staining procedure was done by staining both test and control slides (each in triplicates) with Kinyoun staining. The slides were then allowed to air dry. The lipid staining for test slides with BD and control slides with GLx were done according to the protocol in Section 3.3.4. Images were taken for both Kinyoun/BD and Kinyoun/GLx.

Figure 26 shows example images of BD and GLx LB localization in the BCG cells in combination with Kinyoun staining. Figure 26A shows BD labeling of BCG cells that reflect poor LB labeling compared with GLx labeling of BCG cells from the same culture in Figure 26B. Thus, BD lipid staining shows poor labeling on BCG cells compared with the GLx lipid staining.

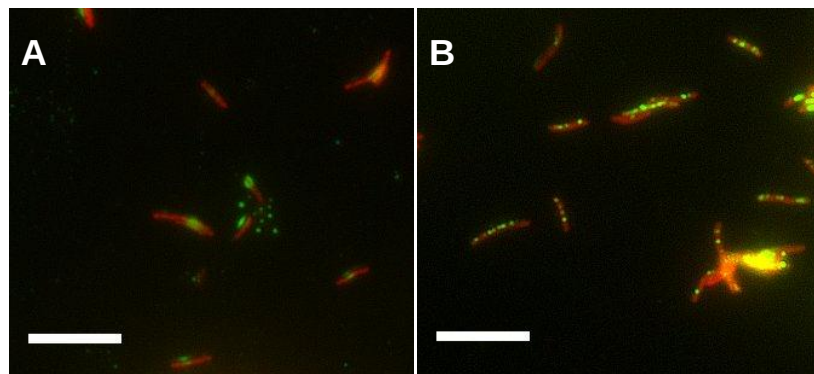


Figure 26: BODIPY Neutral Lipid Staining labelling in combination with Kinyoun.

The images show lipid staining localisation on BCG cells in combination with Kinyoun. The images show *M. bovis* BCG (Glaxo) late-exponential phase grown in Middlebrook 7H9 medium. Image (A) shows BCG cells with Kinyoun/BD staining, whereas image (B) shows control BCG cells with Kinyoun/GLx dual staining. The cells were stained and imaged with lipid staining at the same time. An image of Kinyoun fluorescence (red pseudocolour) is overlaid on the BD or GLx (green pseudocolour) image. Scale bar 5 µm.

3.4.7. Comparison between neutral lipid staining techniques to identify the LB *M. tuberculosis* sub-populations

To identify LB sub-populations from sputum and *in vitro* culture, it is crucial to choose a reliable technique to detect and identify the LBs in combination with modified Kinyoun staining. This was especially the case when choosing between the two green LB staining options of BD and LipidTox Green neutral lipid neutral lipid staining (GLx, a neutral lipid stain has an extremely high affinity for neutral lipid droplets with approximate excitation/emission of 495/505 nm). For a direct comparison of neutral lipid staining techniques, late-exponential phase *M. bovis* BCG strain Glaxo cells were grown in Sauton's medium. The cells were then harvested and slides were prepared according to the protocol in Section 2.4.1. Slides were prepared in triplicate for each staining technique and LB staining was applied for each staining technique at the same time.

At least 100 cells were counted in each replicate. Figure 27A shows that there was a significant difference between the LB detection proportion between of RLx and BD and GLx and BD. Almost 50% of the cells with LB⁺ were undetected by the BD lipid staining in comparison with RLx and GLx. Figure 27B shows images of the different neutral lipid labeling of BCG cells from the same culture.

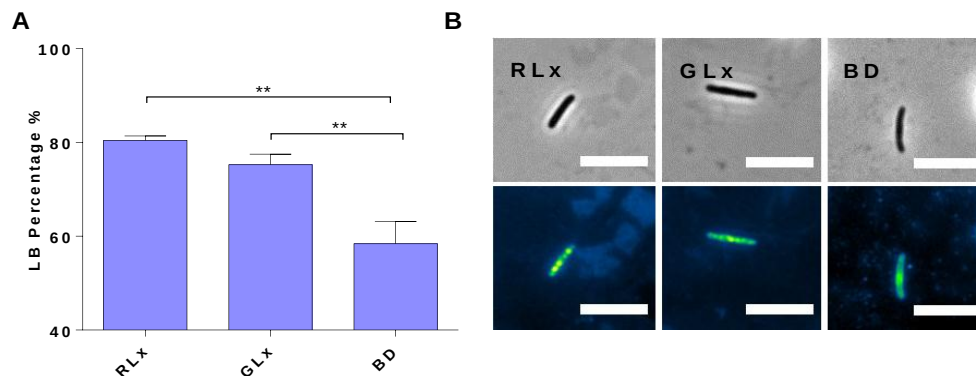


Figure 27: Lipid body proportions comparison between neutral lipid staining on *M. bovis* BCG cells.

Bar chart (A) shows proportions of LB^{+ve} cells of late-exponential phase BCG labelled with RLx, GLx and BD neutral lipid staining techniques. The results indicate that RLx and GLx LB^{+ve} cell proportions are significantly higher than BD staining. Selected cells in (B) were then converted to LUTs to show the LB localization clearly. Asterisks designate significant results ($P < 0.01$).

3.4.8. Comparison between Kinyoun, Auramine O, immunofluorescence and PNA staining techniques to detect *M. tuberculosis*

The main objective of this chapter was to develop a staining technique to detect as larger proportion of Mtb in sputum and pure culture as possible. Four different staining techniques were used in this study: IIF with Rv Ab, PNA-FISH with the OK682 probe, Auramine O, and fluorescence Kinyoun AF staining. Each one of these techniques targeted a specific property of the Mtb cell.

Multiple Mtb populations within sputum samples were detectable with Auramine O, but not by the other techniques. Pk non-Auramine O AF cells were used in the assessment of the staining techniques. Hence, when there were a higher proportion of non-AF cells detected, the more reliable particular staining technique was needed to be for the detection of a greater proportion of the total Mtb cells in sputum. For this reason, PK cells from Mtb H37Rv biofilm (non-AF cells) were stained with the four staining techniques in triplicate for each staining series. A comparison was also done on Mtb cells from sputum using the four staining techniques. Cells which were labeled with the IF and PNA techniques were counted manually. At least 100 cells were counted from each replicate. Mtb cells from sputum were counted by the Mtb total cell number/15 field of view by eye in triplicate (45 fields in total for each staining technique).

Figure 28 shows the detection of Pk cells according to the four different staining techniques. Auramine O AF staining detects the lowest proportion at < 20% of Pk cells, whereas PNA staining detects almost 50% of Pk cells. IF staining detects about 90% of Pk cells, which is high detection proportion. However, Rv Ab shows non-specific binding with non-Mtb sputum cells. Fluorescence Kinyoun AF staining shows nearly 100% detection of Pk cells with very low SD, which is the highest detection proportion. Furthermore, results from sputum show that the highest total cell number is counted by fluorescence Kinyoun staining, whereas the Ab staining shows the lowest number of detection. Auramine O is significantly lower than Kinyoun staining, which indicates that Kinyoun is more specific than Auramine O. The detection levels with PNA are slightly higher than with IIF. Hence, fluorescence Kinyoun staining is considered the staining technique that best detects the greatest proportion of Auramine non-AF Mtb cells.

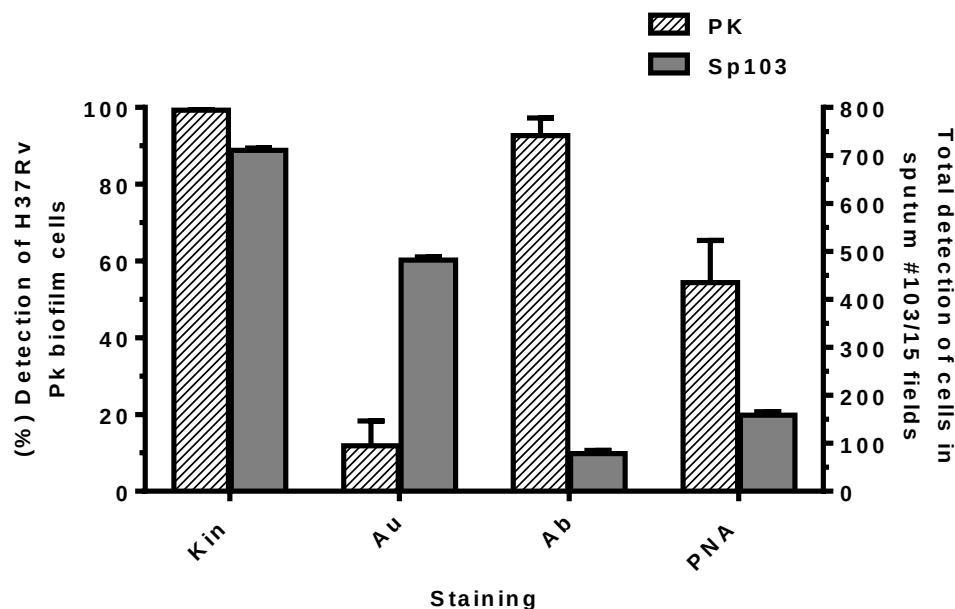


Figure 28: *M. tuberculosis* cells detected by different staining techniques.

Comparison between fluorescence Kinyoun, Auramine O, IIF(Rv Ab) and PNA-FISH (OK682) probe staining techniques to detect Pk non-AF Mtb H37Rv cells (hatched columns) and Mtb from sputum #103 (grey columns). Pk cells were counted in triplicates and almost 100 cells were counted in each replicate, Auramine O and Kinyoun stained cells were counted using image-J based software whereas PNA and IIF stained cells were counted by eye. Mtb from sputum were counted as a total detection number per 15 fields of view in triplicates by eye for all staining techniques, then the average and SD was calculated from the three means for each replicate.

Figure 29 shows a summary image of the detection of Mtb bacilli from the same sputum sample. Image A shows the Auramine O labelling of Mtb, which is much less than the

Kinyoun staining detection on image B, which is from the same sputum sample. Image A shows three Mtb populations (Au^{+ve}/LB^{+ve} , Au^{+ve}/LB^{-ve} and Au^{-}/LB^{+ve}), whereas image B shows two populations from the same sample (Kin^{+ve}/LB^{+ve} and Kin^{+ve}/LB^{-ve}). Thus, in a comparison with Figure 10 in the introduction of this chapter, which shows LB^{+ve}/AFB^{-ve} populations with image A & B in Figure 29, it can be seen that these populations are detected with fluorescence Kinyoun where there is no AFB^{-ve}/LB^{+ve} .

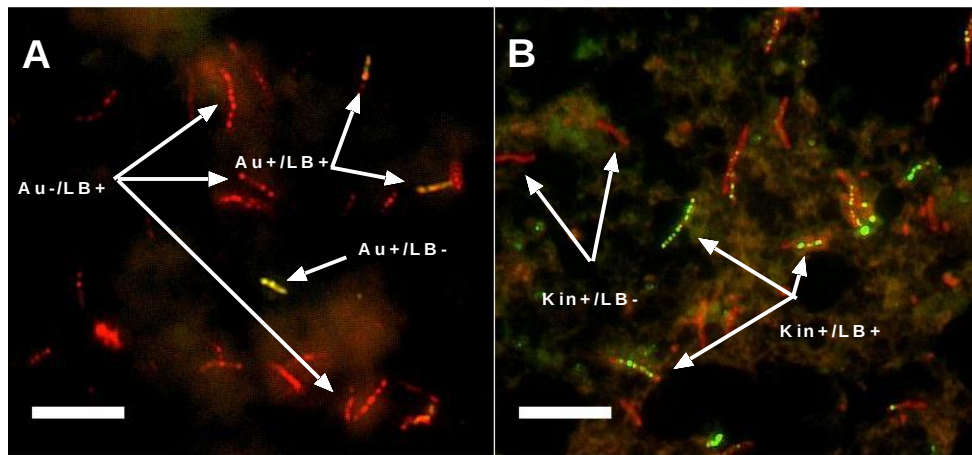


Figure 29: Tubercle sputum sample.

The images show Mtb heterogeneity in the same 4+ sputum sample. (A) Shown Mtb cells with Auramine O (yellow pseudocolour) and RLx (red pseudocolour), whereas (B) shows Mtb cells with Kinyoun (red pseudocolour) and GLx (green pseudocolour). Both images were taken from the same sample with different staining techniques. The images suggest that there are multiple populations of Mtb in sputum that were not detected with Auramine O, but were detected with Kinyoun staining. Scale bar 5 μ m.

3.5. Discussion

3.5.1. Studying acid-fastness of *M. tuberculosis* by Auramine O staining

Auramine O staining is widely used in research and clinical laboratories around the world (Steingart *et al.*, 2006). This technique requires limited effort as the protocol does not require heating, in contrast to Z-N staining. Auramine O has also shown a higher sensitivity for detection of Mtb than transmitted light Z-N and Kinyoun staining (Sawadogo *et al.*, 2012). However, some reports showed that the transmitted light of Z-N was more sensitive for the detection of *Mycobacterium paratuberculosis* than Auramine O (Huntley *et al.*, 2005). Both techniques have lower specificity for the detection of *Nocardia* and *Corynebacteria* (Shinnick & Good, 1994). In this study all the sputum samples were already clinically diagnosed as Mtb samples.

Many conditions are responsible for the loss of acid-fastness. In multiple stress model Mtb was incubated under several conditions including low O₂, 5% CO₂, low nutrient and acidic pH which resulted in loss of acid-fastness (Deb *et al.*, 2009). The existence of a non-AF Mtb phenomenon is well recognised (Mudd & Mudd, 1927). There are several mechanisms that affect AF staining and result in the forming of non-AF Mtb cells, such as cell wall disruption (Yegian & Porter, 1944), UV irradiation (Murohashi & Yoshida, 1965), the lack of the growth nutrients or starvation (Nyka, 1974), mycobacteriophage infection (Gangadharam & Stager, 1975), cell wall alteration resulting in changes in mycolic acid (MA) content (Yuan *et al.*, 1998, Bhatt *et al.*, 2007a) and isoniazid exposure (Bhatt *et al.*, 2007b). Recently, it was shown that a decontamination step with N-Acetyl-L-Cysteine (NALC) decontamination with NaOH to thinned sputum samples significantly reduces the mycobacterial acid-fastness (Garton *et al.*, 2008). Also, it was also shown that dry heating the sputum and culture smears in a heat block might alter the cell wall and as a result reduce acid-fastness (Gokhale *et al.*, 1990). Therefore, the acid-fastness could be affected and lost by many mechanisms which require an alternative staining technique for the identification of Mtb bacilli. The mechanism of the acid-fastness mechanisms was discussed in Section 1.4.1.

Another avenue explored in this project was to study the effect of multiple freeze-thaw cycles on the AF proportion. The current study shows that multiple freeze-thaw cycles over a 24h freeze time sharply reduced the acid-fastness of cells from Mtb H37Rv culture and sputum samples. A previous study done by Bell, (2013) showed that the acid-fastness of the Mtb CH strain was substantially reduced. Due to the extended freezing time of this study (24h), large

ice crystals were developed in freezing and subsequent thawing which affected the cells wall integrity and subsequent AF staining. The effect of freezing-thawing cycles seems to permeabilise the Mtb cells from culture and sputum.

Furthermore, the sputum sample that was used in this study was already decontaminated, which is an additional reduction factor for acid-fastness. This was studied by Nyka (1971), who showed that Z-N AF staining is destroyed by reduction, and that the Mtb bacilli can be recovered again by oxidation.

Another limitation of this study is that counting the AF cells from sputum was done manually by eye in contrast with the cells from culture, which was counted by computer software. However, the bias rate of the manual count is not high, as the AF and non-AF bacilli are reasonably clear and can be differentiated. Overall, it is preferable to examine clinical samples for AFB prior to freezing, as the result will be significantly affected by freeze-thaw cycles.

The other staining methods that are available to detect Mtb include PNA-FISH (Stender *et al.*, 1999 and Lefmann *et al.*, 2006) and IF staining (Brozostec A *et al.*, 2009 and Ryan *et al.*, 2010). AF staining, however, is the most commonly used in clinical laboratories (Ulukanligil *et al.*, 2000). None of these methods are known to detect all Mtb populations in sputum. Thus, it is necessary to develop a staining method to show a higher detection of these populations in sputum.

3.5.2. Immunofluorescence application to detect *M. tuberculosis*

Initially, four anti Mtb Abs techniques that were directed towards the Mtb surface Ags such as RV, PPD and CDC1551 Abs and WCL Ab which directed towards whole cell lysate, were applied on H37Rv culture from a mid-exponential phase. PPD and WCL Abs were prepared in vitro and then injected into Guinea pigs to produce polyclonal Abs. CDC1551 and WCL Abs showed a slightly lower detection against the Mtb H37Rv strain in comparison to PPD and Rv Abs. WCL Ab was used in another study that showed that this Ab does not detect the complete set of H37Rv populations at a mid-exponential phase (Ryan *et al.*, 2010). The study also suggested the reason for the incomplete detection was the late-exponential phase growth used for immunisation, which might miss mid-exponential phase Ags. Another reason might be due to masking of surface Ags by the polysaccharide-rich capsule (Ortalomagne *et al.*, 1995). Additionally, PPD antibody was not be able to detect the majority of H37Rv cells

in the early-exponential phase in this study, which means that the Mtb surface Ags at this stage may be different to the phase of growth used for the production of PPD.

In a study examining the ability of the Mtb to accumulate and utilise cholesterol, the same PPD Ab was not able to detect Mtb cells cultured with cholesterol (Brozostec *et al.*, 2009). The cholesterol may mask the Mtb cell surface Ags preventing detection by the Ab. This finding potentially explains why IIF could not detect the majority of Mtb populations from sputum which is known to have high cholesterol content (Garton, personal communication). However, the exact reasons for why IIF does not detect the complete set of Mtb populations remain unknown (Ryan *et al.*, 2010).

Through this study, IIF succeeded in detecting the majority of H37Rv cells in pure culture. In sputum on the other hand, it was noticed that when the Rv-Ab was used with a 4+ sputum sample, the number of bacilli per field was lower compared with cells detected with Auramine O staining. Therefore, a spiked sputum sample was used with the IIF. The results showed that Mtb bacilli from sputum display different surface Ags than Mtb from culture, as the spiked sputum sample showed a significantly higher detectable cell number than unspiked.

Ag60, the essential constituent of tuberculin, reaches the highest level at the stationary phase, whereas in the early-exponential phase, it is secreted in low levels (Harboe, 1981). An analysis of differentially expressed proteins in mid-exponential, early-stationary and late-stationary H37Rv growth phases showed that ten proteins are differentially expressed in the late-stationary phase, but not in the other phases (Ang *et al.*, 2013). These studies are consistent with the finding that H37Rv labelled differently with Anti-PPD in early-exponential phase from the other growth phases.

3.5.3. PNA-FISH application to detect *M. tuberculosis*

In this study it was shown that the OK682 probe detects Mtb from culture and sputum samples. However, some H37Rv populations in culture lack a fluorescence signal and are not detected. Also, the mycobacterial ribosomal genes are simultaneously downregulated with the cessation of growth (Trauner *et al.*, 2012), this was also shown with mid-exponential phase Mtb cells in the current study. The rRNA number in the earlier exponential phase increases in contrast to the number in the stationary phase, which decreases (Verma &

Davidson, 1994). In the formation of NRP populations, these populations might have downregulated 23S rRNA. As a result, the OK682 probe would give a low signal when hybridized to NRP Mtb populations.

The insufficient signal of the PNA probes is a significant problem. This may reflect downregulation of the rRNA in the bacterial cell during the NRP stat (Amann *et al.*, 1995). Another reason for the lack of the PNA signal may be the insufficient accessibility of the target probe into the mycobacterial cells (Amann *et al.*, 1995). Lysozyme is a N-acetylmuramide glycanhydrolase, which is a glycoside hydrolyses agent (Say *et al.*, 2012) that hydrolyses the glycosidic bonds of the bacterial peptidoglycan (Bidnenko *et al.*, 1998). By using this permeabilising agent, an improvement the PNA labelling average was made and the detection number increased.

Many Mtb cells in sputum were not detected with the OK682 probe in this study, possibly because their rRNA was low (Garton *et al.*, 2008; Trauner *et al.*, 2012). Use of sputum spiked with H37Rv cells in mid-exponential phase supported the hypothesis that unlabelled sputum bacilli had low rRNA content.

A study was done to reveal the multiple Mtb populations in mouse and Guinea pig tissues revealed that FISH yielded a stronger signal of single strand DNA (ssDNA) probes targeting 16S rRNA on H37Rv under a hypoxic condition, a weak signal from mouse, and failure to detect Mtb from Guinea pig (Ryan *et al.*, 2010). Ryan *et al.*, (2010) suggested that the failure of the nucleic acid probes to detect Mtb from Guinea pig tissue as arising from the number of rRNA in necrotic tissue, which is below the limit of FISH detection. Additionally, the cell wall might have been altered and became less permeable to the nucleic acid probes (Ryan *et al.*, 2010). All these findings of FISH on mouse and Guinea pig tissues might justify the variation on the detection percentages between sputum and pure culture.

The PNA detection results from this project on Mtb from sputum were similar to the FISH results of Ryan on a mouse *in vivo* model, where the signal is weaker than the *in vitro* culture. Although, the reported high sensitivity and specificity of PNA probes to detect Mtb in sputum (Stender *et al.*, 1999), the current study showed a limited detection of Mtb by the OK682 PNA probe in sputum. Therefore, it continues to be necessary to find a staining technique that is able to detect the majority of Mtb populations in sputum.

3.5.4. Fluorescence Kinyoun staining to detect *M. tuberculosis* from sputum

Many Mtb positive samples by culture are not positive by Z-N (Dewit *et al.*, 1992). The first reason for this is because the Z-N staining detection limit is 10^4 bacilli per ml or slide (Allen, 1992 and Marks, 1974). It is also proposed that Z-N staining fails to accurately detect Mtb from clinical samples due to the alteration of the mycobacterial cell envelope infection (Seiler *et al.*, 2003).

In the current study it was shown that Auramine O detects some, but not all Mtb populations in sputum and pure culture. With Auramine O/LB dual staining, however, there are LB^{+ve} populations observed that are morphologically similar to Mtb and yet are AF-. Additionally, in culture Auramine O is not able to detect more than 90% of the non-AF populations. Although, recent studies have shown that the sensitivity is increased for TB diagnosis when using fluorescence microscopy (Steingart *et al.*, 2006), the work shown here indicates the potential presence of multiple AF- population. Likewise, PNA-FISH probe and IF staining techniques in this study showed a lower detection for Mtb in sputum.

Examination of Mtb Pk biofilm cells stained with fluorescence Kinyoun in the current study resulted in about a 99% detection percentage in the same sample that gave a below 10% detection proportion with Auramine O (Figure 28). Exactly why assessing fluorescence of fuchsin following Kinyoun results in a higher detection proportion than detection of fluorescence Auramine O is still unknown. Although the staining times of Kinyoun and Auramine are the same, Kinyoun shows a higher sensitivity for the detection of AFB from culture and sputum over Auramine O. Although the equal decolourisation time and higher acid and ethanol concentrations in the decolourisation agent of Kinyoun staining than Auramine O (Kinyoun, 95% v/v ethanol and 3.1% v/v HCl, Auramine O, 35% v/v ethanol and 0.5% v/v HCl), Kinyoun staining showed higher detection proportion than Auramine O. Further, it was reported that staining with potassium permanganate (KMnO₄) for 30 sec has an effect on Mtb acid-fastness (Heimer *et al.*, 1978). The higher phenol concentration used in Kinyoun staining, (3.4% w/v in Auramine O, 6.8% w/v in Kinyoun) might permit more carbol-fuchsin to get into the cell.

Although there is a higher detection proportion with fluorescence Kinyoun, the main drawback is that the background level in the fluorescence field is higher than with Auramine O. This might be due to the effect of the KMnO₄ with Auramine O, which significantly

quenches the background level (Smithwick *et al.*, 1995). This disadvantage, however, does not affect cell counting for fluorescence Kinyoun, which can be easily counted by Image-J based software.

3.6. Conclusions

The work presented here demonstrates that the fluorescence Kinyoun staining technique detects the majority of non-Auramine O AFB Mtb populations in growth from *in vitro* culture and also the Mtb from a sputum sample, which was the ultimate aim. IF and PNA detected the majority of Auramine O non-AFB from the Pk biofilm cultures tested here, in contrast to sputum, where they failed to detect the majority of Mtb population. Auramine O had a lower detection of Pk Mtb from culture and Mtb from sputum. As such, fluorescence Kinyoun staining is the best staining technique for use in detection of majority of Mtb from sputum.

The principle findings of this chapter are:

- Auramine O shows poor detection of Mtb populations in Pk cells and Mtb from sputum compared with fluorescence Kinyoun.
- IF and PNA staining techniques show lower detection of Mtb from sputum than Auramine O and Kinyoun staining.
- Auramine O AFB^{-ve}/LB^{+ve} populations that have the same morphology as Mtb were identified by Kinyoun staining as AFB populations.
- Fluorescence Kinyoun staining shows the highest detection average of non-Auramine O AFB Mtb from culture and sputum when compared with other staining techniques.

Chapter 4

Microscopic studies of
M. tuberculosis in sputum

4.1. Introduction

When *Mtb* bacilli first enter the lung, they are met by alveolar macrophages (Saunders & Cooper, 2000), which are the first line of defence against *Mtb* (Miranda *et al.*, 2012). In most individuals the infection is then controlled by host-cellular granuloma formation (Saunders & Cooper, 2000). It has been thought that margins of liquefied lesions in the lung cavities are the location of tubercle bacillus that are characterised by rapid and extensive bacterial growth (Canetti, 1955b, Young & Duncan, 1995). Once the infection becomes active, sputum expectorated from pulmonary TB patient contains different *Mtb* populations (Garton *et al.*; 2008). Some of these populations may express properties that are required for transmission (Garton *et al.*, 2008).

Recent cytological studies on mycobacteria reported that *Mtb* exists in multiple populations *in vitro* and *in vivo*, even within an apparent single microenvironment (Ryan *et al.*, 2010; Hoff *et al.*, 2011). Ryan *et al.* (2010) found multiple *Mtb* phenotypes in mouse and Guinea pig lung tissues by using Auramine-Rhodamine and immunohistochemistry dual-staining techniques. The study revealed that two populations of *Mtb* from stationary phase were detected with Auramine-Rhodamine/IF dual staining. However, applying this technique to *Mtb* from hypoxic culture revealed three populations, IF (alone), Auramine-Rhodamine (alone), and both Auramine-Rhodamine/IF. Interestingly, IF on *Mtb* from hypoxic culture showed punctuated staining manner. On the other hand, when applying Auramine-Rhodamine/IF dual staining to *Mtb* from lung tissue of Guinea pig, three populations were detected, similar to hypoxic culture (Ryan *et al.*, 2010). Moreover, *Mtb* within lipid-loaded macrophages accumulated lipid droplets and lost acid-fastness (Daniel *et al.*, 2004; Gomez & McKinney, 2004).

These populations may be reflecting the phenotypic features of *Mtb* in human sputum. Hence, any identified bacillary population in sputum may provide clues to recognise these undefined properties and ultimately offer avenues toward improved treatment and prevention of TB disease. Further, defining of *Mtb* phenotypes from sputum provides an idea of stimuli throughout specific phases of infection. Within this laboratory, several *in vitro* models have been developed with H37Rv to reproduce the transcriptional profile and LB contents of *Mtb* from sputum (Lee, 2012). These conditions were chosen to replicate the sputum environment in TB disease.

One of the clear characteristics of Mtb bacilli in sputum is the formation of LBs (Garton *et al.*, 2008 and Garton *et al.*, 2002). LB droplets were first seen in leprosy bacilli in 1946, using the lipid stain Sudan black B (Burdon, 1946). Garton *et al.* succeeded in characterizing Mtb-LB-rich populations in sputum using Nile-Red neutral lipid staining (Garton *et al.*, 2002). Further, study revealed that the LB proportion varies in TB patients from 8 - 80% (Garton *et al.*, 2008). It has also been shown that Mtb could produce LB *in vitro* (Sherratt, 2008). In various stress conditions the morphology and frequency, however, differs from the LB in sputum; LB formed *in vitro* are less distinct.

The sputum transcriptome of Mtb has revealed some important findings related to the LB formation by Mtb in sputum. The transcriptome signatures and presence of LB suggest that LB^{+ve} cell population may exist in a slow or non-growing Mtb in sputum (Garton *et al.*, 2008). Therefore, it is considered a significant challenge to detect and identify mycobacterial phenotypes and their expressed properties in sputum and in culture media. Doing this may enable the development of specific therapeutic agents and ultimately prevent transmission. It has been recorded that many TB cases go undetected with conventional microscopy staining methods, but are positive with molecular-based methods (*i.e.*, PCR) and cultural methods (Kim *et al.*, 2011). Thus, it has been assumed that there is a detection limit in conventional staining methods such as with Auramine O and standard Z-N due to the presence of non-AF population.

The specific reasons why some of these populations go undetected are still unknown (Ryan *et al.*, 2010). It is likely, however, that it is due mainly to cell wall variations that alter the permeability to dyes (Bhatt *et al.*, 2007). Various studies of mutants have revealed that Mtb bacilli with altered cell wall lipid composition loss acid-fastness. The acid-fastness can also be lost through death and injury of Mtb cells (Seiler *et al.*, 2003; Ulrichs *et al.*, 2005). Recently, Shapiro *et al.*, (2008) revealed that there are some Mtb populations that could not be detected with using Z-N transmitted light, but could be detected with fluorescence light (Shapiro & Hänscheid, 2008). This indicates that fluorescence microscopy of carbol-fuchsin staining is more sensitive than bright field examination.

Researchers evaluating Mtb in sputum have revealed populations of LB^{+ve} bacilli (Garton *et al.*, 2008), non-AF Mtb bacilli (Nyka, 1976, Chandrasekhar *et al.*, 1990) and an Mtb population only recovered with Rpfs (Mukamolova *et al.*, 2010). The study of Ryan *et al.*, (2010) showed that the phenotypic characteristics of *in vitro*-grown Mtb

under hypoxia are not the same as those in the hypoxic, necrotic lesions of Guinea pig and mouse *in vivo* models (Ryan *et al.*, 2010).

Relying on the hypothesis that there are multiple Mtb populations in sputum (Nyka, 1969, Garton *et al.*, 2008), a central objective of this work has been to identify these populations using different staining techniques. A number of studies show the multiple Mtb populations in pure culture (Andreu *et al.*, 2008, Deb *et al.*, 2009 and Ryan *et al.*, 2010), whereas there are just a few studies showing the non-AF bacilli in sputum samples (Shapiro & Hänscheid, 2008) and *in vivo* (Nyka & Oneill, 1970).

There is no published study showing the various Mtb populations in sputum using different Mtb cell target staining techniques. Combining different staining techniques with different targets of the Mtb cell effectively provides more opportunity to identify these sub-populations in sputum. The work presented in this chapter will show multiple Mtb populations in pure culture and sputum using different staining approaches and will also further characterise these populations.

4.2. Aims and objectives

To use various cytological staining methods to identify the multiple Mtb sub-populations in pure culture and ultimately in sputum samples.

The specific objectives are to:

- 1. Identify Mtb sub-populations in vitro and sputum using:**
 - I. IIF triple-staining technique**
 - II. PNA triple-staining technique**
 - III. Auramine O/LipidTox Red Neutral Lipid stain dual-staining technique**
 - IV. Kinyoun/ LipidTox Green Neutral Lipid stain dual-staining technique**
- 2. Characterise the Mtb LBs subpopulations using the previous staining techniques**
- 3. Study the validation of manual cell counts with different staining techniques compared with automated cell count**

4.3. Methods

4.3.1. Auramine O, Ab (or) PNA and RLx triple-staining technique

The staining procedures were carried out by initially staining Mtb smears from sputum or pure culture with IIF or PNA. Prior to examining slides under the microscope, the regions of interest were marked with a clear permanent marker and then images were taken. The cover slip was then taken off and the slide left to dry. Auramine O staining was done on top of IIF, and the images were taken from the same regions of interest. The cover slip was then taken off again and the slide left to dry. Finally, RLx staining was carried out on the top of IIF and Auramine O, and images were taken from the same regions of interest. The triple-staining order should be taken under account as IIF fluorophores fade when exposed to the phenol of Auramine O.

4.3.2. Auramine O, Kinyoun and RLx triple-staining technique

The Mtb smear was first stained with Auramine O. Images were captured from marked fields and then the cover slip was taken off. The smear was then stained with Kinyoun stain on top of Auramine O, and the images were recorded from the same regions of interest. The coverslip was then taken off and the smear was stained again with RLx on the top of Auramine O and Kinyoun. The images were then taken from the same regions of interest.

4.3.3. Auramine O/RLx (or) Kinyoun/GLx dual-staining techniques

The dual-staining techniques were done by staining the smears of sputum or pure culture with Auramine O or Kinyoun stain. Slides were then air dried and RLx (with Auramine O) or GLx (with Kinyoun) was applied.

4.3.4. Cell-count analysis

Mtb cells from sputum from IIF/Auramine O/RLx, PNA/Auramine O/RLx triple-staining and Auramine O/RLx, fluorescence Kinyoun dual-staining techniques were counted in triplicate. Cell counts were done in triplicate by assessing each cell individually with the three stains by scoring positive cells as 1 and negative as 0. Statistical analyses were performed using Excel 2010 (Microsoft Corp.). Figure 30 shows the statistical analysis

on the Excel spreadsheet and explains the calculations of the sputum sample stained with the PNA/Auramine O/RLx triple-staining technique.

