

# **Assessing the drug susceptibility of resuscitated differentially culturable tubercle bacilli after tuberculosis treatment.**



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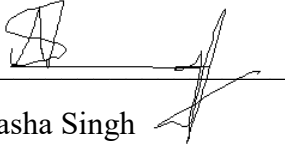
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A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

08 September 2022

## Declaration

I, Natasha Singh, declare that this dissertation is my own unaided work. It is being submitted for the degree of Master of Science in Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.



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08 September 2022

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Date

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## Abstract

*Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent of tuberculosis (TB) and remains the leading cause of death among various infectious agents. The disease is curable as it has both a vaccine and chemotherapy but due to the adaptive nature of *M. tuberculosis*, it is highly capable of evading host immunity by becoming phenotypically drug-tolerant or drug-resistant which negatively impacts the treatment outcomes and increases mortality rates. In addition, various studies have identified a non-replicating, drug-tolerant heterogeneous bacterial population termed differentially culturable tubercle bacilli (DCTB) in TB sputum samples that are most likely adding to the challenges involved in eradicating the disease. These DCTB are only resuscitated and detected in a liquid-based limiting dilutions most probable number assay and not by standard culture methods such as the colony forming unit and MGIT assays. Moreover, these DCTB are shown to be present at diagnosis and throughout the course of treatment indicating that the first-line drugs (rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA)) are unable to eliminate DCTB. Presently, it is unclear how these DCTB adapt and promote their survival during TB treatment therefore, determining whether these DCTB are phenotypically drug-tolerant or genetically resistant will aid in the development of novel drugs and improved treatment regimens in an attempt to lessen the TB burden. In this study, we purified and analyzed stored resuscitated DCTB samples from MPN assays performed in a previous study that quantified the DCTB population in sputa from TB patients on treatment. Once purified, the drug susceptibility of these samples to RIF, INH, EMB and PZA were determined using the broth microdilution assay. The data showed that 4 out of 9 patients had samples displaying drug resistance to one or more of the first-line drugs. The resuscitation of the bacteria in the MPN assay may have resulted in a loss of the DCTB phenotype thus, the resuscitated bacteria were forced back into a reversible non-replicating, drug-tolerant DCTB state using a carbon starvation model. Broth microdilution assays were performed on the DCTB samples to assess the drug tolerance of the bacteria to RIF, INH and EMB compared to the unstarved bacteria. The data showed that under stressful conditions, the bacteria revert to the DCTB state and have a higher drug tolerance than the unstarved bacteria. Lastly, the resistance-conferring regions of RIF, INH and EMB were amplified by polymerase chain reaction (PCR) for all patient samples displaying resistance to any of the drugs and sequenced to identify mutational changes. The sequencing data showed that these DCTB are phenotypically tolerant to RIF, INH and EMB. The data showed that TB bacterial populations are underestimated which may result in misdiagnosis and premature termination of treatment.

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One day you'll leave this world behind so live a life you will remember -Avicii

## Abbreviation List

| Abbreviation           | Full name                                                                |
|------------------------|--------------------------------------------------------------------------|
| ATP                    | Adenosine triphosphate                                                   |
| BCG                    | Bacille calmette-guérin                                                  |
| CAF                    | Central analytical facilities                                            |
| CAS                    | Central asian strain                                                     |
| CF                     | Culture filtrate                                                         |
| CFU                    | Colony forming unit                                                      |
| CoA                    | Coenzyme A                                                               |
| DCTB                   | Differentially culturable tubercle bacilli                               |
| DMSO                   | Dimethylsulfoxide                                                        |
| DNA                    | Deoxyribonucleic acid                                                    |
| EDTA                   | Ethylenediaminetetraacetic acid                                          |
| EMB                    | Ethambutol                                                               |
| gDNA                   | Genomic DNA                                                              |
| HIV                    | Human immunodeficiency virus                                             |
| HyClone™               | HyPure™ molecular biology grade water                                    |
| INH                    | Isoniazid                                                                |
| ISO                    | International organization for standardization                           |
| LTBI                   | Latent TB infection                                                      |
| MDR-TB                 | Multi-drug resistant TB                                                  |
| MGIT                   | Mycobacterial growth indicator tube                                      |
| MIC                    | Minimum inhibitory concentration                                         |
| MPN                    | Most probable number                                                     |
| mRNA                   | Messenger ribonucleic acid                                               |
| <i>M. smegmatis</i>    | <i>Mycobacterium smegmatis</i>                                           |
| <i>M. tuberculosis</i> | <i>Mycobacterium tuberculosis</i>                                        |
| OADC                   | Oleic albumin dextrose catalase                                          |
| OD <sub>600nm</sub>    | Optical density at a wavelength of 600 <sub>nm</sub>                     |
| PANTA                  | Polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin |
| PBS                    | Phosphate-buffered saline                                                |
| PCR                    | Polymerase chain reaction                                                |
| PZA                    | Pyrazinamide                                                             |

|          |                                          |
|----------|------------------------------------------|
| PZase    | Pyrazinamidase                           |
| RI       | Resuscitation index                      |
| RIF      | Rifampicin                               |
| Rpfs     | Resuscitation promoting factors          |
| RNA      | Ribonucleic acid                         |
| RRDR     | Rifampicin resistance determining region |
| RR-TB    | Rifampicin-resistant TB                  |
| TA       | Toxin-antitoxin                          |
| TAE      | Tris-acetate-EDTA                        |
| TB       | Tuberculosis                             |
| TDR-TB   | Totally drug resistant TB                |
| Tx-media | Liquid media supplemented with tyloxapol |
| v/v      | Volume/volume                            |
| w/v      | Weight/volume                            |
| XDR-TB   | Extensively drug resistant TB            |

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# **1. Introduction**

## **1.1 Tuberculosis (TB) background**

*Mycobacterium tuberculosis* (*M. tuberculosis*) is a pathogenic bacterial species first discovered in 1882 by Robert Koch and is the causative agent of tuberculosis (TB) (WHO 2020). Worldwide, TB is one of the leading causes of human death caused by a single infectious agent. In 2019, an estimated 10 million new TB cases and 1.4 million TB deaths were reported worldwide, with Southern Africa accounting for 14% of the total. Men and women aged >15 years accounted for 56 % and 32 % of individuals diagnosed with TB, respectively, whilst children aged <15 years accounted for 12 % (WHO 2020).

### **1.1.2 TB transmission and pathogenesis**

TB is a communicable disease where the bacteria are spread among humans as airborne droplets expelled during coughing and sneezing from an infected individual, and inhaled by uninfected individuals (Figure 1A). The disease primarily affects the lungs (pulmonary tuberculosis), but can also affect other parts of the body (extrapulmonary TB) (WHO 2020). Transmission of the bacteria from an infected to an uninfected individual is dependent on the bacterial load expelled, as well as the proximity and duration of exposure (Escombe et al 2008). Once transmitted, the pathogenesis of *M. tuberculosis* is dependent on various factors such as the strain type, drug resistance, treatment adherence, access to healthcare, co-morbidities and the site of infection (pulmonary or extrapulmonary) (Heemskerk et al 2015). These factors affect whether an individual becomes infected (active TB infection) or whether the infection is contained (latent TB infection) and eventually eradicated by the host immune response (Barry et al 2009; Pai et al 2016).

### **1.1.3 Active TB infection**

Upon infection, the host immune system will recognize the microbe as foreign and recruit various immune cells in an attempt to eliminate the bacteria. The most common immune cells are alveolar macrophages (Schluger and Rom 1998). The macrophages engulf the bacteria by phagocytosis resulting in a vacuole called a phagosome. The phagosome then fuses with a lysosome providing an acidic environment for the elimination of the mycobacteria, thereby preventing disease progression (Sullivan et al 2012). *M. tuberculosis* uses a variety of strategies

which include eliciting an anti-inflammatory response, inhibiting the formation of reactive oxygen and nitrogen intermediates, and lowering the acidity of the phagosome thereby preventing phagosome-lysosome fusion allowing for the replication of *M. tuberculosis* within the macrophage to promote its survival to circumvent the immunological response of the host (Flynn and Chan 2001; Cooper 2009; Russell 2011; Sasindran and Torrelles 2011). If the bacteria manage to evade the macrophage's bactericidal effects, the macrophage activates T cells, which release chemokines that attract other inflammatory cells, such as dendritic cells, B and T cells, natural killer cells, fibroblasts, neutrophils, and macrophages, to the infection site (Ulrichs and Kaufmann 2006; Sasindran and Torrelles 2011; Pagan and Ramakrishnan 2014; Pai et al 2016). Once the immune cells are at the infection site, the activated T cells help cluster and organize the cells to form a granuloma to kill or successfully contain the engulfed bacilli (Figure 1B) (Co et al 2004). Active TB infection occurs when the microbes evade the host's immune response and continue to proliferate, whereas the successful containment of active *M. tuberculosis* is referred to as latent TB infection (LTBI).

#### **1.1.4 Latent TB infection (LTBI) and re-activation**

Latent tuberculosis infection (LTBI) is characterized as an asymptomatic, non-transmissible clinical state, where *M. tuberculosis* is successfully encapsulated by a granuloma (Pai et al 2016). The duration of latency is variable and is dependent on the individual's immunity (Kiazyk and Ball 2017). A delicate balance exists between the individual's immune reaction and the metabolism of the bacteria for the infection to remain latent. If this balance is broken, the infection will re-activate and symptoms will develop (Carranza et al 2006). Human immunodeficiency virus (HIV) infection is the largest recognized risk factor contributing to the re-activation of TB disease from LTBI among several other factors such as failure to produce and/or sustain immunological signals, stress, and old age (Sasindran and Torrelles 2011). Among the factors contributing to the re-activation of TB, such as failure to produce and/or sustain immunological signals, stress, and old age, Human immunodeficiency virus (HIV) infection is the largest recognized risk factor.

#### **1.1.5 TB/HIV Coinfection**

If an individual becomes immunocompromised through a secondary infection, such as HIV, the granuloma can no longer contain the mycobacteria resulting in the re-activation of the TB

disease (Figure 1C) (Scanga et al 2000; Dye and Williams 2010). The re-activated infection is a result of HIV infecting and killing CD4<sup>+</sup> T cells which are responsible for initiating the formation of the granuloma by recruiting inflammatory immune cells to contain and control the TB infection. Therefore, the HIV/TB co-infection contributes to the burden of TB by promoting the transmission of *M. tuberculosis* (Scanga et al 2000). In 2019, a total of 456 426 individuals were found to be co-infected with TB and HIV worldwide, with 88 % receiving antiretrovirals (WHO 2020).

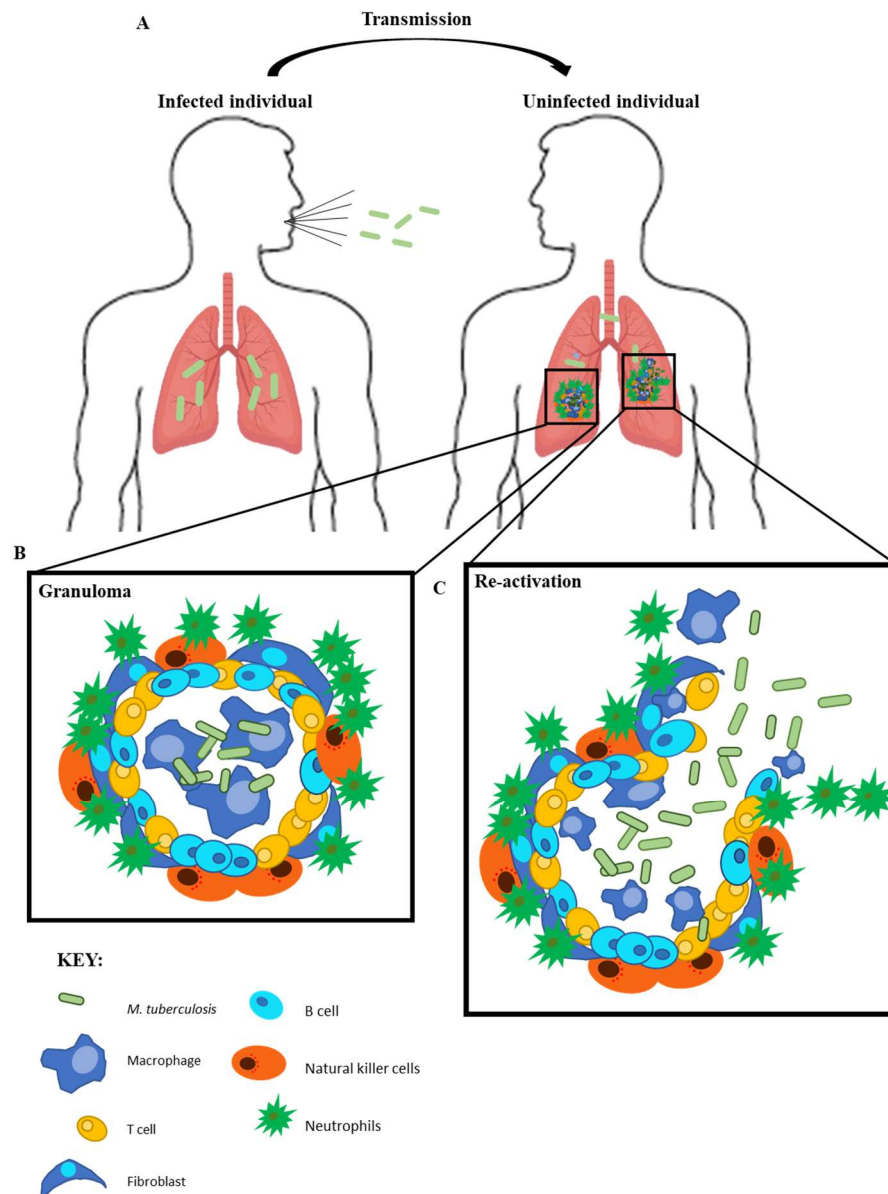


**Figure 1. Pathogenesis, granuloma formation, and re-activation of TB disease.**

(A) *M. tuberculosis* is spread from infected individuals to uninfected individuals through coughing and sneezing. (B) Upon infection, the host immune system will recruit various immune cells such as macrophages, fibroblasts, natural killer cells, neutrophils as well as T and B cells, in an attempt to eliminate or contain the intruding bacteria by forming a granuloma. (C) Any disruption between the immune signals or a secondary infection can disrupt the granuloma formation leading to the re-activation of the disease. This figure was adapted from (Ehrt et al 2018).

## 1.2 TB treatment

Despite the wide range of TB outcomes, the disease is curable and preventable as both a vaccine and chemotherapy are available. Bacille Calmette-Guérin (BCG) is the only approved TB vaccine to date (WHO 2020). BCG is used to effectively prevent TB meningitis, caused by *M. tuberculosis* invading the membranes surrounding the brain and spinal cord, in infants, young children and adolescents as well as miliary TB, which is the widespread dissemination of the bacteria within the body through the bloodstream (WHO 2019). The vaccine, however, does not protect infants, young children and adolescents from pulmonary TB and is less effective in protecting adults. Therefore, active pulmonary drug-susceptible TB is currently treated with a combination of four standard first-line chemotherapy drugs, namely rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA) for 6 months (Heemskerk et al 2015). The treatment regimen consists of a 2-month intensive phase with all four first-line drugs followed by a 4-month continuation phase with only RIF and INH. RIF, along with INH, make up the backbone of the short-course therapy because of its significant bactericidal activity (Kochi et al 1993). From 2015 to 2019, the worldwide number of individuals receiving TB treatment increased from 1 million to 4.1 million (WHO 2020). About 85 % of infected individuals can be successfully treated with the 6-month treatment regimen (WHO 2020). Drug-resistant forms of TB can develop as a result of misdiagnosis, drug overuse, or patient non-compliance to the treatment (Heemskerk et al 2015).

## 1.3 Types of drug-resistant TB and treatment options

Over time, *M. tuberculosis* has developed resistance to several antimicrobial drugs, resulting in multiple forms of drug-resistant TB. There are currently five forms of drug-resistant TB, listed in order of severity, which includes rifampicin-resistant TB (RR-TB), multi-drug resistant TB (MDR-TB), pre-extensive drug-resistant TB, extensively drug-resistant TB (XDR-TB), and eventually totally drug-resistant TB (TDR-TB) (Nguyen 2016; WHO 2020). As the severity of the resistance increases, the bacteria become harder to treat due to limited, less effective second-line drug options. Moreover, the second-line drugs have higher toxicity and increased side effects which negatively impact the treatment outcomes and increase mortality rates. RR-TB is used to describe resistance to RIF, which is one of the most effective antimicrobial TB drugs. MDR-TB is defined as resistance to the two main first-line drugs, RIF or INH. Most individuals with MDR-TB are treated with a combination of a fluoroquinolone (levofloxacin, moxifloxacin, ofloxacin or ciprofloxacin), bedaquiline, linezolid, and one or more effective oral drugs

(ethionamide, cycloserine, or p-aminosalicylic acid) for 18-20 months (Nath and Ryoo 2013). A shorter regime is also available, that requires administration of the above-mentioned drugs for 9-12 months with an additional daily administration of one injectable second-line drug (kanamycin, amikacin, and capreomycin) for 4 months (WHO 2019). Globally, there are approximately 206 030 individuals diagnosed with MDR- or RR- TB (WHO 2020). The second-line drugs have various targets such as cell wall components (ethionamide and cycloserine), important enzymes and proteins involved in replication and translation processes (fluoroquinolones, injectables and linezolid) as well as cellular processes involved in maintaining bacterial growth (p-aminosalicylic acid and bedaquiline). These drugs are effective against *M. tuberculosis* because they can weaken the cell wall which leads to cell death, and can inhibit bacterial growth by hindering replication and protein synthesis as well as cellular processes such as adenosine triphosphate (ATP) synthase and folic acid metabolism (Figure 2) (Nath and Ryoo 2013). Pre-extensive drug-resistant TB is MDR-TB with additional resistance to any fluoroquinolone, whilst XDR-TB in addition is resistant to either bedaquiline, linezolid or levofloxacin/moxifloxacin (WHO 2020). The old definition of XDR-TB was MDR-TB with resistance to one fluoroquinolone and one second-line injectable drug. Globally, there are approximately 12 350 individuals diagnosed with XDR-TB (WHO 2020). The last and most severe form of drug-resistant TB, TDR-TB, is a result of *M. tuberculosis* becoming resistant to all available first- and second-line drugs. There are currently no statistics available for TDR-TB. Individuals can acquire resistance during treatment or be infected with a resistant form of bacteria through transmission. There are two forms of drug resistance: genetic and phenotypic (Dartois 2014). The mechanisms involved in genetic resistance are discussed below.

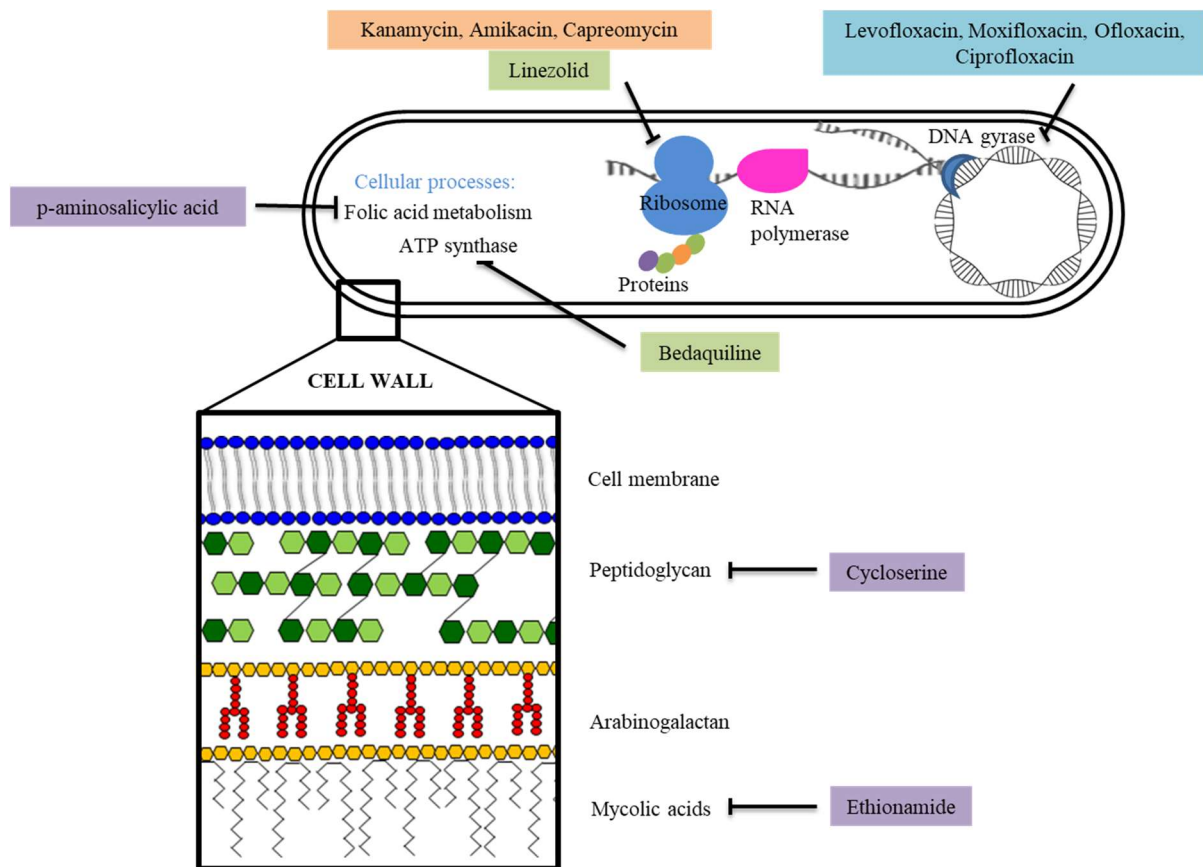


**Figure 2. Schematic showing the drug targets of all the second-line antibiotics.**

*M. tuberculosis* is a rod-shaped bacillus that has an intricate cell wall consisting of arabinogalactan which covalently binds the mycolic acid layer to the peptidoglycan layer in the cell wall. The second-line drugs consist of fluoroquinolones, oral drugs, injectables, as well as the drugs linezolid and bedaquiline. Fluoroquinolones include levofloxacin, moxifloxacin, ofloxacin and ciprofloxacin (blue) which target DNA gyrase, an important enzyme in DNA replication. Ethionamide, cycloserine, and p-aminosalicylic acid make up the oral drugs (purple). Ethionamide and cycloserine target the synthesis of cell wall components – mycolic acids and peptidoglycan, respectively, whilst p-aminosalicylic acid targets folic acid metabolism which is necessary for bacterial growth. The injectable drugs include kanamycin, amikacin, and capreomycin (orange). The last of the second-line drugs are linezolid and bedaquiline (green). All the injectables and linezolid target protein synthesis by inhibiting ribosomal function and formation. Bedaquiline is responsible for ATP synthase which plays a role in bacterial cell growth.

## 1.4 Development of genetic drug resistance

Genetic resistance is associated with chromosomal mutations acquired by *M. tuberculosis* to promote its survival by evading the antimicrobial effects of the drugs (Trauner et al 2014). Unlike other bacterial pathogens, resistance plasmids and horizontal gene transfer play no role in the development of drug resistance in *M. tuberculosis*. (Trauner et al 2014; Gold and Nathan 2017). Multiple mechanisms of drug resistance exist including intrinsic resistance, acquired resistance, compensatory resistance, epistasis, target alteration as well as mutations targeting

drug degradation and modification mechanisms (Nguyen 2016). Drug resistance resulting from mutational changes is often linked to a fitness cost which, for example, affects the rate of bacterial growth (Andersson and Levin 1999).

Intrinsic resistance occurs when the bacteria have naturally developed molecular mechanisms to counteract the cytotoxicity of the antimicrobial drugs (Morris et al 2005; Nguyen 2016). The majority of the mechanisms developed involve the cell wall of *M. tuberculosis* and its permeability to anti-microbial drugs. *M. tuberculosis* has a thick cell wall consisting of multiple layers which include an intracellular/innermost phospholipid bilayer or cell membrane, peptidoglycan, arabinogalactan, mycolic acid layer and an extracellular/outermost layer as shown in Figures 2 and 3. The cell wall creates a barrier for drug uptake, for example, the penetration of  $\beta$ -lactam is 100-fold slower through the cell wall of *M. tuberculosis* compared to *E. coli* (Chambers et al 1995). *M. tuberculosis* uses intrinsic resistance mechanisms to alter transcriptional machinery which in turn can cause downstream alterations in gene expression levels to change the permeability of the cell wall to prevent the drug from reaching its target and activate efflux pumps to siphon out drugs that have entered the cell wall (Goossens et al 2021).

Acquired resistance is when the bacteria obtain mutations that genetically alter and interfere with drug-target binding, compromise prodrug activation, or produce an overexpression of the drug target. Compensatory mutations are those that rectify a fitness loss caused by previous mutations while maintaining the resistance phenotype. Bacteria also evade antimicrobial agents by degrading or modifying the drug or rendering it inactive. Lastly, *M. tuberculosis* can degrade the drugs by hydrolases or chemically modify them through acetylation and phosphorylation (Nguyen 2016). The development of drug resistance to the four first-line drugs has a huge impact on the treatment outcomes as well as the spread of the various forms of resistant TB. These resistance mechanisms with regard to the first-line drugs are discussed below.

## **1.5 Mechanisms of action and resistance development for first-line drugs**

Drug resistance is a major contributor to the TB burden and poses a grave public health risk therefore, understanding the manner in which these bacteria adapt to evade the antimicrobial effects of the first-line drugs can aid in the development of strategies to weaken drug resistance and to identify novel drug targets.

### 1.5.1 Rifampicin (RIF)

Rifampicin (RIF) was first introduced as an antimicrobial drug in 1972 (Nath and Ryoo 2013). The antibiotic targets the mycobacterial DNA-dependent RNA polymerase which controls the transcription process and is essential for gene expression (Herrera-Asmat et al 2017; Zaw et al 2018). RNA polymerase is an oligomer comprised of five subunits,  $\alpha_2\beta\beta'\omega$ , each of which is encoded by *rpoA*, *rpoB*, *rpoC* and *rpoD*, respectively (Gill and Garcia 2011; Bortoluzzi et al 2013). RIF specifically binds to and targets the  $\beta$ -subunit of the RNA polymerase encoded by the *rpoB* gene, thereby hindering the transcription process and killing the bacilli (Figure 3) (Zaw et al 2018).

The *rpoB* gene is essential for the *in vitro* growth of *M. tuberculosis*, as discovered through HimarI transposon mutagenesis (Minato et al 2019). Mutations in the *rpoB* gene lead to defective drug binding and consequent resistance. Approximately 95% of the mutations associated with RR-TB are located in the *rpoB* gene within the 81 base pair region known as the rifampicin resistance determining region (RRDR) (Rattan et al 1998; Gill and Garcia 2011; Zaw et al 2018). The majority of the mutations found within the RRDR are non-synonymous mutations that result in single amino acid substitutions that affect the protein sequence. Frameshift mutations, deletions and insertions occur at lower rates. These mutations occur at several codons (508, 509, 511, 513, 514, 515, 518, 522, and 536) but are more commonly found in codons 531 (serine changes to leucine, glutamine, tryptophan or phenylalanine), 526 (histidine changes to alanine, tyrosine, glutamine, tryptophan, aspartic acid, arginine, asparagine, serine, proline or glutamine) and 516 (aspartic acid changes to valine) in MDR-TB strains (Telenti et al 1993; Zaw et al 2018). It is speculated that RR-TB is due to a small portion of mutations not occurring in the RRDR but in genes encoding for other subunits of the RNA polymerase (Rattan et al 1998).