Total number of counted cells by PNA
 Total cell number detected by x staining
 Total cell number detected by individual staining

	A	B	C	D	E	F	G	H	I	J
1										
2	108									
3	108									
4	12	6	51	30	5	3	1	15	87	46
5	RLx only	PNA only	Au only	Au/RLx	PNA/Au	PNA/RLx	All	PNA	Au	RLx
6	0	0	0	2	0	0	2	0	1	1
7	0	0	0	2	0	0	2	0	1	1
8	0	0	1	1	1	0	1	0	1	0
9	0	0	1	1	1	0	1	0	1	0
10	1	0	0	1	0	1	1	0	0	1
11	0	0	1	1	1	0	1	0	1	0
12	0	0	1	1	1	0	1	0	1	0
13	0	0	1	1	1	0	1	0	1	0
14	0	0	1	1	1	0	1	0	1	0
15	0	0	1	1	1	0	1	0	1	0
16	0	0	1	1	1	0	1	0	1	0
17	0	0	1	1	1	0	1	0	1	0
18	0	0	0	0	2	0	2	1	1	0
19	0	0	1	1	1	0	1	0	1	0
20	0	0	1	1	1	0	1	0	1	0

The sum of cells detected by all staining
 COUNTIF(D6:D195,2)
 COUNTIF(H6:H195,1)

Total cell number detected by Au+RLx
 e.g. =IF(H6=0,I6+J6, 0)

The average number and the Standard deviation of the three replicate was taken

	RLx	PNA	Au	Au/RLx	PNA/Au	PNA/RLx	All
1	12	6	51	30	5	3	1
2	4	6	63	15	12	3	4
3	11	6	53	30	4	3	1
Average	9	6	55.6667	25	7	3	2
SD	4.3589	0	6.4291	8.66025	4.3589	0	1.73205

Figure 30: Example of Excel spreadsheet used to calculate the total cell count.

Image is an example of the Excel spreadsheet depicting PNA/Auramine O/RLx triple-staining cell calculations. The sheet shows detailed data of each statistical analysis as well as example equations. The bottom image shows a spreadsheet of the calculations from three replicates of the sputum sample that ended with calculations for average and standard deviation.

4.4. Results

4.4.1. IIF staining reveals multiple *M. tuberculosis* sub-populations in pure culture

The triple-staining technique using IIF (Rv Ab; raised in Guinea pig live H37Rv)/Auramine O/RLx was applied to a pure Mtb H37Rv culture of stationary-phase cells grown in Sauton`s. As mentioned in Section 4.3.1, all images were taken from the same field of view directly after applying the staining technique. The images of the three staining techniques were merged using Image-Pro-Plus 5.0 software. Mtb cells from pure culture were pseudocoloured as IIF (green), Auramine O (yellow) and RLx (red). AF^{+ve} and LB^{+ve} proportions of H37Rv were counted automatically in triplicate and almost 100 cells were counted from each replicate against the phase contrast, whereas the Ab proportions were counted manually by eye.

Figure 31 shows the multiple populations of Mtb from the stationary-phase culture. The results show that the IIF is able to detect all Mtb populations in pure culture. In comparison, Auramine O can detect only some of the total H37Rv population. Also, there is heterogeneity in neutral lipid staining and presence of LBs within Mtb cells from culture. There are four sub-populations of H37Rv in pure culture detected with IIF/Auramine O/RLx. These populations are Ab^{+ve} /Au^{+ve} /LB^{-ve}, Ab^{+ve} /Au^{-ve} /LB^{+ve}, Ab^{+ve} /Au^{-ve} /LB^{-ve} and Ab^{+ve} /Au^{+ve} /LB^{+ve}.

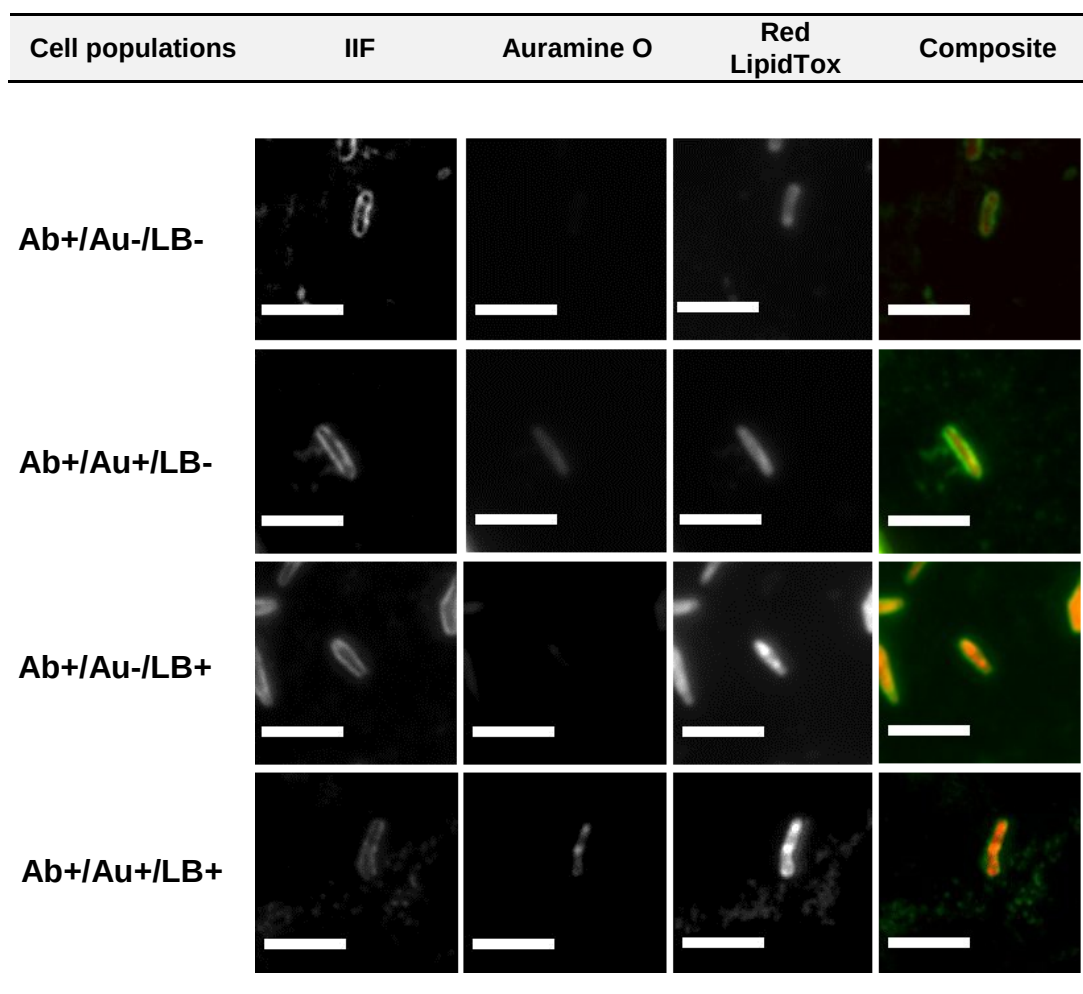


Figure 31: Multiple *M. tuberculosis* populations in pure culture.

Representative images to show the multiple populations in pure Mtb H37Rv stationary-phase culture grown in Sauton's broth. Images show the IIF, Auramine O, RLx triple-staining technique where the images were then merged to show the composite. After aligning to the phase contrast image, each fluorophore was imaged separately and then the images were pseudocoloured as Rv Ab (green), Auramine O (yellow), and RLx (red). Scale bars 4 μ m.

The results in Figure 32 represent the IIF⁺/AF⁺/LB⁺ proportions of the Mtb H37Rv strain during the growth cycle over 23 days. In Sauton's broth almost 100% of H37Rv bacilli were detected by the Rv-Ab in all growth phases, whereas the size for the AF⁺ population decreases in contrast to the LB⁺ population, which increases.

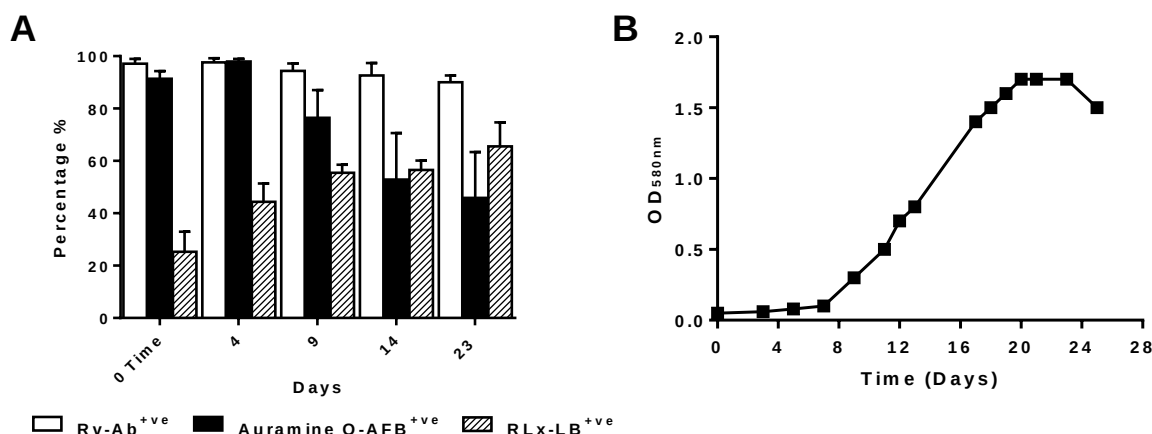


Figure 32: Ab, AF and LB proportions of *M. tuberculosis* H37Rv during growth cycle.

The bar chart shows (A) the proportions of IIF⁺ /AF⁺ /LB⁺ of H37Rv culture grown in Sauton's stained with Rv antibody (open columns), Auramine O (solid columns) and RLx (hatched columns) during the growth cycle. The AF and LB proportions were counted automatically, whereas the Ab proportions were counted by manually eye. Each experiment represents three replicates. Error bars display standard deviation. (B) Shows the linear OD_{580nm} growth curve during 24 days of Mtb H37Rv grown in Sauton's and supplemented with ADC and Tween-80.

4.4.2. Antibody staining shows poor detection of acid-fast bacilli in sputum

IIF staining was performed on four 4+ decontaminated frozen sputum samples. In order to show the different Mtb populations from sputum the triple-staining technique was applied on sputum following the procedure in Section 4.3.1. A comparison between IIF, Auramine O and RLx was done by recording the images of all three staining techniques and then counting by eye. Each cell was assessed individually with the three staining techniques see (Section 4.3.4).

The results in Figure 33 show a comparison between IIF⁺, Auramine O-AFB⁺ and LB⁺ detection of Mtb in sputum. Three replicates were counted and 100 Auramine O-AFB⁺ Mtb cells were counted in each replicate. These cells, which were detected with Auramine O, were then assessed for whether they are IIF⁺ and LB⁺. All results from the four samples show that just a few Auramine O-AFB⁺ cells were detected with IIF. There is just also a small proportion of Auramine O-AFB⁺ /IIF⁺ that is LB⁺. This result indicates that IIF detects much lower Mtb proportions than Auramine O in sputum samples.

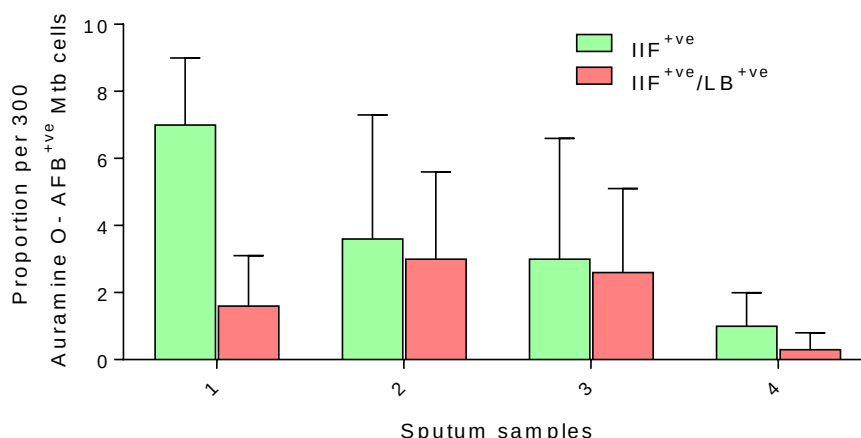


Figure 33: The detection number of IIF^{+ve} and IIF^{+ve}/LB^{+ve} populations per 300 Auramine O-AFB^{+ve} *M. tuberculosis* cells.

The bar chart shows the detection percentages of IIF^{+ve} and IIF^{+ve}/LB^{+ve} populations per 300 Auramine O-AFB^{+ve} Mtb cells in four 4+ sputum samples. Mtb cells were stained with Rv-Ab, Auramine O and RLx triple-staining technique. The AF proportion was counted with Auramine O independently. The same cells then were assessed whether they are IIF^{+ve} and LB^{+ve}. Each sample was counted in three replicates by eye. Error bars display standard deviation.

4.4.3. IIF/Auramine O/RLx triple-staining reveals multiple *M. tuberculosis* sub-populations in pure culture

After revealing that the IIF detects just a small population of Mtb in sputum in comparison with Auramine O, it was necessary to identify these populations. To achieve this aim, the triple-staining technique was performed on a 4+ sputum sample. Figure 34 shows the multiple Mtb populations in sputum that was detected using the IIF/Auramine O/RLx triple-staining technique. There are more Mtb sub-populations detected with triple-staining technique in sputum than pure culture because of the existence of the IIF negative population in sputum. Eight different Mtb sub-populations were detected in sputum. These sub-populations are Ab^{+ve}/Au^{+ve}/LB^{+ve}, Ab^{+ve}/Au^{+ve}/LB^{-ve}, Ab^{+ve}/Au^{-ve}/LB^{-ve}, Ab^{+ve}/Au^{-ve}/LB^{+ve}, Ab^{-ve}/Au^{+ve}/LB^{+ve}, Ab^{-ve}/Au^{+ve}/LB^{-ve}, Ab^{-ve}/Au^{-ve}/LB^{+ve} and Ab^{-ve}/Au^{-ve}/LB^{-ve}. The characterisation of these sub-populations in sputum is shown in the next section.

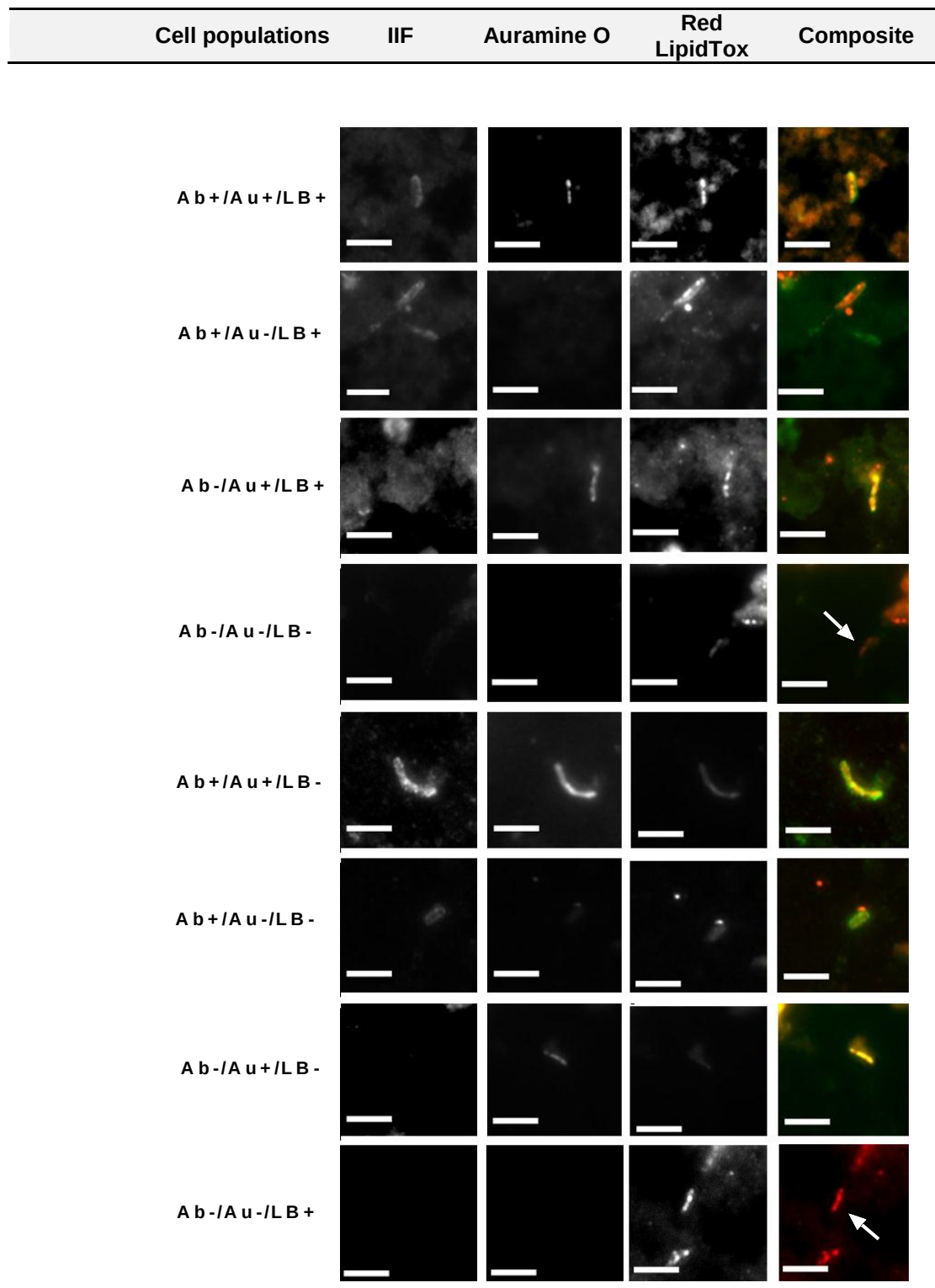


Figure 34: The multiple *M. tuberculosis* populations in sputum.

Representative images show the multiple populations of Mtb in sputum. Images show the IIF, Auramine O, RLx triple-staining technique and then the images were merged to show a composite section. Each fluorophore was imaged separately and then the images were pseudocoloured as Rv Ab (green), Auramine O (yellow) and RLx (red). Scale bars 5 μ m.

4.4.4. Characterising the multiple *M. tuberculosis* sub-populations in sputum using IIF/Auramine O/RLx triple-staining

Three sputum samples were used in this study. Images of IIF, Auramine O and RLx triple-staining were done manually by capturing the field of interest from each staining technique. Three replicate slides were prepared from each sample and from each replicate at least 100 cells were counted. Cell counts were done by assessing each cell individually with the three stains and by scoring positively stained cells as 1 and cells negative for the stain as 0. The total cell number was determined in each sample as the sum of cells that detected by any of staining techniques. Statistical analyses were performed mentioned in Section 4.3.4.

The results in Figure 35 show that IIF/Auramine O/ RLx triple-staining reveals multiple Mtb populations in sputum. IIF staining in all three sputum samples detects lower than 10% of the total cell number of Mtb cell populations. This is in contrast with Auramine O, which detects from 25-60% of the total cell number. The number of cells that are detected with both IIF and Auramine O in all samples is about 2-6, which a significantly low proportion in comparison with the total cell number counted in each sample. The results also show that lower than 4% of the total cell number in all samples show LB in the cells detected with IIF, whereas cells detected with Auramine O show variation in LB cell numbers which is between 13-23%. The LB⁺ cells that were detected with RLx only and which are thought to be Mtb bacilli as they morphologically similar are 34% in sample 103, 16% in sample 96 and 7% in sample 85. The results show that between 0%-5% of the total cell number of Mtb bacilli were stained with all three staining techniques.

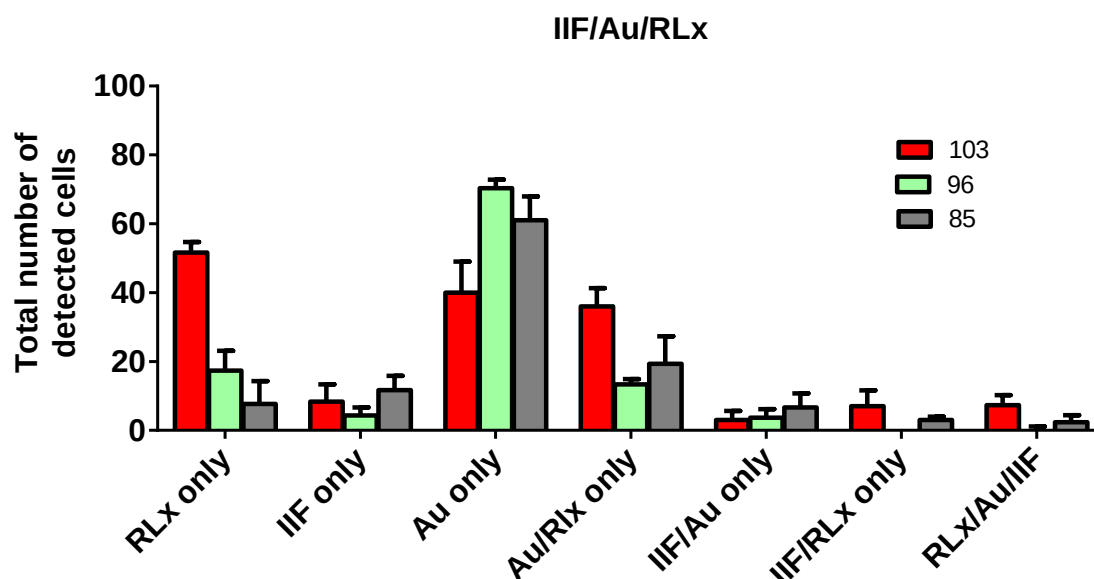


Figure 35: The total number of the multiple populations of *M. tuberculosis* in sputum using IIF/Auramine O/RLx triple-staining technique.

The total number of Mtb cells in three sputum samples, 103 (red bars), 96 (green bars) and 85 (grey bars), are shown in this graph by using IIF/Auramine O/RLx triple-staining technique. From each sample, three replicate slides were stained and at least 100 cells were counted from each replicate and the SD was calculated from the average of the three replicates.

4.4.5. *M. tuberculosis* H37Rv sub-populations using PNA-FISH triple-staining technique

4.4.5.1. The PNA signal intensity is increases in actively growing H37Rv and decreases in the stationary phase of growth

It has been reported that mycobacterial ribosomal genes are simultaneously downregulated with the cessation of growth (Trauner *et al.*, 2012). The rRNA number in the exponential phase increases in contrast to the number in the stationary phase (Verma & Davidson, 1994). Relying on this finding, the hypothesis in this study is that OK682 PNA probe fluorescence intensity will decrease during the growth cycle.

Mtb H37Rv cells were inoculated into Sauton's medium to an initial calculated OD of 0.05. The culture was then harvested at different time points at 4, 7, 10, 15 and 23 days. Cells from all time points were stained with the OK682 PNA-FISH probe. From each time point almost 300 cells were assessed using Image-J-based software developed by Bell, 2013. Samples for negative control were used identically from the

mid-exponential phase of H37Rv cells. For the negative control, the PNA probe in the hybridisation buffer was replaced with d.H₂O.

The results in Figure 36 show that the median fluorescence intensity (MFI) of the OK682 probe after 4 days of growth is significantly higher than the other growth stages. Also, the MFI decreased significantly at each time point until reaching the lowest intensity after 23 days. This finding is identical to that reported by Trauner *et al.*, (2012), showing that rRNA is downregulated with the cessation of growth.

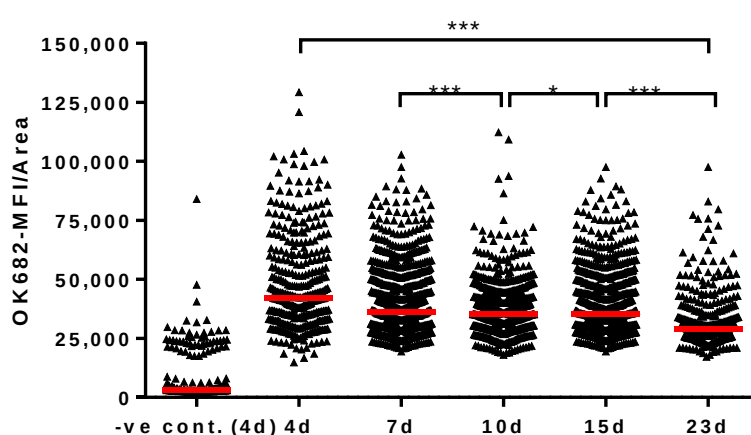


Figure 36: *M. tuberculosis* H37Rv cells with PNA staining during the growth cycle.

The scatter-dot graph shows the OK682 PNA probe median fluorescence intensity of H37Rv cells in Sauton's during the growth cycle. PNA MFIs were assessed from triplicate slides at each time point and (at least 100 cells per slide) using Image-J developed software. A paired t-test was used to compare the PNA MFI between the different variables; asterisks designate significant result.

4.4.5.2. PNA, Auramine O and RLx triple-staining reveals multiple *M. tuberculosis* populations in pure culture

The proportions of PNA/AF/LB by the PNA/Auramine O/RLx triple-staining technique were measured from a late-exponential Mtb strain H37Rv culture in Sauton's. A comparison was done of each staining compared with the phase contrast on triplicate slide preparations. The PNA positivity was scored according to the presence of spots of staining in the cells, meaning that cells with no spots were scored negative.

Figure 37A shows the multiple Mtb populations revealed by PNA/Auramine O/RLx triple-staining. The detected proportions of the total cell population with OK682 PNA probe, Auramine O and RLx staining of Mtb are shown in Figure 37B. The result reveals that the detected proportion with OK682 PNA probe staining is significantly higher than with Auramine O staining. Also, a large Mtb H37Rv proportion was detected with the OK682 PNA probe as non-AFB Mtb cells detectable by PNA-FISH.

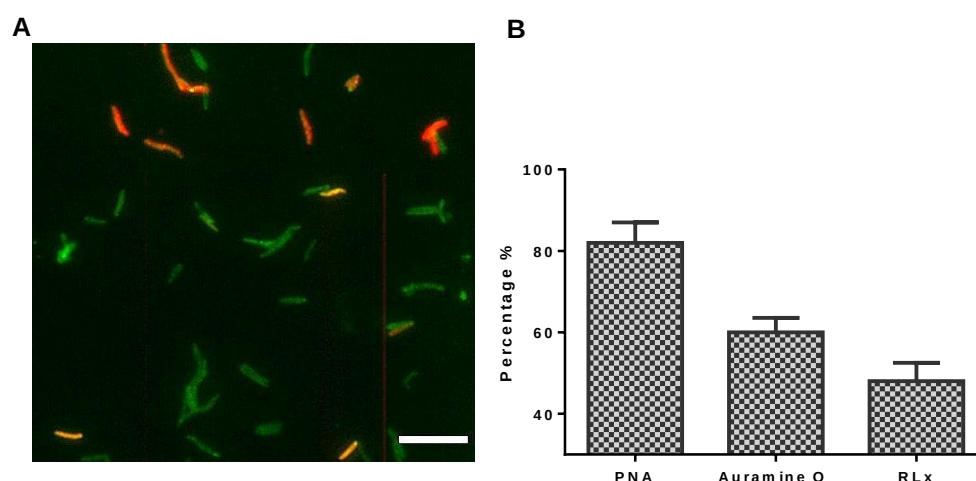


Figure 37: The PNA, Auramine O and RLx proportions of *M. tuberculosis* H37Rv by triple-staining technique.

The bar chart shows: (A) the multiple Mtb H37Rv populations revealed by PNA (green)/Auramine O (yellow)/RLx (red) triple-staining technique; (B) the average PNA, AF and LB staining proportions of Mtb H37Rv strain from the late-exponential phase grown in Sauton's assessed with the triple-staining technique. Each staining was applied to triplicate slides and the error bars represent the SD of the three replicates.

To investigate the hypothesis that there are multiple Mtb populations in sputum sample and in pure culture, the PNA, Auramine O and RLx triple-staining technique was applied to H37Rv cells from the late-exponential phase of growth. Microscopic images were taken following each staining technique and the composite images were prepared. The results in Figure 38 show the multiple H37Rv populations from culture identified with the triple-staining technique. There are eight different populations: PNA^{+ve}/Au^{+ve}/LB^{+ve}, PNA^{+ve}/Au^{-ve}/LB^{+ve}, PNA^{+ve}/Au^{-ve}/LB^{-ve}, PNA^{+ve}/Au^{+ve}/LB^{-ve}, PNA^{-ve}/Au^{+ve}/LB^{+ve}, PNA^{-ve}/Au^{-ve}/LB^{+ve}, PNA^{-ve}/Au^{+ve}/LB^{-ve}, PNA^{-ve}/Au^{-ve}/LB^{-ve}. All these populations were detected by the triple-staining technique and shown separately, with the composite image subsequently done to show how the cell population looks when combined.

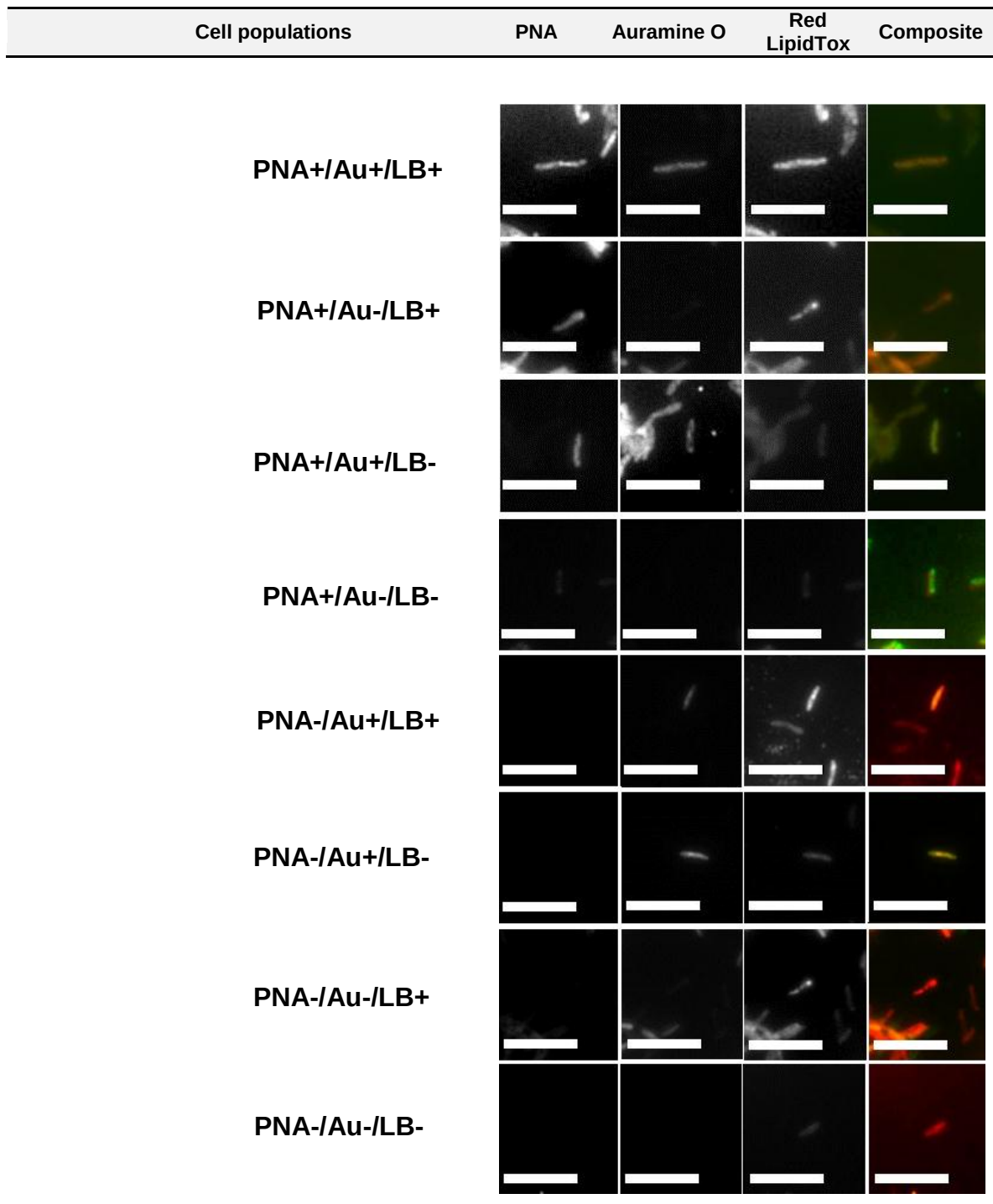


Figure 38: Multiple *M. tuberculosis* populations in pure culture using PNA, Auramine O and RLx triple-staining technique.

Representative images show the multiple Mtb H37Rv populations in Sauton's revealed by the triple-staining technique. Images show the PNA, Auramine O, RLx stains, with the images subsequently merged to show the composite. Each fluorophore was imaged separately and then the images were pseudocoloured as PNA (green), Auramine O (yellow) and RLx (red). Scale bars 5 μ m.

4.4.5.3. PNA, Auramine O and Neutral lipid (Red LipidTox) triple staining reveals multiple *M. tuberculosis* populations in sputum

Three 4+ decontaminated and frozen sputum samples were used in this study according to the protocol in Section 4.3.1. Then a comparison was done between the PNA, Auramine O and RLx by recording the images of all three stains with the counting then done manually by eye.

The results in Table 14 show a comparison between PNA, Auramine O and RLx detection of Mtb in sputum. Three replicates were counted and 100 Auramine⁺ cells were counted in each replicate. Then these cells which were detected with Auramine O were assessed for whether they were PNA⁺ and LB⁺. All results from the four samples show that just a few Auramine O⁺ Mtb cells are PNA⁺ and LB⁺. This result indicates that the PNA detects much lower Mtb proportions than Auramine O.

Table 14: The detection number of PNA and LB populations per 300 Auramine O-AFB+ve *M. tuberculosis* cells

Sputum sample	PNA ⁺ (%)±SD	PNA ⁺ /LB ⁺ (%)±SD
1	35.3±11.5	9.3±8.3
2	19.6±7	11±8.5
3	25.3±12.3	23±5.6

The comparison between PNA, Auramine O and RLx, shows that PNA detects just a proportion of the cells compared with Auramine O. Thus, it was necessary to identify these populations. To achieve this target, the triple-staining technique was performed on a 4+ sputum sample. Figure 39 shows the multiple Mtb populations in sputum which were detected by three different staining techniques, PNA, Auramine O and RLx. Eight different Mtb sub-populations were detected in sputum. These sub-populations are: PNA⁺/Au⁺/LB⁺, PNA⁺/Au⁺/LB⁻, PNA⁺/Au⁻/LB⁻, PNA⁺/Au⁻/LB⁺, PNA⁻/Au⁺/LB⁺, PNA⁻/Au⁺/LB⁻, PNA⁻/Au⁻/LB⁺ and PNA⁻/Au⁻/LB⁻.

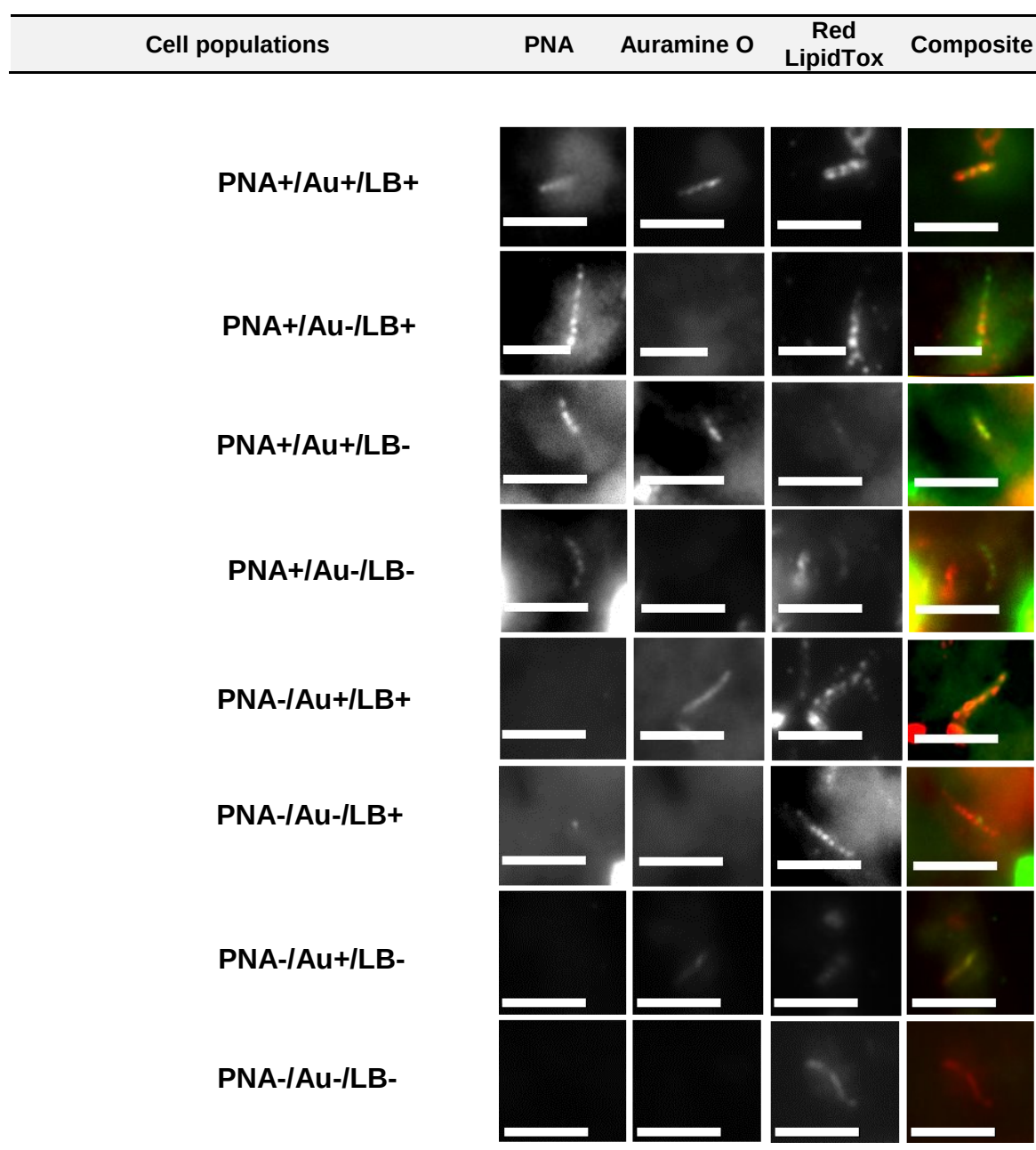


Figure 39: Multiple *M. tuberculosis* populations in sputum

Representative images show the multiple populations of Mtb in sputum. Images show PNA, Auramine O, RLx triple-staining technique, and subsequently the images were merged to show the composite. Each fluorophore was imaged separately and then the images were pseudocoloured as PNA (green), Auramine O (yellow) and RLx (red). Scale bars 5 μ m.

4.4.5.4. Characterising the multiple Mtb sub-populations in sputum using PNA/Auramine O/RLx triple-staining

The same three sputum samples (103, 96 and 85) that were used with IIF were used with PNA, Auramine O and RLx, and triple-staining was done manually by capturing the field of interest from each staining technique. Three replicates from each sample were used. From each replicate almost 100 cells were counted. The cell counts were as mentioned in Section 4.3.4.

The results in Figure 40 show that Mtb cell populations that were detected with PNA was equal to or lower than 9%, which is much lower than the cell populations detected with Auramine O varying between 28%-60%. The results also reveal that just less than 5% of the total cell population was detected in all three samples when assessing cells stained with both PNA and Auramine O. Less than 5% of PNA^{+ve} cell populations are LB^{+ve}, whereas Auramine O^{+ve} populations show variation in LB cell numbers between samples of 18% of sample 103, 24% of sample 96 and 17% of sample 85. The LB^{+ve} cells that were detected with RLx only and thought to be AFB^{-ve} Mtb bacilli according to their similar size and morphology compared with Auramine O-AFB^{+ve} cells, comprised 43% in sample 103, 17% in sample 96 and 4% in sample 85.

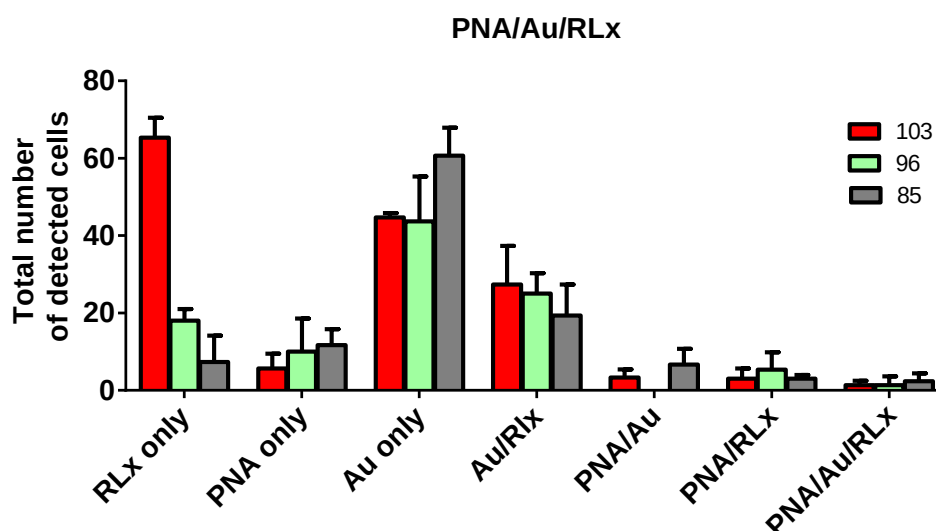


Figure 40: The total number of the multiple populations of *M. tuberculosis* in sputum using PNA/Auramine O/RLx triple-staining technique.

Graph showing the total number of Mtb populations in the three sputum samples, 103, 96 and 85, by using PNA/Auramine O/RLx. From each sample three replicates were counted and the SD was calculated from the average of the three replicates.

4.4.6. Fluorescence Kinyoun staining detects significantly higher proportion of AFB than Auramine O

The previous results show that Auramine O detects higher numbers of Mtb from sputum than either IIF or PNA. However, it has already been shown that Auramine O failed to detect non-AFB in sputum and pure culture. Furthermore, examination of fluorescence following Kinyoun staining was shown to detect a higher proportion of Mtb H37Rv from pure culture and Mtb from sputum. Therefore, a comparison between Auramine O and fluorescent Kinyoun was done in five sputum samples to prove that fluorescence Kinyoun staining detects a higher proportion of Mtb than Auramine O.

The experiment was performed by staining the formaldehyde-fixed sputum smears with a dual-staining technique. Auramine O staining was performed first and then images were captured from marked fields on the slides. The cover slip was then taken out and Kinyoun staining was applied on the same smear. Images of the fluorescence of carbol-fuchsin were then taken from the same marked fields. The counting was done manually by eye by comparing Auramine O count against the count of fluorescence Kinyoun stained cells. In each staining method, cells from 20 fields were counted and then the mean number was calculated in those fields from triplicate slides.

The results in Figure 41 show that there is a variation in the Auramine O detection mean per 20 fields compared to fluorescence Kinyoun staining count in the sputum samples. Some samples, such as sample 41 and 88 and 104 show a significantly higher detection of fluorescence Kinyoun than Auramine O, whereas some show no significant difference in mean cells count per 20 fields, such as sample 23 and 44.

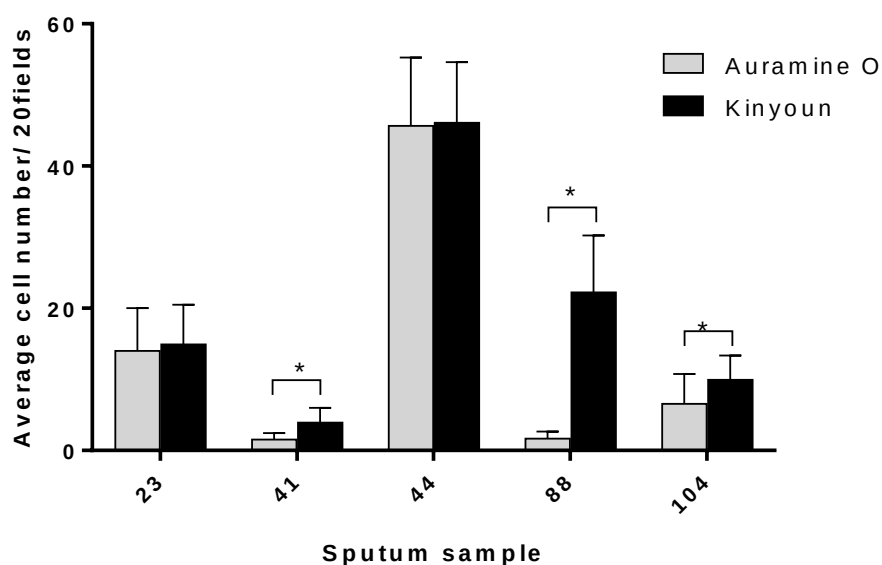


Figure 41: Comparison between fluorescence Auramine O and fluorescence Kinyoun of *M. tuberculosis* from sputum.

The bar chart represents the acid-fast averages of Mtb from five (4+) decontaminated and frozen sputum samples per 20 fields. Samples were stained with an Auramine O and Kinyoun dual-staining technique in three replicate slides for each sample. The AF cells were counted manually by eye. Error bars represent the standard deviation of AF cells from 20 fields of view. Unpaired t-tests were used to compare the fluorescence Auramine O with fluorescence Kinyoun. Asterisks designate significant results.

Identifying Mtb populations in sputum that were not detected with Auramine O (AFB-) might give further clues to the Mtb physiology during transmission. For this purpose, a triple-staining technique comprised of Auramine O, Kinyoun and RLx was done on a 4+ sputum sample following the protocol in Section 4.3.2. Figure 42 shows the multiple Mtb populations using the Auramine O, Kinyoun and RLx triple-staining technique. Images A, B, C and D show the separate fields of the staining techniques, whereas image E is the composite image of the three staining techniques. This technique reveals some Mtb sub-populations detected with fluorescence Kinyoun staining, but not with Auramine O. There are three different populations shown in this image: $Au^{+ve}/Kin^{+ve}/LB^{+ve}$, $Au^{-ve}/Kin^{+ve}/LB^{+ve}$ and $Au^{-ve}/Kin^{+ve}/LB^{-ve}$. This finding reveals the heterogeneity of Mtb bacilli in sputum samples. However, it is essential to classify these sub-populations in terms of showing the predominant population in a number of sputum samples.

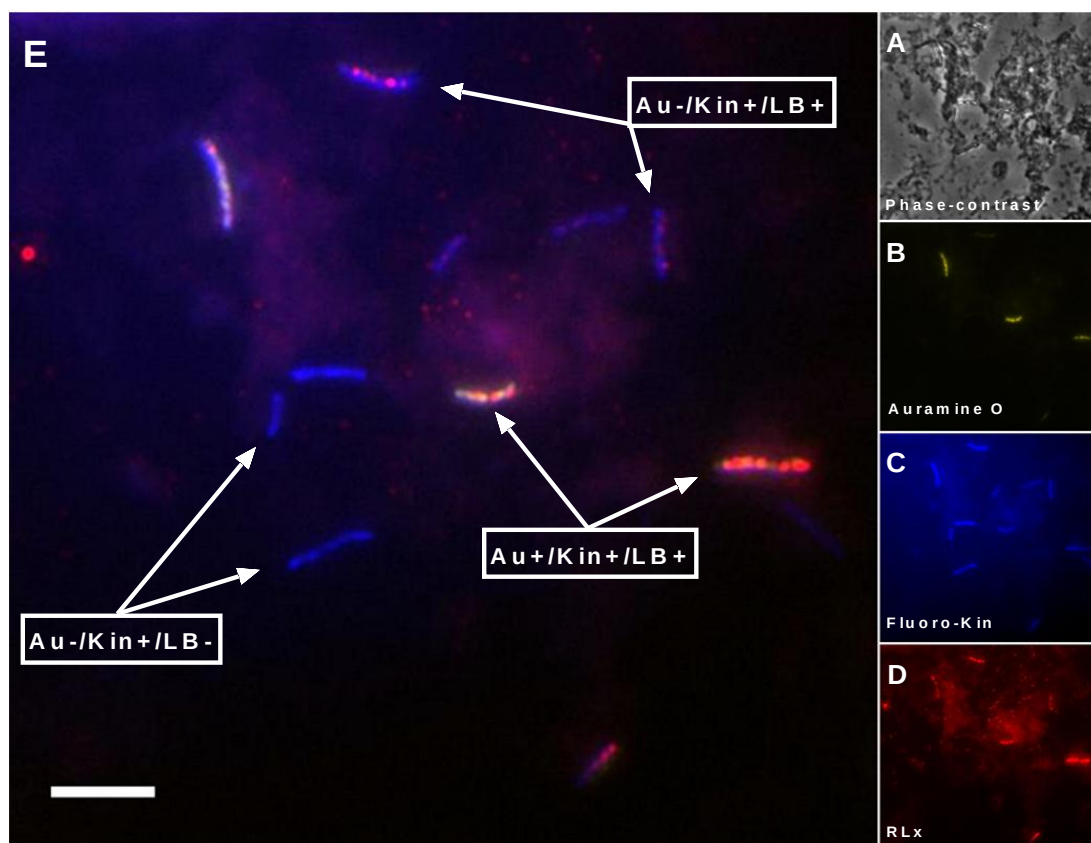


Figure 42: The multiple *M. tuberculosis* populations in sputum revealed by Auramine, Kinyoun and RLx triple-staining technique.

A tuberculous sputum sample is imaged by phase contrast: (A) then stained and imaged sequentially with Auramine O (B), Kinyoun (C) and LipidTox Red Neutral Lipid (D). After aligning to the phase contrast image, each fluorophore was imaged separately and then the images were pseudo-coloured as shown (B-D) before preparing an overlaid composite image (E). Scale bar 5 μm . Note that the Kinyoun stain imaged by fluorescence detects Auramine negative bacilli.

4.4.7. The multiple Mtb populations revealed by Auramine O/RLx and fluorescence Kinyoun/GLx dual-staining techniques

As fluorescence Kinyoun revealed Mtb populations that could not be detected by Auramine O in sputum, it was decided to compare between these two staining techniques. The GLx was combined with Kinyoun as the RLx interferes with fluorescence Kinyoun because both have red fluorophores. Auramine O/RLx and fluorescence Kinyoun/GLx dual-staining techniques were applied on the same three sputum samples: 103, 96 and 85 as assessed with the triple PNA and IIF staining techniques. The staining was done according to the protocol in Section 4.3.3. Three

replicates from each sample were used. From each replicate almost 100 cells were counted. The cell counts were done as mentioned in Section 4.3.4.

The result in Figure 43A reveals that the total number of a cell population that was only detected with RLx becomes higher than when assessed by the IIF (Figure 35) and PNA (Figure 40) triple-staining techniques. For sputum samples 103, 96 and 85, the population proportion that stained with RLx only by using Auramine O/RLx dual staining technique was subsequently 56%, 30% and 8%. This result indicates that IIF and PNA detect non-AFB Mtb populations in sputum. Further, the proportion number of the Auramine O-AFB^{+ve}/RLx-LB^{+ve} population of samples 103, 96 and 85 are of 20%, 31% and 19%. However, sample 96 showed higher cell populations detected with Auramine O/RLx than the other triple-staining techniques. This might be due to bias in the manual counting method of the triple-staining techniques. Also, the proportion of the cells that show RLx only, is inversely proportional to the proportion of the Auramine O cells. This indicates that when the number of AFB^{-ve}/LB^{+ve} cells increases, the number of AFB^{+ve}/LB^{-ve} cells decreases and vice versa.

The results in Figure 43B show the fluorescence Kinyoun/GLx dual-staining technique that was applied on the same sputum samples. This showed that there are almost no Mtb that were detected only with GLx alone. This means that fluorescence Kinyoun was able to detect the majority of the Mtb population in these sputum samples. Also, the proportion number of the Mtb cells that are AFB^{+ve}/LB^{+ve} is much higher than the previous staining techniques. The proportion number of the Kinyoun^{+ve}/GLx^{+ve} population of samples 103, 96 and 85 were subsequently 41, 50 and 44%, respectively. Similarly, as found in the Auramine O/RLx results, sample 96 showed higher cell populations detected with Kinyoun/GLx than the other triple-staining techniques. This might be due to the bias of the manual counting method utilized in the triple-staining techniques. It is significant that using fluorescence Kinyoun/GLx technique reveals that the LB^{+ve}/AFB^{-ve} populations with Auramine O are AFB^{+ve}/LB^{+ve} populations with Kinyoun staining. This result also confirms that LB^{+ve}/AFB^{-ve} populations with Auramine O in sputum are Mtb cells as they detected with carbol-fuchsin.

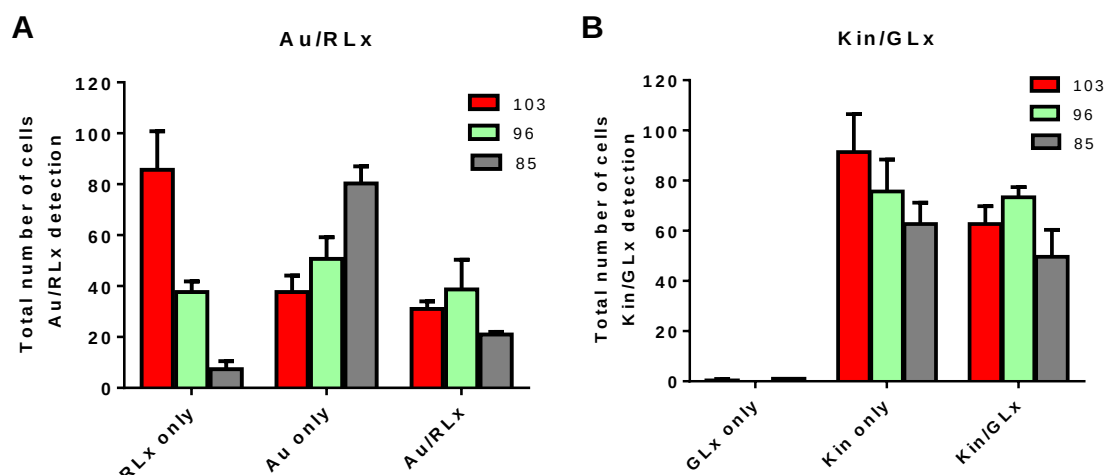


Figure 43: The total number of the multiple populations of *M. tuberculosis* in sputum using Auramine O/RLx and fluorescence Kinyoun/GLx dual-staining techniques.

The total number of Mtb cells in the three sputum samples, 103, 96 and 85, are shown in this graph with application of Auramine O/RLx and fluorescence Kinyoun/GLx. From each sample three replicate slides were stained and were counted and the SD was calculated from the average population sizes of the three replicates.

4.4.8. Comparison between manual and automated lipid body counting using Auramine O/RLx Kinyoun/GLx dual-staining techniques

Counting Mtb staining types, specifically LB^{+ve} populations will provide an assessment regarding the predominant population in the sputum. The Mtb LB^{+ve} phenotypes are worthy of inquiry because it is thought to be transmission adapted populations (Garton *et al*; 2008).

The previous section described the classification of the Mtb populations in sputum using different staining methods. The non-Auramine O AFB population which was LB^{+ve} was detected with the fluorescence Kinyoun staining method. However, the counting was done using a manual counting system. Therefore, it is necessary to develop automated software for the counting of LB populations from sputum to avoid any potential for bias in manual counting. To achieve the non-subjective target of counting LB from different sputum samples by different users, Image-J based software was developed by Andrew Bell (Bell, 2013).

Four decontaminated and frozen sputum samples (96, 104, 115 and 44) were stained with Auramine O/RLx and Kinyoun/GLx dual-staining techniques as shown in Figure 44. Each sample was stained with Auramine O and Kinyoun dual-staining techniques

in triplicate. The manual counting method was done by eye by counting almost 100 cells from each replicate. The automated counting method was done by using the Image-J-based software, which captures the fluorescence field of an individual cell and then assesses the LB result of each cell blindly.

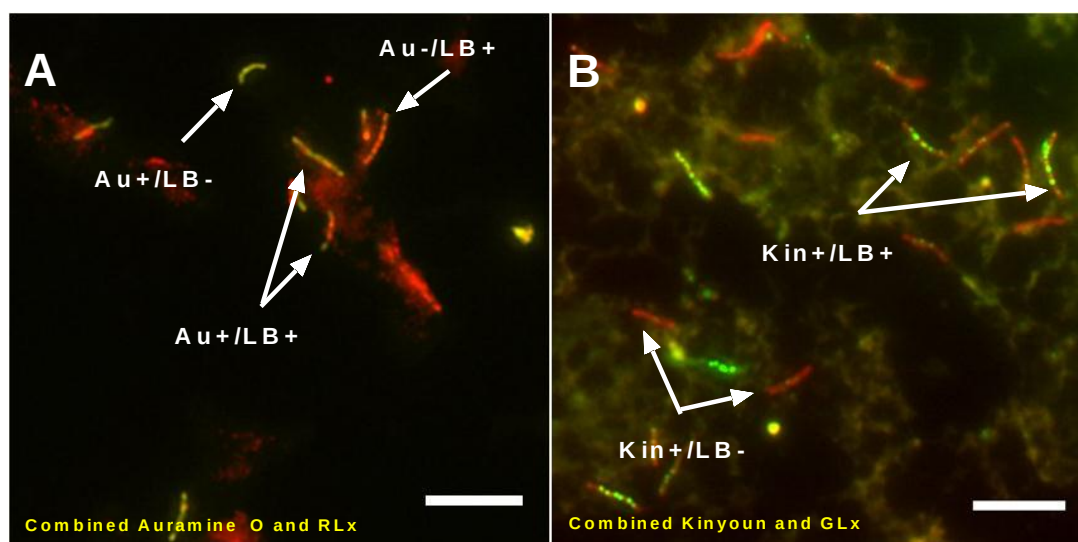


Figure 44: Multiple *M. tuberculosis* populations in sputum revealed by Auramine/RLx, Kinyoun/GLx dual-staining techniques.

Tuberculous sputum sample imaged by: (A) Auramine O combined with RLx; and (B) Fluorescence Kinyoun combined with GLx. The two fluorophores for each dual labelling technique were labelled with different filters then images were pseudo-coloured before preparing an overlaid composite image. Scale bars 5 μ m.

The results in Figure 45 show the LB^{+ve} proportions of the four sputum samples assess with each AF dye. The LB proportions vary between sputum samples with both techniques. The result also shows that the LB proportions with both techniques are significantly higher with the fluorescence Kinyoun dual technique than Auramine O. This is because fluorescence Kinyoun detects all Mtb populations and the counting was done on the whole population. However, Auramine O misses the detection of some LB^{+ve} /AFB^{-ve} populations that results in a lower LB proportion.

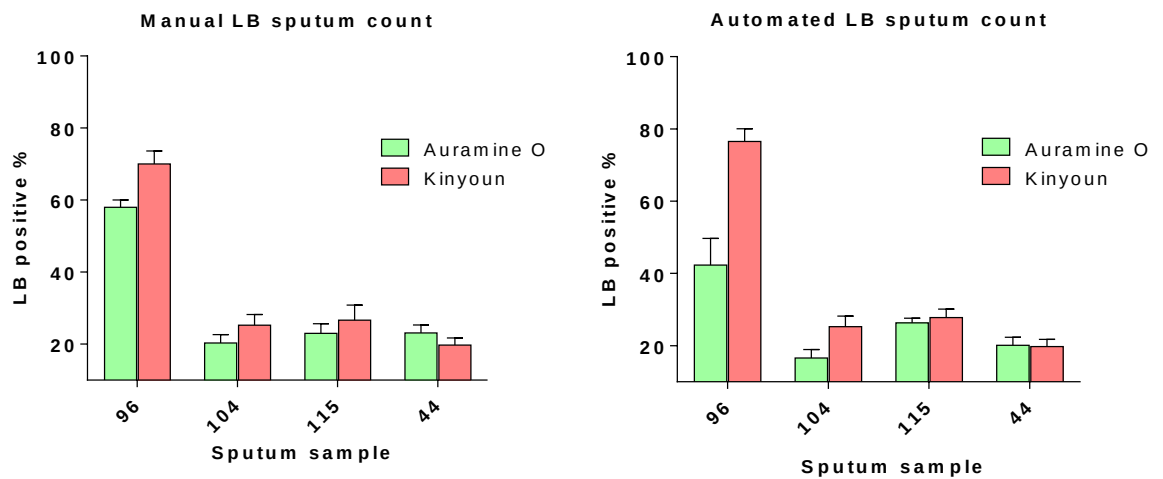


Figure 45: The LB proportions of *M. tuberculosis* from sputum with two staining techniques.

Bar graphs show the LB proportions in four 4+ sputum samples. The counting was done with two methods, manual by eye and automated using Image-J based software.

4.5. Discussion

The main aim of this work is to identify the Mtb sub-populations in sputum and pure culture. To achieve this objective, current detection staining techniques were assessed for their ability to detect the majority of Mtb in pure culture and sputum samples. The detection techniques which were used for the identification of the multiple sub-populations in pure culture and sputum are IIF, which uses the Rv Ab (live infection) detecting the surface Ags, PNA targeting the 23S rRNA, Auramine O, and Kinyoun staining which is believed to target the mycobacterial MAs (Richards, 1941) and nucleic acids (Oster, 1951 and Hanscheid *et al.*, 2007).

In this study new approaches were introduced to detect and identify the Mtb sub-populations in pure culture and sputum. These approaches combine staining techniques to detect and identify Mtb populations simultaneously. These different staining techniques target different sites of the Mtb cell and each has different detection ability. These approaches are IIF-Auramine O-RLx, PNA-Auramine-O-RLx, Auramine-O-RLx and fluorescence Kinyoun-GLx. The intention was that by using each of these approaches, it would be possible to detect both AF and non-AF Mtb. Additionally, there was the aim to show the total cell number of each of these sub-populations.

One of the findings of this study shows that fluorescence Kinyoun staining detects a much greater proportion of Mtb populations in sputum and pure culture in comparison with Auramine O. There are also multiple Mtb populations in sputum and pure culture and each staining technique detects different population types. It was also shown that Auramine O has higher detection of Mtb from sputum than the IIF and PNA staining techniques. Using the four staining approaches on the same three sputum samples revealed that Fluorescence Kinyoun staining shows the total cells number of populations that were found with the total number of the other approaches. It was also shown that the AFB^{-ve}/LB^{+ve} population detected with IIF/Auramine O/RLx or PNA/Auramine O/RLx triple-staining techniques and Auramine O/RLx dual-staining is AFB^{+ve}/LB^{+ve/-ve} following fluorescence Kinyoun/GLx dual-staining technique. However, the total cell number indicates that the majority of this population is AFB^{+ve}/LB^{+ve}.

4.5.1. *M. tuberculosis* populations revealed by IIF, Auramine O and RLx triple-staining from pure culture and sputum

The IIF application using Rv Ab to stain Mtb from pure culture successfully detected almost all populations with reasonably no background auto-fluorescence. It was clearly noted that the IIF application to Mtb from pure culture was highly reproducible and easy to perform. Mtb H37Rv bacilli were shown to be stained exclusively with either IIF or by IIF with and without Auramine-O and RLx. In the H37Rv sample from the stationary phase culture, all Mtb bacilli were stained with IIF staining. Auramine O and RLx show some heterogeneity in the stationary phase culture. As shown in this study, the acid-fastness decreases whilst the LB proportion increases at this stage of growth. The results show that four sub-populations can be observed in H37Rv culture from the mid-exponential phase. The first two populations are both IIF^{+ve} and AFB^{+ve} with and without LBs, (IIF^{+ve} /Auramine O^{+ve} /LB^{+ve}) and (IIF^{+ve} /Auramine O^{+ve} /LB^{-ve}). The second two populations are IIF+ and non-AFB with and without LBs, (IIF^{+ve} /Auramine O^{-ve}/LB^{+ve}) and (IIF^{+ve} /Auramine O^{-ve}/LB^{-ve}). Based on this result, the IIF/Auramine O/RLx triple-staining technique is able to characterize the Mtb populations in pure culture.

Although a number of studies have shown the phenotypic alteration of Mtb under multiple stress conditions (Kussell *et al.*, 2005 and Deb *et al.*, 2009), this is the first study showing the multiple Mtb populations in pure culture using a triple-staining technique. Ryan *et al.*, (2010) did a study showing the multiple Mtb populations in Guinea pig and mouse tissue using a dual-staining technique. The study showed that two populations are observed when H37Rv cells from the stationary phase were stained with IIF (WCL anti-Mtb Ab)/Auramine-Rhodamine dual-staining. These populations were IIF^{+ve} /Auramine-Rhodamine^{+ve} and IIF^{+ve} /Auramine-Rhodamine^{-ve} (Ryan *et al.*, 2010). This result is identical with what was found in the results of this chapter regardless the type of the polyclonal Ab.

In the same study, however, it was shown that when H37Rv cells were exposed to hypoxic condition, there are three populations revealed with the dual-staining technique. These populations are IIF^{+ve} /Auramine-Rhodamine^{+ve}, IIF^{-ve}/Auramine-Rhodamine^{+ve} and IIF^{+ve} /Auramine-Rhodamine^{-ve}. Surprisingly, H37Rv cells in hypoxic culture show a higher acid-fast proportion than cells from the stationary phase culture (Ryan *et al.*, 2010).

Deb *et al.* (2009) established a novel *in vitro* multiple-stress dormancy model for *Mtb* by exposing the bacilli to combined stresses of hypoxia (5% O₂), high CO₂ (10%), nutrient starvation (10% Dubos medium), and acidic pH (5.0), where these conditions are thought to reflect the *Mtb in vivo*. The study revealed that *Mtb* under these stresses stopped replicating, lost acid-fastness, accumulated TAG and WE, and became tolerant to anti-mycobacterial agents (Deb *et al.*, 2009). Another *in vitro* model used H37Rv under gradual O₂ depletion and after 30 days of incubation, the AF percentage dropped from 70% to 40% whereas the LB percentage increased from 30% to 60% (Shi *et al.*, 2010). Recently, Kapoor *et al.* (2013) developed an *in vitro* granuloma model reflecting the *in vitro* granuloma condition and found that the *Mtb* bacilli lost the acid-fastness and accumulated LBs (Kapoor *et al.*, 2013).

In sputum, the current study showed that all sub-populations that have been found in pure culture were found homogeneous over the studied sputum samples. Because it was already known through this work that a tubercle sputum sample has non-AF population, it was therefore expected that this population would be found in most samples. Surprisingly, IIF staining failed to detect all *Mtb* populations in sputum. Three new sub-populations were detected in sputum using the IIF/Auramine O/RLx triple-staining technique in addition to the four that were found in pure culture. The first four populations were detected in both pure culture and sputum and were: (IIF^{+ve} /Auramine O^{+ve} /LB^{+ve}), (IIF^{+ve} /Auramine O^{+ve} /LB^{-ve}), (IIF^{+ve} /Auramine O^{-ve} /LB^{+ve}) and (IIF^{+ve} /Auramine O^{-ve} /LB^{-ve}). The new sub-populations that were found only in sputum sample were: (IIF^{-ve} /Auramine O^{+ve} /LB^{+ve}), (IIF^{-ve} /Auramine O^{-ve} /LB^{+ve}), (IIF^{-ve} /Auramine O^{+ve} /LB^{-ve}) and (IIF^{-ve} /Auramine O^{-ve} /LB^{-ve}).

One of the aims of this study is to identify the AF^{-ve}/LB^{+ve} populations in sputum that have the same morphology as *Mtb* to secure the hypothesis that these are non-AF *Mtb*. In all sputum samples that were used in this study it was observed that the total cell number of the IIF^{-ve}/AF^{-ve}/LB^{+ve} population varied between 8%-33%. Thus, effort was placed into applying this technique to identify this population. Currently, there is no published study that applies the IIF/Auramine O/RLx simultaneous triple-staining technique on *Mtb* from a sputum sample.

This study found the different cell populations that reflect the heterogeneous environment of *Mtb* in sputum, which is coughed up from the cavitory lesion from the lung. The IIF staining failed to detect the AF^{-ve}/LB^{+ve} population in sputum as the total cell number of IIF^{+ve} /LB^{+ve} was lower than 5%. Although, Auramine O is believed to target cell-wall contents (Seiler *et al.*, 2003 and Ulrichs *et al.*, 2005), and IIF is

targeting the surface Ags, it is unclear why the IIF results in poor labelling of Mtb in sputum. Because the Rv antibody that was used to label Mtb in sputum in this study is raised from H37Rv live infection in Guinea pig, the growth stage might be from the mid-exponential phase. Mtb bacilli in sputum are also thought to be at NRP state; therefore, the expressed Ags at this stage might be different than the surface proteins of the mid-exponential phase cells. Also, bacilli in different environment in sputum compared with Guinea pig infection. Guinea pig foci of infection don't breakdown and form cavities as Mtb in human (Clark-Curtiss, 1994). As a result the surface Ags of Mtb in human sputum might be different than Guinea pig infection.