### 1.5.2 Isoniazid (INH)

Isoniazid (INH), also known as isonicotinic acid hydrazide, was first introduced as an antimicrobial drug in 1951 (Jaber et al 1996; Vilcheze et al 2006). INH enters the cell by passive diffusion and targets mycolic acid biosynthesis, which are long-chain  $\alpha$ -branched,  $\beta$ -hydroxylated fatty acids, found in the cell wall of *M. tuberculosis* (Figure 3) (Bardou et al 1998; Mo et al 2004). Since INH is a prodrug, it must be converted into a pharmacologically active drug via the bifunctional heme enzyme catalase-peroxidase, encoded by *katG*, after administration. The peroxidase converts INH into its active form, isonicotinoyl-NAD, which

binds to and inhibits InhA, an NADH-dependent enoyl acyl carrier protein reductase involved in mycolic acid synthesis (Zhang et al 1992; Mo et al 2004; Metcalfe et al 2008). This makes the bacteria more vulnerable to reactive species and other environmental factors (Rattan et al 1998). This bifunctional enzyme primarily acts as a catalase and a peroxidase but can also act as a peroxynitritase and NADH oxidase (Timmins and Deretic 2006). The catalase, peroxynitritase, and NADH oxidase enzymes protect *M. tuberculosis* against the cytotoxicity of reactive intermediates produced by the macrophages by detoxifying them. The catalase enzyme eliminates hydrogen peroxide ( $H_2O_2$ ), while the peroxynitritase enzyme eliminates peroxynitrous acid ( $HNO_3$ ), which is produced when a superoxide anion reacts with nitric oxide (Wengenack et al 1999; Mo et al 2004). The elimination of the reactive intermediates which have various cellular targets promotes the persistence and virulence of *M. tuberculosis* within the macrophages.

INH resistance has been associated with mutations in the *katG* gene and the *ahpC* promoter region. Mutations occur more frequently in *katG* (42-95% of isolates) than in *ahpC* (6-43% of isolates), whilst 10% have mutations in both genes (Bollela et al 2016). Mutations in the *katG* gene have been shown to inhibit the conversion of INH to isonicotinoyl-NAD (Jaber et al 1996). Non-synonymous and null mutations are the most common, with the conversion of serine to threonine at codon 315 (S315T) occurring most frequently. This mutational change has a low fitness cost because it reduces the potential of activating INH while maintaining significant catalase activity (Pym et al 2002; Mo et al 2004). Other mutations which occur are arginine to glutamine at codon 418 (R418Q) or tryptophan to glycine at codon 321 (W321G) (Mo et al 2004). These mutations significantly reduce catalase and peroxidase activity thereby rendering the drug inactive (Mo et al 2004). Mutations in the *ahpC* region prevent the inhibition of mycolic acid synthesis and cause an overexpression in *inhA*, thus requiring higher doses of INH to elicit an inhibitory response (Takayama et al 1972; Vilcheze et al 2006).

### 1.5.3 Ethambutol (EMB)

Ethambutol (EMB) was first introduced into the TB treatment regimen in 1966 (Thomas et al 1961). The drug enters the cell by passive diffusion and targets the enzyme arabinosyl transferases encoded by the *embCAB* operon, consisting of three homologous genes - *embB*, *embA* and *embC* (Xu et al 2015). Arabinosyl transferases are responsible for the biosynthesis of arabinogalactan which covalently binds the mycolic acid layer to peptidoglycan in the cell wall (Figure 3) (Telenti et al 1997; Crick et al 2001). EMB inhibits these enzymes, preventing

the polymerization of arabinose to arabinan, the core element of two cell wall structural components: arabinogalactan and lipoarabinomannan (Xu et al 2015). Due to the lack of arabinan receptors, mycolic acids accumulate in the cell wall which eventually leads to cell death (Telenti et al 1997). Mutations commonly occur in *embB* and occasionally in *embC* genes. The most frequent mutation found in *embB* is at codon 306 and less frequently at codons 406 and 497 (Bakula et al 2013; Safi et al 2013). The mutations present in the operon, *embCAB*, alter the binding site on the protein and prevent the interaction between EMB and the arabinosyl transferases (Telenti et al 1997; Xu et al 2015).

#### 1.5.4 Pyrazinamide (PZA)

The antibacterial effects of pyrazinamide (PZA) were discovered in 1952, but it only became part of the treatment regimen in the 1980s (Rattan et al 1998). Whilst, RIF, INH and EMB are effective against actively growing bacteria, PZA works differently in that it has little to no effect on actively proliferating bacteria and is most effective against non-growing persisters found in acidic environments, pH ~5.5 (Boshoff et al 2002; Nath and Ryoo 2013; Zhang et al 2014). PZA, like INH, is a prodrug that requires the enzyme, pyrazinamidase (PZase), to convert PZA into its pharmacologically active form, pyrazinoic acid (Boshoff et al 2002; Ngo et al 2007; Zimhony et al 2007). Pyrazinamidase is encoded by *pncA*. Pyrazinoic acid binds to and inhibits aspartate decarboxylase PanD which is responsible for the conversion of L-aspartate to beta-alanine which impedes the biosynthesis of coenzyme A (CoA) (Figure 3) (Boshoff et al 2002; Zhang et al 2013). CoA is an essential enzyme that plays various roles in the survival of *M. tuberculosis* such as regulating metabolic enzymes as well as synthesizing and catabolizing lipids (Evans et al 2016). PZA resistance is associated with mutations in the *pncA* gene (72-97%), resulting in the loss of PZase activity and continued alanine and CoA production thereby, promoting the survival of the bacteria (Boshoff et al 2002; Nath and Ryoo 2013). Resistance conferring mutations in the *pncA* gene are highly diverse and scattered throughout the gene (Shi et al 2020)

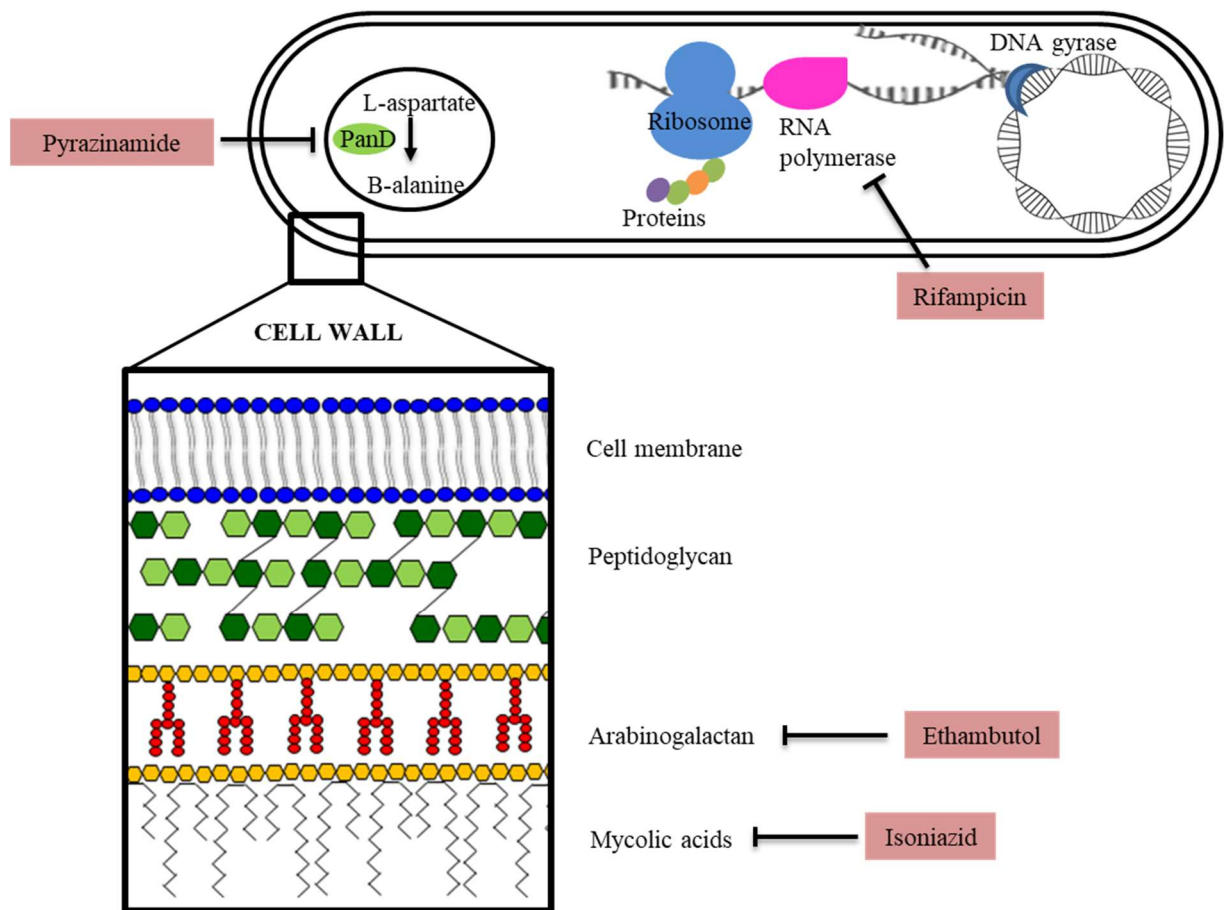


**Figure 3. Schematic showing the drug targets of all the first-line antibiotics.**

*M. tuberculosis* is a rod-shaped bacillus that has an intricate cell wall consisting of arabinogalactan which covalently binds the mycolic acid layer to the peptidoglycan layer in the cell wall. The first-line drugs (red) consist of rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA). RIF targets RNA polymerase which is responsible for gene expression and PZA targets PanD and ultimately impedes the biosynthesis of coenzyme A (CoA) which plays a role in various pathways to ensure the survival of *M. tuberculosis*. INH and EMB target the biosynthesis of mycolic acids and arabinogalactan, respectively, which are responsible for maintaining the cell wall structure of *M. tuberculosis*.

## 1.6 Phenotypic drug tolerance

In addition to drug resistance, *M. tuberculosis* can develop drug tolerance, a phenotypic adaptation that allows the bacteria to withstand the bactericidal effects of the antimicrobial drugs. There is no genetic difference between the drug-tolerant strain and its susceptible counterpart (Smith et al 2013). These tolerant bacteria were first discovered by Hobby et al. (1942) when exposing *Streptococcus* to penicillin which was later termed "persisters" by Bigger (1944) after noticing the same phenomenon with *Staphylococcus* (Hobby et al 1942; Bigger 1944). Stress-induced and stochastic physiological changes are thought to be the cause of this drug-tolerant phenotype (Ayrapetyan et al 2018). Bacteria that encounter stressful conditions

such as hypoxia, nutrient deprivation, change in pH, acidification, starvation, and exposure to reactive oxygen species and nitrogen intermediates are driven into a low metabolic state that is non-replicating and drug-tolerant in order to survive (Zhang et al 2012; Smith et al 2013; Nguyen 2016; Gold and Nathan 2017). There are various *in vitro* models exposing *M. tuberculosis* to single stress conditions to drive the bacteria into this low metabolizing, drug-tolerant state (Betts et al 2002; Voskuil et al 2003; Voskuil et al 2004; Rustad et al 2008). The most well-known *in vitro* model is the Wayne model wherein *M. tuberculosis* was exposed to decreasing oxygen levels in a step-wise manner to transition the bacteria into a non-replicating state (Wayne and Hayes 1996). While in the above-mentioned studies *M. tuberculosis* was subjected to single stresses to drive the bacteria into a non-replicating state, a study conducted by Deb et al. (2009) used an *in vitro* multiple-stress model to better mimic the *in vivo* environment (Betts et al 2002; Voskuil et al 2003; Voskuil et al 2004; Rustad et al 2008; Deb et al 2009). The bacteria were exposed to high carbon dioxide and low oxygen levels, low nutrients and an acidic pH (5.0). Thereafter, microarray hybridisation was used to measure the gene expression levels and the data showed that ATP levels, as well as transcriptional and translational components, were all suppressed, whereas stress-responsive genes were induced (Deb et al 2009). Some studies have also shown an increase in lipid metabolism (Wayne and Hayes 1996; Betts et al 2002). The exact mechanisms behind the *in vivo* development of drug-tolerant persisters are still unknown however, *in vitro* models have shown that biofilm formation and genes regulating transcriptional and post-transcriptional machinery, metabolic slowdown, metabolic shifting, cell wall thickening and efflux pumps are either upregulated or suppressed to promote this persister state (Nguyen 2016).

### **1.6.1 Altered transcription, translation and post-translational modifications**

The *WhiB* genes function at a transcriptional level to respond to stress conditions and drug pressure. The *WhiB* genes play a role in various cellular processes such as cell division, pathogenesis and response pathways to stressors and antibiotic exposure (Zheng et al 2012). Toxin-antitoxin (TA) systems, sigma factors and small regulatory RNAs (srRNAs) respond to drug pressures through post-translational modifications. Several studies have shown that TA systems contribute to the formation of these persisters (Zhang et al 2012). TA systems are comprised of two proteins - a toxin and an antitoxin. Toxin proteins decrease bacterial metabolism by preventing DNA replication and the translation of the associated genes whereas, antitoxin proteins neutralize the effect of the toxin proteins (Lewis 2008; Smith et al 2013). There are various types of TA systems, namely type I – VI. *M. tuberculosis* has 77 TA systems,

the bulk of which are type II (Goossens et al 2021). The types are differentiated based on the nature of the antitoxin and its interaction with the toxin (Ulrichs and Kaufmann 2006). The TA systems have stringent control over the metabolism of *M. tuberculosis* thereby managing the entry and exit of this persister state (Smith et al 2013; Nguyen 2016). To better respond to antibiotic stress, sigma factors influence post-translational changes such as acetylation and phosphorylation. *M. tuberculosis* contains 13 sigma factors (SigA-M). The srRNAs control the expression levels of genes associated with efflux pumps, cell wall synthesis and membrane proteins by binding to messenger ribonucleic acid (mRNA) and inhibiting translation (Goossens et al 2021).

### **1.6.2 Metabolic slowdown and shifting**

The persister state in *M. tuberculosis* results from slowing down its metabolic activity or by shifting between pathways. As most anti-microbial drugs target metabolically active bacteria, *M. tuberculosis* lowers its metabolic activity and growth rate through multiple pathways involved in ATP synthesis, and carbon and lipid metabolism to become undetectable to these drugs (Goossens et al 2021). Additionally, bacteria can achieve hindered growth and increased drug tolerance by converting energy-generating pathways into energy storage pathways through carbon flux. For example, *M. tuberculosis* switches from carbon metabolism using the citric acid cycle to using fatty acids as a carbon storage through triacylglycerol synthesis via the upregulation of the gene *tgsl* (Goossens et al 2021).

### **1.6.3 Cell wall thickening**

The cell wall naturally acts as a barrier to prevent the entry of antimicrobial drugs. Under stressors and drug pressure, genes involved in building and maintaining the cell wall are upregulated whereas, genes involved in cross membrane transport are downregulated to thicken the cell wall to slow down the rate of drug penetration (Deb et al 2009). Additionally, the thickening of the cell wall prevents the access of the drugs via porins, which are located in the outer layer of the cell wall and used to uptake nutrients and other molecules (Niederweis 2003).

## **1.6.4 Upregulation of efflux pumps**

*M. tuberculosis* contains approximately 18 efflux pumps which are channels used to siphon out drugs that have entered the mycobacterial cell wall (Smith et al 2013). Under antibiotic pressure, there is an increase in efflux pump expression to either a single or multiple drugs (Goossens et al 2021). Increasing the activity of efflux pumps decreases intracellular drug concentrations, thereby altering the drug susceptibility of the bacteria to select drugs. Cross-resistance occurs when the upregulation of an efflux pump is induced upon exposure to one drug but reduces the drug susceptibility to another. For example, the upregulation of an efflux pump encoded by *Rv0849* induced by exposure to RIF or INH lowered the drug susceptibility of *M. tuberculosis* to RIF and amikacin (Balganesh et al 2012; Goossens et al 2021).

## **1.6.5 Biofilms**

*M. tuberculosis* can form biofilms which are formed when cells aggregate and are held together by self-secreted polymers composed of polysaccharides, proteins, lipids, and nucleic acids (Esteban and Garcia-Coca 2017). These polymers form a polar mixture around the bacteria which help the bacteria to cluster together. Biofilm formation is induced by environmental pressures that allow the bacteria to withstand these stresses (Esteban and Garcia-Coca 2017). Additionally, a study conducted by Mah and O'Toole has shown that bacterial cells within the biofilm structure are 10-100 times less susceptible to antimicrobial drugs compared to planktonic bacteria (Mah and O'Toole 2001). It is unclear whether these cells are physically protected or enter a metabolically low persister state to resist the effect of the antimicrobial drugs. There are multiple mechanisms used by *M. tuberculosis* to confer the drug-tolerant phenotype. The survival of the bacteria is dependent on how fast they can respond to stressful conditions. Not only does entering this persister state promote bacterial survival, but it also allows the bacteria to evade detection by routine diagnostic tools which are detrimental to TB diagnosis and treatment.

## **1.7 Routine diagnostic tools**

### **1.7.1 Bacterial culturing**

There are various routine diagnostic tools available to detect the presence of *M. tuberculosis* in sputum samples but culturing of the bacteria remains the gold standard. Initially, *M.*

*tuberculosis* was cultured as colonies on solid Löwenstein Jenson medium for 4-6 weeks. *M. tuberculosis* has a slow growth rate, therefore obtaining a diagnosis can take time. To combat this a more sensitive and rapid liquid-based detection test called BACTEC Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickinson) was developed. The MGIT tube contains 7-8 ml of Middlebrook 7H9 liquid media and a fluorescent compound, ruthenium pentahydrate, seeded in silica at the bottom of the tube. The compound fluoresces in response to a decrease in oxygen levels caused by actively respiring bacteria (Barry et al 2009). A positive signal can be produced anywhere between 1-5 weeks but if a signal is not produced after 42 days, it is flagged as negative equating to no growth of the bacteria. The disadvantage of the MGIT test is that it can produce false positives since other bacteria prevalent in sputum can be detected.

### **1.7.2 Smear microscopy**

Sputum smear microscopy is most commonly used to detect pulmonary TB because it is rapid and inexpensive. The technique's fundamental flaw is its insensitivity to low bacterial loads (<1000 bacilli/ml of sputum) (Desikan 2013). Therefore, fluorescence staining using the Ziel-Neelsen or Auramine stain was introduced to improve the results of smear microscopy. The Ziel-Neelsen stain contains carbol fuchsin which is taken up by every cell, however, during the destaining process with acid-alcohol only the acid fast organism (mycobacteria complex) retain the red colour whilst non-acid-fast organisms become blue or green. This is because mycobacteria have a thick, waxy lipid layer. The same principle applies for the Auramine stain whereby the dye binds to the mycolic acids found in the cell wall of the mycobacteria complex and the acid-alcohol destains the non-acid-fast organisms. Both dyes stain bacteria found in the mycobacteria complex which includes *M. bovis*, *M. canetti*, *M. microti*, *M. africanum* and *M. tuberculosis* and therefore, are non-specific to *M. tuberculosis* which can lead to misdiagnosis.

### **1.7.3 GeneXpert**

Currently, Nucleic Acid Amplification Tests (NAATs) which utilize polymerase chain reaction (PCR) to detect the presence of *M. tuberculosis* in sputum have been developed. This molecular diagnostic tool was developed to advance diagnostic tools by increasing the turnaround time for a diagnosis and to detect extrapulmonary TB (Nyendak et al 2009). The most recognized NAAT system is the GeneXpert machine which not only detects *M. tuberculosis* but can also detect RIF resistance. The GeneXpert is a cartridge based system in which the samples are

added. Within this cartridge the DNA is extracted and subjected to qPCR. There are five molecular probes that overlap five distinct regions on the RIF resistance determining region (RRDR) in RIF susceptible TB, however, if there are mutations in any of the regions the probe won't bind and won't fluoresce. The fluorescence signal indicates which region of the RRDR contains the mutation.

#### **1.7.4 The downside in TB diagnostics**

Diagnostics have vastly improved over the years, however, there is one significant downside – only actively replicating bacteria can be detected (Nyendak et al 2009). Several studies have found that sputum from TB patients with active disease contains a high proportion of non-replicating bacteria undetectable by colony-forming units (CFU) and MGIT assays (Garton et al 2008; Mukamolova et al 2009; Chengalroyen et al 2016; Dartois et al 2016). The CFU assay entails enumerating the growth of bacterial colonies on solid agar from diluted suspensions of the sample. The study by Mukamolova et al. (2009) identified a non-replicating bacterial population in sputum samples from 25 drug-susceptible, treatment-naïve patients that could only be detected in a liquid-based limiting dilutions assay, called the most probable number (MPN) assay. The data from this study showed a four-fold higher bacterial load, compared to CFU enumeration, in 20 patient samples in liquid media supplemented with resuscitation-promoting factors (Rpfs). Rpfs are growth-promoting enzymes secreted by *M. tuberculosis* that re-activate non-replicating bacteria (Mukamolova et al 2009). Furthermore, another study conducted by Turapov et al. (2016) on 15 treatment-naïve patients showed that these non-replicating bacteria present in sputum samples are also tolerant to the first-line drugs (Turapov et al 2016). This indicates that during TB treatment the non-replicating bacterial population persists, resulting in a higher bacterial load that can only be detected by the liquid MPN assay and not by CFU enumeration or MGIT assay. The inability to detect all the bacterial populations in sputum samples can lead to misdiagnosis, relapse and increased transmission rates of the disease.

#### **1.8 Differentially culturable tubercle bacilli (DCTB)**

More recently, Chengalroyen et al. (2016) further analyzed the non-replicating bacterial population in a cohort of 110 treatment naïve patients in South Africa and found that it consisted of phenotypically distinct sub-populations which they collectively termed differentially

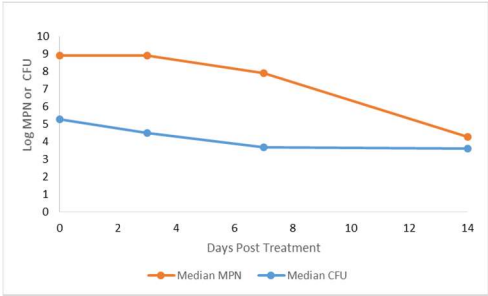
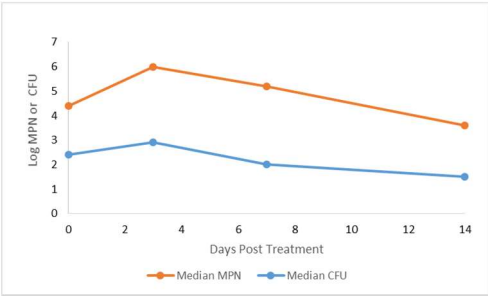
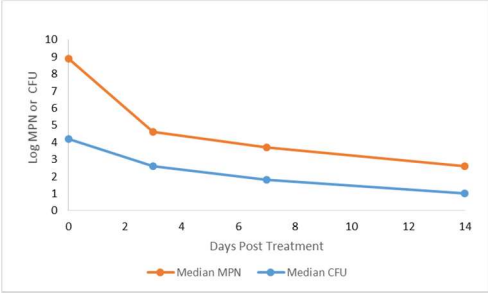
culturable tubercle bacilli (DCTB). The data showed that the non-replicating bacterial population is not only resuscitated by Rpf<sub>s</sub> but also by other growth components present in the culture filtrate (CF) such as fatty acids and cyclic-AMP (Shleeva et al 2013). Moreover, there was a sub-population that did not require any growth factors to replicate. Only 19.1% of the sputum samples required CF containing Rpf<sub>s</sub> for resuscitation (CF-dependent DCTB) whilst 11.8% did not require Rpf<sub>s</sub> for resuscitation (Rpf-independent DCTB). Furthermore, 53.6% of the sputum samples had both CF-dependent and Rpf-independent DCTB indicating that growth factors other than Rpf<sub>s</sub> within the CF are capable of resuscitating the bacteria. Lastly, 1.8% and 13.6% of sputum samples had CF-independent DCTB and no DCTB, respectively (Chengalroyen et al 2016). This longitudinal study demonstrated the complexity and heterogeneity of the DCTB populations in TB sputum samples which could have major implications for TB treatment and transmission.

Another longitudinal study (Biomarkers BMG study, ethics clearance number M140265) conducted at the CBTBR by Peters et al. (Manuscript in preparation) tested sputum samples from 80 individuals infected with RIF-susceptible TB at baseline and over a treatment course of 180 days. Individuals were treated for 56 days with the four standard first-line drugs and then only RIF and INH until the end of treatment at 180 days. The study was aimed at assessing the treatment response of DCTB using the liquid-based MPN assay compared to routine culturing methods (CFU enumeration and MGIT assays). The MPN assay is used to measure the number of bacilli present in the sputum samples by performing a ten-fold dilution of the liquid broth across a 96 well plate. Additionally, the MPN assays were performed using CF-dependent, CF-independent and Rpf<sup>-</sup>CF (obtained from mutant H37Rv strain with quintuple *rpf* deletion) conditions to determine the phenotypically distinct populations present in the sputum samples. After the MPN assays were performed for each sample, the first and last row containing growth was harvested and stored at -80°C for further analysis. In the BMG study, the MPN assay and CFU enumeration identified 37 individuals who had CF-dependent DCTB, 34 individuals had both CF-dependent and CF-independent DCTB and 5 individuals had no DCTB (CFU enumeration had a higher bacterial count compared to the MPN assay). Additionally, 2 individuals had no MPN or CFU counts and 2 had contamination. The Rpf<sup>-</sup>CF supplemented MPN assays yielded a half log decrease in bacterial load compared to CF-dependent MPN assays indicating that the presence of *rpf*<sub>s</sub> in CF promoted bacterial growth.

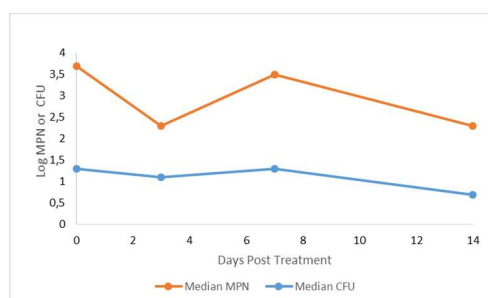
MPN assay data analysis of the DCTB population in sputum samples obtained after 14 days of treatment identified four treatment response patterns classified as early non-responders, paradoxical worsening, classic bi-phasic and non-responders as shown in Table 1. The early

non-responsive group of samples was characterized by a high baseline DCTB count which did not change significantly ( $< 0.5$  logs) until days 3 or 7 and then rapidly decreased as treatment progressed to 14 days. The patient samples in the paradoxical worsening were characterized by an increase in DCTB count on day 3 with a constant decrease until day 14. There was no substantial difference in the bacterial count at baseline and day 14 post-treatment. The classic bi-phasic group of samples was characterized by a high baseline DCTB count which rapidly declined by day 3 followed by a more gradual decrease in bacterial load until day 14. The patient samples in the non-responsive group showed a low baseline DCTB count which only varied slightly in the first 2 weeks post-treatment (Peters et al Manuscript in preparation). The four response group patterns shown below in Table 1 are a representation of the actual data obtained in the BMG study.

**Table 1. The four response group patterns for the DCTB population found in the sputum of RIF susceptible participants on TB treatment (Peters et al Manuscript in preparation)**

| Treatment Response Group | Response Pattern                                                                     | Description                                                                                                                                                                                                                               |
|--------------------------|--------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Early non-responders     |  | <ul style="list-style-type: none"> <li>• A high baseline DCTB count</li> <li>• No significant change (<math>&lt; 0.5</math> log) between baseline and day 3 or 7</li> <li>• Rapid decrease as treatment progressed to 14 days.</li> </ul> |
| Paradoxical worsening    |  | <ul style="list-style-type: none"> <li>• An increase in DCTB count on day 3</li> <li>• A constant decrease until day 14</li> <li>• There is a <math>\sim 1</math> log difference between baseline and day 14</li> </ul>                   |
| Classic bi-phasic        |  | <ul style="list-style-type: none"> <li>• A high baseline DCTB count</li> <li>• Rapid decline by day 3 followed by a more gradual decrease in bacterial load until day 14.</li> </ul>                                                      |

## Non- responsive



- A low baseline DCTB count that only varied slightly in the first 2 weeks post-treatment

In each of the response groups, the DCTB population was not completely eradicated by the end of the treatment course as the MPN assay still detected a  $\pm 1-2$  log higher bacterial count than the CFU assay. As these DCTB are not identified by current standard culture methods, they add to the complexity of TB disease diagnosis and its management. The underestimation of the bacterial population may result in misdiagnosis and premature termination of treatment. Based on the preliminary data in the BMG study it is apparent that the first-line drugs are unable to eliminate the DCTB or perhaps in the presence of antibiotics *M. tuberculosis* has the propensity to revert to a non-replicating state as a survival mechanism (Peters et al Manuscript in preparation). Presently, the mechanism on how these DCTB adapt and promote their survival during TB treatment has not been established. It is possible that these DCTB may have genetically adapted to the four first-line drugs during the treatment regimen to evade elimination. Hence, determining whether these DCTB are phenotypically drug-tolerant or drug-resistant will aid in understanding the underlying mechanisms of DCTB and their role in bacterial heterogeneity and persistence. In addition, this knowledge can advance diagnostic tools which are only able to detect actively replicating TB, improve treatment outcomes and prevent the dissemination of resistant forms of TB, lead to the development of novel drugs and improve treatment regimens in an attempt to lessen the TB burden. The stored DCTB samples from the BMG study were utilized in this study to determine whether these DCTB identified at the various time points during the 180 day treatment period are drug-resistant and/or drug-tolerant to the first-line drugs (RIF, INH, EMB, and PZA). This was achieved by determining the drug susceptibility of the stored samples by broth microdilution assay. Thereafter, the resistance conferring regions of the drugs were amplified by polymerase chain reaction (PCR) using the gDNA isolated from samples showing resistance to RIF, INH, EMB and PZA and sequenced to identify mutations that are causing the observed drug resistance.