Another observation that could be seen in Mtb from sputum when stained with IIF is the punctuate staining manner. This characteristic was observed only with Mtb from sputum. This is in contrast to Auramine O staining which shows uniform labelling on Mtb from sputum. It has been reported that Mtb could show punctuated Ab labelling when it is exposed to a hypoxic condition (Ryan *et al.*; 2010). It has also been reported from the same study that Mtb showed the same staining manner from mice and Guinea pig tissues (Ryan *et al.*, 2010).

It has been hypothesized that Mtb could alter its metabolic activities depending on the environment in order to survive (Jamshidi *et al.*, 2007). Therefore, it might be that the set of surface Ags expressed by Mtb *in vitro* are different to *in vivo* surface Ags. The lack of detection of IIF in sputum might be due to the effect of sputum, which masks the surface Ags of Mtb bacilli. Furthermore, when Mtb cells show punctuate labelling it is more likely to raise the IIF^{-ve} population in contrast to the pure culture where the IIF labelling is uniform and the detection proportion is around 100%. In contrast, Auramine O shows a higher detection of Mtb in sputum. The identification of the AFB^{-ve}/LB^{+ve} population was not achieved by the IIF/Auramine O/RLx triple-staining technique. There is a significantly high proportion of this population in all sputum samples that were used in this study. Hence, the PNA/Auramine O/RLx triple-staining technique was applied in an attempt to identify this population.

4.5.2. *M. tuberculosis* populations revealed by PNA, Auramine O and RLx triple-staining from pure culture and sputum

In the current study, an OK682 PNA probe using FISH was assessed for the ability to label all Mtb populations in pure culture and a sputum sample by targeting the 23S rRNA. It has been observed that the MFI of H37Rv cells differed during the growth cycle. In the mid-exponential phase where growth is maximal active, the signal

reaches the highest intensity then it goes down gradually to the lowest intensity at the stationary phase of growth. It has also been observed that PNA^{+ve} Mtb cells in sputum differ in PNA intensity as some cells look brighter than others. This could reflect cells in sputum are in different growth states. These findings together indicate that Mtb bacilli *in vitro* and *in vivo* environments differ in their ability to be identified by the FISH technique. The lack of fluorescence intensity in a sputum sample may be due to a decrease of the numbers of rRNA in Mtb within the necrotic lesions in the lung cavity that are below the limits of PNA-FISH detection. Another explanation is that Mtb bacilli in sputum came from the NRP state and due to the alternation of the cell wall, might become impermeable to the PNA probe.

In a study to detect multiple Mtb populations *in vivo* and *in vitro* (Ryan *et al.*, 2010), it was observed that the signal of a ssDNA-FISH probe targeting the 16S rRNA is higher in H37Rv under hypoxia, lower in Mtb in mice tissue, and there is no signal in Guinea pig tissue (Ryan *et al.*, 2010). Although, the human granuloma is presumed to be hypoxic (Lenaerts *et al.*, 2007 and Via *et al.*, 2008), it was observed that FISH yielded a strong signal for Mtb in culture under hypoxia (Ryan *et al.*, 2010). This finding indicates that the hypoxic condition is not the only stimulus that decreases the rRNA ssDNA-FISH signal. This finding also indicates that ssDNA-FISH has different staining ability according to the microenvironment. Another *in vitro* study on Mtb reported that the level of rRNA is stable during the stationary phase, aerobic NRP1 and anaerobic NRP2 (Desjardin *et al.*, 2001). However, in this study 23S rRNA PNA-FISH MFI was not the same in all H37Rv growth stages. Furthermore, the signal intensity of Mtb bacilli in sputum differs between the tubercle bacilli. Therefore, PNA-FISH staining can discriminate between cell populations in growth and rRNA content.

According to the findings in this study, the main advantage of using IIF staining in pure culture is to detect all Mtb populations that are labelled with IIF in all growth stages. Thus, because there is no IIF^{-ve} population in pure culture, the triple-staining technique using IIF/Auramine O/RLx showed four different populations. In contrast, the sputum sample has eight populations identified by the IIF/Auramine O/RLx triple-staining technique. PNA/Auramine O/RLx triple-staining also identified four extra populations in addition to the four that IIF detected in pure culture. These populations were detected in all the sputum samples.

The PNA^{-ve} populations might indicate the downregulation of the rRNA in these cells. Also, in a comparison between IIF and PNA triple-staining on sputum, PNA triple-staining detects more Mtb cells than IIF. This might be due to Triton-X, which helps the

PNA probe get into the Mtb cell. It also might be due to the nature of sputum masking the targets of the IIF staining. The main drawback of using PNA staining in this study was the high background that might interfere with the cell detection. The washing time was extended to various time points with no significant improvement in background as a result.

One observation seen clearly in PNA labelling on Mtb from sputum is that the punctuate PNA cellular labelling pattern shows a different appearance on Mtb from pure culture. The discrete spots of the PNA probe on Mtb cells in sputum are brighter than the spots in H37Rv from pure culture and also different in morphology. This might because this probe was designed specifically to detect Mtb from sputum (Stender *et al.*, 1999), where cells have different permeability than pure culture.

The Mtb populations that were detected with PNA triple-staining technique correlate with the populations that were detected with IIF triple-staining. The target population, AFB^{-ve}/LB^{+ve} was still not detected by any of these techniques as the total number of this population is more or less similar by both staining approaches. By using PNA/Auramine O/RLx triple-staining technique, the total cell number that was detected by Auramine O alone is still significantly high compared with the total number detected by PNA. Due to hypoxic stimulus in the lung granuloma, which is the source of Mtb in sputum, protein and RNA synthesis is known to be significantly reduced (Dick, 2001, Wayne *et al.*, 2001 and Voskuil *et al.*, 2004), and this might explain why IIF and PNA failed to detect all Mtb populations from sputum.

The OK682 probe has been shown to have 100% diagnostic sensitivity (Stender *et al.*, 1999). This probe was also used in this project to detect Mtb from sputum and pure culture. However, in Stender's study only two sputum samples were assessed, and there have been no further publications based on this method, which might indicate that other sputum samples may vary in their susceptibility to staining with PNA-FISH. For these reasons, to ameliorate any lack of detection by IIF and PNA, other staining techniques were used in this study to detect and identify putative AFB^{-ve}/LB^{+ve} populations in sputum.

4.5.3. Revealing the multiple *M. tuberculosis* populations in sputum by Auramine O/RLx and fluorescence Kinyoun/GLx dual-staining techniques

Previous sections of this discussion showed that IIF and PNA staining techniques failed to detect the majority of Mtb in sputum. It was also noticed that Auramine O detects a higher number of Mtb than IIF and PNA. However, Auramine O still misses some cell populations that are thought to be Mtb, according to their size and morphology.

A systemic review (Steingart *et al.*, 2006) included 45 studies and compared Auramine O staining with the conventional Z-N staining method (bright-field detection). The review reported that Auramine O is significantly greater diagnostic sensitivity than Z-N staining by an average of 10%. Furthermore, the same review showed that the specificity of Auramine O and conventional Z-N are similar at 98%, with both excluding HIV-infected individuals (Steingart *et al.*, 2006). Our study has shown that using a fluorescence detection of CF raises the sensitivity of the cell detection compared with the Auramine O. This finding is identical with those using Z-N under fluorescence microscopy, instead of the transmitted light approach, which was developed recently (Shapiro *et al.*, 2008). Thus, the Kinyoun staining technique was developed for use by detecting fluorescence of CF detection rather than under transmitted light.

The average cell number of Mtb per 20 fields of fluorescence Kinyoun was significantly higher than Auramine O in 3 sputum samples out of the 5 samples studied. Also, none of the sputum samples showed a higher detection with Auramine O than fluorescence Kinyoun. In order to achieve the aim that AFB^{-ve}/LB^{+ve} should be identified as Mtb by a staining method, the same three sputum samples were used with both IIF and PNA triple-staining techniques, and were used in the Auramine O/RLx and fluorescent Kinyoun/GLx dual-staining techniques. These populations of AFB^{-ve}/LB^{+ve} were identified with the Kinyoun dual technique as the total number of cells staining with alone GLx was almost 0% in all three sputum samples. The hypothesis that the AFB^{-ve}/LB^{+ve} population is Mtb bacilli is positively confirmed by showing that Mtb cells detected by fluorescence Kinyoun/GLx population are significantly higher than the Auramine O/RLx population.

In a study of Mtb in multiple stress conditions the Auramine O staining AFB^{+ve} proportion increased as the LB^{+ve} proportion decreased as the culture aged (Deb *et al.*, 2008), a similar situation is described here. This was shown when the total number of

Mtb cells detected in sputum by RLx in Auramine O/RLx dual-staining increased, the Auramine O total cell number decreased and vice versa. However, this assessment requires more than 10 sputum samples.

Counting Mtb populations from sputum might have some limitations as the process was done manually by eye. Therefore, identifying these populations using a microscopically automated counting method is more reliable than manual counting. Thus, Image-J-based software was developed by (Bell, 2013) to count Mtb LB^{+ve} cells from sputum by comparing the AF staining field with the fluorescence LipidTox field, which are detected by dual-staining techniques. The automated counted method offers more reliable results as it relies less as operator input. The comparison between the manual and the automated counting showed that both counting techniques are highly correlated. Thus, the manual counting results of LB^{+ve} proportion using the triple and dual-staining technique are reliable, but might have high bias probability.

4.6. Conclusions

The work presented in this chapter shows that sputum samples contain multiple Mtb populations. Some sub-populations failed to be stained with conventional fluorescence staining techniques perhaps due to different cell envelope compositions of Mtb bacilli in sputum. It was possible to detect AFB^{-ve}/LB^{+ve} bacilli with fluorescent Kinyoun staining and it was shown to be one of the Mtb sub-populations in sputum. Fluorescence Kinyoun reveals that the majority of the Auramine O-AFB^{-ve}/LB^{+ve} population is fluorescence Kinyoun-AFB^{+ve}/LB^{+ve}. Furthermore, manual counting method is significantly and highly correlated with the automated counting method which reflects reliable counting numbers in this study.

The principle findings of this chapter include:

- Auramine O, IIF and PNA staining techniques failed to detect all Mtb populations from sputum
- The number of Mtb population detected by IIF is different that number of populations detected with PNA
- IIF and PNA staining techniques detected just a few bacilli in sputum compared with Auramine O
- Fluorescence Kinyoun staining was able to detect almost all Mtb populations in sputum
- The non-Auramine O-AFB Mtb cells are more likely to be LB^{+ve}
- The manual counting method of the triple- and dual-staining technique correlated with the automated counting method.

Chapter 5

Characterising *M. tuberculosis* in
biofilms

5.1. Introduction

Numerous studies have been performed to understand Mtb physiology in the pulmonary cavity specifically in granulomatous lesions (Canetti, 1955a, Saunders and Cooper, 2000, Peyron *et al.*, 2008, Kruh *et al.*, 2010; Kumar *et al.*, 2011, Kapoor *et al.*, 2013). Indeed, understanding TB physiology in its microenvironment can offer more efficient treatments and control of the disease (Stokes *et al.*, 2009). Therefore, it is increasingly important to determine the conditions that produced the phenotypes that have been observed in sputum. One way to understand these conditions and give further insight into Mtb physiology is to replicate the Mtb phenotypes *in vitro*.

Lee, (2012) has compared the gene expression of Mtb in sputum against Mtb exposed to *in vitro* stimuli. Data from this study revealed that no obvious growth condition fully replicated the sputum transcriptome. However, gene expression of Mtb cultures exposed to multiple stimuli such as RPMI or PBS medium, cholesterol, nitric oxide (NO), oleic acid (OA) and static incubation correlates significantly to sputum transcriptome (Lee, 2012).

In this part of the project the capacity of an *in vitro* biofilm system to replicate the Mtb sputum phenotypes was explored. Mycobacterial biofilms were discussed in Section 1.8.3.

5.1.1. *M. tuberculosis* features in sputum

The features of Mtb bacilli in sputum have been described in chapter 1. All three aspects, cytological, transcriptional and growth related have been explored in biofilm cultures in this chapter. In the transcriptional studies a subset of genes differentially expressed in sputum was chosen to screen for comparable expression *in vitro*. These had been selected by a previous worker in this lab (Lee, 2012)

5.1.2. Genes selected to represent the sputum transcriptome

Twenty selected genes (10 upregulated and 10 downregulated) from the sputum transcriptome were compared with the gene expression of Mtb exposed to *in vitro* conditions. These 20 genes were selected, as they were the only genes that up or down regulated >2.5-fold in the sputum transcriptome (Figure 46). Each one of these genes reflects function hypothesised to be affected by one or more of the selected

growth conditions in this study. These 20 genes include *DosR* regulon genes, genes involved in lipid and cholesterol metabolisms, virulence, toxin-antitoxin system, aerobic respiration, MA metabolism, and ribosomal genes.

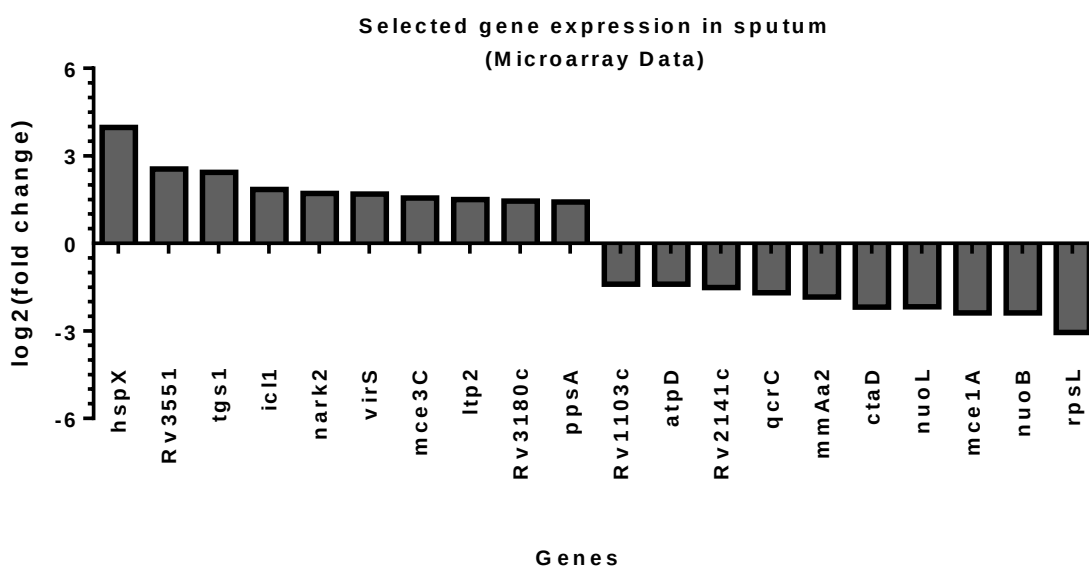


Figure 46: Microarray data of the Selected Gene Expression of *M. tuberculosis* in sputum.

The bar chart shows the microarray data gene expression of the 20 selected target genes of *Mtb* in Sputum (Garton *et al.*; 2008). The X-axis represents the 20 selected genes, whereas the Y-axis represents the Log2-fold change of each individual gene. Genes on the X-axis were arranged from the highest upregulation (the first 10 genes) to the lowest downregulation (the second 10 genes).

In this study, 4 housekeeping genes were selected and used for normalisation. These reference genes were selected according to a previous study by Lee (2012). The study measured and compared the Average Expression Stability (*M*) of 11 candidate reference genes, including *sigA* and 16S rRNA, in different *Mtb* growth conditions. The study concluded that *sigA* and 16S rRNA are the least stable whereas the four housekeeping genes (*thyA*, *dfrA*, *aroA* and *polA*) are the most stable genes. Therefore, these reference genes were used in the current study. Gene products and functions of the selected genes are summarised in Tables 15 and 16. In addition, the use of 5 different housekeeping genes was considered to normalise gene expression ratios. These are summarised in Table 17.

Table 15: Upregulated *M. tuberculosis* genes in sputum transcriptome.

Gene	Gene Number	Product	Function	Reference
<i>hspX</i>	Rv2031c	Heat shock protein, hspX (alpha crystallin homolog)	Virulence, detoxification, adaptation	(Monahan IM <i>et al</i> ; 2001)
Rv3551	Rv3551	Co-enzyme A transferase alpha subunit (cholesterol stimulon)	Essential gene for in vitro growth of H37Rv on cholesterol, intermediary metabolism and respiration	(Rengarajan J <i>et al</i> ; 2005)
<i>tgs1</i>	Rv3130c	Triacylglycerol synthase (diacylglycerolacyltransferase)	Predicted to be in the DosR, lipid metabolism	(Daniel J <i>et al</i> ; 2004)
<i>icl1</i>	Rv0467	Isocitratelase (glyoxylate shunt enzyme)	Intermediary lipid metabolism and respiration, induced 3.61-fold in sputum	(Dubnau E <i>et al</i> ; 2002)
<i>narK2</i>	Rv1737c	Probable Nitrate/Nitrite transporter (excretion of nitrite), narK2	Probable Nitrate/Nitrite transporter (excretion of nitrite), narK2. Predicted to be in the DosR	(Sasseti CM <i>et al</i> ; 2003)
<i>virS</i>	Rv3082c	Virulence-regulating transcriptional regulator, virS	Virulence-regulating transcriptional regulator, virS. Virulence, detoxification, adaptation	(Gupta S <i>et al</i> ; 1999)
<i>mce3C</i>	Rv1968	Mammalian cell entry family protein (possible cell invasion protein)	Thought to be involved in host cell invasion	(Tekaia F <i>et al</i> ; 1999)
<i>ltp2</i>	Rv3540c	Probable lipid transfer protein or Keto Acyl-CoA Thiolase	Supposed involvement in lipid and cholesterol metabolism. Member of the putative kstR regulon that controls cholesterol utilisation	(Sasseti CM <i>et al</i> ; 2003)
Rv3180c	Rv3180c	Conserved hypothetical; possible toxin (part of toxin-antitoxin operon with Rv3181c)	Represents a possible toxin, part of a toxin-antitoxin (TA) operon with Rv3181c. may be induced following exposure to stress conditions, allowing the cell to respond to the adverse conditions	(Sasseti CM <i>et al</i> ; 2003)
<i>ppsA</i>	Rv2931	Phenolphthiocerol Synthesis Type-I Polyketide Synthase	Involved in the biosynthesis of pththioceroldimycocerosate (PDIM), a component of the cell wall. Upregulation of ppsA in sputum may help the bacillus adapt to transmission	(Camacho LR <i>et al</i> ; 2001)

Table 16: Downregulated *M. tuberculosis* genes in sputum transcriptome.

Gene	Gene number	Product	Function	Reference
<i>Rv1103c</i>	Rv1103c	Conserved hypothetical; possible mazE3, antitoxin, part of toxin-antitoxin operon with Rv1102c	A putative antitoxin gene. Virulence, detoxification, adaptation	(Betts JC <i>et al</i> ; 2002)
<i>atpD</i>	Rv1310	Probable ATP synthase beta chain	Produces ATP from ADP in the presence of a proton gradient across the membrane. [Catalytic activity: ATP + H ⁺ (2) O + H ⁺ (in) = ADP + phosphate + H ⁺ (out)]. Intermediary metabolism and respiration	(Sassetti CM <i>et al</i> ; 2003)
<i>Rv2141c</i>	Rv2141c	Conserved hypothetical; possible protease	Identified to be downregulated on nitric oxide exposure to be similarly repressed in sputum	(Sassetti CM <i>et al</i> ; 2003)
<i>qcrC</i>	Rv2194	Probable Ubiquinol-cytochrome C reductase, QcrC (cytochrome C subunit)	Intermediary metabolism and respiration	(Shi L <i>et al</i> ; 2005)
<i>mmaA2</i>	Rv0644c	Methoxy-mycolic acid synthase 2, mmaA2	Involved in mycolic acids modification	(Cole ST <i>et al</i> ; 2001)
<i>ctaD</i>	Rv3043c	Probable cytochrome C oxidase polypeptide I, ctaD	Intermediary metabolism and respiration	(Sassetti CM <i>et al</i> ; 2003)
<i>nuoL</i>	Rv3156	Probable NADH dehydrogenase I (chain L), nuoL	Intermediary metabolism and respiration	(Betts JC <i>et al</i> ; 2002)
<i>mce1A</i>	Rv0169	Mammalian cell entry family protein (possible cell invasion protein)	Unknown, but thought to be involved in host cell invasion (entry and survival inside macrophages). The disruption of the complete mce1 operon led to a hypervirulent mutant	(Flessells B <i>et al</i> ; 1999)
<i>nuoB</i>	Rv3146	Probable NADH dehydrogenase I (chain B) NuoB (NADH-ubiquinone oxidoreductase chain B)	Involved in aerobic/anaerobic respiration [catalytic activity: NADH + ubiquinone = NAD(+) + ubiquinol]	(Betts JC <i>et al</i> ; 2002)
<i>rpsL</i>	Rv0682	30S ribosomal protein S12 RpsL	Protein S12 is involved in the translation initiation step	(Mulder MA <i>et al</i> ; 1997)

Table 17: Housekeeping genes for *M. tuberculosis*

Gene	Gene Number	Product	Function	Reference
16S	<i>rrs</i>	Ribosomal RNA (16S) component of small prokaryotic ribosomal subunit (30S)/interacts with 23S subunit.	Translation, ribosomal structure and biogenesis	(Garton NJ <i>et al</i> ; 2008)
Rv 2703	<i>sigA</i>	The sigma factor is an initiation factor that promotes attachment of the RNA polymerase to specific initiation sites and then is released. This is the primary sigma-factor of these bacteria.	RNA-polymerase sigma factor A – <i>sigA</i> is an initiation factor that promotes attachment of RNA polymerase to specific initiation sites and then is released. <i>sigA</i> is the primary sigma-factor of these bacteria. Supposedly involved in the housekeeping regulons	(Garton NJ <i>et al</i> ; 2008)
Rv2764a	<i>thyA</i>	Probable thymidylate synthase ThyA (ts) (TSASE).	Involved in deoxyribonucleotide biosynthesis. Provides the sole de novo source of dTMP for DNA.	(Institut Pasteur, 2004, Rengarajan <i>et al.</i> , 2004)
Rv2763c	<i>dfrA (folA)</i>	DihydrofolatereductaseDfrA (DHFR) (tetrahydrofolate dehydrogenase).	Essential step for de novo glycine and purine synthesis, DNA precursor synthesis, and for the conversion of dUMP to dTMP.	(Oswaldo Cruz Institute, 2010, Argyrou <i>et al.</i> , 2006)
Rv3227	<i>aroA</i>	3-phosphoshikimate 1-carboxyvinyltransferase AroA (5-enolpyruvylshikimate-3-phosphate synthase) (EPSP synthase) (EPSPS).	Involved in the biosynthesis of chorismate within the biosynthesis of aromatic amino acids (the shikimate pathway). Acts in the sixth step of this pathway	(Parish and Stoker,2002)
Rv1629	<i>polA</i>	Probable DNA polymerase I PolA.	Involved in post-incision events. In addition to DNA polymerase activity, this DNA polymerase exhibits 3' to 5' and 5' to 3' exonuclease activity	(Institut Pasteur, 2004, Huberts and Mizrahi, 1995)

5.2. Aims and objectives

To develop an *in vitro* Mtb biofilm system and determine the degree to which it replicates the Mtb phenotypes that were seen in sputum.

The specific objectives were to:

1. Develop an Mtb biofilm system under various growth conditions.
2. Use of qPCR to analyse the gene expression of Mtb biofilm layers.
3. Compare the biofilm gene expression of Mtb biofilm layers to sputum microarray data.
4. To measure if growth is occurring during biofilm formation by assessing the uptake of ^3H uracil by Mtb biofilm layers.
5. Assess the tolerance of Mtb biofilm layers to INH and RIF.
6. Assess the ability of Mtb biofilm layers to replicate sputum phenotypes in terms of:
 - I. LB and AF proportions
 - II. Tolerance to RIF and INH
 - III. Formation of Rpf-dependant populations

5.3. Methods

5.3.1. *M. tuberculosis* basic biofilm culture development

The Mtb biofilm was developed according to Ojha *et al*, 2008 protocol. Mtb H37Rv and Beijing65 strains from mid-exponential phase were grown in 7H9 Middlebrook broth supplemented with ADC and Tween-80 as a starter culture. Mtb biofilms were grown in 24-well polystyrene tissue culture plates (Corning®Costar® Cell Culture Plates) by inoculating 2 ml of warmed (37°C) Sauton's medium (no Tween-80) with 30 µl of saturated Pk Mtb cells. The plate was sealed with PetriSEAL™ tape properly, double bagged with wet tissue to ensure the humidity and then placed into a plastic box incubated statically at 37°C for 5 wks.

5.3.2. Stimuli added to the biofilm and mid-exponential phase cultures

A number of stimuli were added to the biofilm and mid-exponential phase cultures selected according to the genes that were upregulated in sputum see (Figure 46). These included cholesterol +oleic acid (OA) +nitric oxide (NO) as test conditions that were added to the Mtb cultures. Control conditions were made in parallel to assess the effect of the test conditions and these include Spermine-hydrochloride (Sper) (the endogenous polyamine, inhibits neuronal NO synthase), Methyl-β-cyclodextrin (CD) and BSA as a control condition of the lipid and cholesterol stimulatory conditions. To have the same oxygen exposure when the additional conditions were added, the untreated condition was exposed to sterile PBS.

Then, the gene expression of Mtb biofilm layers and mid-exponential phase cultures under the selected growth conditions was compared with the sputum transcriptome. All biofilm cultures were initially grown in Middlebrook 7H9 starter culture up to mid-exponential phase as described in Section 5.3.1; it was then used to inoculate the biofilm culture in Sauton's. The mid-exponential phase culture (control) was grown identically with the biofilm in Sauton's medium. All Mtb biofilm cultures under the selected growth conditions were developed in 24-well plates whereas all mid-exponential phase cultures were grown in 25ml conical flasks. Mtb H37Rv and Beijing65 cultures used in this study were:

- Biofilm grown with cholesterol and exposed to NO+OA for 4h (Ch+NO+OA) (Test).
- Biofilm grown with CD and exposed to Sper+BSA for 4h (CD+Sper+BSA) (Control).
- Biofilm culture exposed to PBS for 4h (un-treated condition).
- Mid-exponential culture grown with cholesterol and exposed to NO+OA for 4h (Mid-Exp (Ch+NO+OA)).
- Mid-exponential culture grown with CD and exposed to Sper+BSA for 4h (mid-Exp (CD+Sper+BSA)).
- Un-treated mid-exponential culture.

5.3.3. Harvesting of Biofilm layers

The developed biofilm culture was separated into three layers:

- 1- **Pk** biofilm layer, non-attached cells in the middle of the biofilm (see Figure 47), was separated by piercing the top layer of the biofilm with a 1 ml sterile-filtered pipette tip gently and withdrawing the cell suspension. Three-quarters of the biofilm liquid was taken into 5 ml-bijoux tube (Sterillin, Bargoed), to ensure that the top layer did not touch the bottom biofilm layer.
- 2- **Pellicle** (the top air-liquid interface) layer was separated by adding the same original amount of warmed biofilm medium (37°C), onto the biofilm to aid the Pellicle harvesting by resuspending thoroughly using 1 ml sterile-filtered pipette tip into the same liquid medium of the original biofilm.
- 3- **Att** biofilm layer was harvested by washing the Att biofilm with the same original amount of warm biofilm medium 3 times gently to remove the other non-attached biofilm cell layers. The Att cells were then harvested by carefully scraping the surface with a pipette tip into with the same volume of warmed medium. This was then transferred into 5 ml-bijoux tube. The biofilm harvesting process is explained in Figure 47.

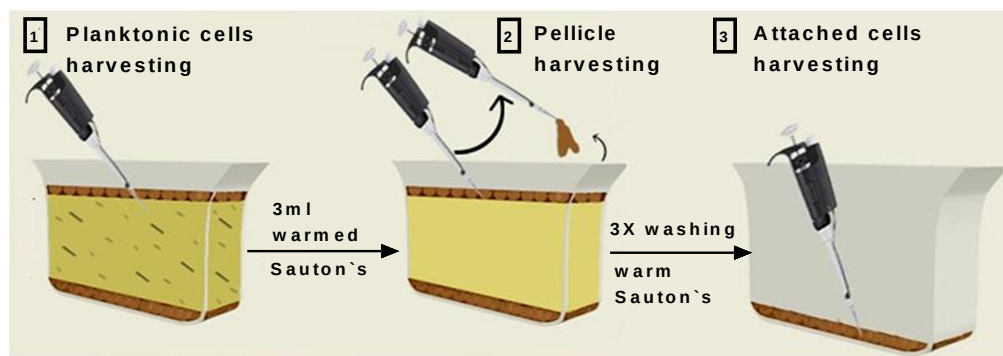


Figure 47: Schematic diagram of biofilm harvesting steps.

The figure shows the 3 harvesting steps of Mtb biofilm. The harvesting protocol was done in 12-wells & 24-wells flat microtitre polystyrene plates (Corning, UK).

5.3.4. Ribonucleic Acid (RNA) Manipulation Technique

RNA precautions

Precautions when handling RNA were taken in order to reduce the RNA degradation due to contamination with ribonuclease (RNase). In all RNA work gloves were worn and were changed frequently. Prior to work, all equipment and working areas were treated with RNase-Zap (Ambion) to remove surface RNase contamination. New RNase-free filter pipette tips and sterile disposable plastic wares were used. Only those RNase free reagent stocks were used for RNA work. All RNA work steps were carried out on ice to minimise RNase activity and the RNA was stored at -80 °C.

M. tuberculosis RNA extraction

RNA was extracted from Mtb biofilm cells and aerated growing cultures in 5 ml aliquots. A volume of Mtb cells harvested from the biofilms or aerated grown cells was added into 4 volumes of 5M GTC solution. Cells were centrifuged at 2000 xg for 30 minutes. The supernatant was discarded and cells were resuspended in 1 ml GTC and are transferred into 2 ml screw-cap microfuge tube. The suspension was centrifuged at 9600 xg for 5 minutes and the supernatant was discarded using pointed-tipped filter pipette tips. Trizol® LS (Invitrogen; 1ml) was added directly to the cell pellet and the samples were kept at -80 °C awaiting RNA extraction.

Samples were defrosted and ceramic silica beads (entire contents of one tube of Lysing Matrix A, Q-Biogene, UK) were added carefully to the cells in Trizol. Cells were then disrupted in a FastPrep bead beater with a speed setting of 6.5 m/s for 45 seconds (FastPrep FP120, Thermosavant). Samples were left at room temperature for 10 minutes to cool down and also to allow dissociation of nucleoprotein from the nucleic acid followed by adding 200 µl of chloroform and 200 µl of RNase free d.H₂O. In order to separate the aqueous phase from the organic phase, samples were vortex well and centrifuged (Heraeus FRESCO 17 centrifuge, Thermo Electron Corporation) at 9600 xg for 15 minutes at RT. The upper aqueous phase containing RNA was transferred to a new 1.5 ml microfuge tube carefully to avoid any carryover of the interphase materials. An equivalent volume (1:1) of chloroform was added to the sample, vortexed well and then centrifuged at 9600 xg for 5 minutes at RT. The upper aqueous phase was transferred to a new microfuge tube which was kept on ice and 0.8 volumes of isopropanol were added to one volume of the sample. Glycoblu (Ambion; 1µl) co-precipitant was added to each sample to reach the final concentration of 150µg/ml and tubes then were mixed by inversion. Tubes were kept at -20°C overnight to allow the RNA to be precipitated. Tubes were taken out from Category 3 laboratory at this stage.

Subsequent to RNA precipitation, the samples were mixed gently by inversion centrifuged at 9600 xg for 15 minutes at 4°C and the resulting RNA pellet was washed once with 1 ml 70% (v/v) ethanol followed by centrifuging at 9600 xg for 2 minutes at 4°C. The supernatant was discarded and the pellet was washed once with 200µl of 95% (v/v) ethanol then centrifuged at 9600 xg for 2 minutes at 4°C. The supernatant was discarded and the pellet was air-dried by leaving the tubes open at room temperature for about 10 minutes. The RNA pellet was then resuspended in 50µl of sterile RNase-free distilled water. If the pellet was difficult to dissolve the amount of water was increased to 100 µl.

Turbo DNase Treatment

Turbo DNase treatment was applied to the sample to remove any residual DNA by adding 5 µl of 10x Turbo DNase buffer (Ambion) and 1 µl of Turbo DNase (Ambion) to 44 µl of the sample. The sample was mixed gently and incubated at 37°C in the hot block (Grant Instruments QBT2, Cambridge, England) for 30 minutes. Tubes were taken to the ice and 1µl of Turbo DNase was added and tubes were incubated at 37°C for 30 minutes. Following incubation, 10 µl of DNase the slurry of inactivation reagent

(Ambion) was added and tubes were mixed gently then centrifuged at 9600 xg for 5 minutes at 4°C to pellet the solid inactivation reagent. The aqueous phase was taken avoiding carryover of activation reagent to a new microfuge tube. Following DNase treatment the RNA was cleaned up using the RNeasy Mini Cleanup Column (Qiagen) following the manufacture`s procedure and including an on-column DNase digestion step. RNA was eluted form the RNeasy column in a volume of 50 µl of RNase-free water.

RNA Quantification method

For each sample the RNA concentration was measured spectroscopically of 1 µl of extract using a Nanodrop spectrometer (Thermo Scientific) at absorbance of 260nm. The blank sample was 1 µl of RNase free distilled water. The RNA concentration was measured using the following equation (A₂₆₀=1 is equivalent to 40µg/µl):

$$C = \frac{A \times 40 \times D}{1000}$$

C = Concentration (µg/µl)

A = Absorbance at 260nm

D = Dilution factor

1000 = Correction (converts ml to µl)

Reverse transcription polymerase chain reaction (RT-PCR)

The DNase treated RNA was reverse transcribed to complimentary DNA (cDNA) using Mycobacterial genome directed primers (mtGDPs, Laboratory stock) and SuperScript II Reverse Transcriptase enzyme (Invitrogen). mtGDPS are a set of random oligonucleotide primers with a GC bias. The RNA sample was divided into RT (Reverse transcribed RNA) and no-RT (NO Reverse transcription) reactions in RNase-free PCR tubes. The following reagents were used the denaturation step:

Buffer	RT (µl)	No RT (µl)
dNTP`s (10 mM)	1.5	1.5
mtGDPs primers (25 pmol/µl)	1	1
RNA	x (0.5ug)	x (0.5ug)
The total volume (made up with H ₂ O)	Up to 18 µl	Up to 18 µl

(x 0.5µg) The concentration of the RNA is varying between samples. If the concentration of the extracted RNA is < 5µg, then 31µl of the RNA was split between RT and no RT tubes.

The solution was heated at 65°C for 5 minutes to denature the secondary structure of the RNA and then placed directly to the ice.

The annealing step was carried out then by adding the following reagents:

Buffer	RT (µl)	No RT (µl)
5X Superscript II buffer	6	6
0.1 M DTT	3	3
RNasin Ribonuclease inhibitor (20 units/µl)	1.5	1.5

Primers were annealed by heating the mixture at 25°C for 2 minutes then placed directly to the ice.

The reverse transcription step was carried out by adding the following reagents:

Buffer	RT (µl)	No RT (µl)
Superscript II transcriptase	1.5 (300units)	0
RNA free d.H ₂ O	0	1.5
Total volume	30	30

The tubes were placed in the PCR machine and incubated at 25°C for 10min, and then the reverse transcription of the RNA was allowed by incubating the tubes at 42°C for 50min. The last step was incubation at 70°C for 15min to inactivate the reverse transcriptase.

When RNA was reverse transcribed, the stock cDNA sample was diluted 1:3 (v/v) with RNase/DNase free distilled water. Samples were stored at -20°C.

Gene expression data analysis of cDNA

Initially, the number of 16S rRNA, *sigA* and (*thyA*, *aroA*, *dfrA* and *polA*) housekeeping genes transcript copies of all Mtb biofilms and mid-exponential phase cultures cDNA were measured. The statistical analyses then performed using Excel 2010 (Microsoft

Corp.). Primers for gene transcripts measured are shown in Table 6 in the appendix. The average transcript copy number of the gene of interest was normalised with that of 16S rRNA or *sigA* directly. For the (*thyA*, *aroA*, *dfrA* and *polA*), housekeeping genes the geometric mean was taken from all genes and then the gene of interest was normalised with the geometric mean. Following normalisation, the resulting numbers were compared with the normalised control mid-exponential phase culture numbers. Finally, the log2 was applied and the average number as well as the SD was calculated.

5.3.5. Deoxyribonucleic Acid (DNA)

DNA Precautions

DNA precautions were done in order to reduce the DNA degradation due to contamination with deoxyribonuclease (DNase). In all DNA work steps gloves were worn and were changed frequently. New DNase-free filter pipette tips and sterile disposable plastic wares were used. It was used special DNase free reagents stocks only for DNA work. DNA work steps were carried out on ice to minimise DNase activity and the DNA was stored at -80 °C.

DNA Extraction

The DNA extraction was done according to the method of Ernesto *et al*, (2005). The *M. tuberculosis* cell suspension was centrifuged at 2000 xg for 10 minutes. The supernatant was discarded and the pellet was resuspended in 800 µl of 10mM Tris-1 mM EDTA, pH8. The cell suspension was transferred to a 2 ml screw cap tube (Alpha labs) with silica beads (Lysing Matrix B, QBiogene) and 200 µl of warm (70 °C) Phenol/Choloroform/Isoamyl alcohol, pH6.7 (Fisher Scientific) was added. Tubes were vortexed and cells were disrupted with a speed setting of 6.5 m/s for 45 seconds using Fast-Prep shaker. Samples then were left at room temperature for 10 minutes to cool down followed by centrifugation at 9600 xg for 10 minutes. The supernatant was transferred to a new 1.5 ml microfuge tube followed by adding 50 µl of 5M NaCl. An equal volume of Phenol/Choloroform/Isoamyl alcohol 1:1 was added to each sample followed by centrifugation at 9600 xg for 5 minutes. The upper aqueous phase was transferred to a new tube and 0.6 volumes of Isopropanol were added to each sample. Tubes were kept at -20 °C overnight to allow the DNA precipitation. Tubes were then defrosted and the DNA was collected by centrifugation at 9600 xg for 5 minutes. The

supernatant was discarded and the pellet was washed once with 200 µl of 70% ethanol followed by washing with 200 µl of 95% ethanol. Finally, pellet was resuspended in 300 µl free DNase/RNase distilled water. The resulting DNA concentration was measured using the Nanodrop quantification method. Samples were stored at -20 °C.

Gene expression data analysis of DNA

The number of 16S rRNA copies of Mtb H37Rv biofilm and mid-exponential phase cultures DNA was measured. Then the statistical analyses then performed using Excel 2010 (Microsoft Corp.).

5.3.6. Direct RIF and isoniazid treatment of *M. tuberculosis* cells in biofilm and growth culture

Mtb H37Rv and Beijing65 biofilm cultures were developed in 24-wells plates according to the protocol in Section 5.3.1. After 5 wks of incubation, the biofilm was taken out from the incubator and the plate separated into 3 parts each part contains 3 wells. The first part was injected with filter sterilised RIF at final concentration of 5 µg/ml⁻¹, the second part was injected with filter sterilised INH at final concentration of 1 µg/ml⁻¹ and the third part was injected with 2 µl of sterile PBS as a negative control. The antibiotic injection was done by using 10µl pointed-filter tips. The biofilm plate then was sealed with plastic tape, double-bagged with wet tissue and re-incubated at 37°C for seven days statically. The control mid-exponential phase culture was grown in Sauton's medium with Tween-80 up to OD of 1. Following, 5 ml of the stock mid-exponential phase culture was separated into 30 ml Universal plastic tubes (Sterilin, Bargoed). RIF and INH antibiotics were added at the same concentration that added to the biofilm in triplicates. The mid-exponential culture negative control was prepared by adding 5 µl of sterile PBS in triplicates. Tubes then were sealed with Nescofilm and incubated at 37°C for seven days with the proper shaking speed.

After seven days of incubation, the biofilm layers were separated and harvested according to the protocol in Section 5.3.2. The biofilm and the mid-exponential phase cells were treated with syringe in order to break the cultural clumps according to the protocol in Section 2.4.4. The CFU count on each biofilm layer and mid-exponential phase Mtb cells were carried out on 7H10 Middlebrook plates according to the protocols in sections 2.3.6. Plates then were sealed with plastic tape and incubated at 37°C statically for 2-6 wks.

5.3.7. Assessing the number of culturable cells of *M. tuberculosis* from biofilm Samples

This experiment aiming to evaluate numbers of *Mtb* H37Rv biofilm cells that were recovered on agar plates or in liquid medium with or without the addition of culture supernatant (SN). The SN solution, Middlebrook 7H9 control plate reagent, SN plate preparations are shown in Section 2.2.2. The *Mtb* H37Rv biofilm was developed and harvested according to the protocols in Sections 5.1.1 and 5.3.2 respectively. The biofilm cells were treated with syringe to break up cultural clumps according to the protocol in Section 2.4.4.

The MPN count was carried out in 48-wells plates and each plate was separated into two sections. Each section contained 4 replicates for each dilution. Into each well, 450µl was added from the 7H9 control and the SN. Thereafter, 50µl of *Mtb* biofilm cells were added to the 10^{-1} dilution of SN and 7H9 control. Then, 50µl was taken from each 10^{-1} dilution, mixed thoroughly by pipetting and added to the next dilution. The serial dilution was repeated with the rest of the wells. Each sample should have at least 6 series of dilutions (10^{-1} - 10^{-6}).

The 7H10 Middlebrook control agar plates were separated into 6 segments in each plate. The CFU was done only from the 7H9-control medium; each sample should have at least 6 independent series of dilutions (10^{-1} - 10^{-6}) as shown in Section 2.3.6.

Next, all 48-wells plates and 7H10 agar plates were sealed with plastic tape, placed in double zip-lock bags and incubated at 37°C statically for 6 wks. The first check was after 5 days to exclude mould contaminated samples.

Once ready, the CFU calculations were done as described in Section 2.3.6. The MPN counts were performed by using a program at:

http://www.wiwiss.fu-berlin.de/institute/iso/mitarbeiter/wilrich/MPN_ver2.xls

5.3.8. ^3H Uracil labelling

Mtb H37Rv from mid-exponential phase culture was grown to reach an OD of 1 as a starter culture. The biofilm was grown in 12-wells polystyrene plate by inoculating 40 µl of mid-exponential phase *Mtb* cells into 3 ml of Sauton's per well. Each plate was divided into two sections, each section contains 6 wells. The first section was labelled

as ^3H Uracil whereas the second section was labelled as CFU, MPN and DNA and RNA extractions. The biofilm plates then were sealed with plastic sealing tape and incubated at 37°C statically. The biofilm harvested in four time points from the 2nd–5th wk. Prior harvesting, each well of the first section was injected with 16.4 μl of ^3H uracil (Perkin Elmer, 36 Ci/mmol) to reach the final radioactive concentration of 1.09 $\mu\text{Ci/well}$ and the second section was injected with 16.4 μl of sterile PBS as a control for the possible disturbance of the Pellicle layer. The biofilm plate was then sealed with plastic sealing tape and incubated at 37°C statically for 30 h. When ready, the biofilm was harvested and processed as following:

Section1: Cells were harvested from the first 6-wells to 3 different layers:

- 1- The **Pk** layer in the middle: 2 ml from each well was harvested using 1ml filter tip into sterile 50ml falcon tube to a final pooled volume of 12ml.
- 2- The **Pellicle** layer: was harvested from each well using 1ml sterile filter tip and resuspended in 18ml of Sauton`s in sterile 50ml falcon tube..
- 3- The **Att** layer: was washed twice with sterile warmed Sauton`s carefully. The layer then harvested from each well by resuspending cells in 3ml Sauton`s medium by scratching the plastic well bottom with a 1ml filter tip. Cells were then transferred into sterile 50ml falcon tube to a final pooled volume of 18ml.

Tubes were centrifuged at 2000 xg for 10 minutes and the supernatant was removed to the radioactive aqueous waste bottle. The pellet was resuspended in PBS to the same original volume, centrifuged at 2000 xg for 10 minutes and the supernatant was removed leaving 1ml in the falcon tube. Cells were then transferred to a sterile 1.5ml microfuge screw cap tube and centrifuged at 9600 xg for 2 minutes. Supernatant was discarded and cells were resuspended in 300 μl PBS.

^3H Uracil measurements

To measure the incorporation of ^3H uracil, 100 μl from each layer was transferred to a plastic scintillation tube. This step was done in triplicate for each layer. 231 μl of absolute ethanol was added to the cells (final concentration of 70% v/v ethanol); the tubes were capped and kept in the cabinet overnight to kill the viable mycobacteria. Prior to measuring the label, 2.5ml of Emulsifier safe scintillation fluid was added to

each tube. Tubes were then capped tightly and were taken to the scintillation counter for measurement.

Harvesting Section2 for CFU, MPN and DNA and RNA extractions: Cells were harvested and separated into 3 layers:

Biofilm cells harvesting was done as mentioned above in Section 5.3.2. Following washing, pellets were resuspended in 2ml PBS. The cell suspension then was used to count the Mtb H37Rv cells by MPN, CFU, RNA and DNA extraction techniques as described previously.

5.4. Results

5.4.1. Biofilm development

Mycobacterial biofilms have been attributed to the pellicle formed at the liquid-air interface in a diversity of synthetic media (Ojha *et al.*, 2008 and Sambandan *et al.*, 2013). However, in these experiments biomass was assessed in of three different layers. Each one of these layers contains different cell numbers. These layers are: the top **Pellicle** layer at the air-interface of the biofilm, the **Pk** layer in the middle of the biofilm mass and the bottom layer with attached to the plastic surface (**Att**) (Figure 48). Each individual layer was analysed in this chapter.

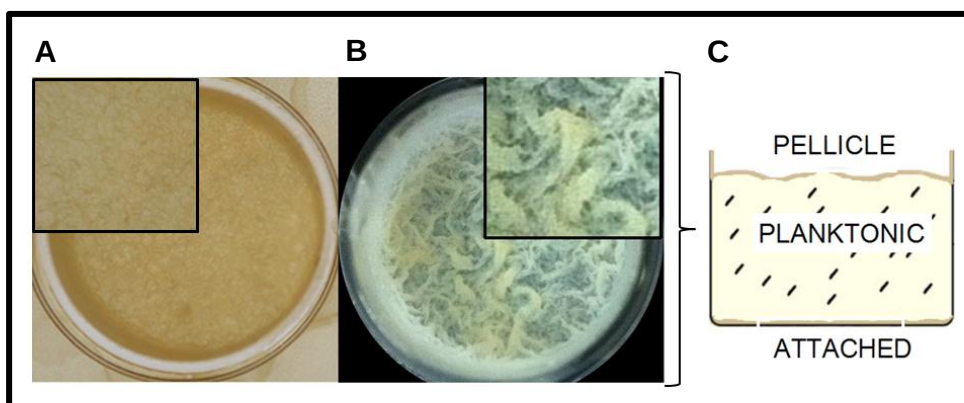


Figure 48: Classification of mycobacterial biofilm layers.

Biofilms were developed in 12-well sealed microtitre plates over 5 wks static incubation according to Ojha *et al.*; 2008. Image (A) shows the Mtb H37Rv biofilm, whereas (B) shows *M. bovis* BCG (Glaxo) biofilm. The two biofilms show distinctive appearance of colour and morphology. The schematic (C) represents the observed layers. Biofilms were harvested separately into three layers as mentioned in section 5.3.3: **Pellicle** (Pellicle) the top layer, **Pk** (Pk) in the middle and cells **Att** (Att) to the plastic surface.

5.4.2. Biofilm layers yield different colony morphologies

The Mtb H37Rv biofilm cells grown in Sauton's and aerated grown mid-exponential H37Rv cells in Sauton's were cultured on sealed Middlebrook 7H10 agar for 30 days. Figure 49 shows distinctive Mtb colonies derived from the different layers compared to a subculture from a mid-exponential culture. Att and Pk biofilm colonies appear smaller in size compared with the Pellicle and mid-exponential phase colonies. Also, the biofilm colonies show rougher colonies compared to the mid-exponential phase derived colonies.

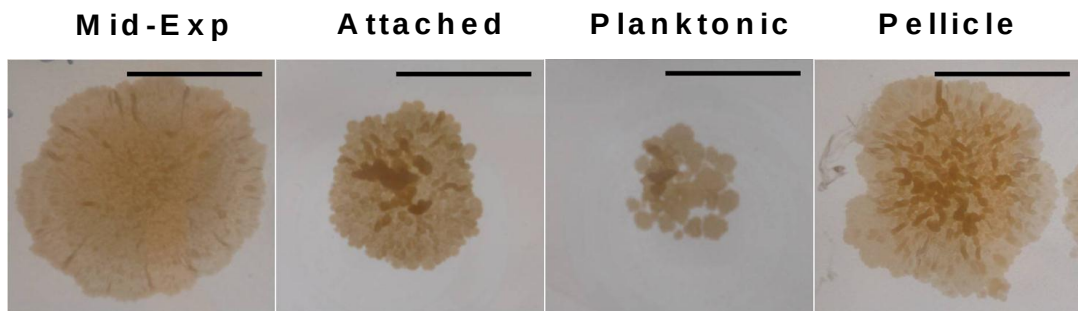


Figure 49: Phenotypic changes of *M. tuberculosis* cells.

Images display the phenotypic changes of Mtb H37Rv cells from the control mid-exponential phase cells, Att, Pk and Pellicle biofilm cells in Sauton's. Cells were cultured from biofilm culture in Sauton's then 20 μ l was spotted in 7H10 agar. Representative colonies were taken after 30 days of incubation. Scale bars 1.5mm.

5.4.3. Biofilm gene expression

5.4.3.1. Experimental strategy

The experimental strategy that was used to measure the Mtb biofilm gene expression is detailed in Figure 50.

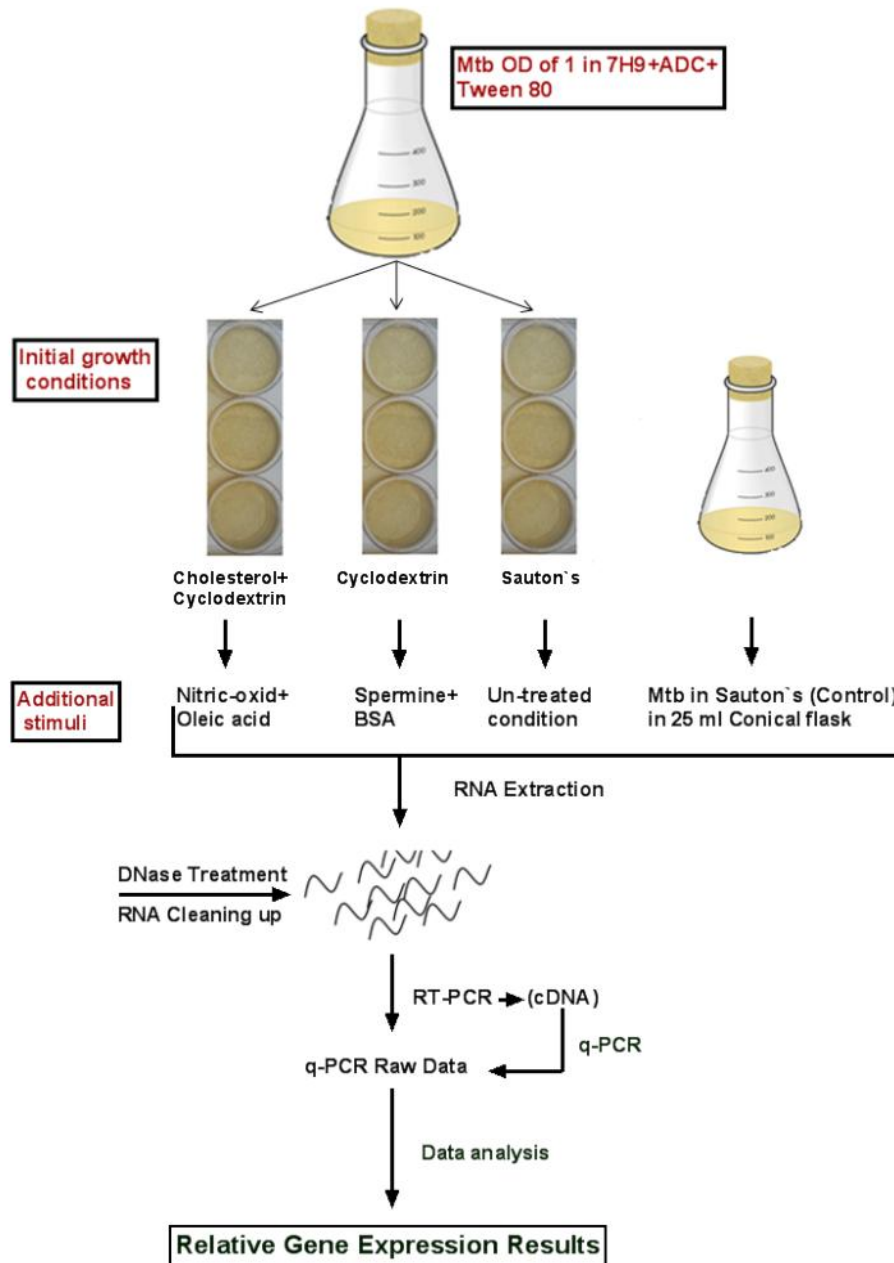


Figure 50: Schematic diagram of the gene expression process.

This diagram represents the gene expression steps starting from the preparation of Mtb starter culture and ends with the q-PCR and data analysis.

This experiment was performed initially as described in Section 5.3.1 by developing the Mtb biofilm and mid-exponential cultures in Sauton's medium under four initial growth conditions (see Section 5.3.2). These conditions are Mtb biofilm with cholesterol+CD, control (CD), un-treated biofilms and mid-exponential phase cultures. The additional stimuli were added to the 5-wk biofilms and mid-exponential phase.

5.4.3.2. Gene expression of *M. tuberculosis* H37Rv biofilms exposed to selected stimuli

After harvesting and separating the biofilm into three layers (Att, Pk and Pellicle) as described in Section 5.3.3, the mRNA of all cultures was extracted, cleaned, and reverse transcribed into cDNA and diluted 1:3 as described in Section 5.3.4. The qPCR was done in triplicate, and then the data analyses including the comparison with the gene expression of the mid-exponential phase were performed as mentioned in Section 5.3.4.

The results of the gene expression patterns for Mtb biofilms under the selected conditions are shown in Figure 51. The results showed that most of the 10 selected genes, which were downregulated in sputum transcriptome, are repressed in all biofilm conditions. Most of them are genes involved in the aerobic respiration. The DosR regulon genes (*hspX*, *tgs1* and *nark2*), which were significantly upregulated in sputum and known to be upregulated under stress conditions, are upregulated in all Att and Pk biofilm conditions. The *tgs1* was significantly upregulated in test condition compared to the control and un-treated conditions in both Att and Pk biofilm cells. Likewise, DosR regulon genes were significantly induced in mid-exponential phase test condition. The FA enzyme *icl1*, the gene originally recognised under NRP conditions by Wayne and Sohaskey (2001) and essential for Mtb pathogenesis and intracellular survival in mice infection (Munoz-Elias and McKinney, 2005), was repressed in all biofilm conditions. However, the *icl1* was significantly repressed in un-treated Pk and Pelli biofilm cells compared to the test and control. In contrast, *icl1* was significantly upregulated in the mid-exponential phase test condition. The ribosomal gene *rpsL* was significantly repressed (>2.5-fold) in all biofilm conditions.

Furthermore, the results showed that *ppsA*, as the component of the cell wall that is involved in the biosynthesis of PDIM and plays a role in countering the early immune response of the host (McKinney, 2000), was downregulated in all biofilm conditions. Similarly, *virS* the gene that is involved in virulence and significantly induced in sputum was downregulated in all biofilm conditions. Surprisingly, most genes in Pellicle biofilm cells under all conditions were downregulated (Figure 51).

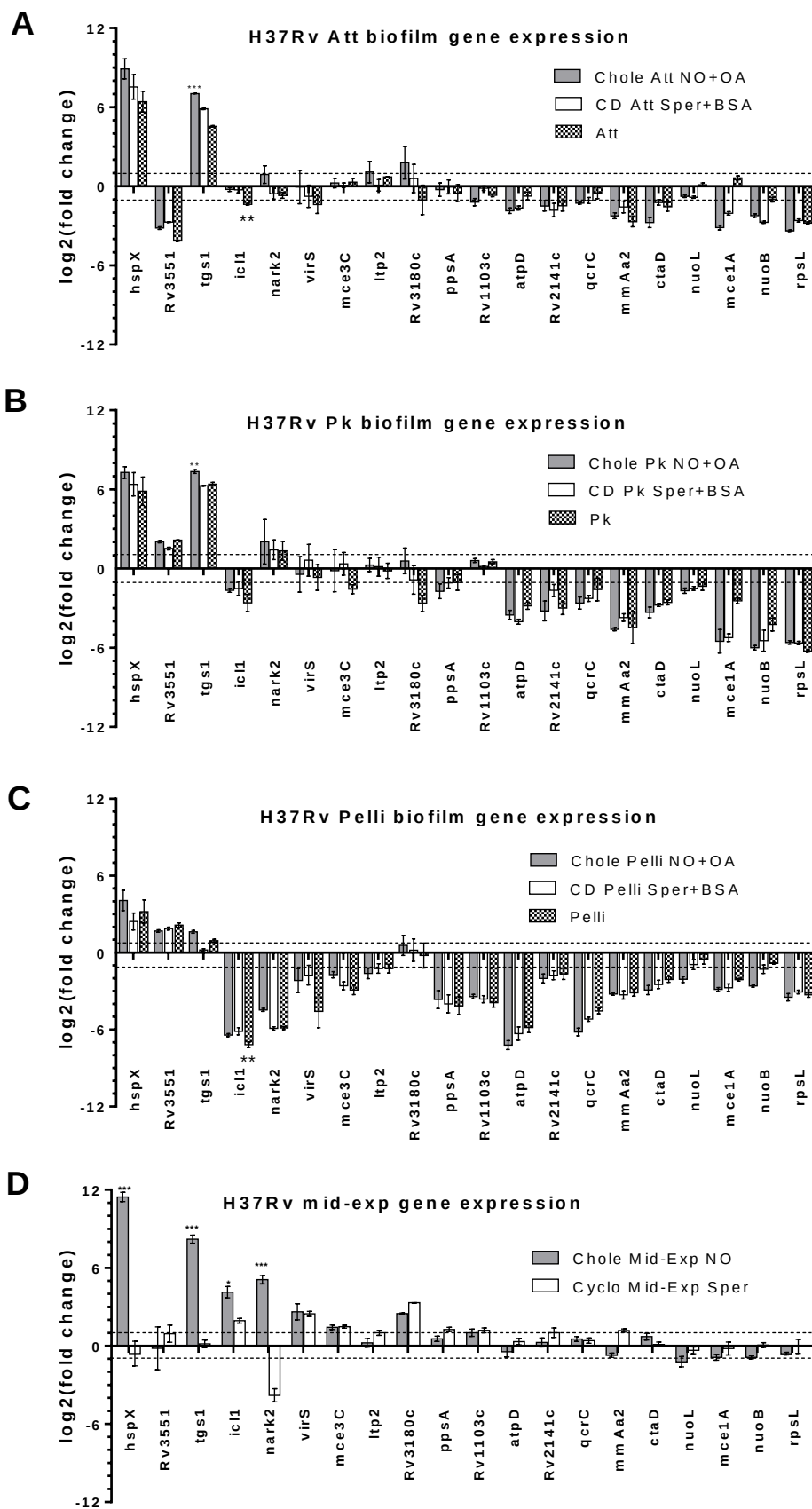


Figure 51: The gene expression of 20 selected genes for Mtb biofilm layers under selected growth conditions

Bar charts display gene expression of 20 selected target genes in *Mtb* H37Rv for (A) Att biofilm with Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, and Sauton's, (B) PK biofilm Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, and Sauton's (C) Pellicle biofilm Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, and Sauton's and (D) Mid-Exp phase cells with Cholesterol/Nitric Oxide/Oleic Acid and CD/Spermine-HCl/BSA. All (log₂) fold changes calculated against aerobic exponential growth. Dotted line represents 2.5-fold (1log₂-fold change). Asterisks indicate significant result.

5.4.3.3. *M. tuberculosis* H37Rv Biofilm correlation with Sputum Transcriptome

To study the correlation of the 20 selected genes on each condition on the biofilm layers to sputum transcriptome, Spearman's rank correlation was used as a parametric measurement. This is a non-parametric test and positive correlation only reflects a monotonic function (a function between ordered sets that preserve the given order), which measures the strength between two ranked variables (Hollander *et al.*, 2004). Therefore, Spearman's correlation statistical analysis does not essentially indicate a linear relationship. The perfect R-Ranking is 1 or closer and the poor R-Ranking is 0 or closer. If the R equals or is greater than 0.8, the correlation is strong, whereas if the r is 0.5 or less, the correlation is weak (Grzegorzewski *et al.*, 2011). This particular measurement was used because the microarray gene expression data of sputum had non-Gaussian's distribution. P-values, R-rank values and 95% confidence intervals were calculated and are displayed in Table 18.

Table 18: Spearman's Rank Correlation R and p-values are displayed for each condition of H37Rv vs. Sputum Gene Expression.

Sample	Ch-NO-OA	Cyclo-Sper.HCl-BSA	No additional stimuli
Att	R = 0.5884, P < 0.0001 CI = (0.3305-0.7645)	R = 0.4614, P = 0.0406 CI = (0.0095-0.7568)	R = 0.08352, P = 0.7263 CI = (-0.3849-0.5177)
PK	R = 0.8360, P < 0.0001 CI = (0.6157-0.9351)	R = 0.8623, P < 0.0001 CI = (0.6711-0.9459)	R = 0.7103, P = 0.0004 CI = (0.3785-0.8804)
Pellicle	R = 0.3785, P = 0.01 CI = (-0.9100-0.7103)	R = 0.2603, P = 0.2676 CI = (-0.2194-0.6387)	R = 0.1053, P = 0.6585 CI = (-0.3660-0.5337)
Mid-exp	R = 0.3213, P = 0.1672 CI = (-0.1552-0.6765)	R = -0.05643, P = 0.8132 CI = (-0.4975-0.4079)	Normalisation sample

CI = 95% confidence intervals

The results show significant correlation between all Pk biofilm conditions studied and sputum microarray gene expression, with P-values of <0.05 and higher R values than all the other conditions examined (Figure 52). Also, each Pk culture harvested showed predominant changes in expression pattern consistent with sputum: Ch+NO+OA biofilm (6/10 up, 9/10 down), CD+Sper+BSA biofilm (7/10 up, 9/10 down) and the Biofilm control (4/10 up, 9/10 down). These patterns are closer to sputum microarray gene expression as indicated by the 95% confidence intervals. The higher correlation with sputum is for the downregulated genes by >2-fold (1 log₂) downregulated in all conditions. The upregulated genes in sputum *tgs1*, *hspX* and *nark2*, which are part of the DosR regulon are shown to be upregulated in all Pk biofilm conditions.

Icl1, which was shown to be significantly upregulated in sputum, is downregulated in all Pk biofilm conditions. The results of the gene expression of the Pk biofilm from all conditions revealed that the exposure to NO for 4 h doesn't affect the level of the *DosR* regulon genes expression significantly with *hspX* and *nark2*, whereas *tgs1* shows significant effect of NO on gene expression. Furthermore, Rv2141c, which is downregulated during exposure to NO, is downregulated in all PK biofilm growth conditions. *Ltp2*, which controls cholesterol utilisation, is upregulated in Pk biofilm test condition that contains cholesterol, whereas the control condition is downregulated. Also, the results show downregulation of the *rpsL* in all Pk biofilm conditions. This indicates that Pk cells might be in an NRP state within the H37Rv biofilm.

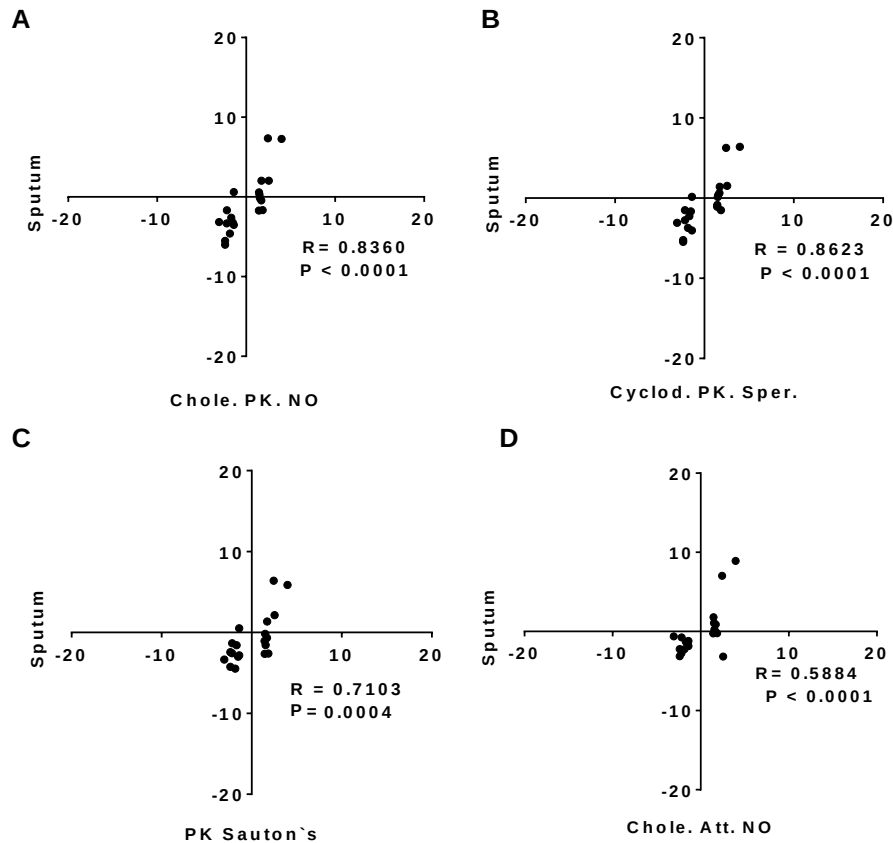


Figure 52: Scatter Plots showing H37Rv biofilm gene expression patterns correlating best with Sputum Microarray Gene Expression.

Scatter plots representing gene expression for *Mtb* H37Rv biofilms with multi-stimuli versus Sputum Microarray gene expression. (A) Pk biofilm Cholesterol/Nitric oxide/OA (B) Pk biofilm CD/Spermine-HCl/BSA, (C) Pk biofilm in Sauton's and (D) Att biofilm Cholesterol/Nitric oxide/OA. Graphs show the closest correlation conditions to Sputum Gene Expression. Spearman's Rank Correlation R and p-values are displayed for each graph.

The H37Rv Att biofilm test culture with Cholesterol+CD and exposed to NO+OA comes after the Pk biofilm layers by correlation with sputum transcriptome with significant P-value of <0.05 . The gene expression pattern of the Att biofilm test condition shows 6 upregulated genes out of 10 and 10 downregulated genes out of 10. Nevertheless, the Pk biofilm layers show higher downregulation level in all respiratory genes (*atpD*, *qcrC*, *ctaD*, *nuoB* and *nuoL*) than the Att biofilm test condition. On the other hand, the Att biofilm control of the test culture shows less correlation than test culture with significant P-value. Furthermore, the *rpsL* gene is downregulated in all Att biofilm conditions, which might indicate the NRP state of the Att layer. However, the H37Rv Pellicle and mid-exponential phase show no correlation with sputum microarray

gene expression. Interestingly, in all Pellicle growth conditions, 10/10 of genes are repressed, while 4/10 of genes on the test and control Pellicle condition and 3/10 in the basic Pellicle culture are upregulated. NO has shown no effect on the biofilm layer's gene expression in contrast to the mid-exponential phase culture, which shows a strong effect on DosR regulon genes. Rv2141c, which is known to be downregulated with NO is shown to be 1 log₂-fold upregulated with no NO exposure than with NO. The lipid metabolism gene *icl1* is known to be upregulated more than 3-fold in sputum and is downregulated in all biofilm layers and conditions. Although the mid-exponential phase test and control cultures show no correlation with gene expression in sputum, the mid-exponential phase test culture with Cholesterol+NO+OA shows high upregulation on the *icl1* gene by 4 log₂-fold. This indicates that the Mtb cells within the biofilm have distinct gene expression from mid-exponential phase growth.