## 1.9 Study Rationale

### 1.9.1 Aim

To determine the drug tolerance levels of resuscitated DCTB isolated from sputum samples of drug susceptible TB patients prior to and during treatment with the first-line drugs, rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA).

### 1.9.2 Objectives

1. To purify *Mycobacterium tuberculosis* (*M. tuberculosis*) from frozen MPN samples at baseline and three treatment time points (days 3, 7 and 14) for each of the four treatment response groups (early non-responsive, paradoxical worsening, classic bi-phasic and non-responsive) as classified in the BMG study (Peters et al Manuscript in preparation).
2. To determine the drug susceptibility of the purified MPN samples to the first-line drugs RIF, INH, EMB, and PZA by the broth microdilution assay.
3. To sequence the RIF resistance determining region (RRDR), *katG* gene, *ahpC* promoter region as well as the *embCAB* operon of all identified RIF, INH and EMB resistant resuscitated DCTB samples, respectively, to establish if a particular mutation is enhanced during treatment and correlate it to the four treatment groups classified in the BMG study (Peters et al Manuscript in preparation).
4. To correlate the drug-resistant patterns observed for select resistant samples to the four treatment response groups classified in the BMG study (Peters et al Manuscript in preparation).
5. To generate DCTB using the carbon starvation model and determine the drug susceptibility of these select DCTB samples to RIF, INH and EMB by broth microdilution assay and compare it to the drug susceptibility obtained for the unstarved samples.

## **2. Materials and Methods**

### **2.1 Materials**

A list of all the materials used and preparation guidelines are under Appendix A.

### **2.2 Collection of samples grown in the Most probable number (MPN) assay**

Of the 73 participants in the BMG study conducted by Peters et al. (Manuscript in preparation), 16 patients were included as part of this study (Peters et al Manuscript in preparation). Each participant had a spot and early morning (overnight) sputum sample which was collected at baseline and throughout the treatment course on days 3, 7, 14, 35, 56 and 180. The samples used in this study were harvested and stored at -80°C from previously performed CF-dependent MPN assays using limiting dilutions of patient sputa (Peters et al Manuscript in preparation). The MPN samples containing the resuscitated DCTB were retrieved from the -80°C freezer for further analysis. A cohort of 16 patients comprised of 7 from the early non-responders group, 5 from the paradoxical worsening group and 4 from the classic bi-phasic group were included in this study. Ethics approval was obtained for the use of these samples in this study, the ethics clearance certificate can be found under Appendix D (ethics clearance number M210448).

Treatment is most effective after 2 weeks, as there is a significant decrease in bacterial load therefore, treatment time points after day 14 were not included in this study. Additionally, based on the data from the principal study, the initial bacterial load of some samples in the non-responders treatment group was low therefore, the outgrowth of *M. tuberculosis* from these samples was unattainable and was excluded from this study. The number of frozen MPN samples analyzed in this study is shown in Table 2 below. The strain type, demographics and HIV status of each patient are listed under Appendix B.

**Table 2. List of MPN samples from the various response groups analyzed in this study.**

| Response Group        | Number of patients | Patient IDs                                     | Total number of samples |
|-----------------------|--------------------|-------------------------------------------------|-------------------------|
| Early non-responders  | 7                  | 59006, 59052, 59041, 59028, 57163, 57158, 57162 | 46                      |
| Paradoxical worsening | 5                  | 59014, 59035, 59127, 59015, 57134               | 43                      |
| Classic bi-phasic     | 4                  | 59007, 59019, 59023, 59032                      | 23                      |

### 2.3 Decontamination of the frozen MPN samples

A frozen aliquot (approximately 400  $\mu$ l) of each MPN sample containing resuscitated DCTB was thawed and added to 5 ml of Difco™ Middlebrook 7H9 liquid media (BD) supplemented with 0.05% volume/volume (v/v) Tween80 and 0.1% (v/v) BBL™ Middlebrook oleic albumin dextrose catalase (OADC) Enrichment (BD) (Figure 4A). The media was further supplemented with 1X polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL™ MGIT™ PANTA™, referred to as PANTA) (BD) to control contaminating bacteria from the lung environment that could have proliferated during the isolation of the DCTB bacteria from these sputum samples. To screen for contamination, 100  $\mu$ l of the inoculated culture was streaked to single colonies on Middlebrook 7H11 solid media (SIGMA®) (Figure 4B). The liquid cultures were incubated at 37°C for 5-7 days whilst the solid plates were incubated under the same conditions for 2-3 weeks. Liquid cultures that did not show contamination on the plates after 5 days were sub-cultured into 5 ml of 7H9 media without PANTA and re-streaked onto 7H11 solid media (Figure 4C). The liquid samples that had contamination on the plates, were re-cultured in 5 ml of 7H9 media with PANTA and re-streaked onto 7H11 solid plates. If the contamination persisted then the liquid culture was serially diluted and spread on 7H11 solid plates to obtain single *Mycobacterium tuberculosis* (*M. tuberculosis*) colonies (Figure 4D). Once the plates were free of contamination (fungus and other bacteria that did not display the classic *M. tuberculosis* morphology), 1 ml of the culture or a single colony was used to inoculate 10 ml of 7H9 media without PANTA supplementation, and grown to an optical density at a wavelength of 600nm (OD<sub>600nm</sub>) of 0.5 to 0.8 (Figure 4E). Cultures were grown within this range since the bacteria enter the log phase and contain a high number of live cells. Cultures below an OD<sub>600nm</sub> of 0.5 have few live cells, whereas beyond the OD<sub>600nm</sub> of 0.8 the bacteria enter the stationary phase where the rate of cell division is equal to the rate of cell death. Once the

OD<sub>600nm</sub> was reached, 1 ml aliquots of the purified samples were dispensed into 1.5 ml O-ring tubes and frozen at -80°C for downstream analyses (Figure 4F).

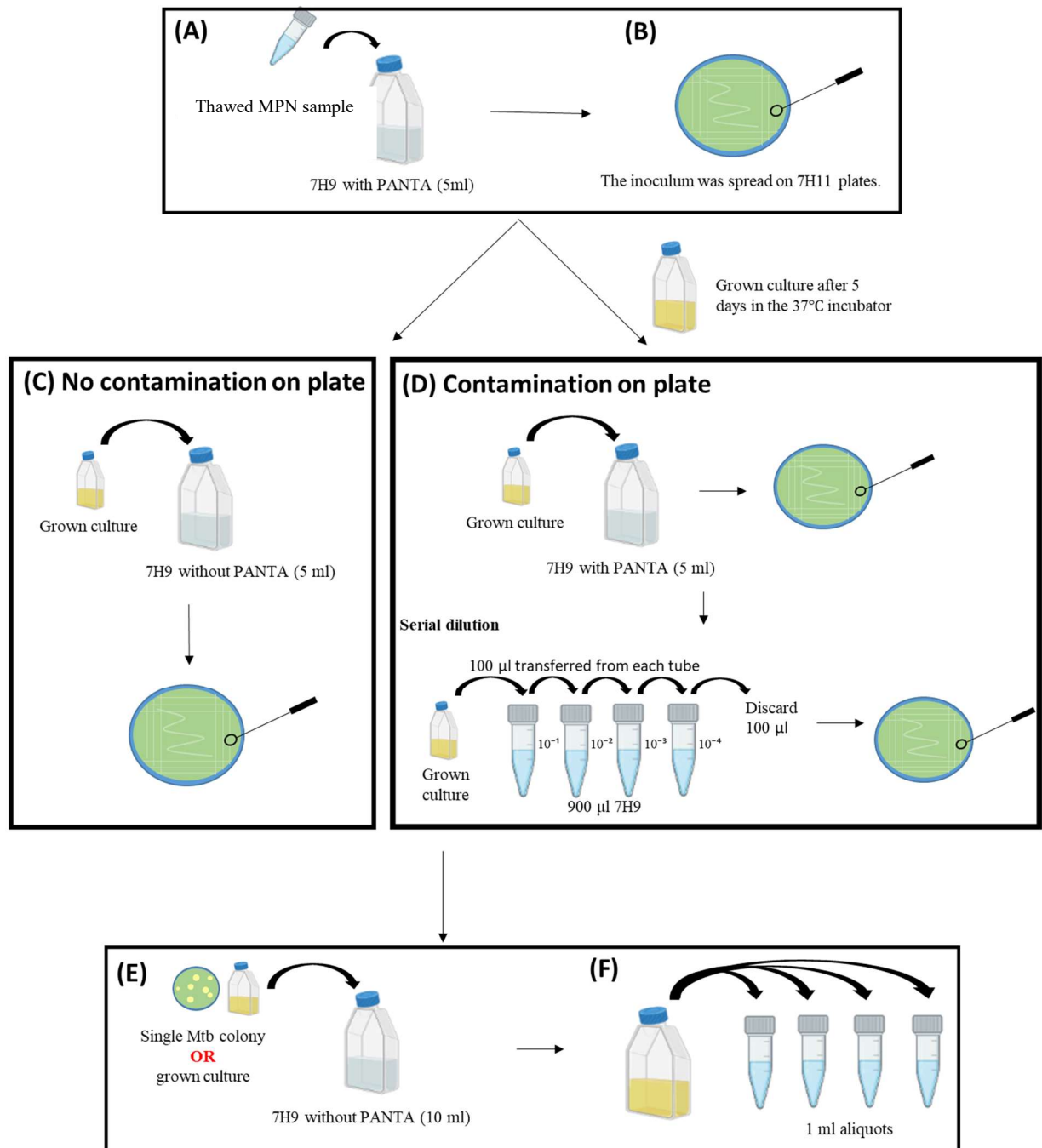


**Figure 4. Decontamination of the MPN samples**

(A) Inoculate 5 ml of supplemented 7H9 media with the thawed DCTB sample. (B) Spread 100 µl of the inoculum on 7H11 plates. (C) Inoculate the grown culture in 5 ml of unsupplemented 7H9 media and spread an aliquot on a 7H11 plate. (D) Inoculate the grown culture in 5 ml of supplemented 7H9 and spread some on a 7H11 plate. If contamination persists, perform a serial dilution of the grown culture and spread each dilution on a 7H11 plate. (E) Once the samples are free of contaminants, inoculate a single colony or the grown culture in 10 ml of unsupplemented 7H9. (F) Once grown to the correct OD<sub>600nm</sub>, 1 ml working stocks of the purified culture were aliquoted and frozen at -80°C.

## 2.4 Broth microdilution assay

The broth microdilution assay was used to determine the lowest concentration of rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) required to inhibit bacterial growth. The assays were first optimized using the wildtype *M. smegmatis* strain, mc<sup>2</sup>155, and the wildtype *M. tuberculosis* strain, H37Rv, before being used to assess the drug susceptibility of the purified MPN samples (Section 2.3) to the four first-line drugs. In addition to performing the broth microdilution assay using the conventional 2-fold dilution, a lower dilution method was performed as described in Section 2.4.4.

### 2.4.1 Drug preparation

All the drugs used in this study were purchased from SIGMA<sup>®</sup> in powder form and stored in the fridge at 4°C. Each drug was dissolved in 99.8% dimethylsulfoxide (DMSO) (SIGMA<sup>®</sup>) or HyPure<sup>™</sup> Molecular Biology Grade Water (HyClone<sup>™</sup>) to make stocks at the desired drug concentration as shown in Table 3.

**Table 3. Drug preparation summary for the four first-line drugs.**

| Drug         | Solvent              | Drug concentration (mg/ml) |                            |
|--------------|----------------------|----------------------------|----------------------------|
|              |                      | mc <sup>2</sup> 155        | H37Rv and purified samples |
| Rifampicin   | DMSO                 | 1 (1)                      | 0.1 (0.01)                 |
| Isoniazid    | HyClone <sup>™</sup> | 1 (10)                     | 0.1 (0.1)                  |
| Ethambutol   | DMSO                 | 10 (1)                     | 10 (10)                    |
| Pyrazinamide | HyClone <sup>™</sup> | 13                         | 13                         |

The values in brackets indicate the drug concentrations made up to perform the lower dilution method.

### 2.4.2 Culture preparation

One aliquot of each frozen purified sample (Section 2.3) was thawed and inoculated into 5 ml of 7H9 liquid media and grown to an OD<sub>600nm</sub> of approximately 0.5-0.8. To ensure the stocks were free of contamination, 50-100 µl of the thawed stock was streaked to single colonies on 7H11 solid media. The broth microdilution assay was set up once the samples had grown to the required OD<sub>600nm</sub> and were confirmed to be free of contamination.

### **2.4.3 Set up of the broth microdilution assay in a 96 well plate using a 2-fold dilution**

In a 96 well round-bottom microtitre plate (Thermo Scientific or SIGMA<sup>®</sup>), 50 µl of 7H9 media was added to all wells in rows 2-12 (Figure 5A). In the first row, 100 µl of 7H9 media was added to the first well, an equivalent concentration of the solvent (DMSO or HyClone<sup>™</sup>) used to dissolve the drugs was added to the second well and in the remaining 6 wells, 3 of the respective drugs diluted in 7H9 to the required concentration and added in duplicate (Figure 5A). Once the drugs were aliquoted in the first row, a 2- fold serial dilution was performed by removing 50 µl from row 1 using a multichannel pipette and adding to row 2. Following this, the liquid in row 2 was mixed and transferred to row 3. This process was followed until row 12 and then 50 µl was discarded from this well so that the volume in each well was 50 µl. Thereafter, 50 µl of a 1:500 diluted respective purified culture was added to all the wells thereby halving the drug concentrations (Figure 5B). The wild-type *M. tuberculosis* strain (H37Rv) was used as the control. The purified samples that can withstand a higher concentration of any of the first-line drugs compared to the H37Rv strain were considered resistant and those that can withstand the same concentration as the H37Rv strain was classified as tolerant. The inoculated plates were placed into zip lock bags and incubated at 37 °C in a CO<sub>2</sub> incubator for 14 days (Figure 5C).

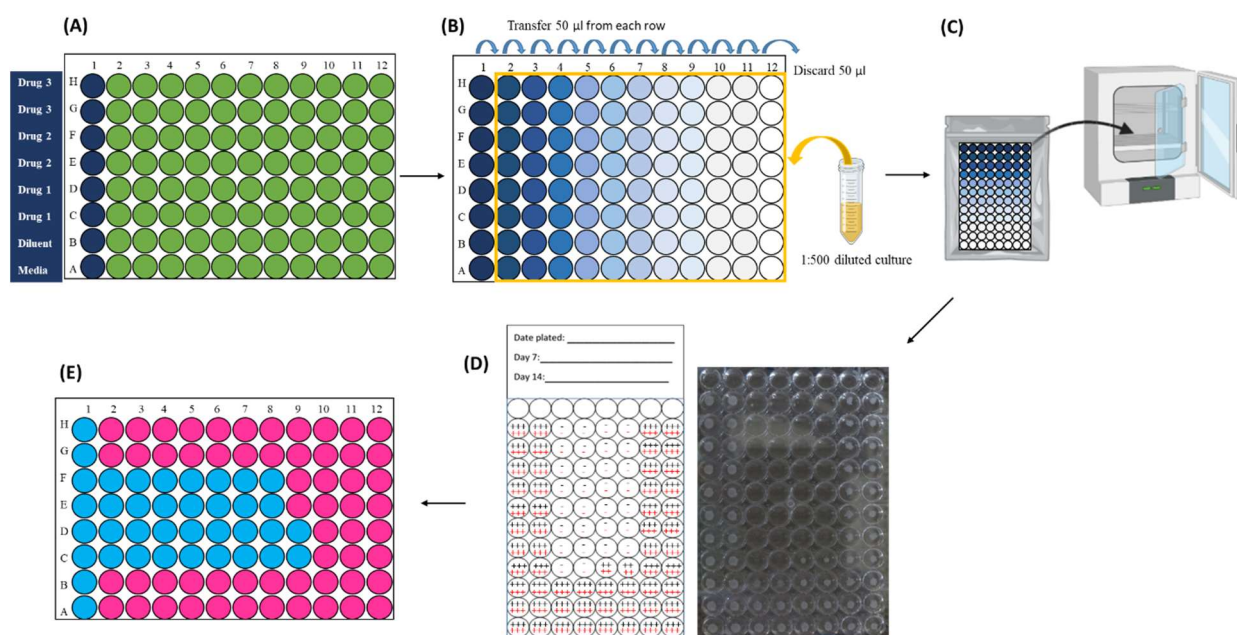


**Figure 5. The 96 well plate set up for the liquid broth microdilution assay.**

(A) In a 96 well plate, media was added to the wells in all the rows except for the first row (green). In the first row, media was added to the first well (A1), the diluent was added to the second well (B1) and the drugs were added to the remaining wells in duplicate (C1-H1) (dark-blue). (B) The dilution series was performed (shown by the curved arrows) and then 50 µl of the respective diluted cells were added to the wells in all the rows except for the first row which is outlined in yellow. (C) The plates were incubated at 37°C for 14 days (D) The plates were scored visually with an inverted mirror at day 7 (black) and day 14 (red) before AlamarBlue® was added to each well. (E) After 16-18 hours, bacterial growth was visualized on the plate, pink = bacterial growth whilst light blue = no growth. The first row displaying no bacterial growth represents the MIC for that drug. The figure was adapted from (Padarath 2020).

#### 2.4.4 Set-up of the broth microdilution assay in a 96 well plate using a 1.41 and 1.26 dilution method

A lower dilution series was assessed based on a study conducted by Colangeli et al. (2018) which utilized a 1.41 dilution and 1.26 dilution series to obtain MIC values for RIF and INH, respectively, instead of the conventional 2-fold dilution (Colangeli et al 2018). The lower dilution series overlaps with the 2-fold dilution with additional points in between to obtain a more precise MIC value. The 1.26-fold dilution was derived from the formula  $\log_2 x = 1/3$  and adds two values between the points in the 2-fold dilution. Whilst the 1.41-fold dilution was derived from the formula  $\log_2 x = 1/2$  and adds a single value between the points in the 2-fold dilution series. Colangeli et al. (2018) did not establish a lower dilution series for EMB and as such the 1.41 dilution series was used to determine the MIC values for EMB because this dilution series overlapped better with the conventional 2-fold dilution series than the 1.26 dilution series. The lower dilution series was performed similarly to the 2-fold dilution except the following volumes were used to set up the 96 well plates:

**Table 4. Lower dilution series volumes to perform the broth microdilution assay.**

| <b>Dilution series</b> | <b>Volume of media added to rows 2-12</b> | <b>Volume of media, diluent and drugs added to row 1</b> | <b>Volume transferred between rows</b> | <b>Volume of diluted purified culture</b> |
|------------------------|-------------------------------------------|----------------------------------------------------------|----------------------------------------|-------------------------------------------|
| 1.41 fold              | 58                                        | 200                                                      | 142                                    | 58                                        |
| 1.26 fold              | 41                                        | 200                                                      | 159                                    | 41                                        |

### **2.4.5 Scoring and analysis of the broth microdilution assay plates**

The bacterial growth on the plates was visually scored on days 7 and 14 using an inverted mirror based on the size of the pellet, where the largest pellet was scored as +++ and as the pellet decreased in size the score decreased to ++ and +. Partial growth was scored as +/- and no growth was scored as minus (-) (Figure 5D). On day 14, 10 µl (1/10 of the total volume in each well) of AlamarBlue® was added to each of the wells and the plates were incubated at 37°C in a CO<sub>2</sub> incubator for 16 to 18 hours. The AlamarBlue® measures the concentration of viable cells in a sample by quantitatively measuring the proliferation of the bacteria. An oxidation-reduction indicator is included in the dye, which fluoresces and changes colour in response to the chemical reduction caused by cell growth. The oxidized form of the dye is blue and non-fluorescent, whereas, growing cells cause a chemical reduction of the dye from non-fluorescent blue to fluorescent pink (Figure 5E). Following the incubation time, the dye absorbance was measured using the SpectraMax® Gemini™ EM Microplate Spectrofluorometer (Molecular Devices®) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The last row which does not contain growth represents the MIC for that drug.

## **2.5 Characterization of drug-resistant bacteria identified from the broth microdilution assay**

### **2.5.1 Small scale genomic DNA (gDNA) extraction**

The genomic DNA (gDNA) was extracted from the purified samples displaying drug resistance to RIF, INH or EMB using the colony boil method. Depending on the colony size, a single colony or roughly 2-3 colonies were selected from the 7H11 solid plates used to check the purified stocks for contamination during the cell preparation step for the broth microdilution assay (Section 2.4.2). The colony/colonies were resuspended in 100 µl H<sub>2</sub>O and then heat killed

for 5 minutes at 95°C in a heating block. After the tubes had cooled, 100 µl of chloroform was added, vortexed for a few seconds then centrifuged using the Microcentrifuge 5417 R (Eppendorf) for 5 minutes at 900 ×g. Thereafter, ±80 µl of the aqueous phase containing the gDNA was removed into a sterile O-ring tube and 1 ul was used for polymerase chain reaction (PCR) to amplify the resistance-conferring genes of RIF, INH and EMB.

### **2.5.2 Polymerase chain reaction (PCR)**

The rifampicin resistance determining region (RRDR) of the *rpoB* gene, the *katG* gene and *ahpC* promoter region, which confers INH resistance, and the *embC*, *embB* and *embA* genes, which confers EMB resistance, were all amplified using PCR. A high-fidelity enzyme, Q5, was used to obtain low error rates during amplification. The reverse and forward primer sequences for amplifying each of these genes/regions are listed in Table 5. The primer sequences were taken from Tekwu et al. (2014) (Tekwu et al 2014). The reagents and the amount of each component used for a 20 ul PCR reaction are shown in Table 6 and the cycling conditions are listed in Table 7. In a PCR reaction, the template DNA is denatured from double stranded to single stranded so the primers can bind to the template DNA. Once bound, the Q5 polymerase extends the primers by adding the dNTPs which synthesizes new DNA strands. The annealing temperature and annealing time are dependent on the primer set as shown in Table 8. The extension time was calculated as 30 sec for every 1000 base pairs (1 kb).

**Table 5. List of PCR primers used to amplify the resistance conferring genes for RIF, INH and EMB.**

| Gene        | Primer type | Primer sequence (5'→ 3') | Fragment size (base pairs) |
|-------------|-------------|--------------------------|----------------------------|
| <i>rpoB</i> | F           | TCGCCGCGATCAAGGAGT       | 158                        |
|             | R           | GTGCACGTCGCGGACCTCCA     |                            |
| <i>katG</i> | F           | GAAACAGCGGCGCTGATCGT     | 210                        |
|             | R           | GTTGTCCCATTTTCGTCGGGG    |                            |
| <i>ahpC</i> | F           | ACCACTGCTTTGCCGCCACC     | 237                        |
|             | R           | CCGATGAGAGCGGTGAGCTG     |                            |
| <i>embA</i> | F           | GCCGGCTATGTAGCCAACTA     | 338                        |
|             | R           | GACCGTTCCACCAACACC       |                            |
| <i>embB</i> | F           | CCGACCACGCTGAAACTG       | 363                        |
|             | R           | GTAATACCAGCCGAAGGGATCCT  |                            |
| <i>embC</i> | F           | GTTCGACAAGCGCGCCACAC     | 346                        |
|             | R           | CGGAGGTAGATGGTAGCCGG     |                            |

F: forward, R: reverse

**Table 6. List of PCR components.**

| PCR component                          | Volume (µl) | Concentration |
|----------------------------------------|-------------|---------------|
| 5X Q5 Reaction Buffer                  | 4.0         | 1X            |
| 5X Q5 high GC enhancer                 | 4.0         | 1X            |
| 1.25 mM dNTPs                          | 3.2         | 0.25 mM       |
| 10 mM Forward primer <sup>o</sup>      | 1.0         | 0.5 mM        |
| 10 mM Reverse primer <sup>o</sup>      | 1.0         | 0.5 mM        |
| 2 U/µl Q5 High fidelity DNA polymerase | 0.2         | 0.4 U         |
| Template DNA                           | 1.0         | -             |
| Nuclease-Free Water                    | 5.6         | -             |
| <b>Total Volume</b>                    | <b>20</b>   |               |

<sup>o</sup>The primer volume was halved when using the *ahpC* and *embC* set to decrease the number of primer dimers formed during the reaction.

**Table 7. Cycling conditions for the PCR reaction.**

| Step                 | Number of Cycles | Temperature (°C) | Time (seconds)   |
|----------------------|------------------|------------------|------------------|
| Initial denaturation | 1                | 98               | 30               |
| Denaturation         | 34               | 98               | 10               |
| Annealing            | 34               | Refer to Table 8 | Refer to Table 8 |
| Initial elongation   | 34               | 72               | 30 sec=1 kb      |
| Elongation           | 1                | 72               | 120              |

**Table 8. Primer annealing temperature and time**

| Gene        | Annealing temperature (°C) | Annealing time (seconds) |
|-------------|----------------------------|--------------------------|
| <i>rpoB</i> | 72                         | 10                       |
| <i>katG</i> | 68                         | 10                       |
| <i>ahpC</i> | 70                         | 10                       |
| <i>embA</i> | 68                         | 20                       |
| <i>embB</i> | 72                         | 20                       |
| <i>embC</i> | 72                         | 10                       |

### 2.5.3 Agarose gel electrophoresis

The size of each of the PCR products was confirmed using agarose gel electrophoresis which is a technique used to separate nucleic acids based on their molecular size by applying an electric current. A 1.5 % gel was made by melting 0.45 g of SeaKem™ LE Agarose (Lonza™) in 30 ml of 1X Tris-acetate-EDTA (TAE) buffer in a microwave. The gel mixture was allowed to cool before adding 1X SYBR™ Safe gel stain (Invitrogen by Thermo Fisher Scientific, 10 000X concentrate). SYBR™ Safe intercalates the DNA so it can be visualized when exposed to UV light. The gel mixture was swirled until homogenous and then poured into a gel casting tray with an appropriate size comb. Once the gel was set it was transferred to the gel tank containing 1X TAE buffer. The amplified DNA (2 µl) was combined with 2 µl of 6X loading dye and loaded into the gel wells. The DNA was separated for 90 minutes at 90V using the PowerPac™ High-Current Power Supply (BioRad). The integrity and quality of the DNA were visualized using the Syngene G: Box equipped with the Syngene GeneSnap version 7.12. Bands with clear edges indicate intact DNA whereas blurred bands and streaks indicate sheared DNA. The brightness of the band correlated with the amount of DNA. The brighter the band, the

higher the amount of DNA present. For each primer set, a single DNA band was expected. Multiple bands indicate non-specific binding of the primers to the gDNA which required optimization.

## 2.5.4 Sequencing

The amplified PCR products with each of the primer sets were sent to the Central Analytical Facilities (CAF) DNA sequencing unit in Stellenbosch for sequencing. Once the sequence data was received, the sequences were analyzed and aligned to the wildtype laboratory strain, H37Rv as well as reference loci sequences obtained from Mycobrowser using FinchTV version 1.4.0 (Geospiza Inc.) and Jalview version 2.11.1.7, respectively, to identify single nucleotide polymorphisms (SNPs) (Table 9).

**Table 9. The reference sequences from Mycobrowser were used to analyze and align the resistance conferring regions of RIF, INH and EMB.**

| Drug | Gene               | Locus   |
|------|--------------------|---------|
| RIF  | <i>RpoB</i> (RRDR) | Rv0667  |
| INH  | <i>katG</i>        | Rv1908c |
|      | <i>ahpC</i>        | Rv2428  |
| EMB  | <i>embA</i>        | Rv3794  |
|      | <i>embB</i>        | Rv3795  |
|      | <i>embC</i>        | Rv3793  |

## 2.6 Carbon starvation assay

The purified samples used in this study were DCTB derived from the MPN assay done on sputum obtained from patients as part of the BMG study (Peters et al Manuscript in preparation). As these purified samples were re-cultured *in vitro* it is possible that the resuscitated DCTB may no longer possess the DCTB phenotype. Therefore, the carbon starvation assay was used to force the bacteria back into a reversible non-replicating DCTB state by growing the bacteria in phosphate-buffered saline (PBS), which provides a stable environment for bacterial preservation but lacks the nutrients required for bacterial growth. PBS lacks a carbon source which lowers the respiration levels of the bacteria thereby forcing it into

a reversible DCTB state. The starvation conditions were modified from a study published by Saito et al. (2017) (Saito et al 2017).

### **2.6.1 Cell preparation**

Select purified samples were thawed and inoculated in 8 ml of 7H9 liquid media and grown for 2-3 days to an OD<sub>600nm</sub> of 0.5-1.0. Once grown, the culture was sub-cultured into two 20 ml cultures using 7H9 liquid media supplemented with 0.02 % (v/v) tyloxapol (Tx-media), which is a surfactant to prevent cell clumping and grown to an OD<sub>600nm</sub> of 0.4-0.8. Thereafter, each culture was transferred into a sterile 50 ml Falcon tube and centrifuged at 4000 ×g for 8 minutes using the Allegra® X-30 Benchtop Centrifuge (Beckham Coulter Life Sciences). The pellet obtained from one tube was washed twice with 20 ml of Tx-media whilst the other pellet was washed twice with the same volume of PBS by repeated centrifugation and resuspension. After the wash steps, the pellets were resuspended in an equal volume of the respective medium (Tx-media or PBS) and centrifuged at 200 ×g for 8 min with zero deceleration to create a single-cell suspension. Approximately 15 ml of the PBS single cell suspension was transferred into a sterile 50 ml Falcon tube. Thereafter, the PBS suspension was diluted in 7 ml of PBS to an OD<sub>600nm</sub> of 0.1 in triplicate and incubated at 37 °C for one week to starve the bacteria before performing MPN and CFU assays. The OD<sub>600nm</sub> of the starved cell culture after 1 week decreased from 0.1 to 0.02 suggesting that the cells have entered a non-replicating, drug-tolerant state. The PBS lacks a carbon source which lowers the respiration levels of the bacteria which forces it into the DCTB state. The decrease in the OD<sub>600nm</sub> does not indicate cell death because the ‘starved’ bacteria grow in the MPN and CFU plates. A volume of 1 ml Tx-media single cell suspension was transferred into a sterile 1.5 ml O-ring tube to perform the MPN and CFU assays to determine the number of bacteria before starvation.

### **2.6.2 Set-up of the 96 well MPN plate**

In a 96 well flat-bottom microtiter plate (Thermo Scientific), 180 µl of Tx-media was added to every well after which 20 µl of the unstarved cells (resuspended in TX-media) were added to wells B2-G2 (Figures 6A1 and 6B1). A 10-fold serial dilution was then conducted by transferring 20 µl of the cell suspension from wells B2-G2 into wells B3-G3 using a multichannel pipette. The process was continued until wells B11-G11 with the removal of 20 µl from the last wells (Figure 6C1). The same process was carried out for the starved PBS

cultures (Figures 6A2 to 6C2), the only exception to the process was that culture filtrate (CF) was added to wells E2-11, F2-11 and G2-11 instead of Tx-media (Figure 6A2). The plates were sealed with biohazard tape and incubated at 37 °C for 4-5 weeks to minimize evaporation during the long incubation period.

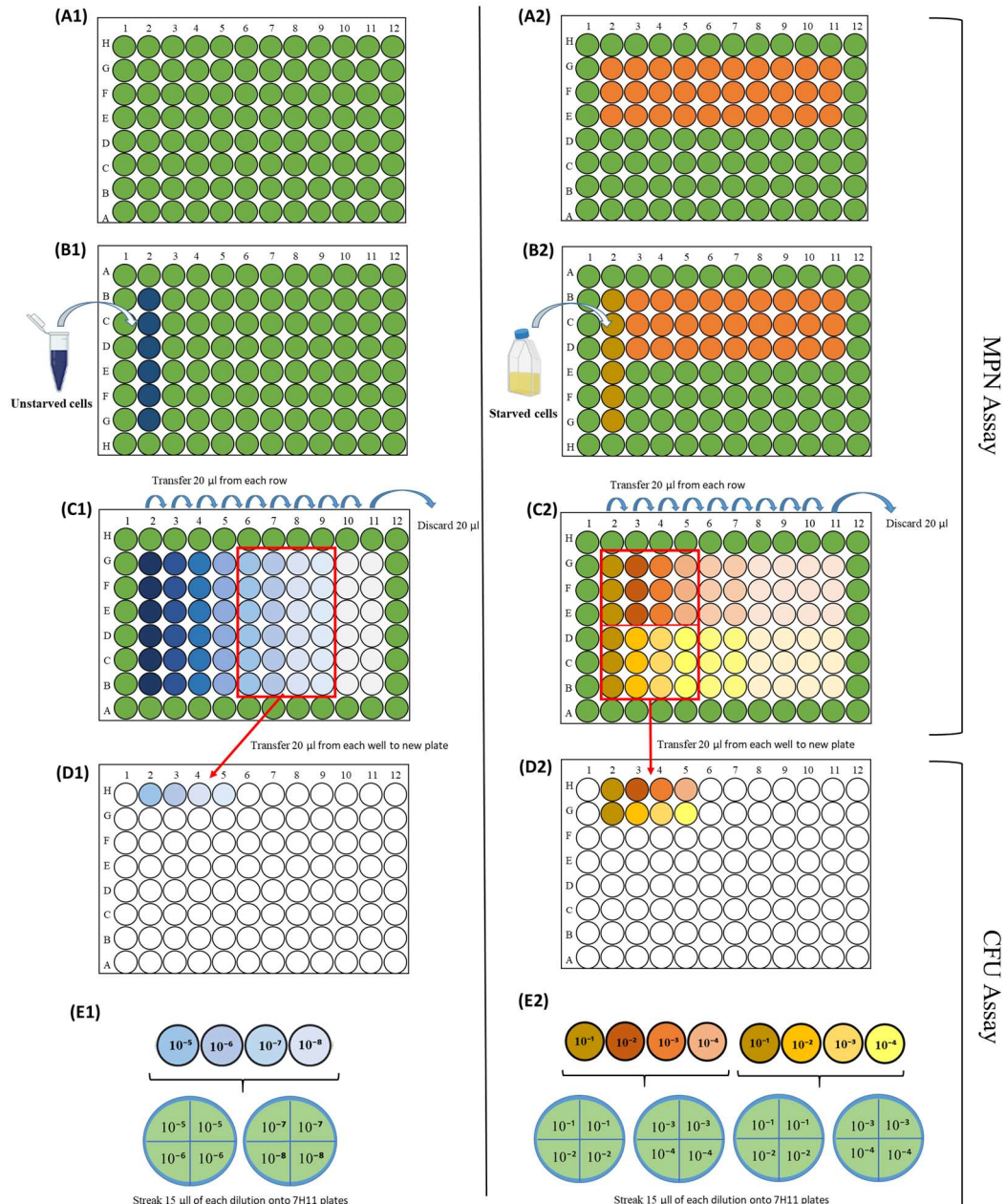


**Figure 6. The experimental set up for the MPN and CFU assay for carbon starvation.**

(A1) In a 96 well plate, media (green) was added to all the wells. (A2) In a 96 well plate, media (green) was added to all the wells except for E2-11, F2-11 and G2-11 to which culture filtrate (orange) was added. (B1 and 2) The starved cells (yellow) or unstarved cells (dark blue) (20 µl) were added to wells B2-G2. (C1 and 2) The 10-fold serial dilution was performed as shown by the curved arrows. (D1 and 2) After the MPN assay was performed, 20 µl was removed from each well in rows 6-9 (dilutions  $10^{-5}$  to  $10^{-8}$ ) for the unstarved cells and rows 2-5 for starved cells (dilutions  $10^{-1}$  to  $10^{-4}$ ) (red boxes in C1 and C2) and placed in a new sterile 96 well microtiter plate. (E1 and 2) Each dilution (15 µl) was spread in duplicate on 7H11 quarter plates.

### 2.6.3 Preparation of culture Filtrate (CF)

A 1 ml freezer stock of H37Rv was thawed and inoculated in 8 ml of 7H9 media and grown to an OD<sub>600nm</sub> of 0.5-0.8. The culture was then sub-cultured into a larger volume of 7H9 and grown to an OD<sub>600nm</sub> of 0.5-0.8. The cells from the grown culture were harvested by centrifugation at 3000 ×g for 8 minutes at 22 °C using the Allegra® X-30 Benchtop Centrifuge (Beckham Coulter Life Sciences). The supernatant was filtered-sterilized using a 0.22 µm Millex-GP syringe filter unit (Merck) and diluted in a 1:1 ratio with 7H9 media. The filtrate was then supplemented with 3.8 % (v/v) 1X PANTA to prevent the growth of contaminants. The pellet, supernatant, and CF were streaked on 7H11 solid plates and incubated at 37 °C for 4 weeks for quality control. In addition, an aliquot of CF was incubated at 37 °C for 4 weeks to check for contamination.

### 2.6.4 Colony-forming unit (CFU) assay

Once the MPN assay dilution series was complete, 20 µl was removed from each well in rows 6-9 (dilutions 10<sup>-5</sup> to 10<sup>-8</sup>) for unstarved cells and rows 2-5 for starved cells (dilutions 10<sup>-1</sup> to 10<sup>-4</sup>) for each column and combined each row in a new sterile microtiter plate (Figures 6D1 and D2). Thereafter, 15 µl of each dilution was spread in duplicate on 7H11 plates to obtain single *M. tuberculosis* colonies (Figures 6E1 and 6E2). The CFU plates were sealed in black bags and incubated at 37°C for 3-4 weeks.

### 2.6.5 Scoring and analysis of MPN and CFU plates

The MPN plates were scored visually for growth after 4-5 weeks using an inverted mirror. Wells which contained growth were scored as positive. The total number of bacteria recovered using the MPN assay was calculated using an MPN calculator (available at <http://www.wiwiss.fuberlin.de/fachbereich/vwl/iso/ehemalige/wilrich/index.html>).

The colonies on the 7H11 plates after 3-4 weeks were counted and as each dilution was performed in duplicate the number of colonies for each dilution was averaged. The CFU/ml was calculated using the following formula:

$$\text{CFU/ml} = \frac{\text{The number of colonies} \times \text{Dilution factor}}{\text{Volume spread on plates}}$$

### **2.6.6 Resuscitation index (RI) calculation**

The resuscitation index (RI) was calculated by comparing the number of bacteria recovered from the MPN assay (liquid) versus CFU enumeration (solid). The RI represents the amount of DCTB present after the starvation process. The index was calculated using the following formula:

$$\text{Resuscitation index (RI)} = \frac{\text{MPN value}}{\text{CFU/ml}}$$

A RI value greater than 1 indicates that DCTB was produced after the starvation process as these bacteria are only recoverable from the MPN assay. If no DCTB was produced after the starvation process then the RI would be less than 1.

### **2.6.7 Broth microdilution assay using starved cells**

The MIC for the starved cells were assessed against RIF, INH and EMB using the broth microdilution assay described in Sections 2.4 using the lower (1.41 and 1.26) dilution method. Two assays were performed for each starved culture, one assay with 7H9 liquid media (CF-independent) and the second assay with CF (CF-dependent) to determine whether the stimulatory molecules present in CF aid in the resuscitation of the bacteria. Additionally, the starved PBS cultures were not diluted in a 1:500 ratio before being inoculated in the broth microdilution plates. The inoculated plates were placed into zip lock bags and incubated at 37°C in a CO<sub>2</sub> incubator for 14 days. The plates were scored visually for growth on days 7 and 14 using an inverted mirror and the bacterial growth on the plates was measured based on the size of the pellet as described in Section 2.4.5.

## **2.7 Data analysis**

Microsoft Excel 2013 (Microsoft Corporation) was used to construct all graphs and statistical analyses in this study. The student's two-tailed *t*-test was performed at a 95% confidence level to compare the significance between the MIC values obtained for the unstarved cultures versus starved cultures under CF-independent and CF-dependent conditions (Section 2.6.7).

### 3. Results

#### 3.1 Decontamination outcomes of the frozen MPN samples

The select frozen MPN samples harvested and stored from previously performed MPN assays in the BMG study were retrieved from the -80 °C freezer and decontaminated as per Sections 2.2 and 2.3. All samples were sub-cultured 3-4 times before stocks were made. The number of samples analyzed in the study and the growth outcomes after the decontamination of the samples in the various response groups are listed in Table 10 below. For the early non-responsive group, 35 out of the 46 samples (76.1%) were successfully decontaminated. Whilst for the paradoxical worsening and classic bi-phasic groups, 26 out of 43 (60.5%) and 4 out of 23 (17.4%) of the samples were respectively decontaminated (Table 10). The DCTB samples either had growth, no growth or contamination when inoculated and grown in the PANTA-supplemented liquid media as per Section 2.3. Once a pure culture was obtained for all the samples, the drug susceptibility of each culture was assessed using the broth microdilution assay using the four first-line drugs. A more detailed analysis of the growth response for each patient's purified DCTB sample at each time point is under Appendix C.

**Table 10. Decontamination outcomes of the stored MPN samples from the various response groups.**

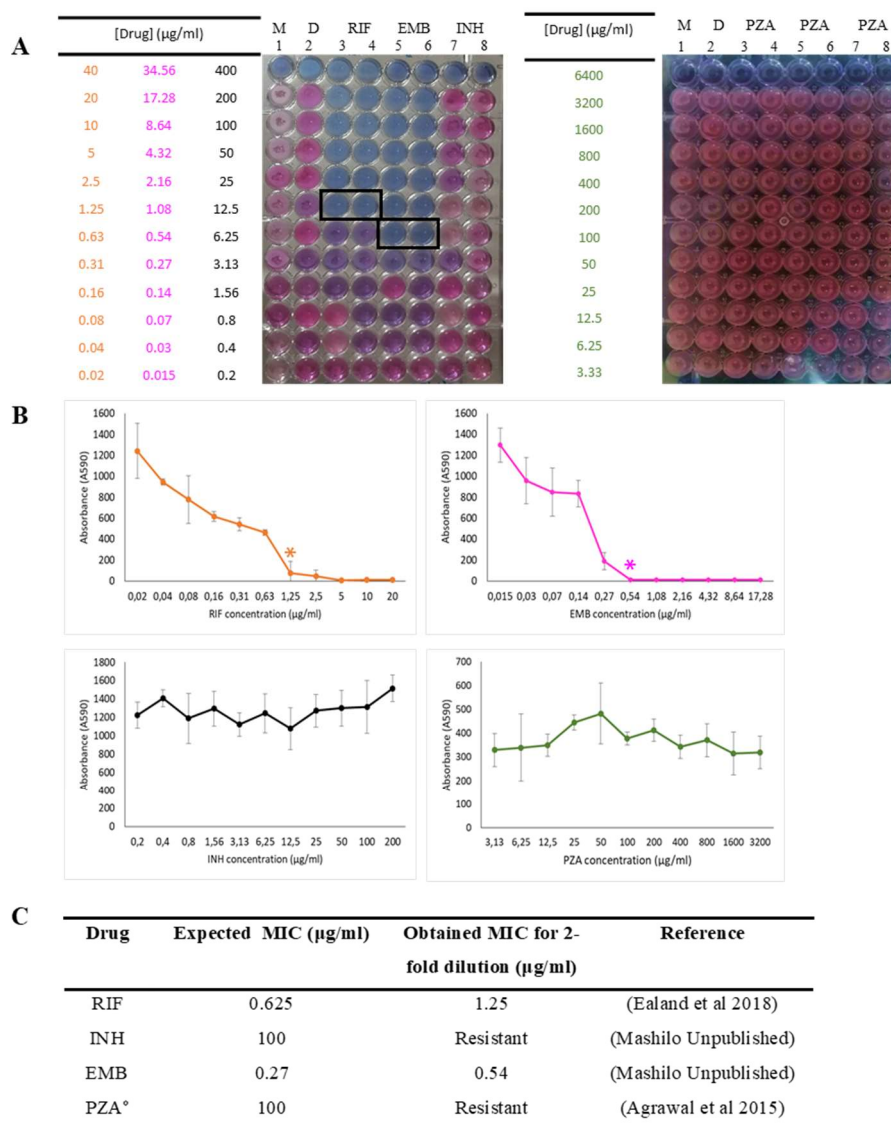
| <b>Response Group</b> | <b>Number of patients</b> | <b>Number of samples<sup>×</sup></b> | <b>Number of samples that grew</b> | <b>Number of samples that were contaminated or had no growth</b> |
|-----------------------|---------------------------|--------------------------------------|------------------------------------|------------------------------------------------------------------|
| Early non-responsive  | 7                         | 46                                   | 35 (76.1%)                         | 11 (23.9%)                                                       |
| Paradoxical worsening | 5                         | 43                                   | 26 (60.5%)                         | 17 (39.5%)                                                       |
| Classic bi-phasic     | 4                         | 23                                   | 4 (17.4%)                          | 19 (82.6%)                                                       |

<sup>×</sup> The total number of spot and early morning (overnight) MPN derived DCTB samples collected at baseline and on days 3, 7, 14 for each patient in the various response groups.

## **3.2 Both microdilution assays in *Mycobacterium smegmatis* (*M. smegmatis*)**

### **3.2.1 Determination of MICs for the four first-line drugs against *M. smegmatis* using the standard 2-fold dilution method**

The broth microdilution assay was first performed and optimized using the non-pathogenic wildtype *Mycobacterium smegmatis* strain, mc<sup>2</sup>155. The assay was performed using the protocol described in Section 2.4. After 14 days, AlamarBlue® was added to the plates to obtain the absorbance readings and to visualize the bacterial growth on the plates (Figure 7A1 and A2). The absorbance readings were plotted against the drug concentrations of each of the first-line drugs to determine the lowest concentration that inhibits the growth of the microorganism (Figure 7B). The desired outcome was to obtain an MIC value close to previously reported values in the literature (Agrawal et al 2015; Ealand et al 2018; Mashilo Unpublished) (Figure 7C). The starting drug concentrations for RIF, INH, EMB and PZA were 40 µg/ml, 400 µg/ml, 34.56 µg/ml and 6400 µg/ml, respectively. The obtained MIC for RIF and EMB for *M. smegmatis*, mc<sup>2</sup>155 was 1.25 µg/ml and 0.54 µg/ml, respectively. The bacteria showed resistance to INH and PZA as at the highest drug concentration each drug was unable to inhibit bacterial growth (Figure 7C). The RIF and EMB MICs were one-fold higher than the expected value of 0.625 µg/ml and 0.27 µg/ml (Ealand et al 2018; Mashilo Unpublished). The difference between the obtained and expected MICs was insignificant and is acceptable according to the standard set by the International Organization for Standardization, ISO, (ISO 20776-2) which states that a MIC deviation of two dilution factors is acceptable (Mouton et al 2018). Therefore, the broth microdilution assay was successfully performed and optimized for RIF and EMB using mc<sup>2</sup>155.



\* MIC was carried out in pH adjusted 7H9 media, pH 5.5.

**Figure 7. Determination of the MICs for the four first-line drugs against *M. smegmatis*, mc<sup>2</sup>155, using 2-fold dilutions in the broth microdilution assay.**

After 14 days of growth at 37°C, AlamarBlue® was added to each of the wells to quantitatively assess the viable cells present (A) The 96 well plates after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black), EMB (pink) and PZA (green) are shown in the tables next to the 96 well plates. (A1) RIF is represented in columns 3 and 4, EMB in columns 5 and 6, and INH in columns 7 and 8. (A2) PZA is in columns 3-8. The pink represents bacterial growth while blue indicates no growth. The dye absorbance was measured on a plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The absorbance readings are a direct correlation of the amount of live cells present in each well. The absorbance readings were read after the 16-hour incubation using a plate reader. (B) The lowest drug concentration that inhibits the growth of the microorganism was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. (C) Summary of the obtained MIC values compared to the previously reported values in the literature (Agrawal et al 2015; Ealand et al 2018; Mashilo Unpublished). The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

### **3.2.2 Determination of MICs for the rifampicin (RIF), isoniazid (INH) and ethambutol (EMB) against *M. smegmatis* using the lower dilution method**

After the standard 2-fold dilution was optimized, the lower dilution series (1.41 and 1.26) was assessed in the broth dilution assay as it corresponds to and is between the more common 2-fold dilution, which gives a more precise MIC value. The assay was carried out according to Section 2.4, whereby RIF and EMB were tested using a 1.41-fold dilution and INH was tested using the 1.26 dilution. These dilution series were established by Colangeli et al. (2018) which measured fine gradations in MIC values that fell below accepted resistance breakpoints (INH: 0.1 µg/ml and RIF: 1.0 µg/ml). A breakpoint is a selected antibiotic concentration that determines whether a bacterial species is susceptible or resistant to the antibiotic. The starting drug concentrations for RIF, INH and EMB were 4.91 µg/ml, 2.1 µg/ml, 400 µg/ml, respectively. After 14 days, AlamarBlue® was added to the plates to obtain the absorbance readings and to visualize the bacterial growth on the plate (Figure 8A). The absorbance readings were plotted against the drug concentration of RIF, INH and EMB to determine the MIC for that drug (Figure 8B). The MIC for RIF and EMB using the lower dilution series was respectively 1.25 µg/ml and 0.19 µg/ml as shown in Figure 8C. The MIC for RIF was the same as the MIC for the 2-fold dilution, whereas the EMB MIC was found to be lower than the one obtained for the 2-fold dilution. The EMB MIC for the lower dilution (0.19 µg/ml), however, is closer to the expected MIC of 0.27 µg/ml than the MIC obtained for the 2-fold dilution (0.54 µg/ml) indicating that the lower dilution produced a more precise MIC than the 2-fold dilution. The difference between the MICs is insignificant and acceptable according to ISO (ISO 20776-2) as described in Section 3.2.1 (Mouton et al 2018). A comparison of the 2-fold dilution series and the lower dilution series for the various drug concentrations is shown in Table 11.

**Table 11. The standard 2-fold versus the lower dilution series for RIF, INH and EMB for *M. smegmatis*.**

| RIF             |                    | EMB             |                    | INH             |                    |
|-----------------|--------------------|-----------------|--------------------|-----------------|--------------------|
| 2-fold dilution | 1.41-fold dilution | 2-fold dilution | 1.41-fold dilution | 2-fold Dilution | 1.26-fold dilution |
| 20              | 3.49               | 17.28           | 1.51               | 200             | 317.58             |
| 10              | 2.47               | 8.64            | 1.07               | 100             | 252.05             |
| 5               | 1.75               | 4.32            | 0.76               | 50              | 200                |
| 2.5             | 1.25               | 2.16            | 0.54               | 25              | 158.8              |
| 1.25            | 0.88               | 1.08            | 0.38               | 12.5            | 126                |
| 0.63            | 0.63               | 0.54            | 0.27               | 6.25            | 100                |
| 0.31            | 0.44               | 0.27            | 0.19               | 3.13            | 79                 |
| 0.16            | 0.32               | 0.14            | 0.14               | 1.56            | 63                 |
| 0.08            | 0.22               | 0.07            | 0.10               | 0.8             | 50                 |
| 0.04            | 0.16               | 0.03            | 0.07               | 0.4             | 40                 |
| 0.02            | 0.11               | 0.0015          | 0.05               | 0.2             | 31.5               |

Red indicates the additional values the lower dilution series added between the conventional 2-fold dilution series which is in black bold. The drug concentrations are represented in µg/ml.

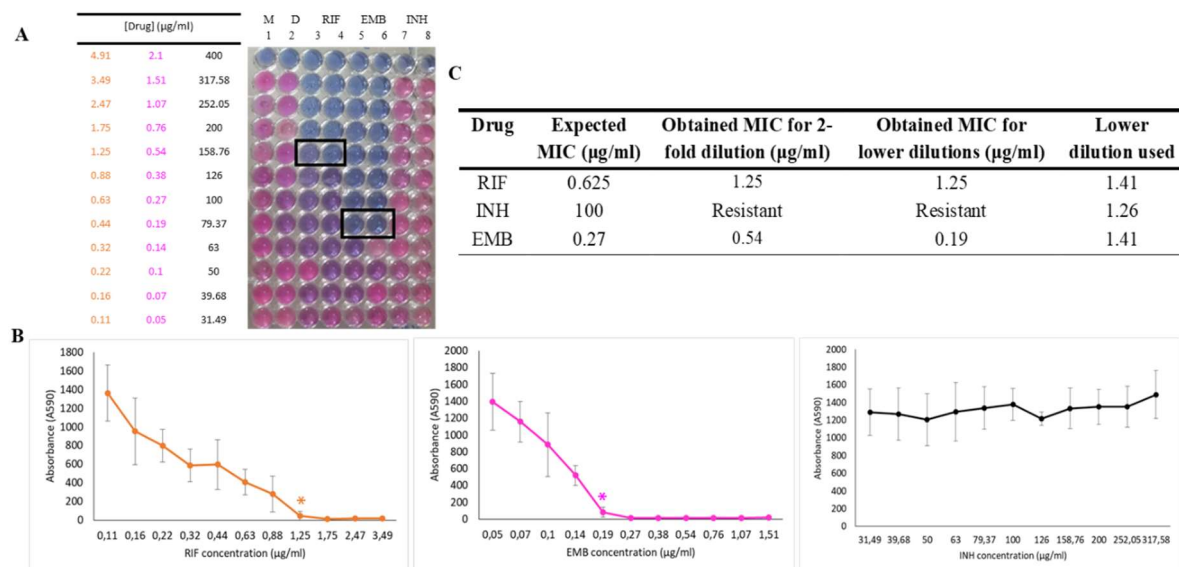


**Figure 8. Determination of MICs of rifampicin (RIF), isoniazid (INH) and ethambutol (EMB) for *M. smegmatis*, mc<sup>2</sup>155, utilizing the lower dilution method.**

After 14 days of growth at 37°C, AlamarBlue® was added to each of the wells to quantitatively assess the viable cells present. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plate. RIF is represented in columns 3 and 4, EMB in columns 5 and 6, and INH in columns 7 and 8. The pink represents bacterial growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The lowest drug concentration that inhibits the growth of the microorganism was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs **(C)** Summary of the obtained MICs for RIF, INH and EMB compared to the previously reported values in the literature and the MICs obtained for the 2-fold dilution (Agrawal et al 2015; Ealand et al 2018; Mashilo Unpublished). The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

### **3.3 MIC testing of the four first-line drugs against *Mycobacterium tuberculosis* (*M. tuberculosis*)**

#### **3.3.1 Optimisation of the appropriate diluent for INH and testing for the MIC against *M. tuberculosis* using the 2-fold dilution series**

Since *M. smegmatis* was resistant to INH it was essential to optimize the assay for this antimicrobial drug in the pathogenic *M. tuberculosis* wild-type laboratory strain, H37Rv, as per Section 2.4. Two drug stocks of INH dissolved in different solvents at the required concentration were tested; one batch of INH was dissolved in HyPure™ Molecular Biology Grade Water (HyClone™) and another in dimethylsulfoxide (DMSO). Due to the photosensitivity of INH, the stocks were kept in the dark until the assay was performed. The starting drug concentrations for RIF and INH were 0.384 µg/ml and 3.2 µg/ml, respectively. RIF was added to the plate as a positive control as the MIC was established prior to this experiment and is shown in Section 3.3.2. The MIC for INH dissolved in DMSO was 0.2 µg/ml, whilst the MIC for INH dissolved in HyClone™ was 0.05 µg/ml (Figures 9). The latter was closer to a previously reported value of 0.025 µg/ml in the literature hence, all further downstream broth microdilution assays were conducted with the INH stock dissolved in HyClone™ (Kana et al 2010). Additionally, the drug stocks were made fresh for each experiment.