5.4.3.4. Gene expression of *M. tuberculosis* Beijing65 biofilms exposed to selected stimuli

One of the aims of the current study is to compare between sputum transcriptome gene expression and Mtb biofilm layers gene expression *in vitro*. It was used in this comparison Mtb H37Rv laboratory strain. However, using laboratory strain may not be the best choice to compare with Mtb gene expression from sputum. This is because H37Rv strain has been passaged for many decades outside the host. Consequently, the relevance of the H37Rv genome sequence to clinical Mtb strains has been minimised (Fleischmann *et al.*, 2002). A preliminary exploration of this hypothesis was made using a more recently isolated Mtb Beijing strain.

Beijing clinical strain is a member family of Mtb that was thought to be originated in Asia before spreading around the world (van Soolingen *et al.*, 1995). This strain has genetic advantages (Bifani *et al.*, 2002), which allows it to be implicated to many outbreaks and drug resistance around the world (Glynn *et al.*, 2002). In the current study, Mtb Beijing65 strain was prepared in a biofilm culture to compare gene expression with the sputum pattern. The gene expression for Mtb biofilm under selected conditions of the 20 selected genes is shown in Figure 53.

Because Beijing biofilm might have different phenotypic features than H37Rv, the gene expression profile has different patterns. One of the obvious differences is that genes involved in the aerobic respiration in all H37Rv Pk biofilm conditions seemed to

repress more compared to Beijing Pk biofilm conditions. The PDIM synthesis gene *ppsA* was upregulated in all Beijing biofilm conditions and it is clearly induced in Att and Pelli biofilm layers (>2.5-fold; > 1-log2 fold change) as shown in Figure 53. The ribosomal gene *rpsL* was highly downregulated in H37Rv biofilm cells, in contrast to the Beijing biofilm. The gene expression pattern of Pelli Beijing biofilm in all conditions differed from H37Rv where most genes were downregulated. Moreover, genes involved in aerobic respiration, such as *nuoB*, *nuoL*, *ctaD*, and *qcrC*, are downregulated in Beijing Pk layer in all conditions in contrast to the Beijing Pelli biofilm layer where these genes are upregulated in all conditions. In Beijing Att biofilm layer, aerobic respiration genes are upregulated in the un-treated condition (*atpD*, *nuoB* and *nuoL* are significantly induced) while in the test and control conditions these genes are repressed (Figure 53).

One of the main similarities in the gene expression between Beijing and H37Rv strains is the downregulation of *icl1*. Furthermore, the DosR regulation genes are induced in all Beijing biofilm conditions. The results in all Beijing biofilm conditions showed no obvious effect of the NO on the gene expression profile. The same observation was noticed in the H37Rv gene expression profile (See section 5.4.3.2).

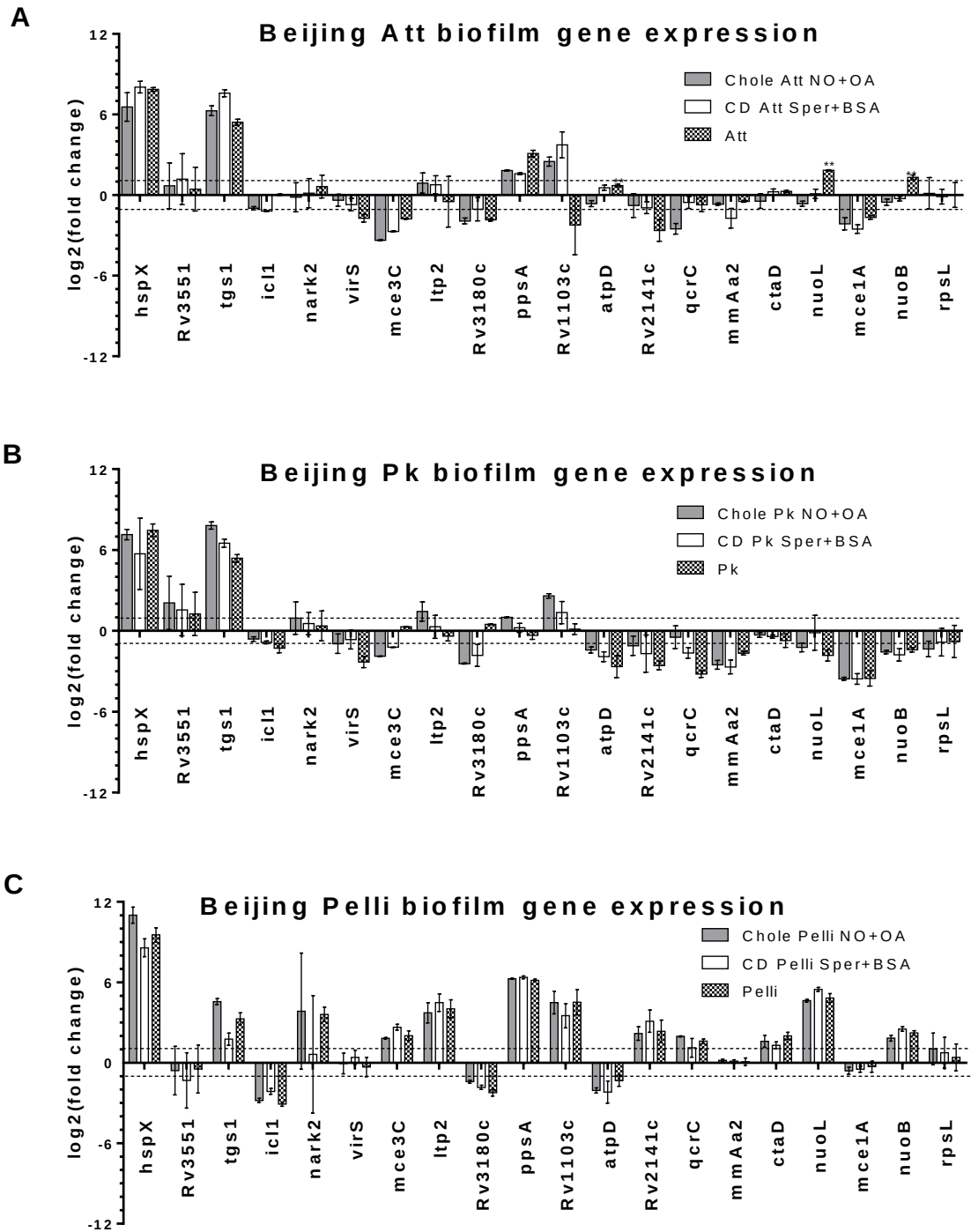


Figure 53: The gene expression of 20 selected genes for *Mtb* Beijing65 strain biofilm layers under selected growth conditions.

Bar charts display gene expression of 20 selected target genes in *Mtb* Beijing65 strain for (A) Att biofilm with Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, Sauton's (B) Pk biofilm with Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, Sauton's and (C) Pelli biofilm with Cholesterol/Nitric Oxide/Oleic Acid, CD/Spermine-HCl/BSA, Sauton's. All ($\log_2$) fold changes calculated against aerobic exponential growth. Dotted line represents 2.5-fold ($1\log_2$ -fold change). Asterisks indicate significant result.

5.4.3.5. *M. tuberculosis* Beijing65 Biofilm Cell layers' correlation to Sputum Transcriptome

The correlation of the gene expression between Mtb Beijing biofilm layers and the sputum transcriptome targeting 20 selected genes was done using Spearman's correlation measurement. Table 17 represents p-values, R-rank values and 95% confidence intervals of the correlation between each Beijing biofilm layer under specific condition and the sputum transcriptome.

Results in Table 19 reveal that the Pk biofilm layer from all conditions correlates significantly with sputum transcriptome. The gene expression results of all Pk biofilm conditions show that there are 9 downregulated genes out of 10 and 6 upregulated genes out of 10 (Figure 53). Also, all aerobic respiration genes and ribosomal genes are downregulated in all Pk biofilm conditions. The *DosR* regulon genes (*hspX*, *tgs1* and *nark2*) are highly upregulated in all Beijing65 Pk biofilm conditions. As for H37Rv Pk biofilm the *icl1* gene is downregulated in all Beijing65 Pk biofilm conditions. The other lipid and cholesterol metabolism genes *ltp2*, *ppsA* and *Rv3551* are upregulated in all Pk biofilm conditions except for the basic condition in which the *ltp2* cholesterol utilisation gene is downregulated.

Using Spearman's ranking to compare Att Beijing65 biofilm conditions with the sputum transcriptome reveals that none of the Att Beijing65 biofilm conditions show significant correlation with the sputum transcriptome (Table 19). Surprisingly, the basic Att biofilm shows an upregulation of the *icl1* gene (Figure 53). However, the upregulation might not be significant as it is lower than 2-fold.

There is no correlation between Beijing Pellicle biofilm conditions and sputum transcriptome as Spearman's ranking shows in Table 19. In contrast to the H37Rv Pellicle biofilm conditions, all Beijing65 Pellicle biofilm conditions show upregulation of most of the 20 selected genes (Figure 53). The obvious findings are the upregulation of the respiratory genes in all Beijing65 Pellicle biofilm conditions. Moreover, the ribosomal gene *rpsL* is upregulated in all Beijing65 Pellicle biofilm conditions. Both upregulation in the respiratory and ribosomal genes are expected as the Pellicle layer closer to the air (air-interface).

Table 19: Spearman's Rank Correlation R and p-values are displayed for each condition vs. Sputum Gene Expression.

Sample	Ch-NO-OA	Cyclo-Sper.HCl-BSA	Sauton`s
Att	R = 0.3589 P = 0.1202 CI = (-0.1134-0.6989)	R = 0.3499 P = 0.1305 CI = (-0.1236-0.6936)	R = 0.2024 P = 0.3921 CI = (-0.2769-0.6010)
Pk	R = 0.5929 P = 0.0059 CI = (0.1903-0.8284)	R = 0.5974 P = 0.0054 CI = (0.1970-0.8270)	R = 0.6501 P = 0.0019 CI = (0.2784-0.8524)
Pellicle	R = 0.1242 P = 0.6020 CI = (-0.3494-0.5472)	R = -0.01279 P = 0.9573 CI = (-0.4639-0.4436)	R = 0.04816 P = 0.8402 CI = (-0.4148-0.4913)

CI = 95% confidence intervals

In general, all Beijing65 biofilm layers under selected conditions show no significant correlation with the sputum transcriptome except the Pk layer (Figure 54). Thus, the result of Beijing65 gene expression correlation with sputum transcriptome is identical with what was shown with H37Rv biofilm in this Chapter. Furthermore, results of H37Rv biofilm gene expression and Beijing65 biofilm gene expression revealed higher correlation of the laboratory strain than the clinical strain with sputum transcriptome.

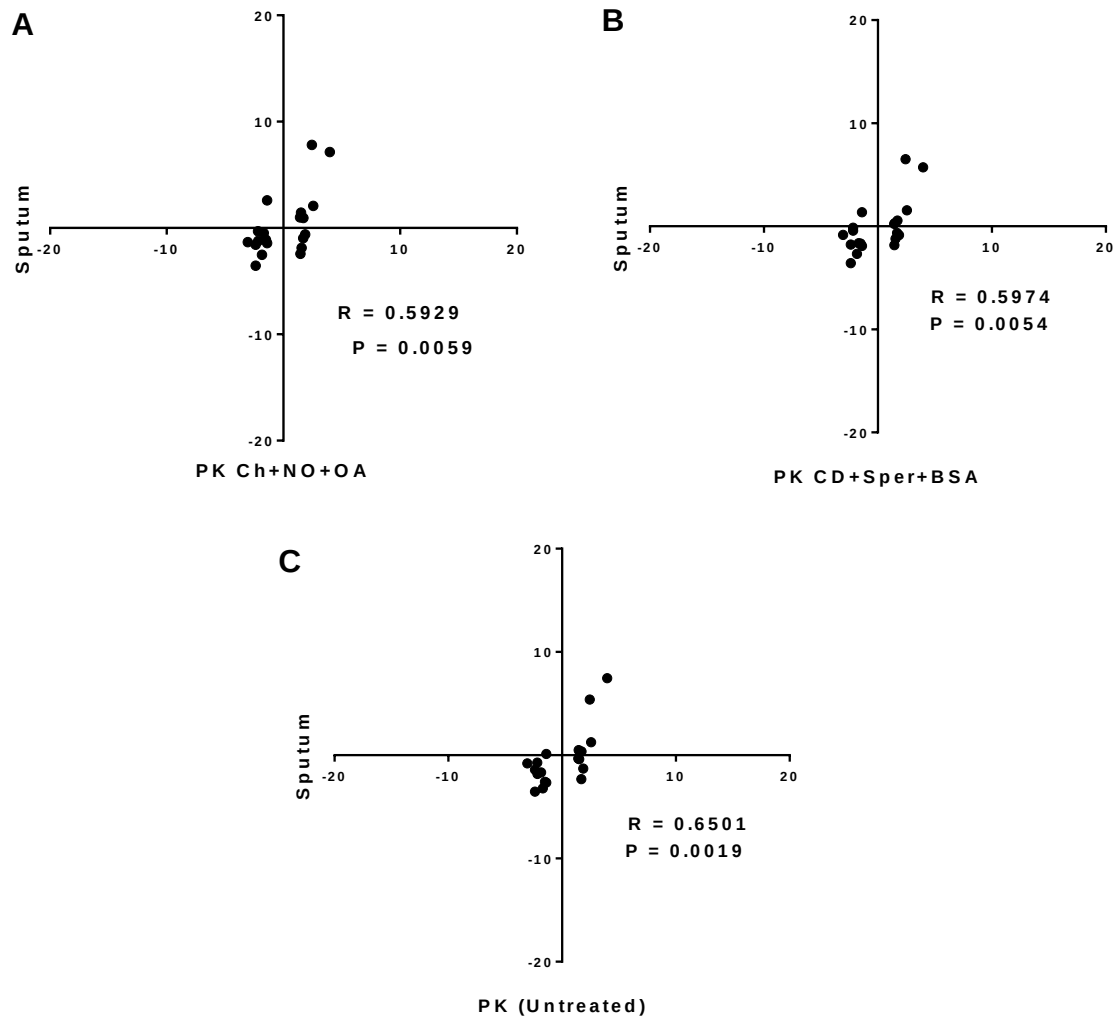


Figure 54: Scatter Plots comparing Pk Beijing65 biofilm in the three stimuli vs. Sputum Gene Expression.

Scatter plots display gene expression of Pk *Mtb* Beijing65 biofilms with and without multi-stimuli versus Sputum Gene Expression. Graphs show Spearman's Rank Correlation R and p-values for each graph.

5.4.3.6. Comparison of gene expression levels with different normalisation procedures

The bacterial biofilm is known to be genetically and phenotypically altered due to environmental and nutritional conditions (Stoodley *et al.*, 2004). This stress may change the gene expression of the ribosomal genes such as 16S rRNA and *sigA* (Larsson *et al.*, 2012), although these genes have been used as housekeeping genes of many studies (Wu *et al.*, 2009, Garton *et al.*, 2008 and Larsson *et al.*, 2012). Lee,

(2012) compared the gene expression stability of 12 candidate genes in stationary phase, SDS, PBS, exponential phase and RPMI growth conditions. The study revealed that the most stable genes were *thyA*, *polA*, *dfrA* and *aroA* in different H37Rv growth conditions. Ribosomal genes 16S rRNA and *sigA* were two of three least-stable genes (Lee, 2012). Therefore, it was required to compare the gene expression level of the six housekeeping genes.

The comparison was done with H37Rv Pk biofilm cells with Spermine-HCl, as it is the closer condition to sputum transcriptome. Three DosR regulon genes *hspX*, *tgs1* and *narK2* were used in this study, as they were known to be highly induced during stress. Results in Figure 55 shows that the 16S rRNA housekeeping gene is significantly upregulated by 3 log₂-folds than *sigA*, *thyA*, *polA*, *dfrA* and *aroA* housekeeping genes (4HKGs). Furthermore, *sigA* housekeeping shows significant upregulation of *tgs1* gene compared with the 4HKGs. However, *sigA* has shown closer gene expression level to the 4HKGs.

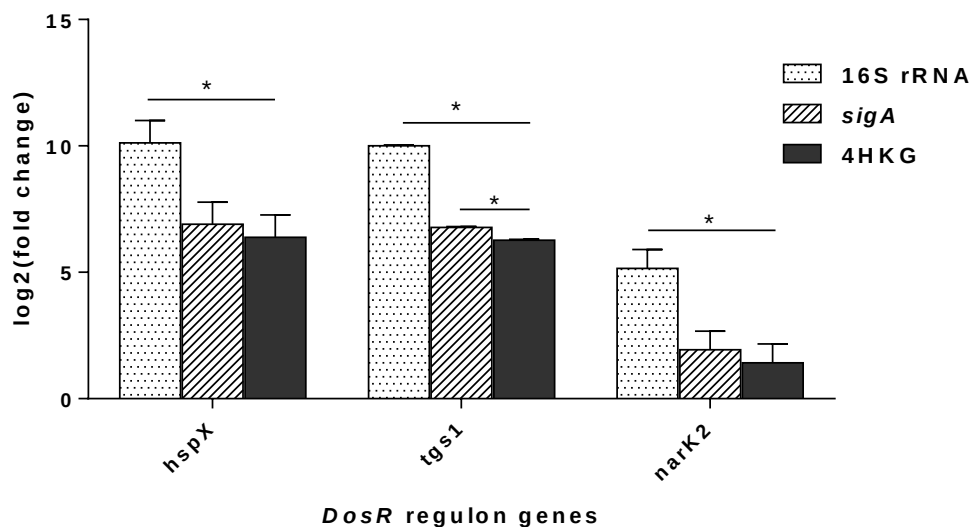


Figure 55: Comparison of DosR regulated gene expression levels with different normalisations.

Bar graphs comparing the *DosR* regulon selected genes (*hspX*, *tgs1* and *narK2*) of Pk Mtb H37Rv biofilm with multi-stimuli (the highest correlated condition with sputum) in different optimisation housekeeping genes. Displayed columns show the gene expression of normalisation with 16S rRNA (green columns), *sigA* (red columns), and 4HKG (white columns). Error bars represent the SD of three (log₂) values of each gene. Unpaired t-tests were used to compare the different growth conditions; asterisks indicate significant results.

5.4.4. *M. tuberculosis* biofilm antibiotic sensitivities

In the current work, it was revealed that Pk and Att Mtb biofilm cells from both H37Rv and Beijing65 strains have closer correlation with sputum transcriptome. This leads to a question whether Mtb biofilm cells share another phenotypic characteristic with sputum. The bacterial biofilm is known to be non-responsive to antimicrobial drugs, which may be due to the persisters that arise as a result of the phenotypic heterogeneity in the bacterial biofilm (Spoering *et al.*, 2001; Dhar *et al.*, 2007). Hence, to assess other phenotypic similarities between Mtb biofilm and sputum, a drug tolerance assay was performed.

Previously, Corper and Cohn (1933) studied the *in vitro* growth of human and bovine Mtb isolates in sealed containers and they noticed that 24 out of 56 cultures included culturable bacilli even after 12 years of incubation. From this study it was revealed the *in vitro* characteristics of Mtb persist in a bacteriostatic environment (Corper & Cohn, 1933). However, the evidence of the mycobacterial biofilm formation in the human lung cavity is still not proven.

H37Rv and Beijing65 were used to assess biofilm-associated drug tolerance. Biofilm and mid-exponential phase cultures were incubated for 7 days with direct exposure to 5µg/ml⁻¹ RIF and 1µg/ml⁻¹ of INH. After incubation, the biofilm layers as well as the mid-exponential phase Mtb cells were harvested then CFU analysis was carried out.

Results in Table 20 represent the log₁₀ data and CFU proportions of the three Mtb biofilm layers and mid-exponential phase cells of H37Rv and Beijing65 strains in 7 days exposure to RIF and INH antibiotics. The results in the table also represent the relative tolerance to mid-exponential phase cells.

In mid-exponential phase for H37Rv and Beijing65 Mtb cells, incubation with either RIF or INH lead to an increased rate of death after 7 days of exposure. In contrast, the H37Rv Pellicle biofilm cells show a high proportion of cells (45%) remaining alive with INH. On the other hand, the majority of H37Rv Pellicle cell population is killed by 5µg/ml⁻¹ RIF. Although, Beijing65 Pellicle shows different reading in the viability proportion after exposure to INH and RIF, the killing trend is similar to H37Rv Pellicle as RIF kills a higher proportion than INH.

Mtb H37Rv and Beijing65 Pk biofilm cells show higher response to RIF as ~98% and 95.4% of cell population is killed subsequently. However, the H37Rv and Beijing65 Pk biofilm cells show less response to the INH as shown in Table 18. It has been

observed that about 4% of the H37Rv Pk biofilm cell population are drug-tolerant to the INH, whereas ~10% of Beijing65 Pk biofilm cell population are drug-tolerant to INH.

The Att biofilm H37Rv and Beijing strains show higher persistence to the RIF and INH exposure. Mtb H37Rv Att biofilm shows ~27% of the cell population is still alive even after 7 days of exposure to $1\mu\text{g/ml}^{-1}$ of INH. Also, the greater proportion of drug-tolerant persists to high RIF concentration is observed in the Mtb H37Rv Att biofilm cells with a viability proportion of 8%. The Mtb Beijing65 Att biofilm cells show about 30% of cell populations persist against RIF, whereas a lower proportion of 0.74% persists against INH.

The relative tolerance of Mtb H37Rv biofilm cells compared to the mid-exponential phase cells shows higher tolerance with Pellicle and attached cells against INH. The same trend is shown with Beijing65 Pellicle and Att cells against INH except the Pellicle which shows lower tolerance compared to mid-exponential phase cells. Pk and Att cells in both H37Rv and Beijing65 show much higher tolerance to RIF than Pellicle cells in both strains compared to mid-exponential phase cells.

These results suggest that cells in biofilm show drug-tolerance persistence against high INH and RIF concentrations. This indicates that cells within the Mtb biofilm may not be actively growing. In addition, there are obvious variations in the cell response to the antimicrobial treatments between the different biofilm layers due to the heterogeneity of the Mtb biofilm.

Table 20: *M. tuberculosis* biofilm cells' drug sensitivity performed by colony-forming unit (CFU) counts.

Cells	Treatment	H37Rv Log10 CFU ($\pm$ SD)	CFU %	Relative tolerance	Beijing65 Log10 CFU ($\pm$ SD)	CFU %	Relative tolerance
Mid-Exp phase	No drug	8.7 ($\pm$ 0.5)	100	-	8.4 ($\pm$ 0.3)	100	-
	INH	6.4 ($\pm$ 1.6)	0.5	-	6.2 ($\pm$ 0.1)	0.6	-
	RIF	3.8 ($\pm$ 0.5)	0.001	-	3.2 ($\pm$ 0.2)	0.000 5	-
Pellicle	No drug	6.8 ($\pm$ 0.3)	100	1	7.9 ($\pm$ 0.8)	100	1
	INH	6.5 ($\pm$ 0.1)	45	90	5.3 ($\pm$ 0.1)	0.2	0.3333
	RIF	4.1 ($\pm$ 0.4)	0.2	200	4.9 ($\pm$ 0.3)	0.08	160
Planktonic	No drug	4.6 ($\pm$ 0.4)	100	1	4.8 ($\pm$ 0.3)	100	1
	INH	3.2 ($\pm$ 0.1)	4	8	3.8 ($\pm$ 0.1)	9.2	15.333
	RIF	2.8 ($\pm$ 0.3)	1.4	1400	3.5 ($\pm$ 0.1)	4.6	9200
Attached	No drug	7 ($\pm$ 0.01)	100	1	6.9($\pm$ 0.3)	100	1
	INH	6.4 ($\pm$ 0.8)	26.7	53.4	6.3 ($\pm$ 0.2)	30	50
	RIF	5.8 ($\pm$ 0.57)	8	8000	4.7 ($\pm$ 0.5)	0.74	1480

The table shows the effect of RIF and isoniazid treatments on *in vitro* viability of Mtb biofilm cells. Log10 CFU values and drug tolerance ratios of H37Rv and Beijing65 biofilm and Mid-Exponential cells after 7 days of incubation with isoniazid ($1 \mu\text{g ml}^{-1}$) and RIF ($5 \mu\text{g ml}^{-1}$) for 7 days. Relative tolerance values of the biofilm were calculated by deviding the %CFU of the biofilm by the %CFU of the control (mid-exponential phase cells). Antibiotics were injected directly into the Pk layer of biofilm cultures. SD displays the mean of three biological replicates.

5.4.5. *M. tuberculosis* H37Rv Resuscitation Promoting Factors (Rpf) Assessment

5.4.5.1. Experimental Strategy

The experimental strategy that was used to assess the Mtb H37Rv Rpf-dependency is detailed in Figure 56.

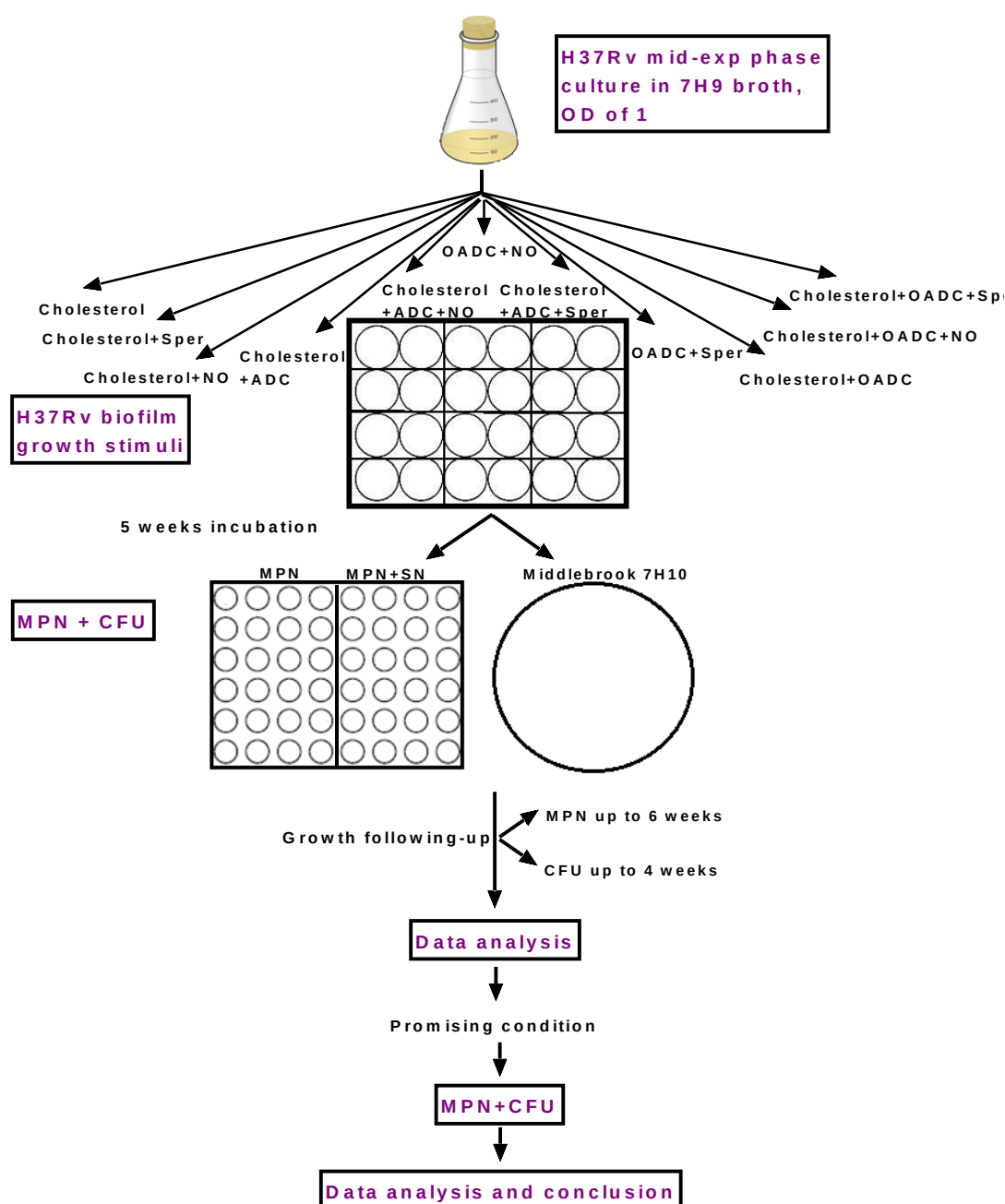


Figure 56: Pictorial representation of the Rpf-dependency assessment experiment.

The schematic diagram demonstrates the Mtb H37Rv Att and Pk biofilm Rpf dependency assessment starting from the biofilm preparation in multiple growth stimuli through to the MPN and CFU of the initial promising condition and ending with the data analysis and conclusions.

In the current work, it was revealed that the Mtb biofilm shows drug-tolerance to RIF and INH. This added some weight to the hypothesis that Mtb biofilm might replicate sputum phenotypes. However, it was necessary to assess the other phenotypes that Mtb biofilm might share with Mtb from sputum.

One of the most important characteristics of Mtb in sputum is the Rpf-dependency. Mukamolova *et al.*, (2010) have shown that smear-positive human sputum is dominated by a population that requires Rpf proteins to grow. Furthermore, in the same study, it was revealed that these Rpf-dependent populations are RIF tolerant (Mukamolova *et al.*, 2010). Thus, it was essential to assess Mtb biofilm cells Rpf-dependency to assess if they share this characteristic with Mtb from sputum.

This experiment was performed initially by developing the Mtb H37Rv biofilm under 11 conditions. The reason behind using multiple stimuli was to mimic the Mtb environment in the lung cavity. Table 21 represents all conditions that were used. The statistical analyses for CFU and MPN were done independently. The resuscitation index (RI) was calculated by subtracting the log₁₀ culture supernatant (SN) by the log₁₀ CFU values. The more significant RI should be 1 and more.

Table 21: Growth conditions and additional stimuli of the Mtb H37Rv biofilm.

Test culture	Control culture	Without NO/Sper.HCl
culture resuspended in Sauton`s/ADC pre-prepared with 0.01% (w/v) cholesterol/CD with added spermine NOnoate solution (4h time-point)	culture resuspended in Sauton`s/ADC pre-prepared with 0.01% (w/v) cholesterol/CD with added Spermine hydrochloride solution (4h time-point)	culture resuspended in Sauton`s/ADC pre-prepared with 0.01% (w/v) cholesterol/CD
culture resuspended in Sauton`s/OADC (BD BBL) pre-prepared with 0.01% (w/v) cholesterol/CD with added spermine NOnoate solution (4h time-point)	culture resuspended in Sauton`s/OADC (BD BBL) pre-prepared with 0.01% (w/v) cholesterol/CD with added Spermine hydrochloride solution (4h time-point)	culture resuspended in Sauton`s/OADC (BD BBL) pre-prepared with 0.01% (w/v) cholesterol/CD
culture resuspended in Sauton`s/OADC (BD BBL) with added spermine NOnoate solution (4h time-point)	culture resuspended in Sauton`s/OADC (BD BBL) with added Spermine hydrochloride solution (4h time-point)	culture resuspended in Sauton`s/OADC (BD BBL)
culture resuspended in Sauton`s pre-prepared with 0.01% (w/v) cholesterol/CD with added spermine NOnoate solution (4h time-point)	culture resuspended in Sauton`s pre-prepared with 0.01% (w/v) cholesterol/CD with added Spermine hydrochloride solution (4h time-point)	culture resuspended in Sauton`s pre-prepared with 0.01% (w/v) cholesterol/CD

5.4.5.2. The Rpf-dependency assessment of *M. tuberculosis* Pk and Att biofilm cells under multiple growth conditions

This work was performed on Mtb Pk and Att biofilms, which were grown under multiple stimuli as shown in Table 21. This experiment was done in large scale in order to choose the candidate condition for further investigation. All samples were done in duplicate and two users performed the work and the statistical analyses independently. Some samples were mould-contaminated, and were therefore excluded and indicated as N/A in Table 22.

Table 22: Rpf-dependancy assessment assay of *M. tuberculosis* H37Rv Att and Pk biofilm cells with multiple stimuli.

Pk (Log10)					Att (Log10)				
Condition	CFU-1	MPN-1	MPN+SN-1	RI-1	Condition	CFU-1	MPN-1	MPN+SN-1	RI-1
	CFU-2	MPN-2	MPN+SN -2	RI-2		CFU-2	MPN-2	MPN+SN -2	RI-2
Ch	5	5.5	5.9	0.9	Ch	5.4	5.1	5.5	0.1
	5.5	5.5	6.1	0.6		6.4	6.4	5.9	-0.5
Ch + NO	4.8	4.9	5.1	0.3	Ch + NO	5.9	5.4	6.4	0.5
	4.9	5.1	5.3	0.4		7.6	7.4	7.9	0.3
Ch + Sper	5	5.1	5.1	0.1	Ch + Sper	4.8	3.9	4.9	0.1
	5.5	5.5	5.5	0		6.4	7.1	6.8	0.6
ADC + Ch	4.6	5	5.9	1.3	ADC + Ch	5.1	5.1	5.9	0.8
	5.4	5.5	5.1	-0.3		N+A	7.1	6.5	N+A
ADC + Ch + NO	4.7	4.9	5.3	0.6	ADC + Ch + NO	5.7	5.7	5.9	0.2
	5.3	5.3	5.9	0.6		N+A	N+A	N+A	N+A
ADC + Ch + Sper	4.8	4.7	5.5	0.7	ADC + Ch + Sper	5.8	5.1	5.9	0.1
	4.7	4.3	5.1	0.4		6.4	6.5	6.9	0.5
OADC	N+A	N+A	N+A	N+A	OADC	N+A	N+A	N+A	N+A
	N+A	N+A	N+A	N+A		N+A	N+A	N+A	N+A
OADC + Ch	5.7	4.4	4.6	-1.1	OADC + Ch	5.4	4.7	5.3	-0.1
	5.7	4.4	4.6	-1.1		N+A	N+A	N+A	N+A
OADC + NO	4.6	5.4	4.4	-0.2	OADC + NO	6.4	6.9	5.6	-0.8
	N+A	N+A	N+A	N+A		N+A	N+A	N+A	N+A
OADC + Sper	4	5.4	5.5	1.5	OADC + Sper	5	5.1	4.9	-0.1
	5.8	5.9	6.1	0.3		5.5	6.1	6.1	0.6
OADC + Ch + NO	4.7	5.1	5.3	0.6	OADC + Ch + NO*	5.3	5.4	4.9	-0.4
	4.5	4.9	4.6	0.1		6.5	8.1	7.5	1
OADC + Ch + Sper	3.9	4.7	5.4	1.5	OADC + Ch + Sper	3.4	5.1	5.3	1.9
	5.4	5.1	5.5	0.1		7	7.1	8.1	1

Results in Table 22 represent log10 MPN (with and without SN), CFU and the RI values of Att and Pk H37Rv biofilm cells. Also, results observe large variations between the two user's readings of MPN, SN and CFU counts. Although there was a

large variation between replicates, the aim was to identify the best conditions to form Rpf-dependent Att and Pk H37Rv biofilm cells.