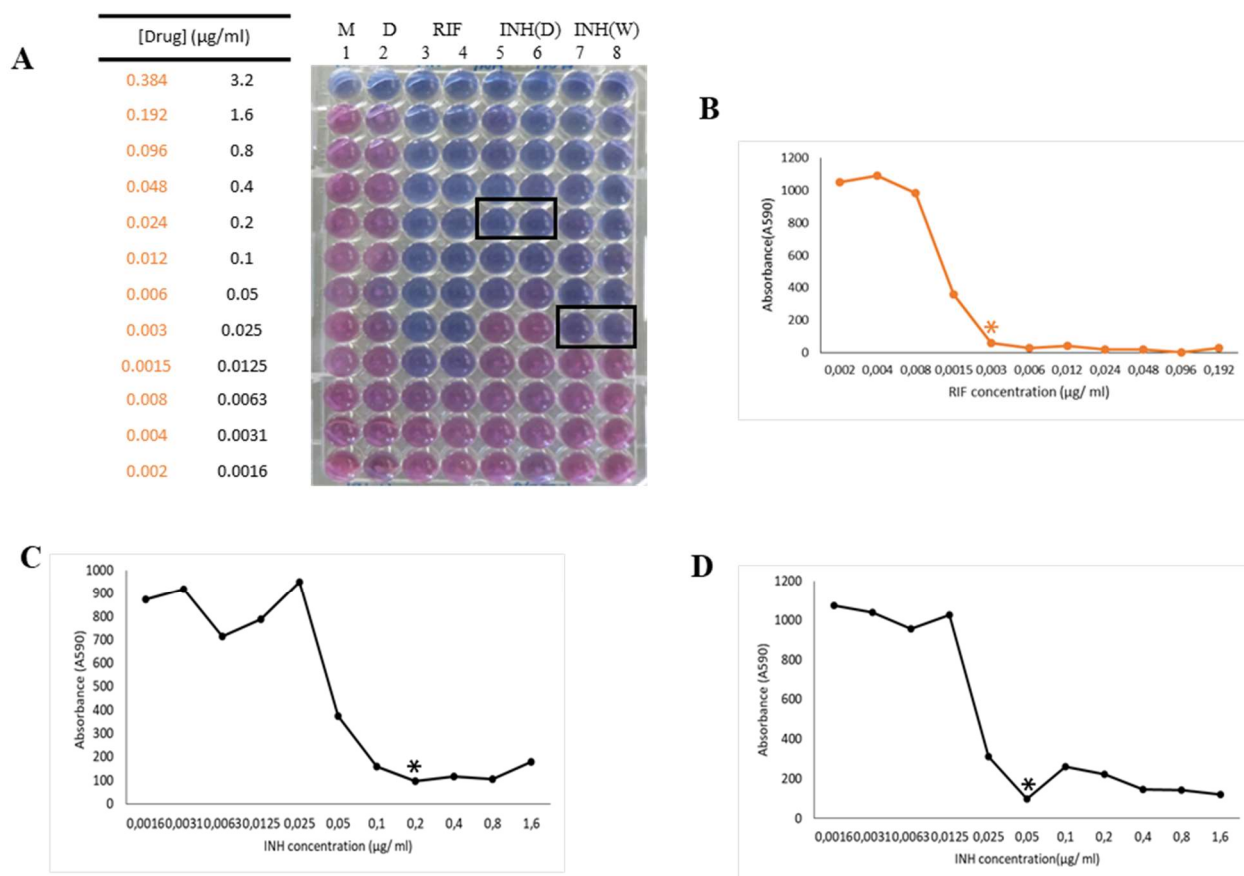


**Figure 9. Analysis of the absorbance readings for different INH drug concentrations dissolved in HyClone™ nuclease-free water or dimethyl sulfoxide against *M. tuberculosis*, H37Rv.**

After 14 days of growth at 37°C AlamarBlue® was added to each of the wells to quantitatively assess the viable cells present. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange) and INH (black) are shown in the table next to the 96 well plate. RIF is represented in columns 3 and 4, INH dissolved in DMSO in columns 5 and 6, whilst, and INH dissolved in HyClone™ in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. The lowest drug concentration that inhibits the growth of the microorganism was determined by plotting the absorbance readings versus the drug concentrations for each condition. **(B)** MIC analysis for the positive control, RIF. **(C)** MIC analysis for INH dissolved in DMSO. **(D)** MIC analysis for INH dissolved in water. The black boxes indicate the MIC values obtained in the broth microdilution assay in the 96 well plate and stars indicate the MIC values for INH from the analysis of the absorbance readings versus the drug concentrations. This experiment was performed once, thus no standard deviation was represented.

### **3.3.2 Determination of MICs for RIF, INH and EMB against *M. tuberculosis* using the 2-fold dilution method**

Next, the MICs for RIF, INH, and EMB were determined using the pathogenic, *M. tuberculosis* wild type strain, H37Rv, to ensure that the previously reported MICs for each of the drugs were effectively produced (Kana et al 2010). The MICs for RIF, INH and EMB using the 2 fold-dilution had to be established first in order to set a benchmark to determine whether the lower dilution series produced a more precise MIC value. The starting drug concentrations for RIF, INH and EMB were 0.384 µg/ml, 3.2 µg/ml and 100 µg/ml, respectively. The MIC for RIF was 0.003 µg/ml which was expected according to MIC range of 0.003-0.006 µg/ml published in literature (Figure 10) (Kana et al 2010). Additionally, the MICs for INH (0.05 µg/ml) and EMB (0.78 µg/ml) were respectively one-fold higher and one-fold lower than expected MIC values (INH- 0.025 µg/ml and EMB- 1.6-3.1) µg/ml) published in literature (Figure 10) (Kana et al 2010). The broth microdilution assay was successfully performed as the obtained MICs for RIF, INH and EMB were close to the expected MICs (Kana et al 2010). The difference between the obtained and expected MIC was insignificant and is acceptable according to ISO (ISO 20776-2) as described in section 3.2.1 (Mouton et al 2018).

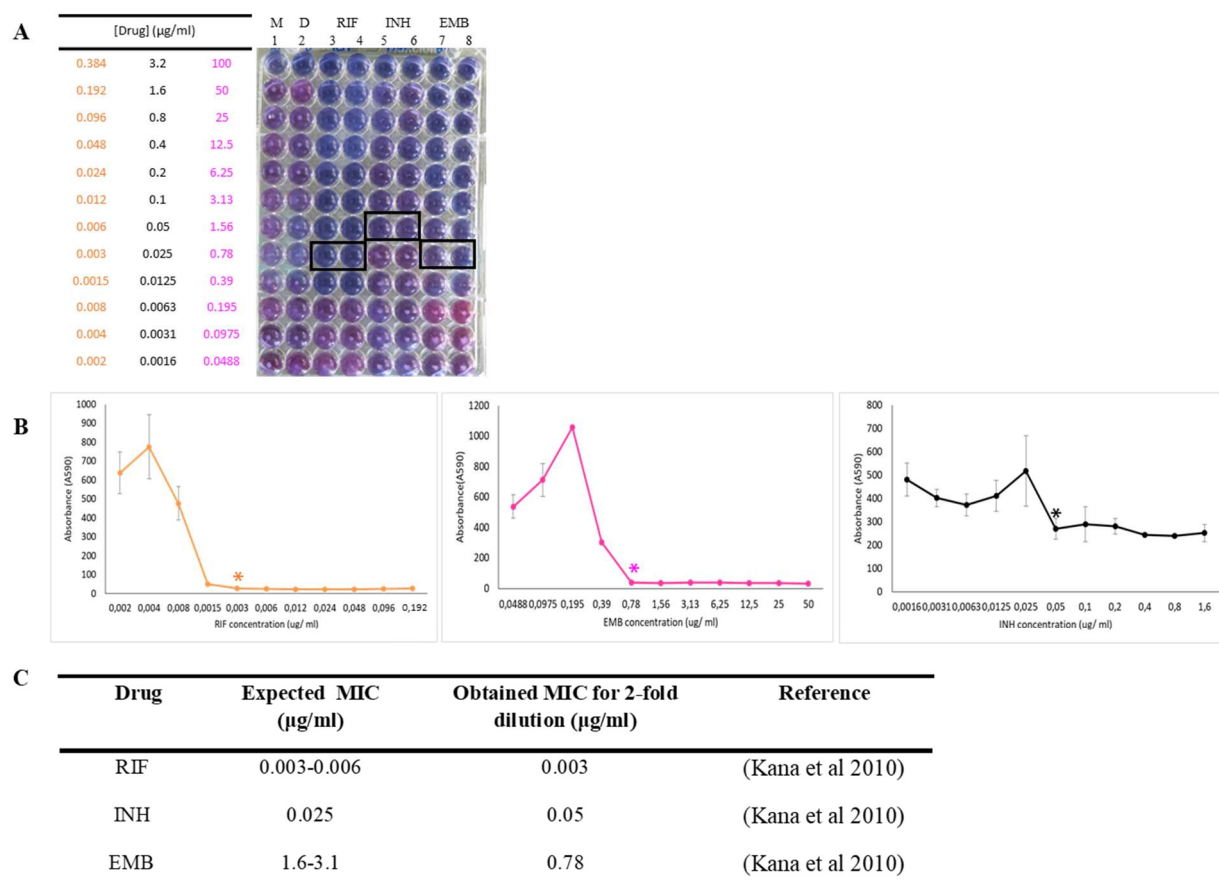


**Figure 10. Determination of MICs of RIF, INH and EMB for *M. tuberculosis*, H37Rv, utilizing the 2-fold dilution.**

After 14 days of growth at 37°C, AlamarBlue® was added to each of the wells to quantitatively assess the viable cells present. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plate. RIF is represented in columns 3 and 4, INH in columns 5 and 6, and EMB in columns 7 and 8. The pink represents growth while blue indicates no growth. The dye absorbance was measured on a plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The absorbance readings are a direct correlation of the amount of live cells present in each well. The absorbance readings were read after the 16-hour incubation using a plate reader. **(B)** The lowest drug concentration that inhibits the growth of the microorganism was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values compared to the previously reported values in the literature (Kana et al 2010). The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

### 3.3.3 Optimisation of MIC for PZA against *M. tuberculosis* using the 2-fold dilution series

Pyrazinamide, unlike the other first-line drugs, requires acidic conditions to convert PZA into its active drug form (Zhang et al 2014). The pH of the media was adjusted using hydrochloric acid and sodium hydroxide. Due to the specific nature of PZA, the assay needed to be optimized as it did not work according to the standard MIC assay method against *M. smegmatis* described in section 2.4. Firstly, different dilutions of H37Rv were investigated, shown in Figure 11, since the standard 1:500 dilution failed to grow after the two-week incubation period. The different dilution factors tested were 1:100, 1:250 and 1:350. Using the dilution factors- 1:350, 1:250, 1:100 resulted in MICs of 400 µg/ml, 800 µg/ml, and 800 µg/ml, respectively as shown by the absorbance versus drug concentration plots in Figure 11. The absorbance readings after 14 days of exposure of *M. tuberculosis* to PZA were too low for the 1:350 dilution compared to the lower dilution factors. Both the 1:250 and 1:100 dilutions resulted in clear MIC values and as such either dilution could be used to conduct all further PZA broth microdilution assays. The 1:250 dilution was used in the remaining experiments because the dilution factor was the closest to the standard 1:500 dilution used in the assays using RIF, INH and EMB.

Secondly, different pH-adjusted liquid media were tested as the MIC is pH-dependent (Figure 12) (Boshoff and Mizrahi 2000). The PZA MIC obtained for different pH media 5.3, 5.5 and 6.0 was 12.5 µg/ml, 100 µg/ml, >400 µg/ml, respectively suggesting that there is a direct relationship between the pH of the media and the MIC for PZA. Media adjusted to pH 5.5 gave a MIC for PZA that was closest to a previously reported value of 50 µg/ml (Boshoff and Mizrahi 2000). Therefore, all further PZA broth microdilution assays were conducted using media adjusted to 5.5. Additionally, due to time constraints, all remaining PZA MIC assays were performed with the 2-fold dilution.

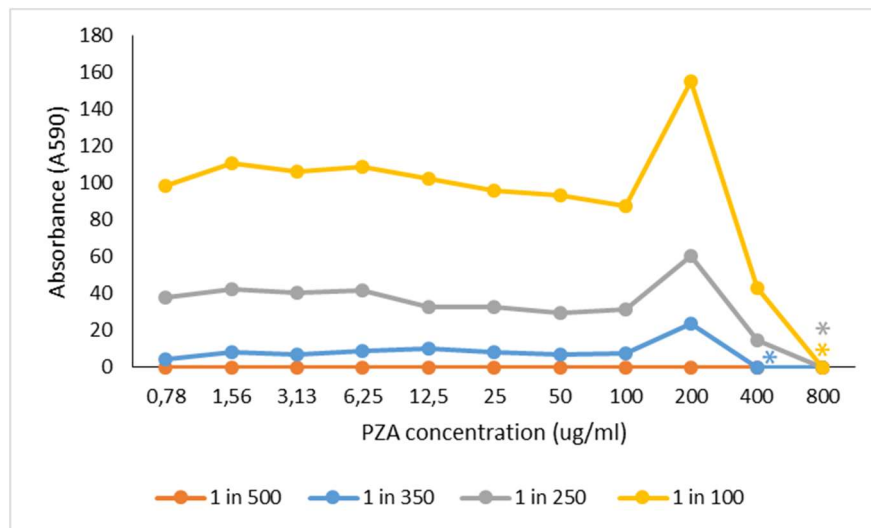


**Figure 11. Determination of MIC of pyrazinamide (PZA) for different dilutions of *M. tuberculosis*, H37Rv.**

The MIC assay was performed using various dilutions of the H37Rv culture, 1:500 (orange), 1:350 (blue), 1:250 (grey) and 1:100 (yellow) in pH adjusted media (pH 6.0). After 14 days of growth at 37°C AlamarBlue® was added to each well in the 96 well plate. The absorbance readings were taken after 16 hours of incubation using a plate reader. The MIC was determined by plotting the absorbance readings versus the drug concentrations for PZA. The stars represent the MIC obtained for each condition tested. This experiment was performed once, thus no standard deviation was represented.

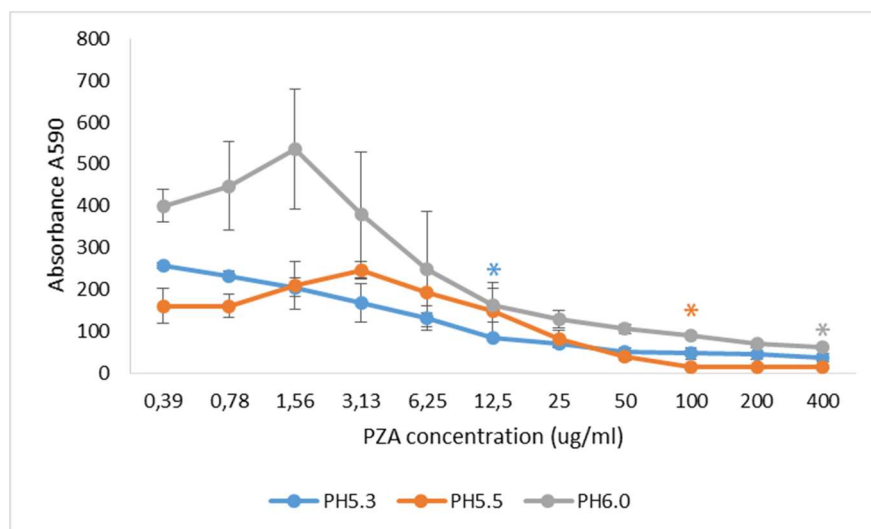


**Figure 12. Determination of PZA MIC at varying pH levels for *M. tuberculosis*, H37Rv.**

The MIC assay was performed using pH-adjusted 7H9 media, pH 5.3 (blue), pH 5.5 (orange) and pH 6.0 (grey). Mid-log phase cells of H37RV at a dilution of 1:250 were used, and after 14 days of growth at 37°C, AlamarBlue® was added to each well in the 96 well plate. The absorbance readings were taken after 16 hours of incubation using a plate reader. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments. The stars represent the MIC for each condition.

### **3.3.4 Determination of MICs for RIF, INH and EMB against *M. tuberculosis* using a lower dilution method**

After the 2-fold dilution broth microdilution assay was optimized for H37Rv and reproducible MICs for the RIF, INH and EMB were obtained, it was necessary to assess a lower dilution series to obtain more precise MICs that lie within the 2-fold range (Colangeli et al 2018). The MIC for RIF and EMB was assessed using a 1.41-fold dilution series whereas, the 1.26 dilution series was used to obtain the MIC for INH. The starting drug concentrations for RIF, INH and EMB were 0.022 µg/ml, 0.25 µg/ml and 17.40 µg/ml, respectively. The lower dilution series resulted in a MIC of 0.004 µg/ml for RIF, which lies between the expected MIC range of 0.0003-0.006 µg/ml for RIF (Figure 13) (Kana et al 2010). The lower dilution series obtained a MIC for RIF which was more precise than the MIC obtained for the 2-fold dilution which was 0.0003 µg/ml for RIF. The MIC for INH utilizing the lower dilution series was 0.032 µg/ml which was as expected and was also between the expected MIC (0.025 µg/ml) and the obtained MIC (0.05 µg/ml) for the 2- fold dilution (Figure 13) (Kana et al 2010). Lastly, the MIC for EMB was the same regardless of whether a 2-fold or 1.41 dilution series was used suggesting that the MIC of EMB is highly specific (Figure 13). The MIC deviations observed were insignificant and are acceptable according to ISO (ISO 20776-2) as described in section 3.2.1 (Mouton et al 2018). These results indicate that the broth microdilution assay performed with the lower dilution series gives a more precise MIC than the conventional 2-fold dilution. A comparison of the 2-fold dilution series and the lower dilution series for the various drug concentrations is shown in Table 12. The drug concentration range for the various drugs using the lower fold dilution series was slightly adjusted when determining the MIC for the purified samples derived from sputum obtained from patients.

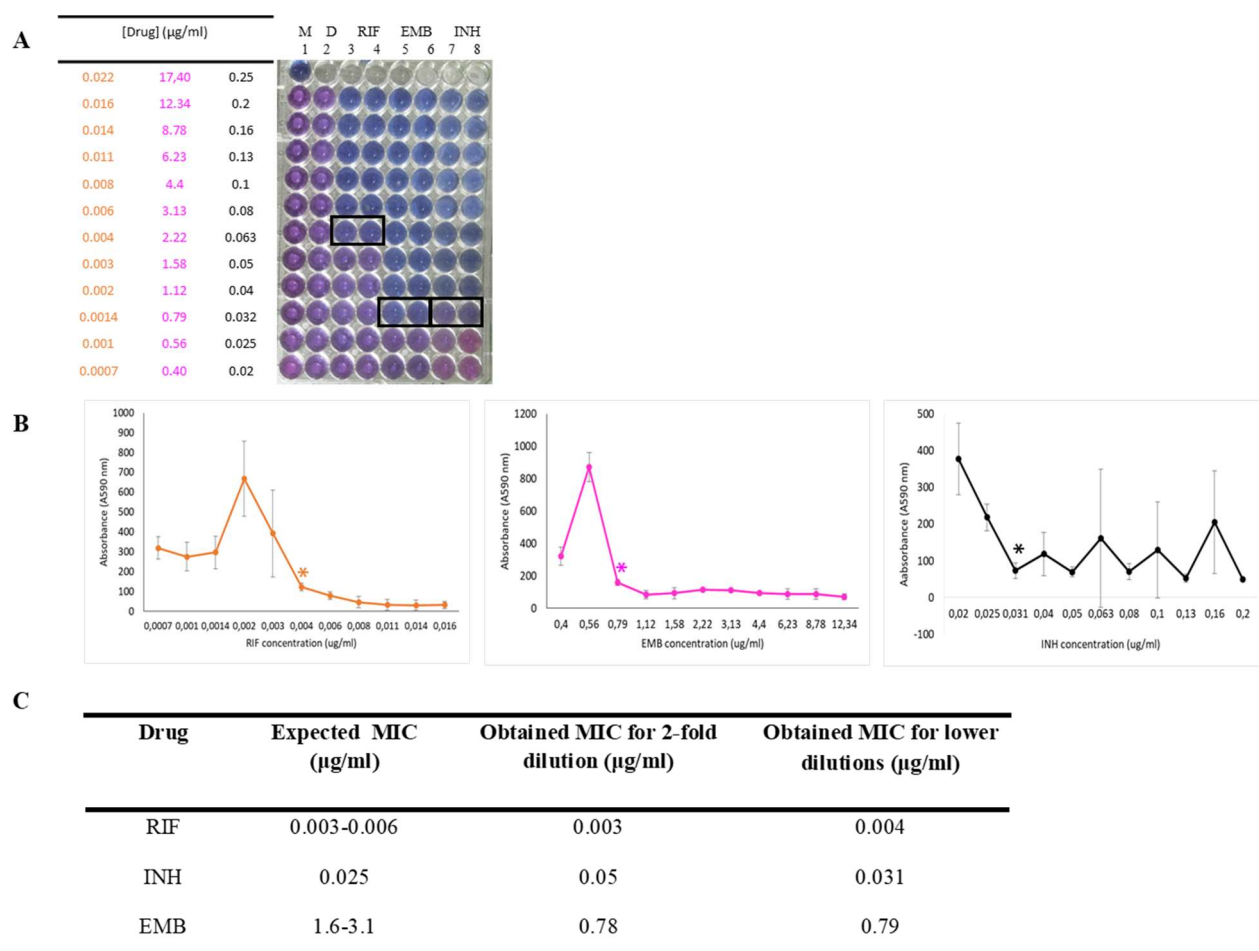


**Figure 13. Determination of MICs for RIF, INH and EMB against *M. tuberculosis*, H37Rv, utilizing the 1.41 and 1.26 dilution series.**

After 14 days of growth at 37°C, AlamarBlue® was added to each of the wells to quantitatively assess the viable cells present. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plate. RIF is represented in columns 3 and 4, EMB in columns 5 and 6, and INH in columns 7 and 8. The pink represents growth while blue indicates no growth. The dye absorbance was measured on a plate reader at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The lowest drug concentration that inhibits the growth of the microorganism was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MICs for RIF, INH and EMB compared to the previously reported values in the literature and the MICs obtained for the 2-fold dilution. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

**Table 12. The standard 2-fold versus the lower dilution series for RIF, INH and EMB for *M. tuberculosis*.**

| RIF             |                    | EMB             |                    | INH             |                    |
|-----------------|--------------------|-----------------|--------------------|-----------------|--------------------|
| 2-fold dilution | 1,41-fold dilution | 2-fold dilution | 1,41-fold dilution | 2-fold dilution | 1,26-fold Dilution |
| 0.192           | <b>0.022</b>       | 50              | <b>12.34</b>       | 1.6             | <b>0.1</b>         |
| 0.096           | <b>0.016</b>       | 25              | <b>8.78</b>        | 0.8             | <b>0.08</b>        |
| 0.048           | <b>0.011</b>       | 12.5            | <b>6.23</b>        | 0.4             | <b>0.063</b>       |
| <b>0.024</b>    | <b>0.008</b>       | <b>6.25</b>     | <b>4.4</b>         | 0.2             | <b>0.05</b>        |
| <b>0.012</b>    | <b>0.006</b>       | <b>3.13</b>     | <b>3.13</b>        | <b>0.1</b>      | <b>0.04</b>        |
| <b>0.006</b>    | <b>0.004</b>       | <b>1.56</b>     | <b>2.22</b>        | <b>0.05</b>     | <b>0.031</b>       |
| <b>0.003</b>    | <b>0.003</b>       | <b>0.78</b>     | <b>1.58</b>        | <b>0.025</b>    | <b>0.025</b>       |
| <b>0.0015</b>   | <b>0.002</b>       | <b>0.39</b>     | <b>1.12</b>        | <b>0.013</b>    | <b>0.02</b>        |
| <b>0.0008</b>   | <b>0.0014</b>      | 0.195           | <b>0.79</b>        | 0.0063          | <b>0.016</b>       |
| 0.0004          | <b>0.0010</b>      | 0.0975          | <b>0.56</b>        | 0.0031          | <b>0.013</b>       |
| 0.0002          | <b>0.0007</b>      | 0.0488          | <b>0.4</b>         | 0.0016          | <b>0.012</b>       |

Red indicates the additional values the lower dilution series added between the conventional 2-fold dilution series which is in black bold. The drug concentrations are represented in µg/ml.

### **3.4 Determination of MICs of the four first-line drugs for purified patient samples using the broth microdilution assay**

Once the H37Rv MICs were established and optimized for all four drugs, the assay was performed using the purified samples from each of the response groups (Section 2.3). Only patients who had 3 or more treatment time points successfully purified were used in the broth microdilution assay to identify the drug susceptibility of the samples from baseline to day 14 of treatment in order to identify any resistance patterns between the time points and response groups. One exception was a patient in the classic bi-phasic group who had two purified samples that were included in the study, as all other classic bi-phasic patients had less than 2 successfully purified samples. The samples in this study were derived from MPN assays conducted in the BMG study using either an overnight or spot sputum sample collected for each TB infected patient at baseline and days 3, 7, and 14. The broth microdilution assay was used to determine the MIC for the four first-line drugs using purified MPN cultures derived from overnight sputum collected in the MPN assay, however, if the overnight sample was not available then the purified MPN culture derived from the spot sample was used. The resistance or susceptibility levels of the samples to RIF, INH and EMB were assessed with the broth microdilution assay using the lower dilution series whilst a 2-fold dilution series was used to assess PZA due to time constraints (Figures 14-17).

The MICs of the four drugs obtained for the wildtype strain, H37Rv, were used to create a benchmark to compare the drug susceptibility or resistance levels of the purified samples to these drugs (Sections 3.3.4 and 3.3.5). To account for the variability between each broth microdilution assay, a standard was set by ISO (ISO 20776-2) which states that a MIC deviation of two dilution factors is acceptable (Mouton et al 2018). Therefore, a drug concentration two dilution factors higher than the H37Rv MICs obtained for the four drugs were used to categorize samples as resistant or susceptible. The orange line in Figures 14-17 accounts for these two dilution factor deviations. Samples that showed a MIC greater than the orange line was considered resistant whilst samples that displayed a MIC similar to or below the orange line were classified as susceptible. Additionally, reading the MIC value off the plate is subjective as the colour produced is not completely distinct. The more accurate value would be the MIC value taken from the absorbance versus concentration graphs as it an empirical measurement taken with a spectrometer.

The samples from patients 57158 and 57163 in the early non-responsive group had no clear growth on the MIC plates for all first-line drugs and H37Rv controls at all time points and were therefore excluded from further analysis. Analyzing the MICs obtained for RIF using the purified patient samples, it was observed that 1 out of 9 patients displayed resistance to RIF (Figure 14). The baseline to day 3 samples for patient 59041 showed resistance to RIF. Of the 9 patients, 2 displayed resistance to INH, patient 59041 from the early non-responders group showed resistance in the day 3 sample and patient 59014 from the paradoxical worsening group displayed resistance in all samples (Figure 15). Analyzing the MICs obtained for EMB using the purified samples, it was observed that 2 out of the 9 patients displayed resistance to EMB. The 2 patients belonged to the non-responders group (patient 59041) and the classic bi-phasic group (patient 59032). EMB resistance was only observed in the day 3 sample for patient 59041 (Figure 16). Regarding PZA, 3 out of 9 patients showed resistance to the antimicrobial drug, these were patients 59041 (early non-responders group), 59127 (paradoxical worsening group) and 59032 (classic bi-phasic group). The baseline and day 3 samples for patient 59041, the day 14 sample for patient 59127, and baseline and day 7 samples for patient 59032 showed resistance to PZA. The data in Figures 14-17 are summarized under Appendix A8.

The data showed no resistance patterns for the samples displaying resistance to the four treatment response groups classified in the BMG study. However, patients who had any samples showing resistance to RIF, INH and EMB were further analyzed to determine the cause for the observed resistance to the drugs. The resistance-conferring genes for RIF, INH and EMB were amplified using the gDNA of all the samples in patients 59041, 59014, 57134 and 59032 by

polymerase chain reaction (PCR). Patient 57134 was further analysed because the MIC values obtained were between the two standards set for RIF, INH and EMB. Due to time constraints, resistance conferring regions for samples showing resistance to PZA were not amplified by PCR or sequenced. The obtained MICs for the patients who had any samples showing resistance to RIF, INH and EMB observed in more detail in Figures 18-21.

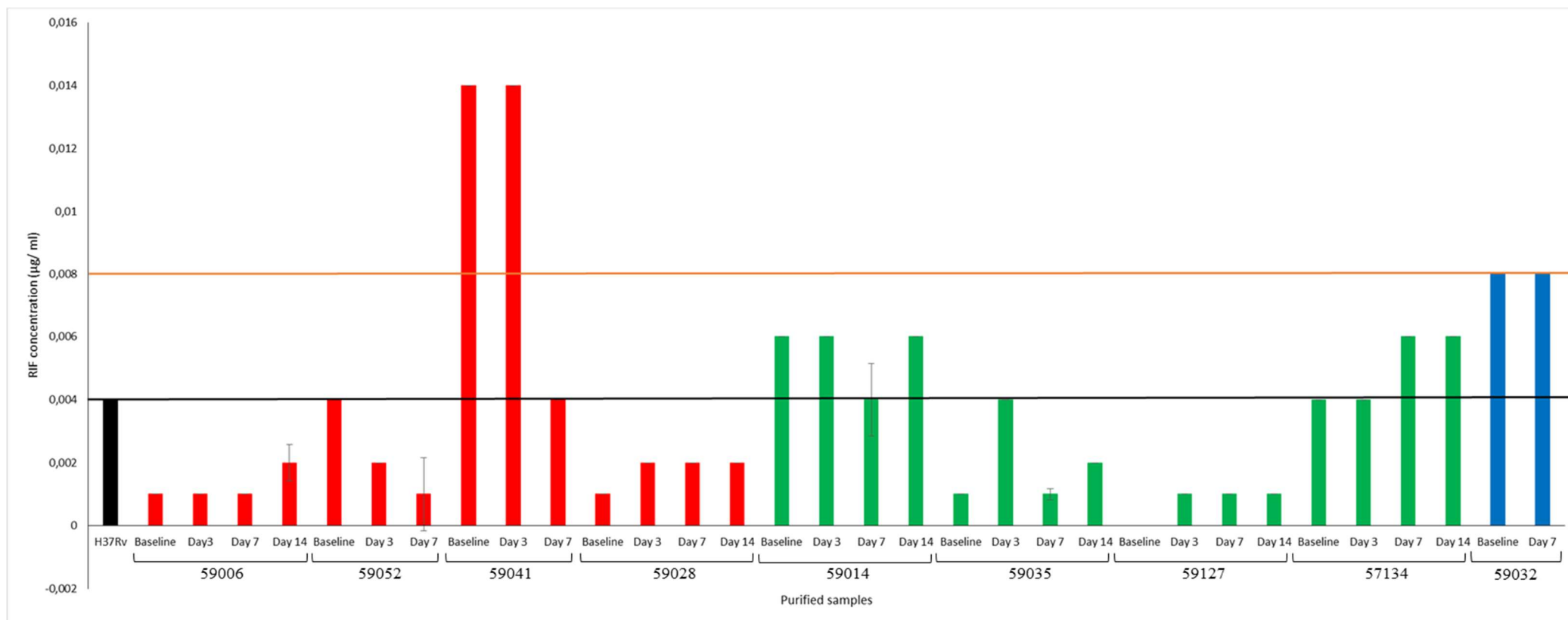


**Figure 14. Analysis of RIF susceptibility of patient samples compared to H37Rv.**

The drug susceptibility of the control, H37Rv, and the patient samples to RIF were assessed using the broth microdilution assay. The MIC values are shown for H37Rv (black), the early non-responsive group (red), the paradoxical worsening group (green), and the classic bi-phasic group (blue). MIC values above the MIC obtained for H37Rv (black line) were interpreted as samples displaying resistance to RIF whilst values below the black line show that the strain is susceptible to RIF. The experiment was performed in triplicate and the error bars represent the standard deviation. Statistical analysis could not be performed on some samples because the values of the pairs within the groups were equal.

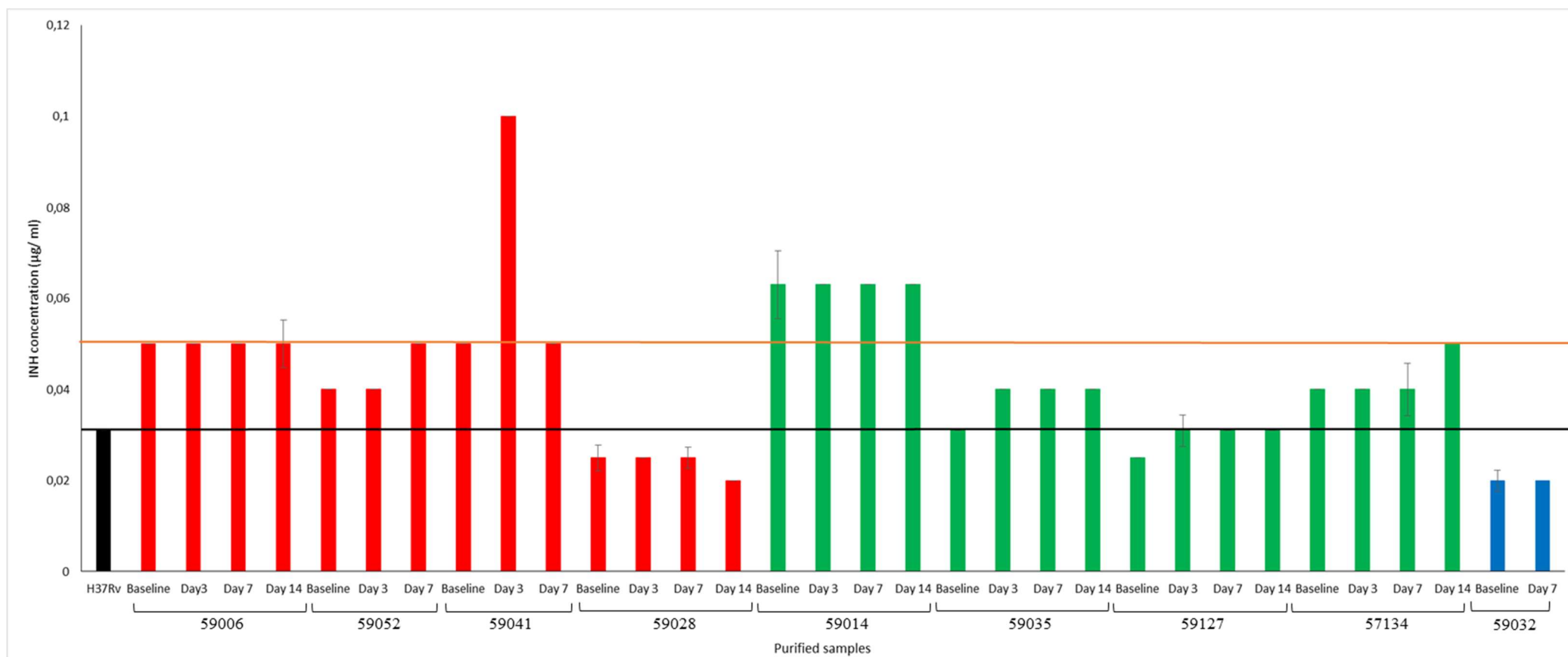


**Figure 15. Analysis of INH susceptibility of patient samples compared to H37Rv.**

The drug susceptibility of the control, H37Rv, and the patient samples to INH were assessed using the broth-microdilution MIC assay. The MIC values are shown for H37Rv (black), the early non-responsive group (red), the paradoxical worsening group (green), and the classic bi-phasic group (blue). MIC values above the MIC obtained for H37Rv (black line) were interpreted as samples displaying resistance to INH whilst values below the black line show that the strain is susceptible to INH. The orange line represents the acceptable two-fold change. The experiment was performed in triplicate and the error bars represent the standard deviation. Statistical analysis could not be performed on some samples because the values of the pairs within the groups were equal.

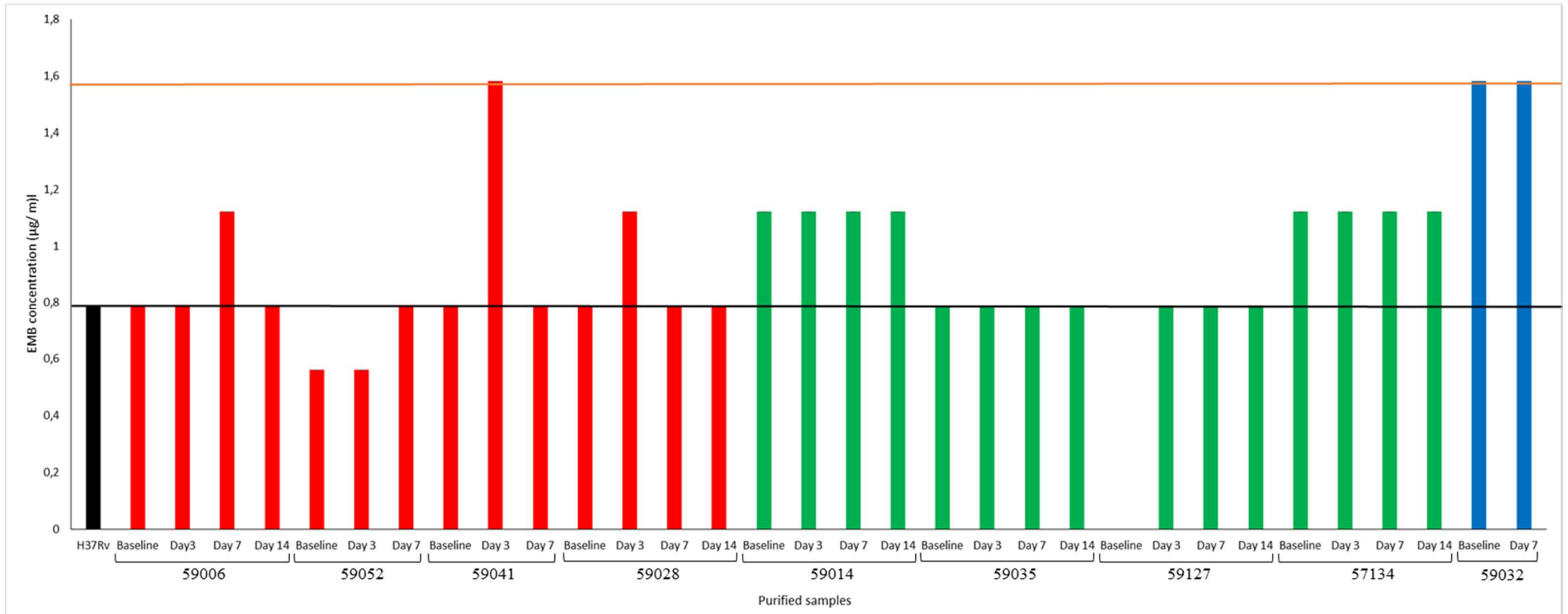


**Figure 16. Analysis of EMB susceptibility of patient samples compared to H37Rv.**

The drug susceptibility of the control, H37Rv, and the patient samples to EMB were assessed using the broth-microdilution MIC assay. The MIC values are shown for H37Rv (black), the early non-responsive group (red), the paradoxical worsening group (green), and the classic bi-phasic group (blue). MIC values above the MIC obtained for H37Rv (black line) were interpreted as samples displaying resistance to EMB whilst values below the black line show that the strain is susceptible to EMB. The orange line represents the acceptable two dilution change. The experiment was performed in triplicate and statistical analysis could not be performed on some samples because the values of the pairs within the groups were equal.

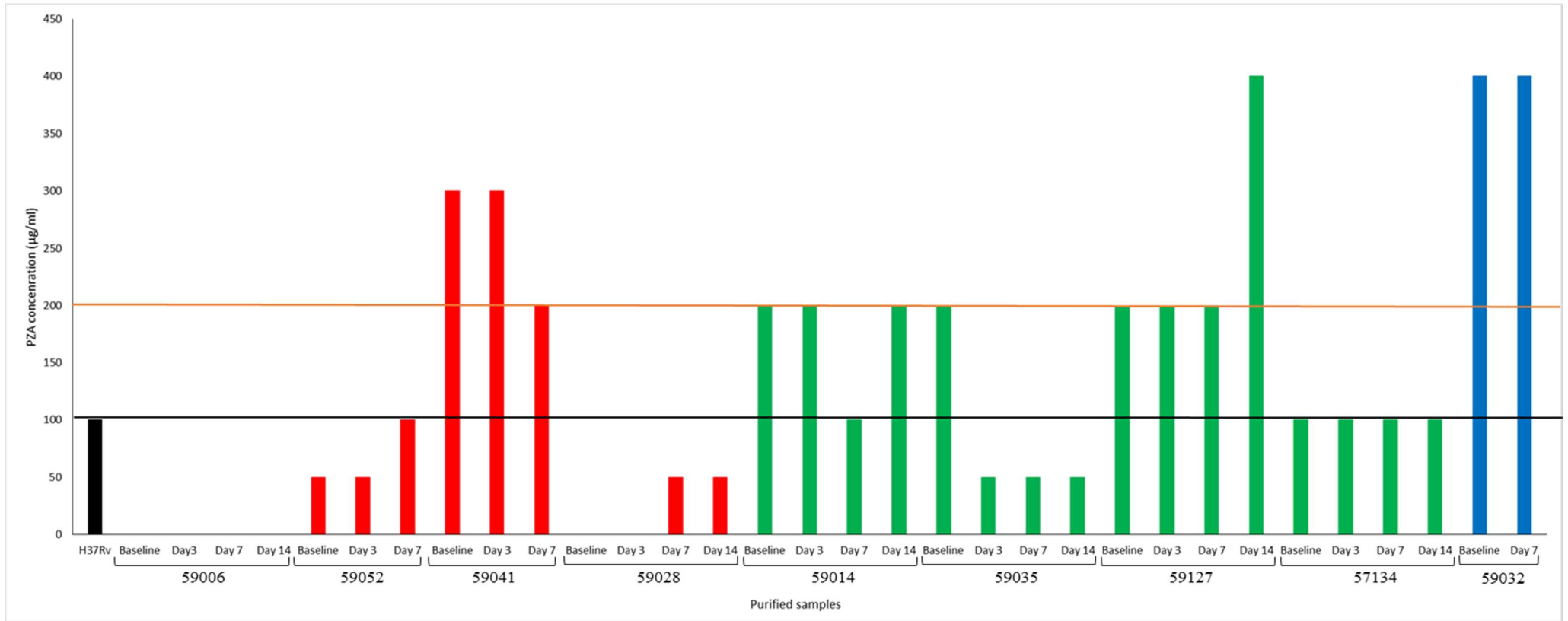
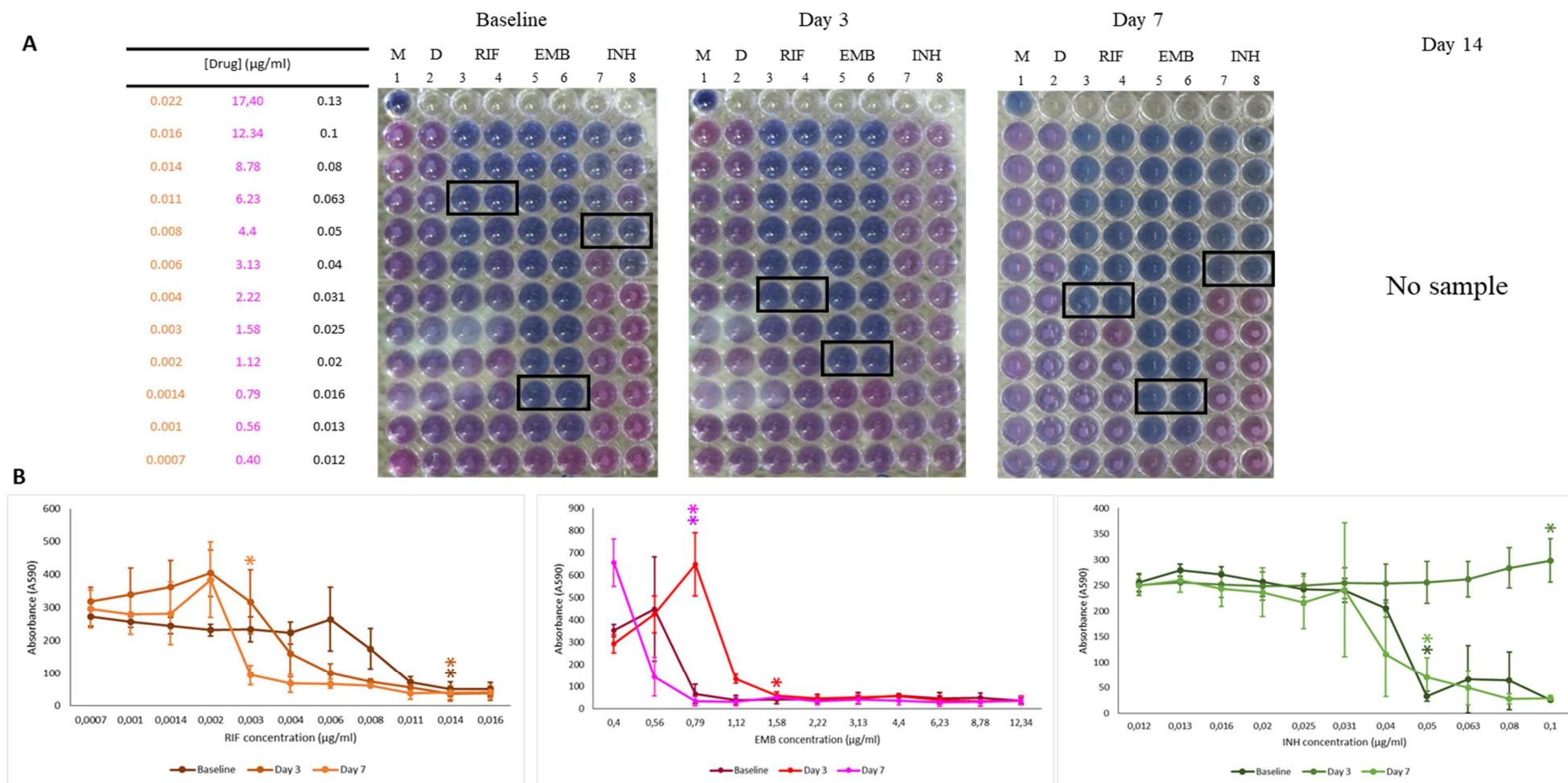


**Figure 17. Analysis of PZA susceptibility of patient samples compared to H37Rv**

The drug susceptibility of the control, H37Rv, and the patient samples to PZA were assessed using the broth-dilution MIC assay. The MIC values are shown for H37Rv (black), early non-responsive group (red), paradoxical worsening group (green), and classic bi-phasic (blue). MIC values above the MIC obtained for H37Rv (black line) were interpreted as samples displaying resistance to PZA whilst values below the black line show that the strain is susceptible to PZA. The orange line represents the acceptable two-fold change. The experiment was performed in triplicate and statistical analysis could not be performed on some samples because the values of the pairs within the groups were equal.



**C**

| Drug     | RIF MIC (µg/ml) | EMB MIC (µg/ml) | INH MIC (µg/ml) |
|----------|-----------------|-----------------|-----------------|
| Baseline | 0.014           | 0.79            | 0.05            |
| Day 3    | 0.014           | 1.58            | > 0.1           |
| Day 7    | 0.004           | 0.79            | 0.05            |
| Day 14   | -               | -               | -               |

The dash (-) means that no sample was collected for that patient at that time point.

**Figure 18. MIC determination of purified samples from Patient 59041 against RIF, INH and EMB using utilizing the lower dilution series in the broth microdilution assay.**

The assays were performed using DCTB samples genrated from sputum samples taken at baseline, day 3 and day 7. **(A)** The 96 well plates after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is represented in columns 3 and 4, EMB in columns 5 and 6, and INH in columns 7 and 8. The pink represents growth whilst blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MICs were determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

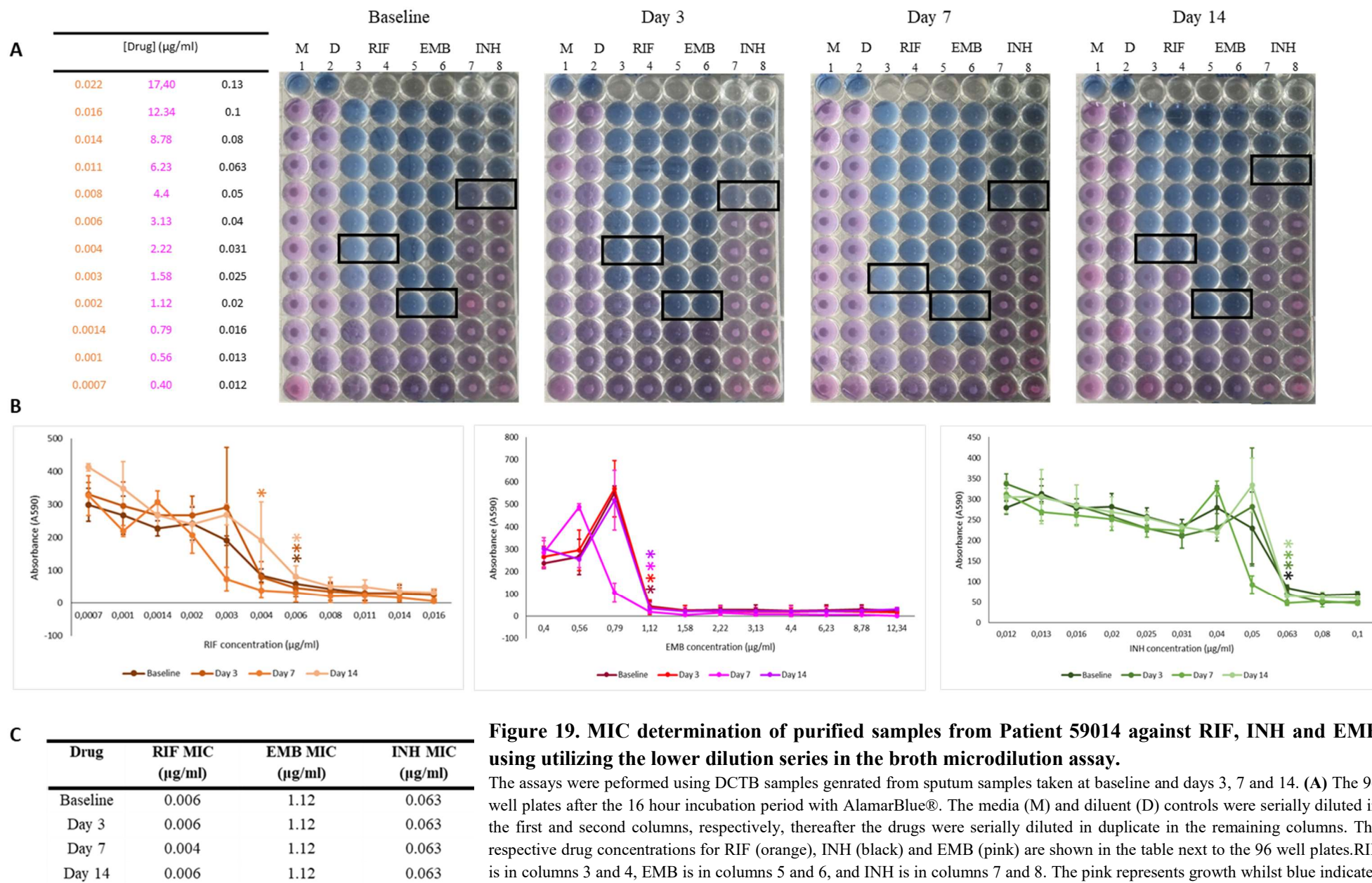


**Figure 19. MIC determination of purified samples from Patient 59014 against RIF, INH and EMB using utilizing the lower dilution series in the broth microdilution assay.**

The assays were performed using DCTB samples generated from sputum samples taken at baseline and days 3, 7 and 14. (A) The 96 well plates after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth whilst blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. (B) The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs.. (C) Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.



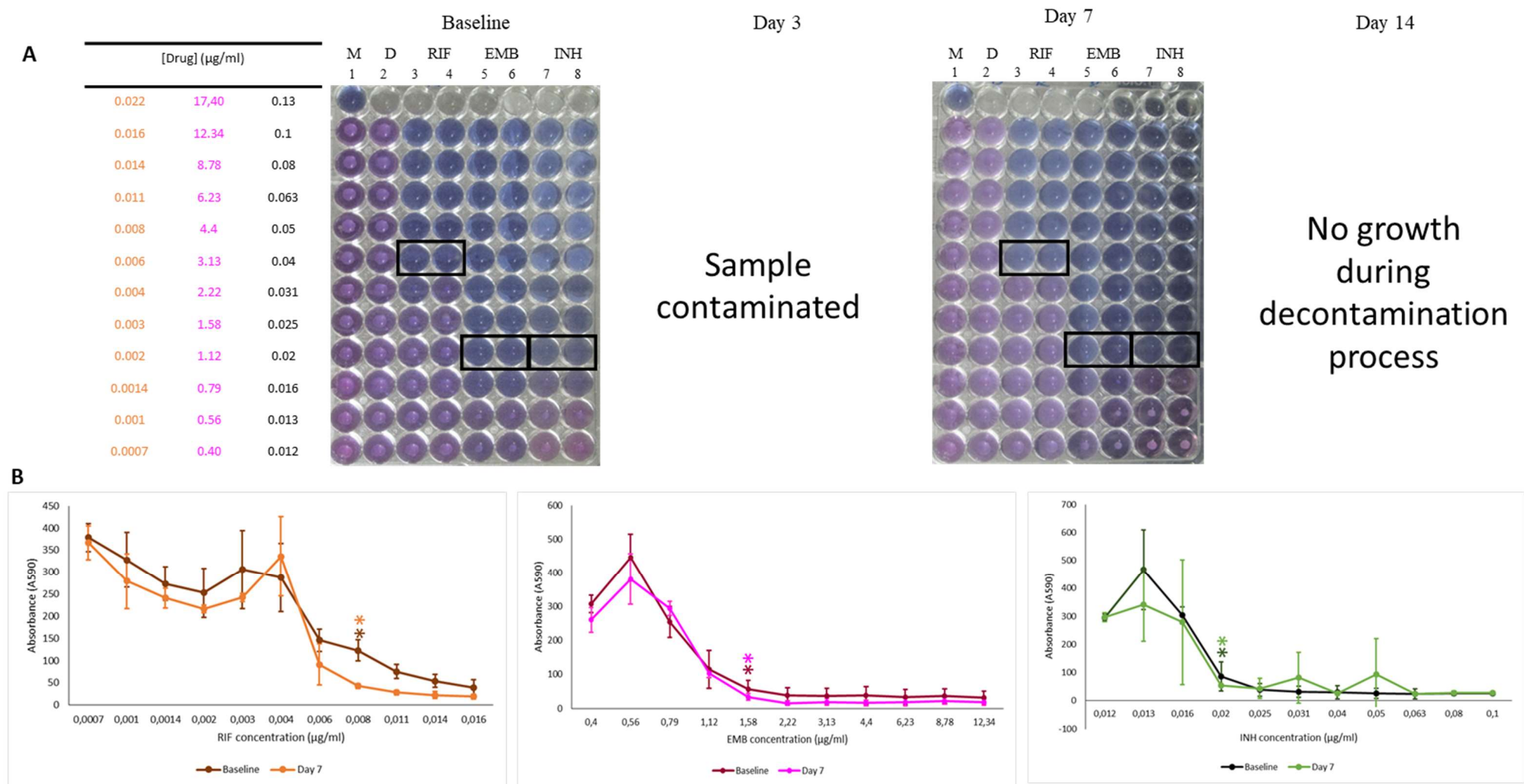


**Figure 21. MIC determination of purified samples from Patient 59032 against RIF, INH and EMB using utilizing the lower dilution series in the broth microdilution assay.**

The assays were performed using DCTB samples generated from sputum samples taken at baseline and day 7. (A) The 96 well plates after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth whilst blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. (B) The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. (C) Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

### 3.5 Analysis of resistance-conferring regions/ genes for RIF, INH and EMB

All samples from patients that showed drug resistance to either RIF, INH or EMB in the broth microdilution assay were analyzed further by sequencing their respective resistance-conferring regions to identify mutations responsible for the observed resistance to the drugs. The gDNA from the samples of select patients (59041, 59014, 57134, and 59032) were isolated using the colony boil method described in Section 2.6.1. For RIF, the RRDR (rifampicin resistance determining region) which is an 81 base pair region in the *rpoB* gene that harbors 95% of the RIF resistant mutations was amplified by polymerase chain reaction (PCR) using primers in Section 2.6.2, Table 5. Similarly, the *katG* and *ahpC* genes were amplified for INH and the *embCAB* operon was amplified for EMB using the primers listed in Section 2.6.2, Table 5. Due to time constraints, the samples that displayed drug resistance to PZA were not sequenced and analyzed further. Additionally, the resistance conferring regions/genes for RIF, INH and EMB were amplified for wild type strain, H37Rv, using the respective primers listed in Section 2.6.2, Table 5. The amplified fragments ranged from 158 to 368 base pairs in length as shown in Section 2.6.2, Table 5 and were therefore, analyzed on a high-concentration (1.5%) agarose gel (Figure 22). Single, clear bands were produced for each of the primer sets thereby indicating no presence of non-specific binding (Figure 22). The amplified PCR products were sequenced at the Central Analytical Facilities (CAF) DNA Sequencing Unit at Stellenbosch University to analyze the mutations in the various resistance-conferring regions. The sequences obtained from the resistance conferring genes (*rpoB*, *katG*, *ahpC*, *embA*, *embB*, *embC*) were analyzed and aligned to the reference sequences - H37Rv and the reference sequences taken from Mycobrowser using FinchTV and Jalview version 2.11. The alignments are shown in Figures 23-28. The sequences obtained for the RIF and EMB resistance samples showed no genetic alteration in their respective resistance-conferring regions compared to the same regions in the reference sequences (H37Rv lab strain and the reference sequences taken from Mycobrowser) (Figures 23, 26-28). These results indicate that the drug resistance in these samples is not attributable to genotypic alterations in the predicted drug target regions, but perhaps to mutations in other genes in the genome, which would require whole genome sequencing to identify. The sequences obtained for INH resistant samples showed no genetic alteration in *katG*, however, a point mutation was found in the *ahpC* gene at position 46 upstream of the transcriptional start site (position -46) for the baseline and day 7 samples from patient 59041 (Figures 24 and 25). Literature has shown that this genotypic change is found in both INH drug-resistant and drug susceptible isolates but does not confer genetic resistance to INH (Sreevatsan et al 1996; Zhang et al 1996; Kiepiela et al 2000; Baker et al 2005; Hosek et al 2006).