By looking at the RI in Table 22, it can be observed that adding OADC+Ch+Sper and to the biofilm medium enhances the formation of Rpf-dependent populations in both Att and Pk biofilm layers where the SN above the 7H9 and 7H10 controls (RI is ~1). Further, the Att biofilm cells, which were supplemented with OADC+Ch+Sper, showed higher RI than Pk cells with the same condition. Hence, by presenting these results to Rpf experts, the decision was to choose Att biofilm cells supplemented with OADC+Ch and exposed to NO as it was thought that NO should have an effect on Mtb Att biofilm cells in contrast to the control Sper.

5.4.5.3. Rpf-dependency of *M. tuberculosis* Attached biofilm cells with multiple stimuli

Att H37Rv biofilm cells were grown in Sauton's medium supplemented with OADC and Cholesterol and exposed to NO. The experiment was done in four biological replicates, and each replicate was taken from independent 12-well plates. After 5 wks of incubation, the biofilm was harvested according to the protocol in Section 5.3.3. MPNs and CFUs were performed in 4 technical replicates.

Figure 57a shows that SN has significant stimulation above CFU and 7H9 Middlebrook controls on resuscitating the Att H37Rv biofilm cells in 3 replicates out of 4. The fourth replicate did not show significant stimulation above the CFU control. However, by taking the mean and the SD results in Figure 57b, there is no significant difference between the SN and the CFU count in contrast to the Middlebrook 7H9 control, where the SN exceeded the cell count obtained.

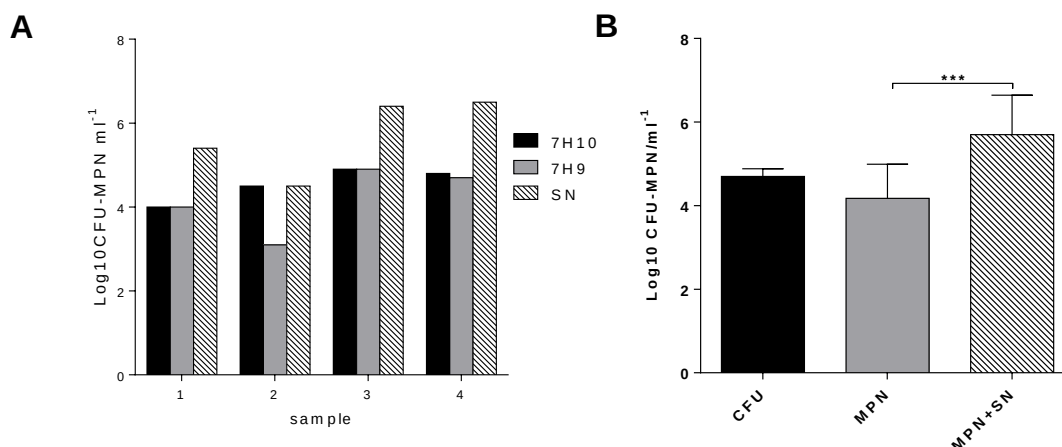


Figure 57: Effect of culture supernatant on the Att *M. tuberculosis* biofilm cells.

Graphs show the effect of the 7H9+SN on the Att Mtb H37Rv cells grown with Cholesterol/NO/OADC. (A) Shows 4 Att biofilm replicates compare the effect of the SN+MPN on the Att biofilm cells in comparison to the MPN and CFU. (B) Shows the average log₁₀ of the 4 replicates. Error bars represent the SD of three (log₁₀) values for each replicate. Asterisks indicate significant data.

Table 23 shows the log₁₀ MPN, CFU and MPN+SN counts of each replicate of the biofilms. The RI was also accounted for (SN-CFU and SN-MPN). Results in the table signify that out of 4 replicates, 2 replicates are shown between 1.5-1.7 log₁₀ difference between the SN and CFU. However, the RI of the other two replicates shows no significant stimulation of the SN on the Att biofilm cells. Hence, the difference between the CFU control and the SN is not significant if the RI mean is taken of the 4 replicates.

The RI of the SN-MPN indicates that the difference between the SN and the MPN control is significant. The RI of all 4 replicates shows above 1 value of SN+MPN than MPN-free supernatant. Therefore, the Att biofilm cells in this condition are inhibited in MPN-SN and stimulated by MPN+SN.

The trend for MPN with no SN log₁₀ counts to be lower than CFU log₁₀ counts is represented in Table 23 by derivation of an inhibitory index (II = log₁₀ MPN/CFU). The II counts in Table 23 show that there is more than 0.5 log₁₀ inhibition in two replicates, compared with the other two.

Table 23: Rpf-dependancy assessment assay of *M. tuberculosis* H37Rv Att biofilm cells with OADC+Cholesterol+NO.

Sample	Log10 CFU	Log10 MPN	MPN+SN	RI (SN-CFU)	RI (SN-MPN)	II (MPN/CFU)
1	4.6	4	5.4	0.8	1.4	-0.6
2	4.5	3.1	4.5	0	1.4	-1.4
3	4.9	4.9	6.4	1.5	1.5	0
4	4.8	4.7	6.5	1.7	1.8	-0.1
Mean±SD	4.7±0.18	4.1±0.81	5.7±0.94	1±0.77	1.5±0.18	-0.52

5.4.6. Assessment of *M. tuberculosis* activity within the biofilm by ³H uracil incorporation

In order to further study the condition of bacilli within the biofilms, ³H uracil incorporation was studied see 5.3.8. 30 h prior to harvesting, ³H uracil was injected into the liquid phase and uptake was assessed in the 3 biofilm layers. Figure 58 shows log10 counts of ³H uracil uptake, CFU and MPN counts and qPCR of 16S rRNA, and 16S rDNA copy numbers in the Att, Pk and Pellicle layers at 4 time points during biofilm development. The CFU count of the Att and Pellicle biofilm layers were below the limit of detection at 2 wks.

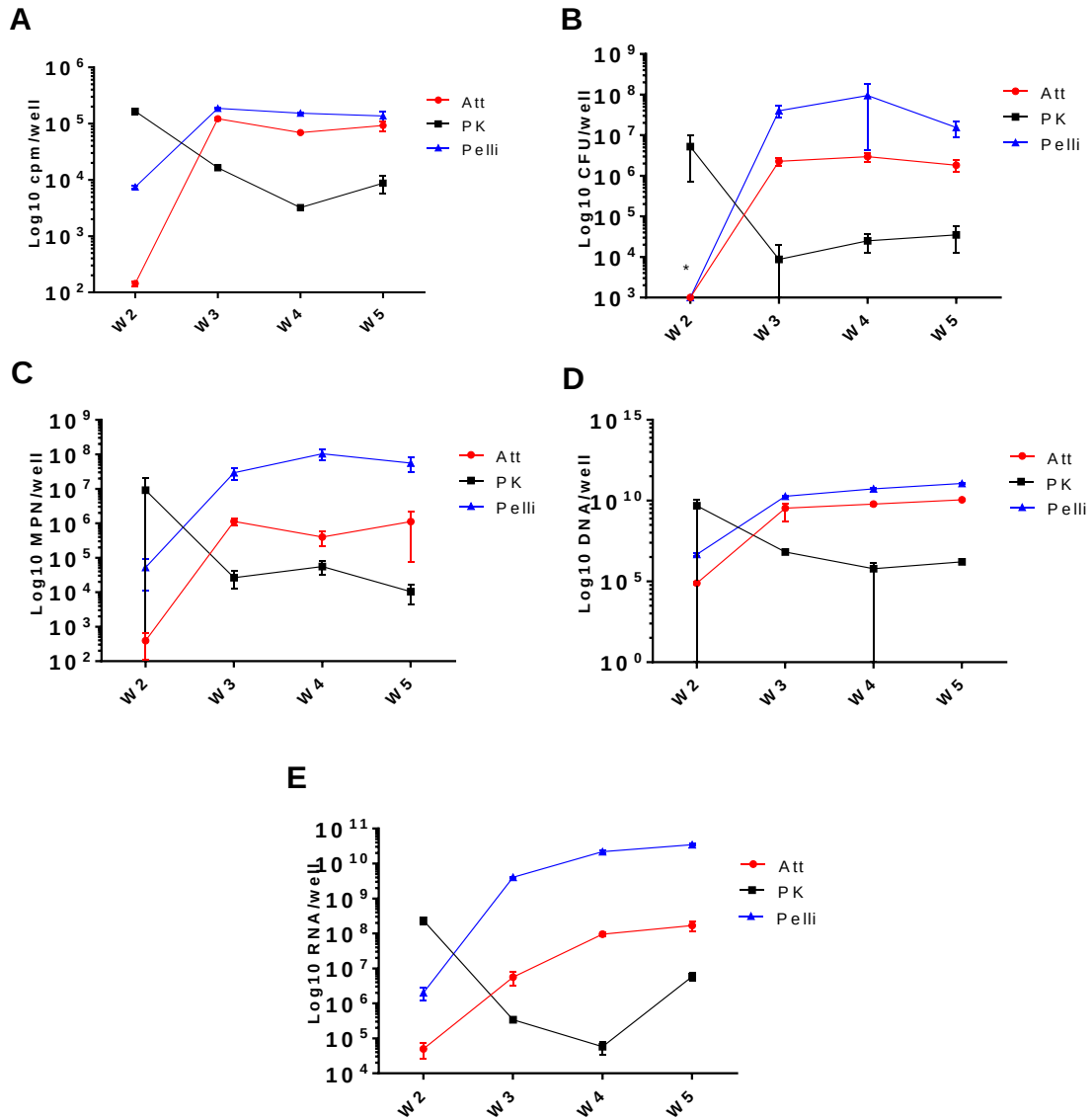


Figure 58: Development *M. tuberculosis* biofilm layers assessed by different methods.

Graphs represent the different growth count methods of Mtb biofilm layers during 4 wks. Graphs (A) Log10 ³H uracil uptake count, (B) Log10 CFU, (C) Log10 MPN, (D) Log10 RNA count and (E) Log10 DNA count. Error bars represent the SD. Asterisk points reading below detection limit.

The relative count of ³H uracil uptake compared to CFU, MPN, DNA and RNA reveals that Pellicle biofilm layer has the lowest ³H uracil uptake followed by the Att and the highest uptake is in Pk biofilm cells (Figure 59). The Pellicle biofilm layer also shows sharp drop in RNA and DNA relative counts to reach the lowest incorporation at wk 5.

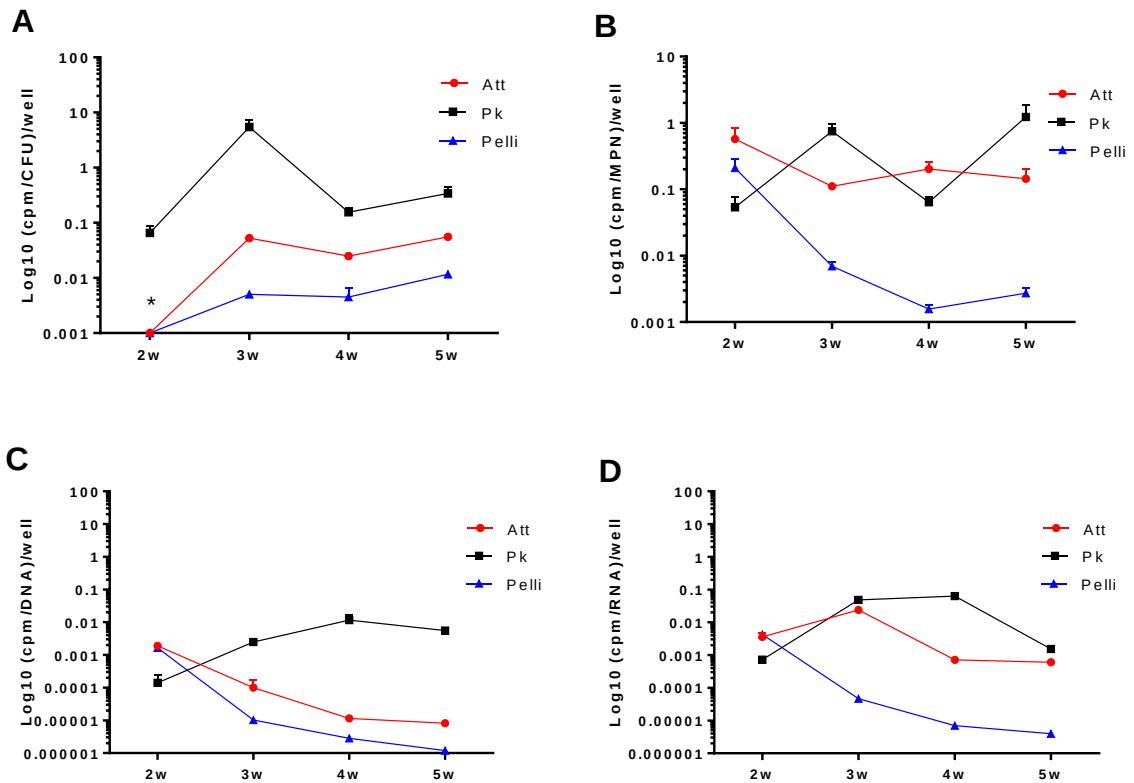


Figure 59: Measurement of ^3H Uracil uptake in biofilm layers.

Graphs show Log10 relative counts of ^3H uracil of Att, Pk and Pellicle biofilm cells per (A) CFU, (B) MPN, (C) DNA and (D) RNA. Error bars represent the 95% confidence interval. Asterisk points reading below detection limit.

Figure 60 shows the relative counts of MPN, DNA and RNA per CFU as well as the relative count of RNA per DNA. The result shows that DNA and RNA relatively increase from wk 2 to reach the highest number at wk 5 for Att and Pellicle layers. The accumulation of cells in Pellicle and Att layers is indicated by this finding as Figure 60b&c show the highest accumulation of Pellicle cells followed by Att.

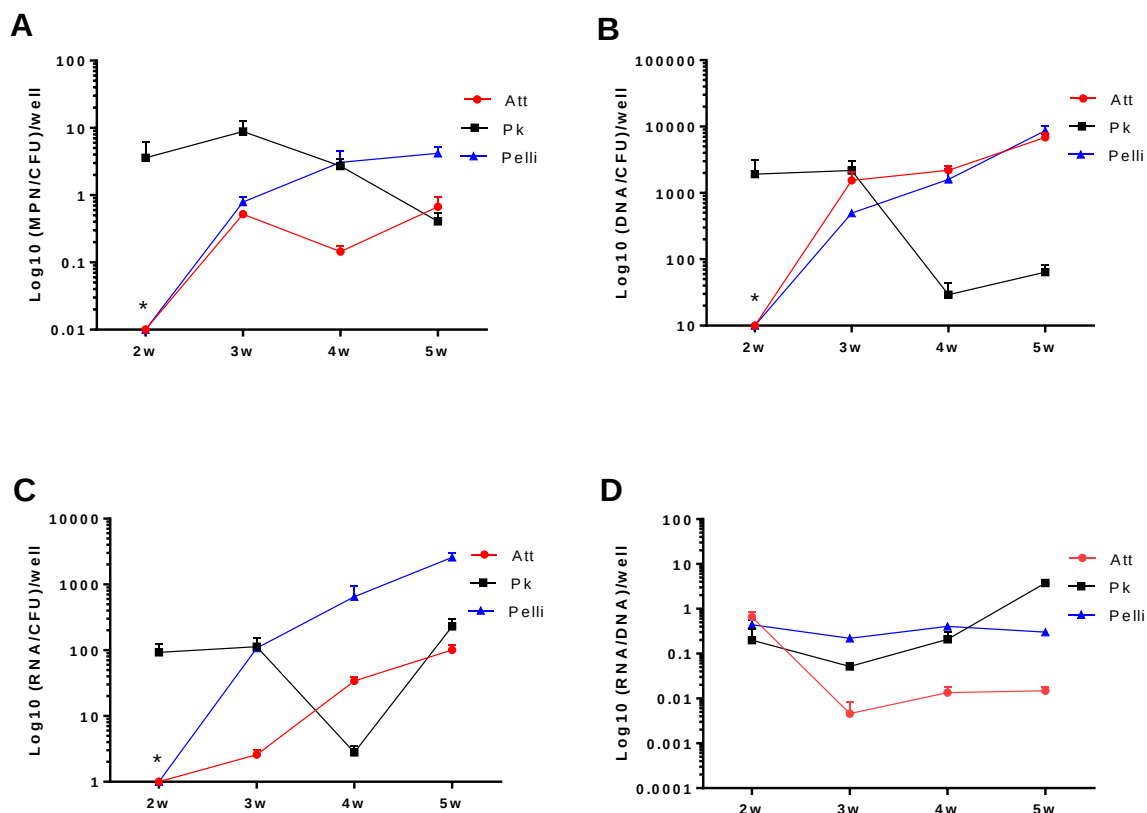


Figure 60: The relative count of CFU in biofilm layers measured by different techniques.

Graphs show the (A) MPN, (B) DNA, (C) RNA relative counts of Att, Pk and Pellicle biofilm layers compared to the CFU counts. Graph (D) shows the relative count of RNA per DNA. Error bars represent the 95% confidence interval. Asterisk points reading below detection limit.

5.4.7. Cytological studies on *M. tuberculosis* biofilms

5.4.7.1. Integrity and activity of biofilm cells

Table 24 shows percentages of un-treated Mtb biofilm Att, Pk and Pellicle layers and mid-exponential phase cells grown in Sauton's with SYTO9, PI and INT staining. Results show that the percentage of mid-exponential phase cells stained with SYTO9 is about 90% which is significantly higher than all biofilm layers. Pellicle biofilm layer contains the higher percentage of PI with about 40% compared with the Att and Pk layers which have 29% and 25% respectively. About 61% of mid-exponential cells form fromazan droplets which is significantly higher than Att and Pellicle biofilm cells.

Table 24: Averages and SD of biofilm and mid-exponential phase cells with SYTO9, PI and INT

Cells	SYTO9 Average $\pm$ (SD)	PI Average($\pm$ SD)	Formazan Average($\pm$ SD)
Mid-Exp	90 $\pm$ 4.1	10 $\pm$ 4.1	61 $\pm$ 17
Attached	71 $\pm$ 4.0**	29 $\pm$ 4.0	26 $\pm$ 7.0*
Planktonic	75 $\pm$ 5.4*	25 $\pm$ 5.5	36 $\pm$ 4.0
Pellicle	60 $\pm$ 1.4***	40 $\pm$ 1.3	17 $\pm$ 5.0*

Table shows the proportions of 5-wks grown Att, Pk and Pellicle biofilm cells and the mid-exponential phase control cells with SYTO9 and PI nucleic acid stain and INT staining by counting the formazan droplets. Asterisks indicate significant data compared with the mid-exponential phase culture.

5.4.7.2. rRNA content of biofilm cells

Figure 61 shows that the PNA median signal intensity (MFI) per area of mid-exponential phase cells is significantly higher than PNA MFI of all biofilm layers. There is no significant difference of the PNA MFI between the three biofilm layers. The result also shows that the PNA MFI of the Pellicle biofilm layer is significantly higher than the PNA MFI of the Att biofilm layer. Moreover, there is no significant difference of the PNA MFI between the Att layer and the Pk layer or between the Pk layer and the Pellicle.

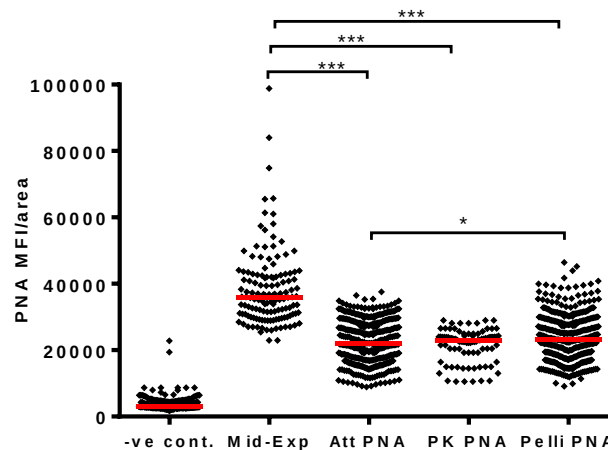


Figure 61: 5 wk biofilm cells show lower rRNA signals than mid exponential cells

Scatter dot graph shows the OK682 PNA probe median signal intensity of Att, Pk and Pellicle biofilm cells in comparison with mid-exponential phase (positive control) grown cells. PNA median signal intensities were counted automatically in triplicates for each sample (almost 100 cells per slide). Unpaired t-tests were used to compare the PNA MFI of the mid-exponential phase cells with the different variables, whereas paired t-tests were used for the comparison between the different biofilm layers. Asterisks indicate significant results.

5.4.7.3. LB frequencies in the biofilm layers

Table 25 and Figure 62 show comparisons between NO treatment, Sper control and un-treated cells in biofilm and mid-exponential phase. Results represent that the 4 h of NO incubation increases the LB proportion in mid-exponential phase by 2- to 4-fold compared to the Sper and un-treated cells.

On the other hand, that there is no effect attributable to NO exposure in the biofilm cells from all layers. Results also show heterogeneity within the biofilm as Att and Pk cells show higher LB proportions than Pellicle.

The lower LB proportions in Pellicle indicate that the oxygen exposure or the air-interface might reduce the LB proportion. The LB proportion of the Pellicle biofilm cells is in between 42-49%. This LB proportion of Pellicle biofilm cells in basic condition is much higher than the mid-exponential phase cells in the basic condition, which indicates that the extreme condition within the biofilm matrix induces the LB proportion.

Table 25: . The LB proportions (%) of Mtb H37Rv biofilm layers and mid-exponential phase cells in different culture stimuli.

Sample	LB%	#cells
Mid-Exp NO	41.2	485
Mid-Exp Sper	21.8	352
Mid-Exp	11.2	398
Att NO	89.4	270
Att Sper	75.7	288
Att	79.0	315
Pk NO	88.9	210
Pk Sper	80.2	268
Pk	82.3	245
Pellicle NO	49.5	652
Pellicle Sper	43.1	498
Pellicle	42.1	510

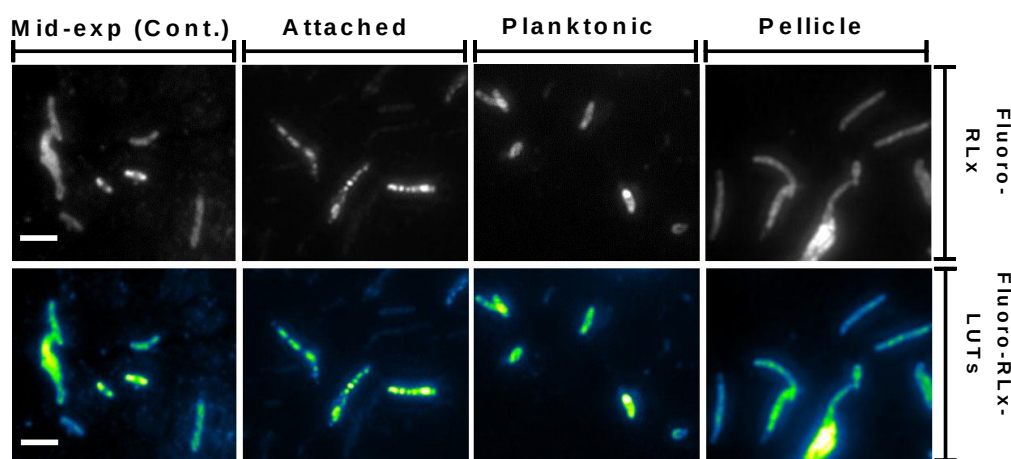


Figure 62: *M. tuberculosis* H37Rv biofilm cells LBs.

Displayed example images are LipidTox Red stained biofilm cells and the control mid-exponential phase cells in basic biofilm condition. Green-Fire-Blue LUT applied. Scale bar 3µm.

5.4.7.4. Measuring the acid-fast proportions of *M. tuberculosis* biofilm

Table 26 and Figure 63 show comparisons between NO treatment, Sper control and un-treated cells in biofilm and mid-exponential phase. Results demonstrated that biofilm cells are much less AF than short term broth cultures. Mid-exponential phase cells have the highest AF proportions (>95%) in all conditions studied while the Pellicle biofilm cells have the highest AF proportion (35-41%) amongst the biofilm layers. Att and Pk biofilm cells have very low AF proportions. NO exposure has no discernible effect on acid fastness in the short term.

Table 26: The acid-fast proportions (%) of Mtb H37Rv biofilm cells under different conditions.

Sample	Acid-fast%	#Cells
Mid-Exp NO	97.7	531
Mid-Exp Sper	95.6	511
Mid-Exp	95.7	432
Att NO	8.1	298
Att Sper	10.1	310
Att	10.5	305
Pk NO	4.0	290
Pk Sper	4.1	288
Pk	3.0	287
Pellicle NO	35.2	640
Pellicle Sper	41.9	486
Pellicle	38.0	496

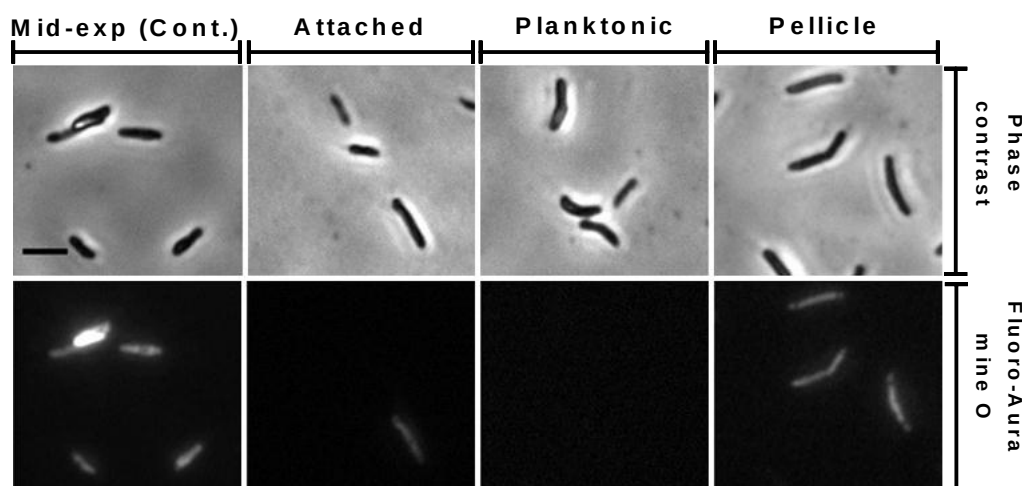


Figure 63: *M. tuberculosis* H37Rv biofilm cells acid-fastness.

Displayed example images are Auramine-O-stained biofilm cells and the control mid-exponential phase cells. Scale bar 3µm.

5.4.7.5. Auramine O staining mechanism on Pk *M. tuberculosis* biofilm cells

Pk Mtb biofilm cells are known to be non-AF bacilli. This was shown in Section 2.8.8.4, as the AF proportion of the PK H37Rv biofilm cells are about 4% under all culturing stimuli. In contrast, the mid-exponential phase Mtb cells show >95% AF proportion under all culture conditions. Thus, the question that can be addressed is does Auramine O wash off or was it difficult to penetrate the Pk biofilm cells during the staining procedure?

To answer this question, Pk H37Rv biofilm cells as well as the positive mid-exponential phase H37Rv control, both under basic conditions, were stained with Auramine O. In order to assess whether Auramine O was washed off the Mtb cells or was difficult to penetrate, the decolourisation step was performed at different time points. The first time point was using the d.H₂O for decolourisation instead of ethanol-HCl as a negative control for both Pk biofilm and the mid-exponential phase cells. Then, the decolourisation time points for Pk biofilm and the mid-exponential phase cells with ethanol-HCl were conducted for 0 (washed directly), 3, 6, 9, 12 and 15 minutes. It was analysed using three replicates from each sample and almost 100 Mtb cells were counted per sample.

Results in Figure 64 reveal that the AF proportion of the Pk biofilm cells decreases from 98% washing with water for 15 minutes to 93% after decolourisation with acid-alcohol for 0 minutes. Also, results show a dramatic decrease of the AF proportion of the Pk biofilm cells after decolourisation with acid-alcohol for 3 minutes. The AF proportion then decreases gradually with the decolourisation with acid-alcohol for 6, 9, 12 and 15 minutes. In contrast, the AF proportion of the mid-exponential phase H37Rv cells shows no difference in during different decolourisation time points. This result reveals that the Auramine O staining is washed off the Pk biofilm H37Rv cells compared with the mid-exponential phase H37Rv cells, which retain the Auramine O.

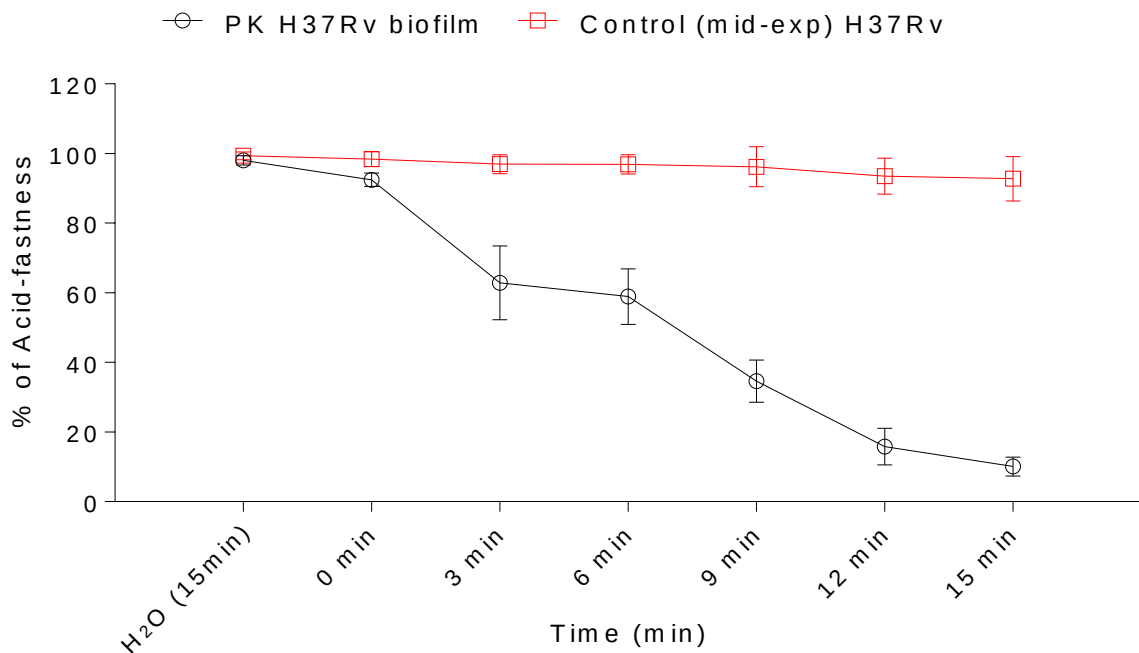


Figure 64: Decolourisation of Auramine O in different time points.

The line graph illustrates Auramine-O-stained Pk Mtb H37Rv biofilm cells (black line) and the positive control mid-exponential phase H37Rv cells (red line) at different decolourisation time points. Samples and controls were stained in triplicates at each time point and the error bars represent the SD of three replicates.

5.4.7.6. The increase of LB proportion correlates strongly with AF proportion reduction

According to the finding of Deb *et al.* (2008), the LB proportion increase indicates decrease in AF proportion; the correlation between the proportions was measured.

Pearson's correlation coefficient was performed with LB proportion values of mid-exponential phase Att, Pk and Pellicle cells under multiple stimuli against the AF proportions under the same condition.

Results in Figure 65 show the linear correlation between the AF and LB proportions of mid-exponential and stationary phase cells, Att, Pk and Pellicle cells. The linear regression shows significant correlation between the AF and LB proportions with high r^2 value of ($r^2 = 0.8206$; $P < 0.0001$). This finding indicates that when the LB proportion increases, the AF proportion decreases in Mtb H37Rv in biofilm layers and mid-exponential phase under multiple conditions.

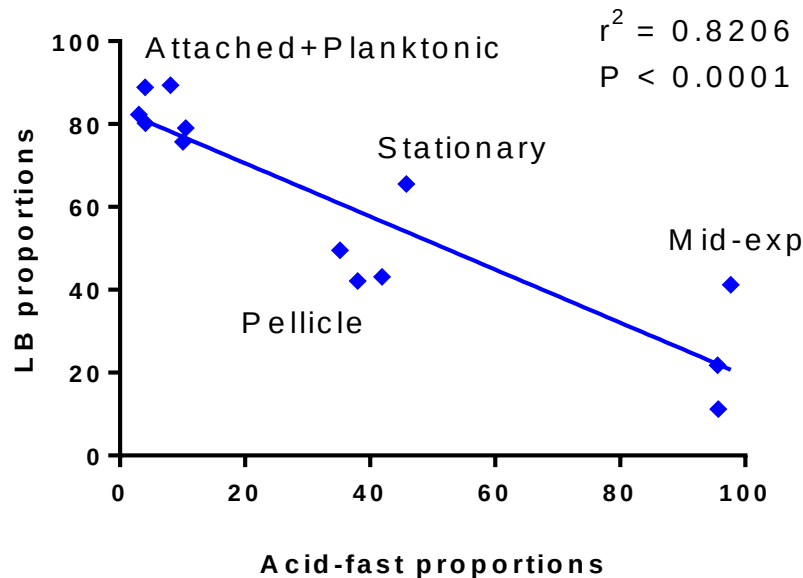


Figure 65: The correlation between LB and AF proportions.