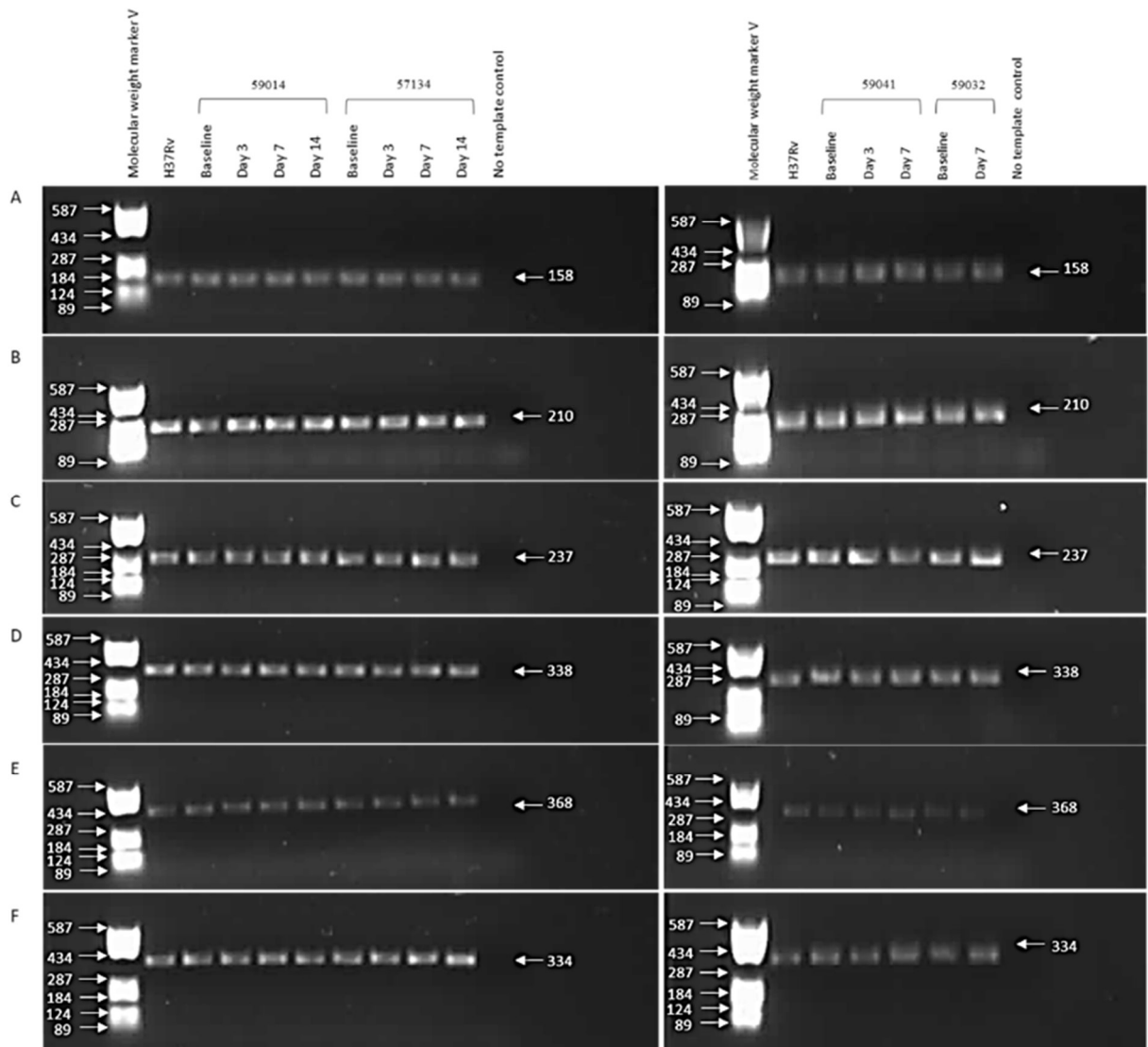


**Figure 22. Size confirmation of PCR products for the resistance conferring regions of RIF, INH and EMB.**

The DNA extracted from samples for patients 59014, 57134, 59041 and 59032 were used to amplify the resistance conferring regions of RIF, INH and EMB. The sizes of the amplified regions are as follows: (A) *rpoB*, 158 bps (B) *katG*, 210 bps (C) *ahpC*, 237 bps (D) *embA*, 338 bps (E) *embB*, 363 bps (F) *embC*, 346 bps. In addition, each gel shows the molecular weight marker V, amplification of the respective genes from H37Rv as the control, amplification of the respective genes from select samples, and the no template control which does not contain DNA.



**Figure 23. Sequence alignment of the RRDR region.**

The sample sequences were aligned against H37Rv and the reference sequence taken from Mycobrowser. The RRDR is an 81-base pair region in the *rpoB* gene where 95% of mutations associated with RIF resistance occur. No mutations were identified. The primers are not part of the RRDR regions and were therefore, left out of the Figure.

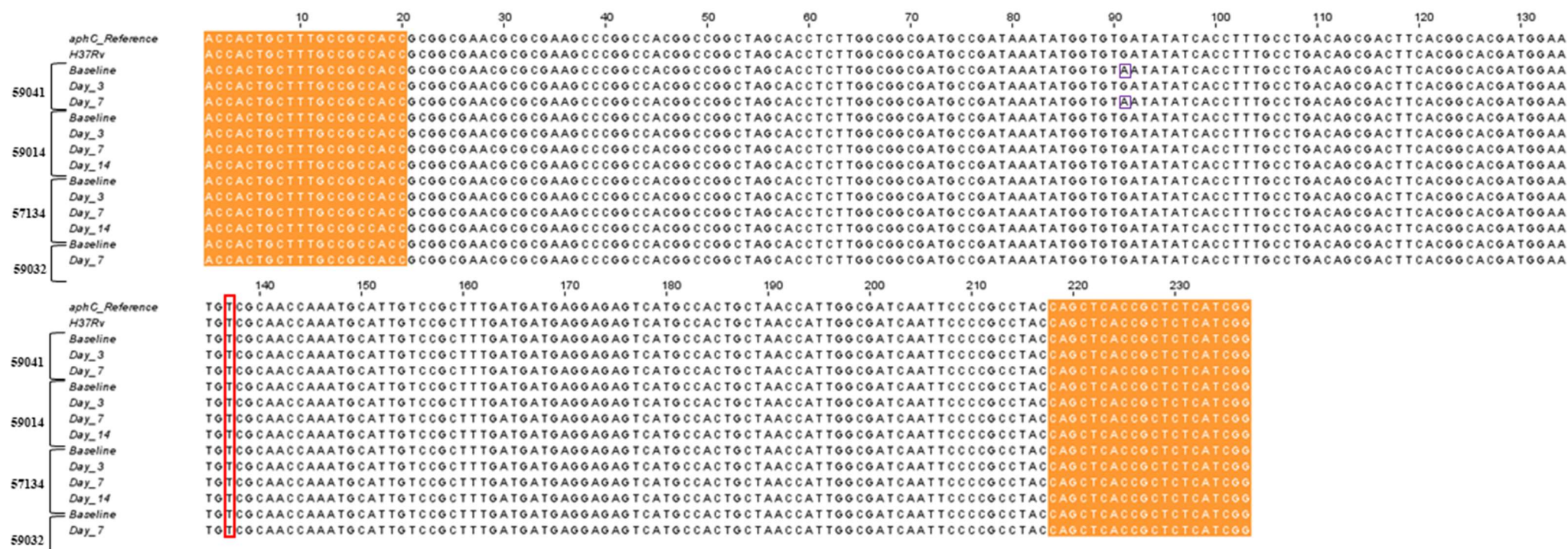


**Figure 24. Sequence alignment for the *aphC* gene.**

The sample sequences were aligned against H37Rv and the reference *aphC* sequence taken from Mycobrowser. A point mutation was identified at position 46 (small black box) upstream of the messenger ribonucleic acid start site (red box). The forward and reverse primers are highlighted in orange. The point mutation occurred for the baseline and day 7 sample for patient 59041.

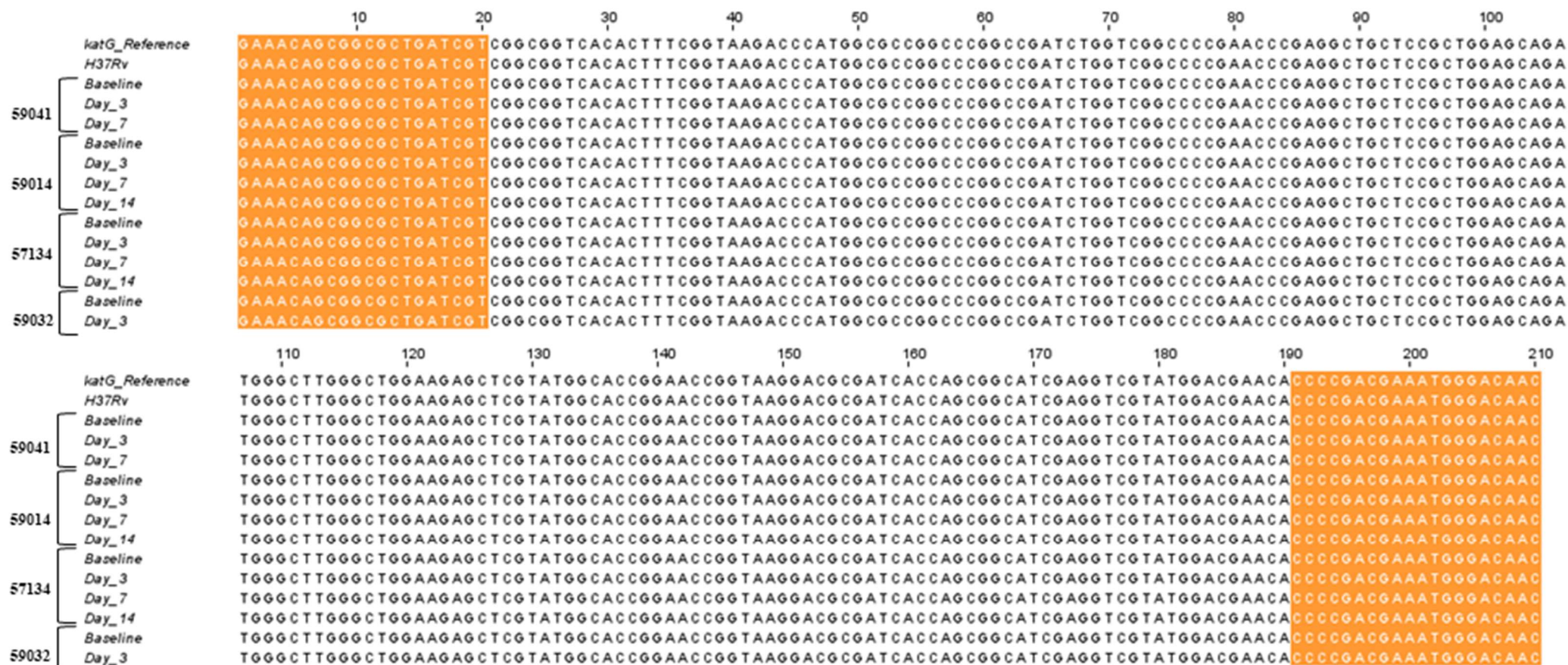


**Figure 25. Sequence alignment for the *katG* gene.**

The sample sequences were aligned against H37Rv and the reference *katG* sequence taken from Mycobrowser. No mutations were identified in the *katG* gene which confers INH resistance. The forward and reverse primers are highlighted in orange. No mutations were identified.

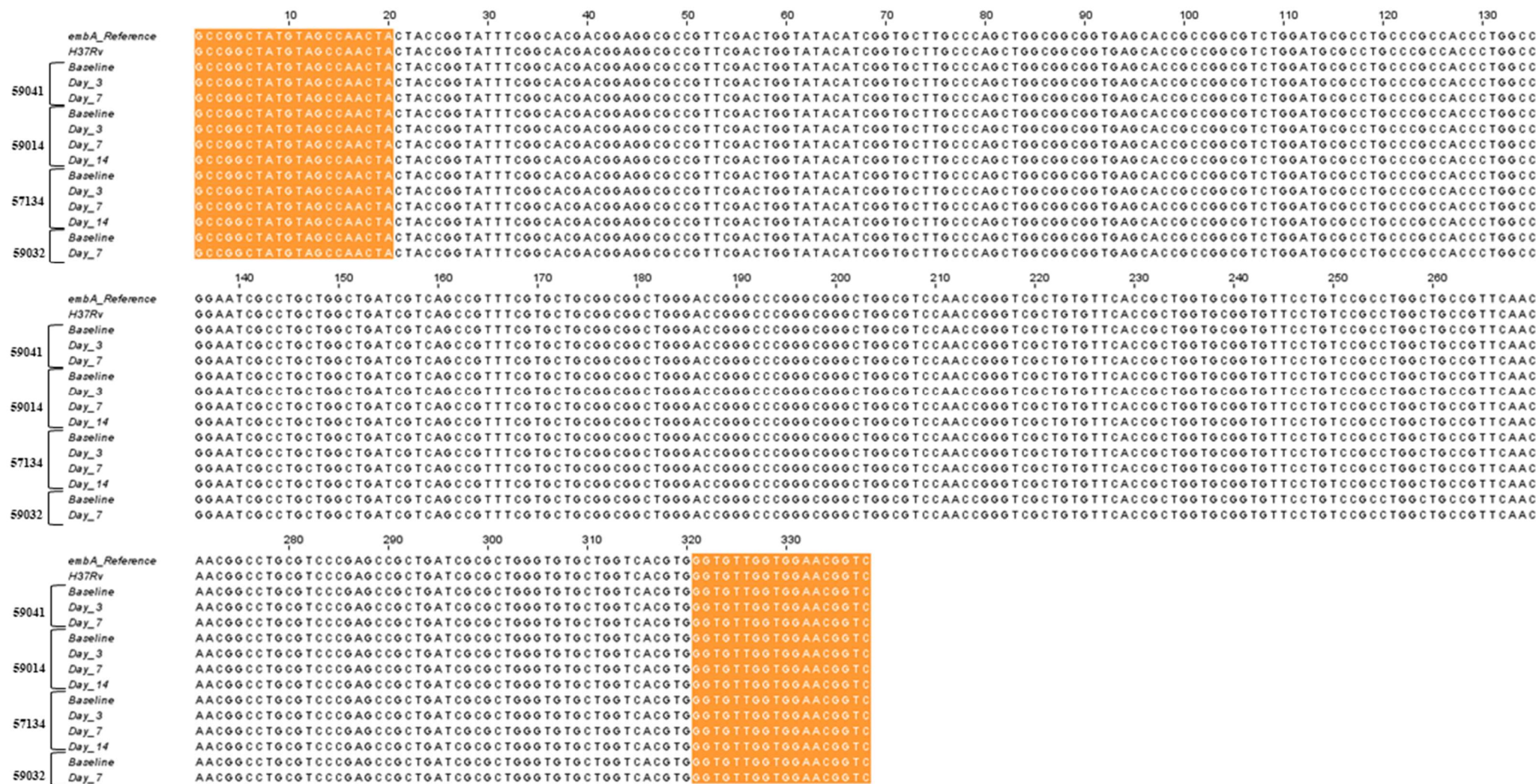


**Figure 26. Sequence alignment for the *embA* gene.**

The sample sequences were aligned against H37Rv and the reference *embA* sequence taken from Mycobrowser. No mutations were identified in the *embA* gene which confers EMB resistance. The forward and reverse primers are highlighted in orange. No mutations were identified.

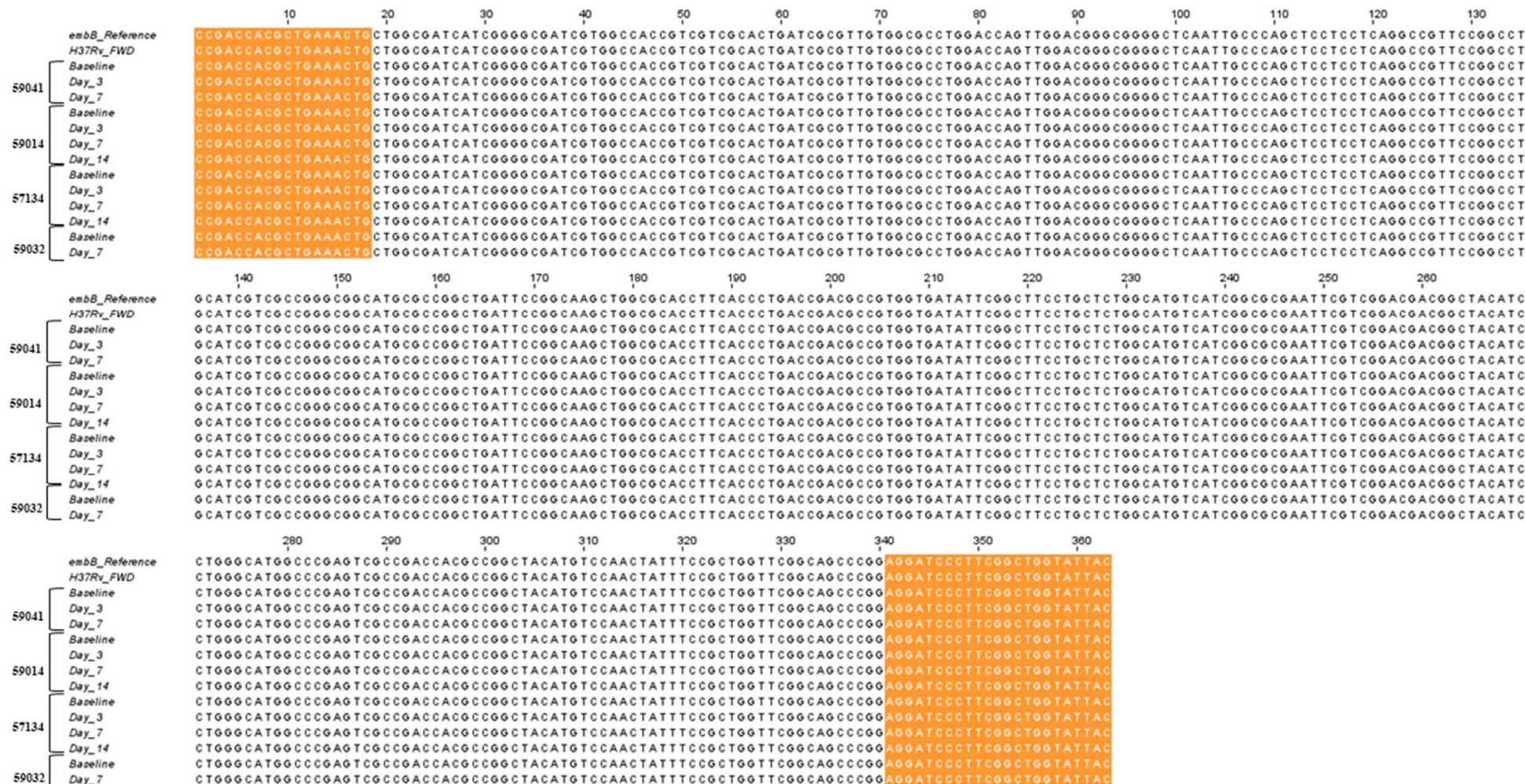


**Figure 27. Sequence alignment for the *embB* gene.**

The sample sequences were aligned against H37Rv and the reference *embB* sequence taken from Mycobrowser. No mutations were identified in the *embB* gene which confers EMB resistance. The forward and reverse primers are highlighted in orange. No mutations were identified.

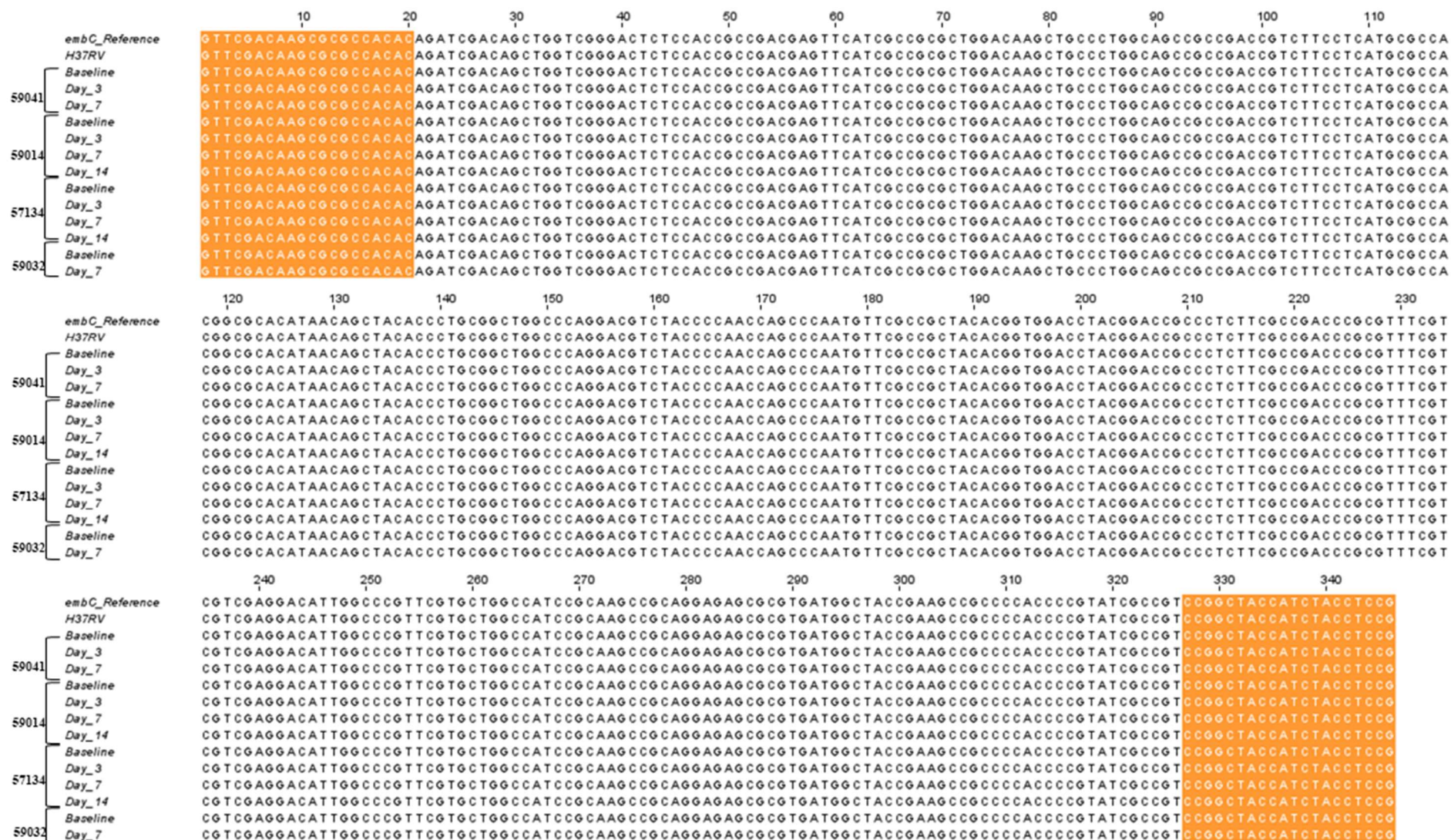


**Figure 28. Sequence alignment for the *embC* gene.**

The sample sequences were aligned against H37Rv and the reference *embC* sequence taken from Mycobrowser. No mutations were identified in the *embC* gene which confers EMB resistance. The forward and reverse primers are highlighted in orange. No mutations were identified.

### **3.6 Testing the MIC of starved select patient samples against RIF, INH and EMB**

The samples used throughout this study were derived from sputum obtained from patients in the principal study that was assessing the presence of differentially culturable tubercle bacilli (DCTB) during the course of treatment (Peters et al Manuscript in preparation). These DCTB are not identified by current standard culture methods (bacterial culturing, smear microscopy and GeneXpert) and are only resuscitated in liquid-based assays such as the most probable number (MPN) assay (Garton et al 2008; Mukamolova et al 2009; Chengalroyen et al 2016; Dartois et al 2016). The resuscitation of the bacteria in the MPN assay from the BMG study may have resulted in a loss of the DCTB phenotype, thus, the carbon starvation assay was performed as per Section 2.5 in order to drive the resuscitated bacteria back into a reversible non-replicating, drug-tolerant DCTB state (Betts et al 2002; Voskuil et al 2003; Voskuil et al 2004; Rustad et al 2008). The assay was performed on the samples of select patients (patient 59041, 59014, 57134, and 59032) that displayed resistance to RIF, INH or EMB in Section 3.4. The select samples were starved in phosphate buffered saline (PBS) for 1 week before assessing the MIC to determine whether the forced differentially culturable state had an impact on the MIC to RIF, INH and EMB. Confirmation that DCTB was produced using the carbon starvation assay is shown in Figure 29. All patient samples produced DCTB except for the day 3 sample for patient 59041 using the CF-independent media, however, DCTB was produced when CF-dependent media was used. This indicates that in this sample, DCTB required the growth factors present in CF to assist in the resuscitation process.