Graph shows Pearson's correlation coefficient of LB proportions of mid-exponential phase Att, Pk and Pellicle H37Rv cells under multiple stimuli against the AF proportions under the same condition.

5.5. Discussion

Published studies on Mtb biofilm have shown that the most prominent features are Pellicle which is characterised by free MAs and a lipid-rich structure as well as high tolerance to RIF and INH. However, these studies focused on studying the whole biomass of the Mtb biofilm (Ojha *et al.*, 2008; Sambandan *et al.*, 2013). There is no published study comparing the Mtb biofilm microenvironment genetically or morphologically with the Mtb in sputum. Hence, the current study provides further detail in this regard. It was hypothesised that populations underneath the Pellicle might have distinctive phenotypic and genetic features. Thus, the biofilm was separated into three different layers.

5.5.1. Gene expression of *M. tuberculosis* under selected growth conditions

5.5.1.1. Selection of *M. tuberculosis* biofilm growth stimuli

Mtb bacilli are known to form clumping or cording structure in detergent-free liquid culture and in certain conditions such as long term starvation they form Pellicle structure (Kim *et al.*, 1976; Hunter *et al.*, 2006). It was not possible to use cholesterol/tyloxapol complex with the Mtb biofilm as tyloxapol has a detergent action by preventing cell clumping. Thus CDs, the cyclic water-soluble oligomers of glucose (Duchene, 1987), was used instead to help cholesterol absorption by Mtb cells (Christian *et al.*, 1997).

Bacteria within the biofilm are facing stresses such as nutrient starvation, hypoxia and low pH (Fux *et al.*, 2003). However, there are a number of stressors that are thought to be in the lung cavity that are not included in the current study. These stressors comprise reactive oxygen species (ROs) and phosphate deprivation (Van der Vliet *et al.*, 1999). Furthermore, the host immune system stress plays a major role in Mtb gene expression profile (Muttucumaru *et al.*, 2004). However, as an initial study the Mtb biofilm was studied under selected stimuli and the other stressors could be done in the future.

5.5.1.2. *M. tuberculosis* H37Rv Pk biofilm gene expression correlates to the sputum transcriptome

In the current study, it was shown that 16S rRNA reference gene is overexpressed from DosR regulon genes in Pk biofilm cells compared with *sigA* and the 4HKGs. In addition to overexpression, it was also shown that the 23S rRNA show lower PNA signal of all biofilm cells compared with the mid-exponential-phase-grown cells. Furthermore, it was shown that *sigA* is not constant in stationary phase and low oxygen level (Manganelli *et al.*, 1999). Thus,

the 4HKGs were used as reference genes to measure the gene expression from Mtb biofilm layers in the current study.

When Mtb H37Rv biofilm layers as well as mid-exponential phase culture in three conditions were compared with sputum microarray, the Pk biofilm layers appear to be significantly the most similar gene expression by using Spearman's ranking. The gene expression from all H37Rv Pk and Att biofilm conditions show upregulation of DosR regulated genes. However, the mid-exponential phase H37Rv gene expression when exposed to NO and OA shows higher upregulation of the DosR regulon genes. Clearly, Att and Pk biofilm cells are not responding to the NO stress. One reason might be that Pk and Att biofilm cells are already in the NRP state, therefore the NO exposure is not significantly affecting the cells gene expression.

It is noted that three genes significantly induced in sputum (*icl1*, *virS* and *ppsA*) appear to be repressed in Pk biofilm gene expression. *icl1* gene was shown to be repressed in end-stage human granuloma (Rachman *et al.*, 2006), which indicates that *icl1* is essential for early-stage growth but not late-stage persistence (Savvi *et al.*, 2008). Perhaps the cells here were well established in a NRP state. *VirS* is known to be upregulated in macrophage infection in acidic conditions (Singh *et al.*, 2003). However, it was downregulated here which might indicate that the pH of the Mtb biofilm is not reaching the level of acidity required for induction. No explanation is offered for the lack of *ppsA* induction at this stage.

The Att H37Rv biofilm cells show significant correlation with sputum transcriptome for test and control conditions, whereas the un-treated stimulus shows no correlation. However, this correlation was lower than with Pk biofilm cells. The result shows that all downregulated genes in the sputum transcriptome are also downregulated in Att biofilm test and control conditions. Rv3551, *icl1*, *virS* and *ppsA*, are all downregulated in all Att biofilm conditions. The downregulation of Rv3551, which involves in the catabolic gene cluster encoding cholesterol catabolism in macrophages as well as virulence, was not expected. However, *ltp2* the cholesterol metabolism gene is upregulated, which means that Rv3551 might be affected by the cholesterol concentration as the Att biofilm cells consumed the cholesterol in the medium for 5 wks.

The H37Rv Pellicle biofilm gene expression exposes predominantly downregulation of 16 selected genes out of 20. The DosR regulon gene *narK2* which is known to be essential for nitrate respiration during anaerobic dormancy was repressed in all Pellicle biofilm conditions. In addition, the other DosR regulon genes showed lower upregulation in all Pellicle biofilm conditions compared with the Att and Pk biofilm layers. This difference might be due to the location of the Pellicle cells, where they are exposed to the air-interface layer where the

upper side is exposed to air and the inner side exposed liquid, there might be heterogeneity in transcriptional features of Pellicle biofilm cells. The Pellicle H37Rv cells have distinctive and modified gene expression compare to Att and Pk biofilm layers. The gene expression of all H37Rv biofilm layers revealed downregulation in *rpsL* gene which indicates that biofilm cells may be in a NRP state.

Using the same 20 genes, the gene expression of Mtb H37Rv Pk biofilm exposed to Cholesterol+NO+OA and CD+Sper+BSA in the current study showed better correlation to sputum transcriptome than PBS and RPMI exposed Colesterol+NO+OA which were tested by Lee, (2012). The LB percentage of Pk biofilm cells from all conditions was higher than PBS and RPMI exposed Colesterol+NO+OA.

Furthermore, it has been hypothesised that bacteria in biofilms are stationary-phase like cells (Beloin *et al.*, 2005). By comparing the gene expression of H37Rv biofilm layers of the 20 selected genes and growth conditions with the gene expression of an un-treated stationary phase culture tested by Lee, (2012), there was no correlation with any of the biofilm layers (data not shown). The LB percentage of the stationary phase culture is lower than those of Pk biofilm cells. Relying on these comparisons, biofilm cells differ in their gene expression from stationary phase cells. Moreover, the biofilm system represents an improved *in vitro* system to replicate sputum phenotypes than the conditions tested by Lee, (2012).

5.5.1.3. *M. tuberculosis* Beijing65 Pk biofilm gene expression correlates to the sputum transcriptome

Interestingly, all Mtb Beijing65 biofilm layers' gene expression shows no correlation with sputum transcriptome except Pk biofilm layers. However, Beijing65 Pk biofilm gene expression showed lower correlation with sputum transcriptome than Pk H37Rv biofilm. This result may reflect the fact that the DosR dormancy regulon in Mtb Beijing strain is constitutively upregulated (Reed *et al.*, 2007). This was shown clearly in the current study as *hspX* is highly upregulated in Pellicle Beijing65 biofilm layer compared with all H37Rv biofilm conditions. Furthermore, *tgs1* is shown to be overexpressed in all Att and Pk Beijing65 biofilm conditions than Pellicle biofilm layer in all conditions. One of the observations in the current study also is the *tgs1* gene in Beijing65 Pellicle from all conditions is significantly higher than the *tgs1* of the Pellicle H37Rv layers. This supports the finding that the W-Beijing lineage overproduces triglycerides (Reed *et al.*, 2007).

The aerobic respiration genes are shown to be upregulated in the Beijing65 Pellicle layer from all conditions. This finding supports the hypothesis that the air-interface cells are closer to the oxygen exposure and therefore they show upregulation in aerobic respiration genes. In all Pellicle culture conditions the ribosomal gene *rpsL* is induced which indicates that Beijing65 Pellicle cells may be metabolically active in contrast to the Att and Pk biofilm layers. However, *rpsL* was repressed in all H37Rv biofilm layers. These findings might indicate that Mtb strains have different response to stress conditions. Hence, choosing another clinical strain might give better correlation with sputum transcriptome than Beijing.

5.5.2. *M. tuberculosis* biofilm cell sensitivity

Bacterial biofilm is known to comprise heterogeneous cell populations with non-responsive antibacterial drugs characteristics (Spoering *et al.*, 2001; Lewis, 2007; Dhar *et al.*, 2007). Both Mtb biofilm strains H37Rv and Beijing65 show drug tolerance to RIF and INH in all layers even after 7 days of incubation. In contrast to H37Rv and Beijing65 mid-exponentially grown cells show fully response to the RIF and INH over 7 days period. The reasons why biofilm cells are tolerance to RIF and INH antibiotics can be summarised in two points: first, structured growth of Mtb in clusters is able to stimulate phenotypic persistence of essential bacilli over physical protection from the environmental pressures. This feature happened as occupant microbes within the bacterial biofilm are self-organized into 3D, matrix-encapsulated structures with internal water networks and cavities (Stoodley *et al.*, 2002; Branda *et al.*, 2005). Second, the environmental stress might alternate the mycobacterial cell wall to be less permeable.

There are three features that could protect bacteria from the antibiotic effect. These features are biofilm formation, low oxygen level and low metabolic activity which were found particularly in *Pseudomonas aeruginosa* biofilm (Walters *et al.*, 2003). This could be seen in the current study as the mid-exponential phase cells are less resistant to RIF and INH drugs. In the biofilm cells, the H37Rv Pellicle layer shows higher tolerance to INH and RIF than Beijing65. To link that with the gene expression profile of Pellicle in both strains it can be observed that *rpsL* gene is repressed in H37Rv in contrast to Beijing65. From this finding it can be speculated that because H37Rv Pellicle cells may be non-replicating cells and RIF targets the RNA polymerase, thus, Beijing65 Pellicle cells are more susceptible to RIF than H37Rv Pellicle cells. The Att biofilm cells also from both strains have higher tolerance to both RIF and INH than Pk cells. This may be because of the extracellular matrix (ECM) barrier which minimises the antibiotic concentration in the Att cells in addition to the direct exposure in the Pk biofilm cells.

Ojha *et al.*, (2008) revealed through their Mtb mc²700 biofilm that cells were shown to be resistant to high RIF (50µg ml⁻¹) and INH (1µg ml⁻¹) concentrations over 5 days of exposure (Ojha *et al.*, 2008). However, Ojha *et al.*, (2008) used the whole biofilm mass whereas in the current study the Mtb biofilm was separated into three layers and each layer was assessed independently. These findings reflect one of the features that Mtb strains *in vitro* share with Mtb in sputum.

5.5.3. The Rpf-dependancy assessment of *M. tuberculosis* H37Rv biofilm cells

In the current work, Mtb H37Rv Att and Pk biofilm cells were assessed for Rpf-dependant cells. Pellicle biofilm cells were excluded as they were previously evaluated to be actively growing in solid Middlebrook 7H10 agar with CFU of 10⁻⁸ (data not shown). The discrepancy of the CFU and MPN results between the two users readings made the assessment more difficult than what was expected. The reason behind this discrepancy of CFUs and MPNs may be because when Pk and Att biofilm layers were harvested, the upper Pellicle layer contaminated them, therefore readings show differences between the two replicates. This discrepancy could be solved by using a wider range of replicates (i.e. at least 10 replicates) to minimize the discrepancy.

It was clear that ADC+Cholesterol with 4 h exposure to NO or Sper has no influence on Pk and Att biofilm cells to stimulate the formation of Rpf-dependant cells. The RI shows no difference between the CFU and the MPN+SN results. However, using the commercial OADC+Cholesterol with 4 h exposure to NO or Sper seems to have an effect on stimulating Att biofilm cells to form Rpf-dependant cells with RI of ≥ 1. This effect was clear when Att biofilm cells exposed to OADC+Cholesterol+Sper. However, following discussion with Dr Mukamolova it was recommended to examine OADC+Cholesterol+NO

As shown in the RI in Table 22 two replicates show clear stimulation by SN and the other two did not. Repeating this experiment with more than 10 replicates may give more reliable results. However, there is a strong evidence of that adding SN to the MPN stimulates the growth of Att H37Rv cells > 1.5 log₁₀ difference. The reason why Att biofilm cells grow in solid better than liquid in two samples as shown in the II might be because of the growth inhibitors in the liquid medium, which inhibits Mtb cells' growth without adding the SN. Another reason for that may be the two MPN+SN plates, which show lower readings have some growth inhibitors in contrast to the other two replicates. In order to remove or at least minimize the effect of the inhibitory activity against Mtb Att biofilm cells growth additional washing steps from one to three times might show effective action. This finding was observed in the Mtb cells in decontaminated sputum as sputum cells require for additional

washing steps to remove the cell-bound inhibitory activity (Mukamolova *et al.*, 2010). This experiment is promising as there is an indication of the presence of Rpf-dependant cells in Att biofilm under OADC+Cholesterol+NO conditions, however, further investigation is required.

5.5.4. Assessment of inhibition of *M. tuberculosis* growth within the biofilm by ³H uracil incorporation

In order to assess the activity of each biofilm layer to uptake the ³H uracil, the experiment was done at 5 wks using CFU, MPN, DNA and RNA counting methods. The ³H uracil uptake measurement has been used to measure bacterial replication within the microenvironment (Cho *et al.*, 2005).

The results of the current study show that Pellicle and Att biofilm cells show the lowest relative ³H uracil uptake compared to Pk cells with all counting techniques. This was not expected as the Pk and Att H37Rv biofilm cells show closer correlation with sputum transcriptome than Pellicle. The log₁₀ CFU counts of Att and Pk biofilm cells also were lower than Pellicle biofilm cells. All these findings suggest that Att and Pk biofilm cells are less active than Pellicle. One of the possible explanations of that the direct exposure of Att and Pk biofilm cells to the ³H uracil isotopes may be higher than Pellicle layer. Furthermore, the ³H uracil isotopes may be recruited into Att biofilm cells. Therefore, the uptake of Pellicle cells was relatively lower than Att and Pk biofilm cells. The exposure of Att biofilm cells might be similar to the Pk biofilm cells as they settle beneath the liquid, however, the uptake of Pk cells was higher. This may reflect the ECM within the Att layer restricting the amount of the ³H uracil that reached to Att cells.

Results of the DNA and RNA relative counts suggested that the metabolic activity of Mtb biofilm cells decreases after 4 wks in Pk and Pellicle cells and remained the same in Att cells. This supports the idea that the Mtb biofilm maturation is established after 5 wks.

Taken together, these findings indicate that there is heterogeneity within the biofilm layers in terms of the growth activity as Pellicle and Att layers showed less activity than the Pk biofilm cells as shown by the relative ³H uracil uptake results.

5.5.5. *M. tuberculosis* biofilm contains higher proportion of SYTO9 nucleic acid stained cells and lower ability to reduce INT salt

Retaining the cell membrane integrity is determining features of viable cells. Hence, SYTO9 (nucleic acid dye) and PI (penetrates only damaged membranes) were used to assess the integrity of H37Rv biofilm cells and the mid-exponential phase control. SYTO9 staining could penetrate both damaged and intact cell membranes whereas PI is only permeant to damaged cells. Therefore, cells with damaged membranes will be labelled with both staining but SYTO9 fluorescence is reduced by the PI and as a result cells appear red under fluorescence light.

Tetrazolium salt reduction offers an alternate electron acceptor to oxygen within bacterial cells and can be used to evaluate the respiratory activity of mycobacterial cells (Bridge *et al.*, 2005). Once the INT reduced in the bacterial cell, whichever enzymatically or via direct reaction with NADH or NADPH, the classical tetrazolium salt produce an insoluble formazan (Vistica *et al.*, 1991). According to the net positive charge of the tetrazolium salt, it helps the tetrazolium salt to be accumulated successfully into the bacterial cell (Bridge *et al.*, 2005).

Cells in all Mtb H37Rv biofilm layers show significantly low INT reduction (determined by formazan deposit) compared with mid-exponential phase cells. Also, the lower reduction was shown in Pellicle biofilm cells followed by Att and Pk biofilm cells. However, as the thick ECM of Pellicle contains many broken and dead cells, the percentage of formazan droplets which were counted under phase contrast is low compared with the total cell number. Likewise, Att cells are located within ECM which may be thinner than Pellicle, thus the percentage of formazan is lower than Pk cells. In contrast, the Pk biofilm cells are freely floating in liquid, therefore, the percentage of formazan droplets is more reliable compared with Pellicle cells.

The lower reduction of biofilm cells compared with mid-exponential phase cells is identical with the gene expression profiling which confirms down regulation of the aerobic respiration genes (*nuoB*, *ctaD*, *qcrC*, and *atpD*) in PK, Att and Pellicle H37Rv biofilm layers compared with exponential phase cells.

5.5.6. Measuring the acid-fast and LB proportions of *M. tuberculosis* biofilm cells

The high LB and low AF percentages with Auramine O were seen in both Pk and Att H37Rv biofilm cells. In contrast, the Pellicle biofilm cells show lower LB percentage and higher AF percentage compared with Att and Pk biofilm cells. This means that H37Rv Att and Pk

biofilm cells within the biofilm may be exposed to cellular alteration as a result of the lower oxygen and starvation stressors as they are covered by the Pellicle.

The ECM of mycobacterial biofilms is characterised by their free MAs (Ohja *et al.*, 2008; Ojha *et al.*, 2010). This might be speculated that Att and Pk biofilm cells are released from the Pellicle layer, leaving rich amount of MAs in the ECM. In contrast, cells in Pellicle are preserving their MAs therefore; the AF proportion is higher than Pk and Att biofilm cells. Att and Pk biofilm cells also face lower oxygen and starvation stresses

There are two genes: *kasA* and *kasB*, were shown recently to be involved in the loss of the mycobacterial acid-fastness. *kasA* and *kasB* encode distinct FASII β -ketoacyl-ACP synthases which is implemented in MA synthesis (Bhatt *et al.*, 2005; Bhatt *et al.*, 2007). This was shown in *KasB* Mtb mutant that shows complete loss of the acid-fastness (Bhatt *et al.*, 2007). According to these findings it can be suggested that Att and Pk biofilm cells possibly have a defect in *KasB* gene. The result in current study also constant with Deb *et al.*, (2009) finding that when the LB proportion increased, the AF proportion decreased.

The finding that Pk and Att biofilm cells have low Auramine O AF rates suggests increased decolourisation or reduced staining due to impermeability. The result showed that the Auramine O washed off the Pk biofilm bacilli after decolourisation with acid-alcohol in different time points. There was dramatic decline in the AF proportion with 3 min decolourisation time.

5.6. Conclusions

Although several features of the sputum phenotype have been replicated here (correlated gene expression, LB⁺ populations, loss of Auramine acid fastness, RIF and INH drug tolerance and low respiration activity), there are important features to be examined. These include the generation of Rpf-dependent cells, which requires further work. Although progress has been made to replicate some sputum phenotypes *in vitro*, the environmental signals required to generate all the Mtb phenotypes that have been observed in sputum remain obscure and require further work.

The principle findings of this chapter are:

- The Pk biofilm phase in both H37Rv and Beijing65 Mtb strains are significantly correlated with sputum transcriptome but *icl1* is notable for its apparent repression and *virS* through to *ppsA* for their lack of induction.
- No single condition tested here completely replicates the sputum transcriptome.
- Poor DosR activation (*hspX* and *tgs1*) by NO was noted in all H37Rv biofilm cultures in clear contrast to the response of the mid-exponential culture.
- Mtb H37Rv and Beijing65 biofilm strains show high tolerance to RIF and INH in contrast to the mid-exponential culture.
- There is an initial indication of the presence of Rpf-dependant populations in the Att H37Rv biofilm layer grown in Cholesterol, OADC and exposed to NO.
- Spermine seems to have influence in biofilm gene expression and Rpf-dependant cells formation.
- The relative CFU, MPN, DNA and RNA counting techniques revealed that Mtb biofilm layers are different in ³H uracil uptake.
- The staining procedures indicate that Mtb in sputum exist in a variety of distinguishable physiological states. Mtb H37Rv Att and Pk biofilm cells showed high LB and low AF percentages, which may indicate that these cells are slow or non-

growing. The Pelli biofilm may be a mixture of these two growth statuses, but the predominant population was the non-growing one, as the INT staining represented. Collectively, this indicates that Mtb in sputum is a mixture of phenotypes which include metabolically active with the potential disseminate, or to persist in a dormant state until conditions are more favourable for resumption of active growth and host colonisation.

Chapter 6

General discussion, future work and
conclusions

6.1. General Discussion

Characterisation of *Mtb* populations in expectorated sputum and replicating these phenotypes *in vitro* has been the topic of this research. Based upon the observation of Sloan (2008) that LB⁺_{ve}/AF⁻_{ve} populations in sputum have the same morphology as *Mtb*, this study was directed to improve the identification of LB⁺_{ve} cells as *Mtb*. Work was also aimed at establishing the best condition to replicate what was observed of *Mtb* features in sputum in *in vitro* settings.

To achieve these objectives, the current cytological techniques were first developed and evaluated for their capability to detect *Mtb* in culture and sputum samples. To characterise the various *Mtb* populations in sputum, two staining approaches were developed and used in this study. These approaches were IIF and PNA triple-staining techniques and Auramine O and fluorescence Kinyoun dual-staining techniques. Once these populations were characterised, an *in vitro* biofilm system was successfully developed under multiple conditions to find the best condition replicating sputum phenotypes.

6.1.1. The multiple *M. tuberculosis* populations in sputum

According to the current study, fluorescence Kinyoun was able to detect both the highest total cell number of *Mtb* in sputum and the highest percentage of Pk biofilm cells compared with IIF, PNA and Auramine O. The classification of *Mtb* sub-populations in three sputum samples by dual- and triple-staining techniques clearly revealed multiple *Mtb* populations in sputum. The main observation was that between 6-55% of LB⁺_{ve} *Mtb* cells that were recognised with fluorescence Kinyoun were not recognised with Auramine O dual- or triple-staining techniques. Concurrently, the *Mtb* populations which were detected with fluorescence Kinyoun/GLx dual-staining reflect the sum of detected populations by the other staining techniques. This may indicate that fluorescence Kinyoun was able to detect the majority *Mtb* sub-populations that were not detected by the other techniques. However, sputum sample 96 showed a higher total number with dual staining techniques than triple staining techniques and this may reflect a technical error or cross contamination during washing steps.

Although this finding is important to classify *Mtb* populations in sputum, the samples used came from TB patients in Gambia, which means that the strain could be *M. africanum*. Hence, further analysis required use of other clinical *Mtb* strains in sputum

such as Mtb Beijing strain to show whether there are strain differences in terms of Mtb detection and classification in sputum. Moreover, the limited number of samples used in this study may affect the strength of these findings as larger numbers of samples should be assessed in order to provide more reliable findings. Although there are few studies revealed multiple Mtb populations in sputum (Shapiro *et al.*, 2008), culture and tissue samples (Ryan *et al.*, 2010), these provided qualitative description rather than counting the different populations as in the current study.

6.1.2. The reproducibility of dual- and triple-staining techniques

The development of dual- and triple-staining techniques was shown in Chapter 4. Indeed, triple-staining is challenging and has many disadvantages. The main drawback is the loss of cells when removing the cover slip or in washing steps. Because it is difficult to visualise Mtb cells under phase contrast in sputum, pointing to a particular cell to ensure its presence was really time consuming. For this reason, to the extent possible, cells were chosen in low background fields so that cell loss could be easily recognised. Because at least 100 cells per replicate should be counted in this study, and the cover slip should be removed and then the following staining applied, the matter of losing cells is potentially significant in this kind of experiment. Further, in order to ensure a return to the same field of view after applying the next staining, slides were marked with a permanent marker. Nevertheless, this mark was occasionally washed off with phenol or acid-alcohol, making the slide unusable and requiring the preparation of another set.

This technique, however, was worth the effort as the results were extremely valuable. There is no other study available showing multiple Mtb populations in sputum using more than two staining techniques combined together. Further development of this approach in terms of reproducibility will open a new window in the study of multiple Mtb populations in sputum with high reproducibility.

Dual-staining has been used previously to visualise Mtb from sputum or pure culture. Ryan and colleagues (2010) combined AF staining with immunological staining using Auramine-Rhodamine in combination with IIF on mouse and Guinea pig lung tissues (Ryan *et al.*, 2010). However, the ultimate value of dual staining is less than the triple staining because it represents fewer Mtb populations compared with three triple-staining.

6.1.3. Is an *in vitro* *M. tuberculosis* biofilm system replicating phenotypes seen in sputum?

The exact mechanism by which *Mtb* bacilli produce different phenotypes in sputum is unclear. Researchers have attempted to mimic the *in vivo* environment of *Mtb* using *in vitro* settings. This might be the best way to study the exact mechanism of *Mtb* and production of these phenotypes. Most of these studies have focused on designing *in vitro* granuloma models in order to recruit an exact environment mimicking *Mtb in vitro*. These models normally use human macrophages or leukocytes host cells that are then infected with *Mtb* (Kapoor *et al.*, 2013, Puissegur *et al.*, 2004). However, in addition to immune system defence, *Mtb* bacilli in human granuloma face many factors such as nitrosative stress, hypoxia, low pH, iron limits and nutrient starvation (Talaat *et al.*, 2004, Voskuil *et al.*, 2004, Timm *et al.*, 2003). Therefore, the production of sputum phenotypes might not reflect exposure to a single environment.

Results of previous study (Lee, 2012) showed no growth condition or stimulus that fully replicated the sputum transcriptome *in vitro*. However, when *Mtb* added to RPMI or PBS and exposed to multiple stimuli for 4 hr improved correlation was observed. Moreover, the induction of LBs did not match with sputum or the gene expression results (Lee, 2012). Rpf-dependancy, drug tolerance and the acid-fastness under these conditions and stimuli were not investigated.

In the current study, the *Mtb* biofilm shed some light on conditions that may be involved in the sputum phenotypes. However, as before, no single stimulus tested completely replicated the sputum transcriptome of the 20 selected genes. The Pk phase from both *Mtb* strains (H37Rv and Beijing65) was significantly correlated with sputum transcriptome. The pattern of expression of the H37Rv Pk phase shows slight matching with sputum transcriptome, particularly in repressed genes. Interestingly, there was no remarkable effect of NO on *Mtb* biofilm cells as was reflected by the poor DosR regulon gene activation. This was noticed once there was no significant difference between biofilm cells exposed to NO and the control Sper. *Mtb* biofilm cells may resist the NO effect or cells may have already entered the NRP state, hence the NO does not show a significant effect compared with those of mid-exponential phase. The poor effect of NO on *Mtb* biofilm cells was also shown in LB percentages of cells treated with NO and Sper revealed no differences in LB percentages between these two conditions. On the other hand, Sper seems to have the same effect that NO does on *Mtb* biofilm cells. Both NO and Sper show almost similar gene expression pattern

especially with Pk biofilm cells those are strongly correlated with sputum transcriptome. Furthermore, the Rpf-dependancy assessment assay on Att biofilm cells revealed that Sper has the strongest influence on Mtb H37Rv cells than NO among conditions tested. Putting all these findings together, Sper may exert significant influence on Mtb biofilm cells.

Similarly, the poor response to NO exposure was also shown in the Att and Pelli gene expression as well as the LB percentages. This raised the question - are biofilm layers growing in the same pattern? This was addressed measuring the ^3H uptake by the three biofilm layers. The result of this study was surprising as the relative activity from the 3rd to the 5th wk was lower in Pellicle biofilm cells followed by Att even though these layers contained most cells. The Pk biofilm cells showed the highest relative activity especially at wk 5. This finding is consistent with the respiratory activity result maintained by INT reduction. The measurement of formazan droplets of biofilm layers revealed that Pellicle biofilm cells show the lowest respiratory activity followed by the Att and the Pk biofilm cells were the most active. To compare these findings with what is known about Mtb in sputum it can be speculated that Mtb cells in sputum may be mixture of the three biofilm layers.

6.1.4. Are Rpf-dependant populations present in *M. tuberculosis* biofilm?

Rpf-dependancy is another significant phenotype shown to be present in smear positive sputum samples. In the Rpf-dependancy assessment of Att biofilm cells under cholesterol, nitric oxide and oleic acid stimuli slightly indicated the presence of this population in biofilm. However, a major issue was the reproducibility of harvesting pure Att biofilm cells with no contamination from the other phases. This requires further analysis.

In the current study, the Rpf-dependency of Pellicle cells was assessed at wk 5 of the biofilm growth. The result revealed that the MPN readings were similar to the MPN+SN which suggest that there are no Rpf-dependant cells when the pellicle has matured. Although the Pellicle layer could be seen macroscopically as a transparent thin layer on top of the biofilm at wk 2, there were no colonies on the solid agar medium. However, at this stage of Pellicle development, MPN, DNA and RNA counts were above the limit of the detection. This might suggest the presence of the Rpf-dependant cells on the surface at this stage; a finding that requires further confirmation.

Taking all together, Mtb biofilm under multiple stimuli have a heterogeneous nature by means of phenotypic properties. Moreover, the physiological state of biofilm cells at early stage may be distinct than late stage in the biofilm development. There are highly significant similarities revealed in this study as correlated to sputum. These findings are a loss of acid-fastness, high LB proportions, drug tolerance, slow or NRP populations, and a close correlation pattern to sputum gene expression. Further analysis should be carried out to study Mtb biofilm and apply more conditions for assessment.

6.2. Future Work

Through the work in this thesis, it should be possible to develop a cytological staining technique that reveals the multiple Mtb populations in sputum. A reproducible technique requires a short processing time and needs to generate reproducible results. According to the finding in Chapter 3, multiple freeze-thaw cycles affects the AF proportion of Mtb in sputum and pure culture. Therefore, working with fresh (non-frozen) sputum samples is required for more reliable results. It is also possible that the fluorescent Kinyoun method will not suffer from this problem and this should be tested. It is possible that different Mtb strains may have different cell properties and as a result their staining properties and transcriptional responses might be different. Hence, studying other Mtb strains, such as the Beijing strain, may show different populations. The preliminary results comparing H37Rv and a Beijing strain in this study reinforce this point.

The genes selected in this study were chosen carefully based on the previous study, and these genes reflect the key features seen in sputum. As the gene expression of Mtb biofilm cells showed that the H37Rv strain correlated better than the Beijing65 strain with sputum transcriptome, and the DosR regulon in the latter is known to be constitutively induced, it is thus essential to study other clinical strains. Furthermore, LB formation in the Beijing strain is known to be high even in normal *in vitro* growth conditions. Hence, this strain may be not be condition or stimuli dependent when producing LBs *in vitro*, which make it not the best strain to compare with sputum.

The comparison between Mtb biofilm and sputum might be not identical in some ways. This is due to the different techniques that were used in the comparison, as Mtb biofilm gene expression was measured by q-PCR and the gene expression of Mtb from sputum was measured using a microarray. Therefore, in terms of identical results, the gene expression of Mtb from sputum should be measured using the same technique as the Mtb in biofilm. One significant work that should be done is to study genes that are essential in Mtb biofilm formation and examine whether these genes are induced in Mtb from sputum. This will give direct evidence of the presence or absence of biofilm in a tubercle lung cavity.

Kapoor *et al.*, (2013) established an *in vitro* granuloma model showing that there are many phenotypes in sputum. Reproducing this model and measuring the gene

expression and comparing it with gene expression from sputum using the same selected genes may give a better correlation. The use of Mtb biofilm Pk or Att phases to infect an *in vitro* granuloma model or human macrophages would also be worthwhile as they show a better correlation with sputum transcriptome. Another suggestion is that Pellicle biofilm shows no growth in solid medium at wk 2, hence it is essential to study the Pellicle cells at this stage. Finally, all biofilm layers show different features that can be replicating features in sputum, therefore further of these cultures may give some clues in replicating sputum phenotypes *in vitro*.

6.3. Conclusions

The work presented in this thesis has fulfilled the main aims which included developing a staining technique to detect, identify and characterise multiple Mtb populations in sputum, as well as developing an *in vitro* system replicating sputum phenotypes. Specific results and conclusions were briefly summarized in each chapter. The main findings of this report are as follows:

- ❖ The best cytological staining technique to detect Mtb bacilli in sputum is modified fluorescence Kinyoun staining as it detects the highest proportions of Mtb populations in sputum and pure culture compared with Auramine O, IIF and PNA staining.
- ❖ Each staining approach used in this study showed different Mtb populations in sputum.
- ❖ Mtb bacilli present in multiple populations in sputum are distinct from those in pure culture.
- ❖ Repeating freeze-thaw cycles significantly reduces the Auramine O acid-fastness of Mtb bacilli in sputum and pure culture.
- ❖ No single biofilm condition or stimulus tested in this report completely replicates the sputum transcriptome.
- ❖ The Pk biofilm phase (H37Rv strain) is significantly correlated with sputum.
- ❖ It has been noted that poor DosR activation by NO in biofilm cultures is in clear contrast to the response of the mid-exponential phase culture.
- ❖ Sper seems to have an influence on Mtb biofilm in terms of gene expression and Rpf-dependency.
- ❖ Mtb biofilm cells from all phases have shown high tolerance to RIF and INH antibiotics.

- ❖ The findings show some indications of the presence of Rpf-dependant populations in attached biofilm cells, but further experiments are required to confirm this conclusively.
- ❖ Data in this study suggest that Mtb biofilm layers have different growth activity.
- ❖ The cytological biofilm results suggest that the Att and Pk may be slow or non-growing cell whereas Pelli cells may be a mixture of both growth statuses.
- ❖ Combining all biofilm phases (Att, Pk and Pellicle) and the cytological results, it can be speculated that Mtb populations in sputum represent mixtures of different biofilm growth layers *in vivo*.

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