The broth microdilution assay using these starved cells was tested with 7H9 liquid media (CF-independent) as well as 7H9 media supplemented with culture filtrate (CF) obtained from a log-phase culture of H37Rv in a 1:1 ratio (CF-dependent) (Figures 30-32). The CF contains molecules/growth factors that assist in the resuscitation of the phenotypically distinct subpopulations (DCTB) generated during starvation. Comparison of the MICs obtained using starved cells under CF-independent and CF-dependent conditions were compared to the MICs obtained for these resistant samples before starvation (Section 3.4). The broth microdilution assays were performed for RIF, EMB and INH using the select starved cultures in CF-independent and CF-dependent conditions are shown in Figures 33-40. All starved samples displayed higher drug tolerance indicated by a higher MIC, to RIF, EMB and INH compared to the unstarved samples (Figures 30-32). The starved samples were able to withstand a significantly higher concentration of RIF and INH compared to EMB as the MICs for EMB did not increase significantly (Figures 30-32). The MIC for EMB showed a 1-2 fold change under

both CF-independent and CF-dependent conditions compared to the unstarved MIC, except for the day 7 sample from patient 59032, there was a 4-fold increase in the MIC. These data indicate that when DCTB are exposed to stressful environments such as the starvation model used in this study, the bacteria re-enter this state and become drug-tolerant, a main characteristic of the DCTB phenotype (Figures 30-32, dashed and checkered bars). Additionally, when these DCTB are resuscitated, the cells lose the DCTB phenotype and become more susceptible to the drug (Figure 30-32, solid bars). The extent to which this DCTB state is lost upon resuscitation remains unclear. Our data also correlates to various studies that show that these bacteria are driven into a low metabolizing, drug-tolerant state (Betts et al 2002; Voskuil et al 2003; Voskuil et al 2004; Rustad et al 2008). Statistical analysis could not be performed on some samples because the MIC values obtained for the pairs within the CF dependent and CF-independent groups were equal.

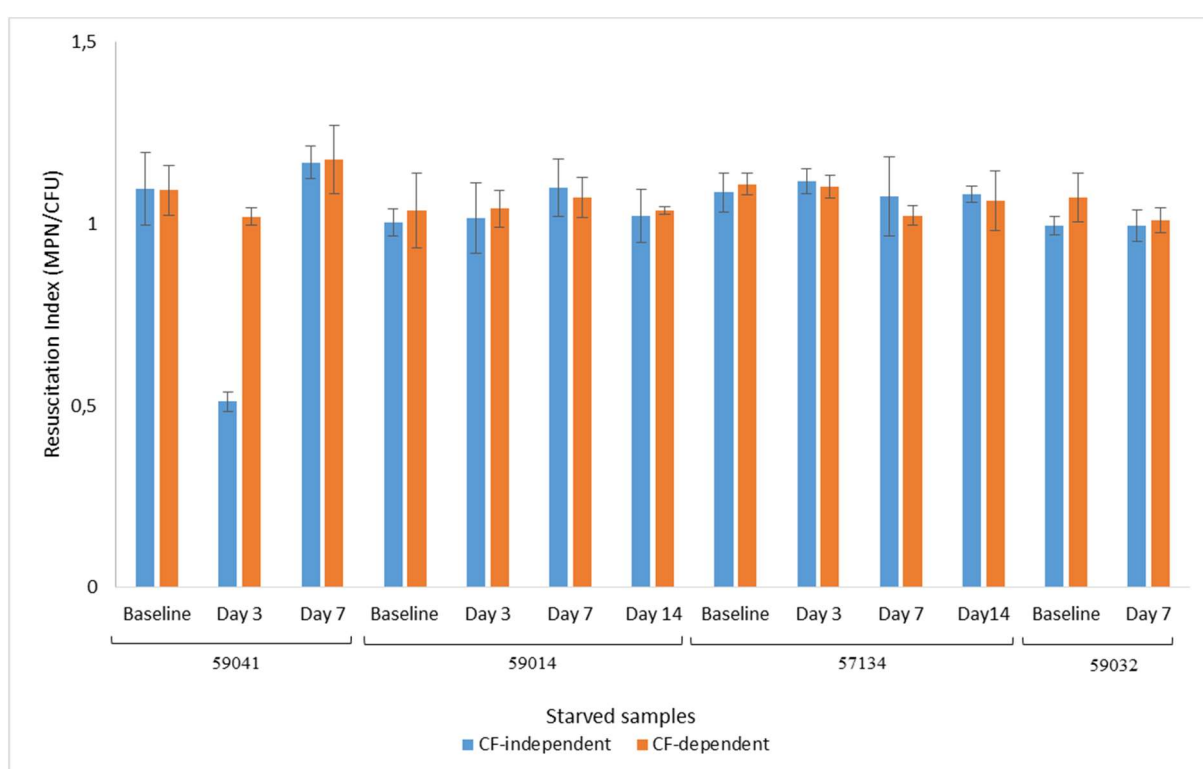


**Figure 29. The generation of DCTB after carbon starvation was assessed using the MPN assay and CFU enumeration.**

The bars present the amount of DCTB resuscitated in CF-independent shown in blue versus CF-dependent media shown in orange. The resuscitation index (RI) represents the log DCTB amount present after the starvation process and was calculated by comparing the number of bacteria recovered from the MPN assay versus CFU enumeration, i.e.  $RI = \text{MPN value} / \text{CFU per ml}$ . A RI value greater than 1 indicates that DCTB was produced after the starvation process whilst a RI value less than 1 indicates that DCTB was not produced. The experiment was performed in triplicate and the error bars represent the standard deviation.

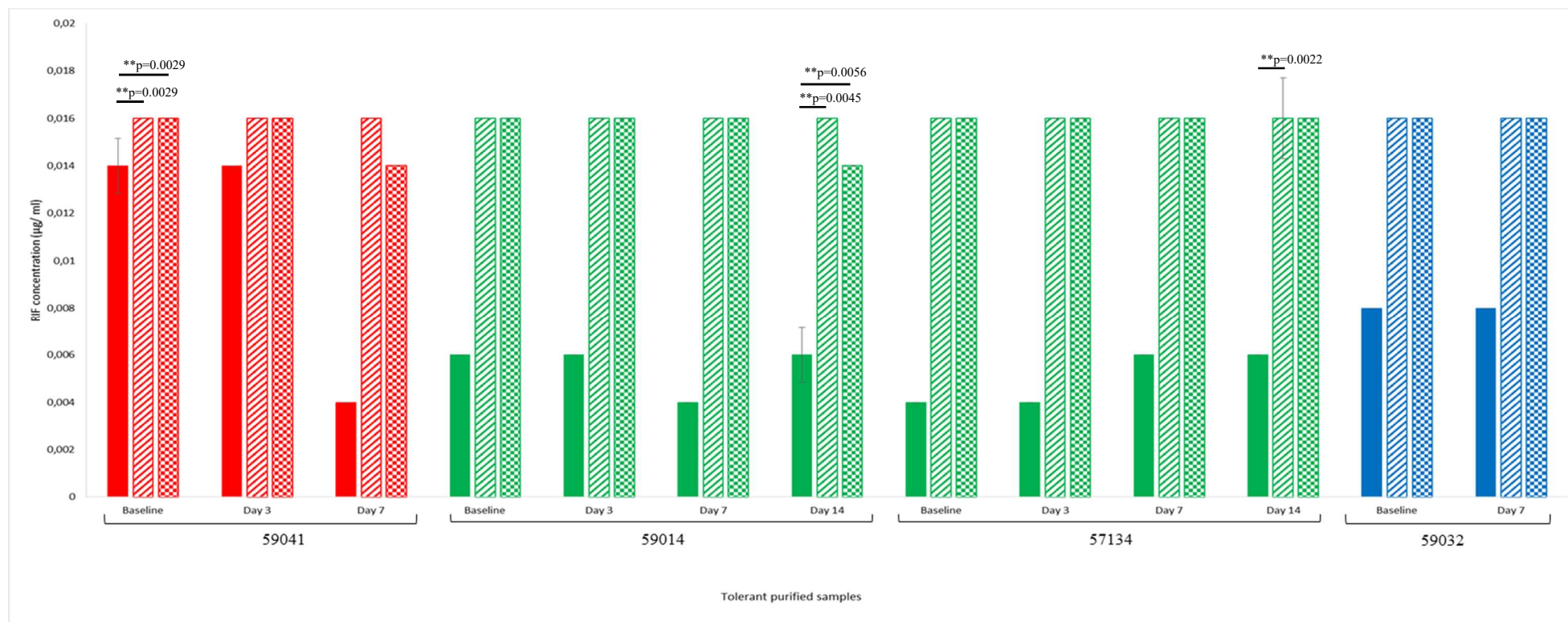


**Figure 30. Analysis of RIF susceptibility of patient samples before and after starvation.**

The drug susceptibility of the starved cultures to RIF was assessed using the broth microdilution assay under CF-independent (media) and CF-dependent conditions (1:1 media:CF). The MICs obtained for the unstarved cultures are shown as solid bars and the MICs obtained for the starved cells under CF-independent and CF-dependent conditions are shown as dashed and checkered bars, respectively. The experiment was performed in triplicate and the error bars represent the standard deviation. Statistical analysis was performed using student's *t*-test; \**p*<0.05, \*\**p*<0.01 and \*\*\**p*<0.001.

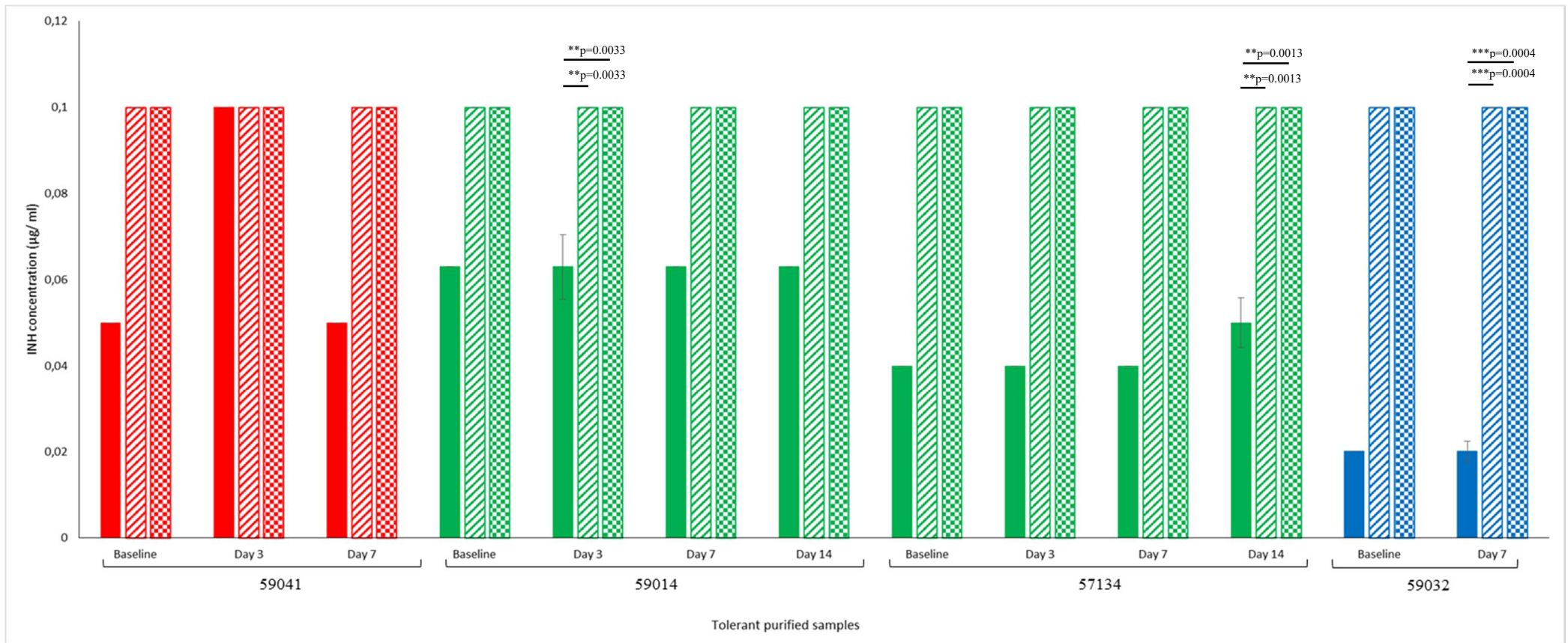


**Figure 31. Analysis of INH susceptibility of patient samples before and after starvation.**

The drug susceptibility of the starved cultures to INH was assessed using the broth microdilution assay under CF-independent (media) and CF-dependent (1:1 media:CF) conditions. The MICs obtained for the unstarved cultures are shown as solid bars and the MICs obtained for the starved cells under CF-independent and CF-dependent conditions are shown as dashed and checked bars, respectively. The experiment was performed in triplicate and the error bars represent the standard deviation. Statistical analysis was performed using student's *t*-test; \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ .

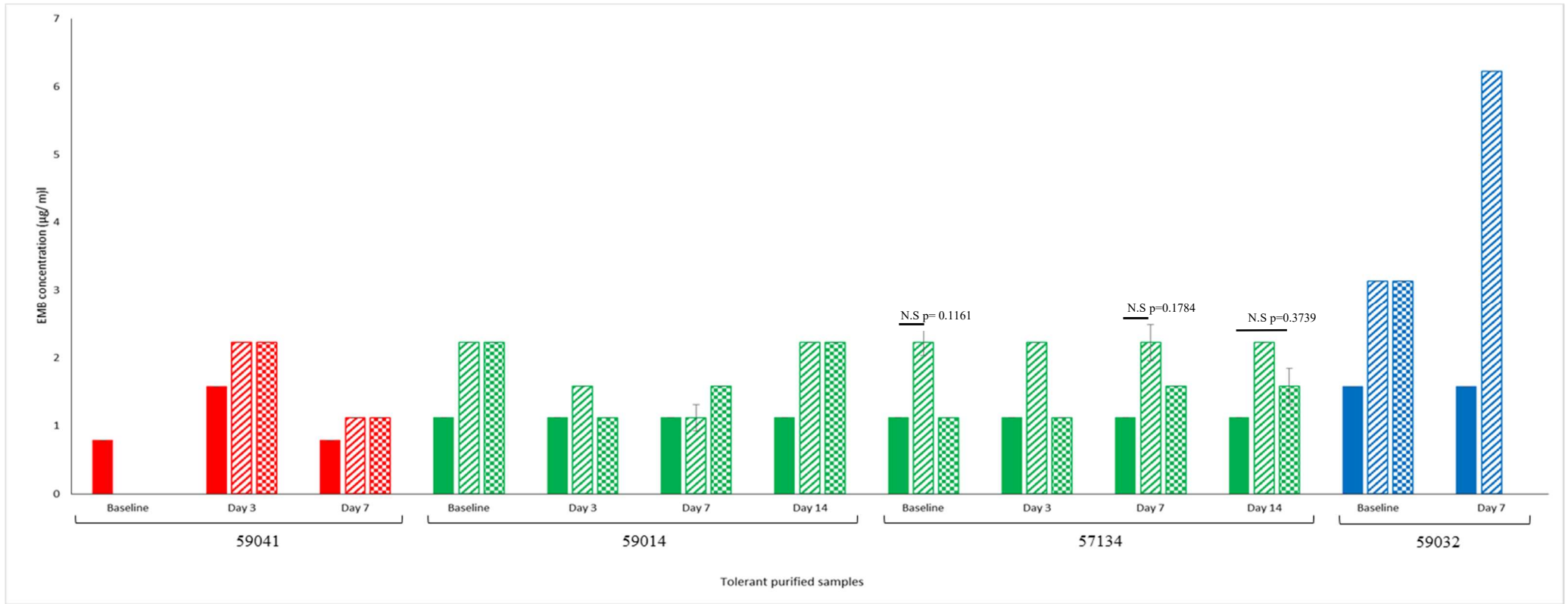


**Figure 32. Analysis of EMB susceptibility of patient samples before and after starvation.**

The drug susceptibility of the starved cultures to EMB was assessed using the broth microdilution assay using CF-independent (media) and CF-dependent (1:1 media: CF) conditions. The MICs obtained for the unstarved cultures are shown as solid bars and MICs obtained for the starved cells under CF-independent and CF-dependent conditions are shown as dashed and checkered bars, respectively. The experiment was performed in triplicate and the error bars represent the standard deviation. Statistical analysis was performed using student's *t*-test; Not significant (N.S)  $p > 0.05$ .

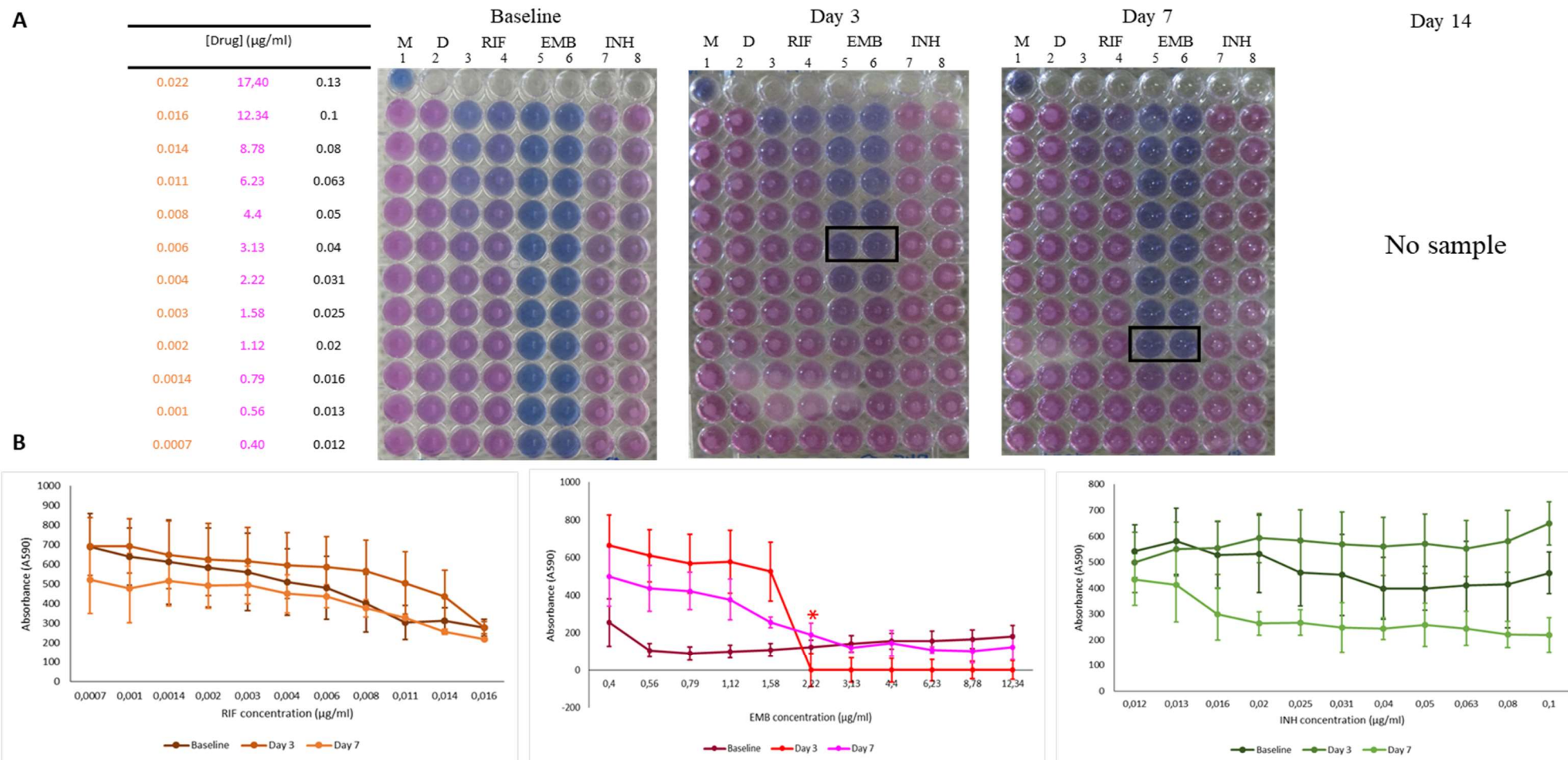


**Figure 33. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59041 using the lower dilution series in the broth microdilution assay with CF-independent media**

The assays were performed using samples taken at baseline, day 3 and day 7. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

The dash (-) means that no sample was collected for that patient at that time point.

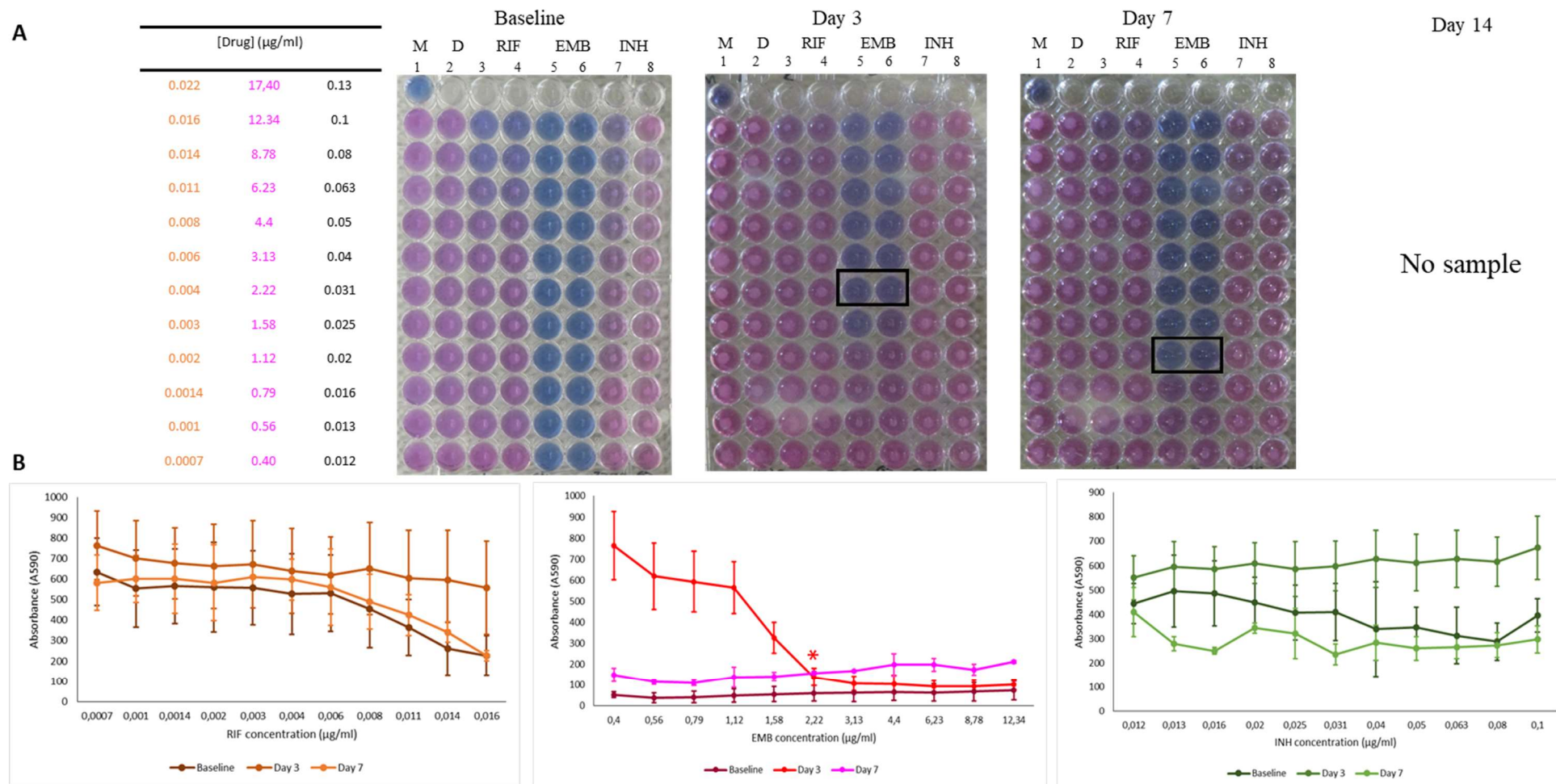


**Figure 34. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59041 using the lower dilution series in the broth microdilution assay with CF-dependent media**

The assays were performed using samples taken at baseline, day 3 and day 7. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

The dash (-) means that no sample was collected for that patient at that time point.

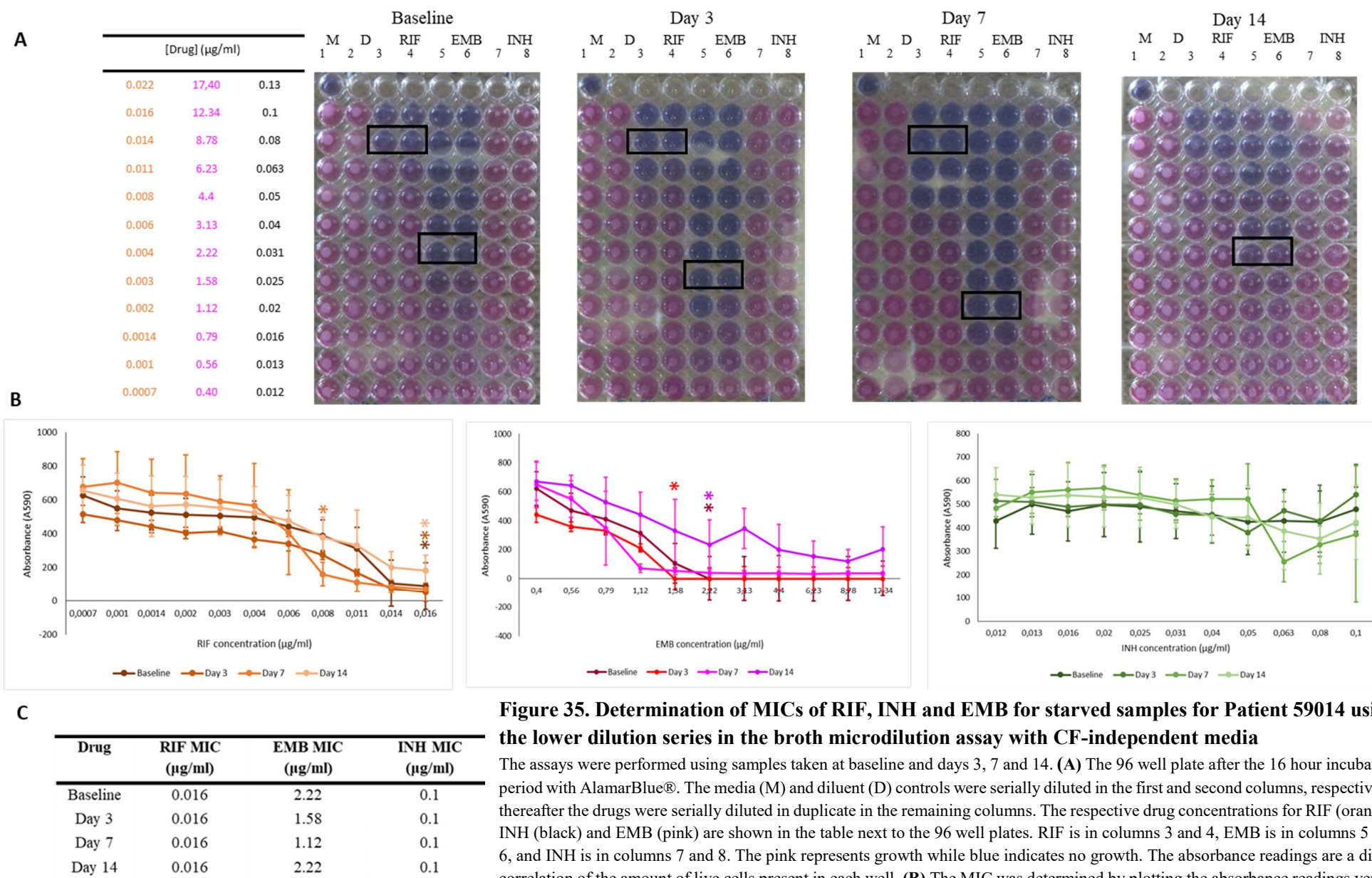


**Figure 35. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59014 using the lower dilution series in the broth microdilution assay with CF-independent media**

The assays were performed using samples taken at baseline and days 3, 7 and 14. (A) The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. (B) The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. (C) Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

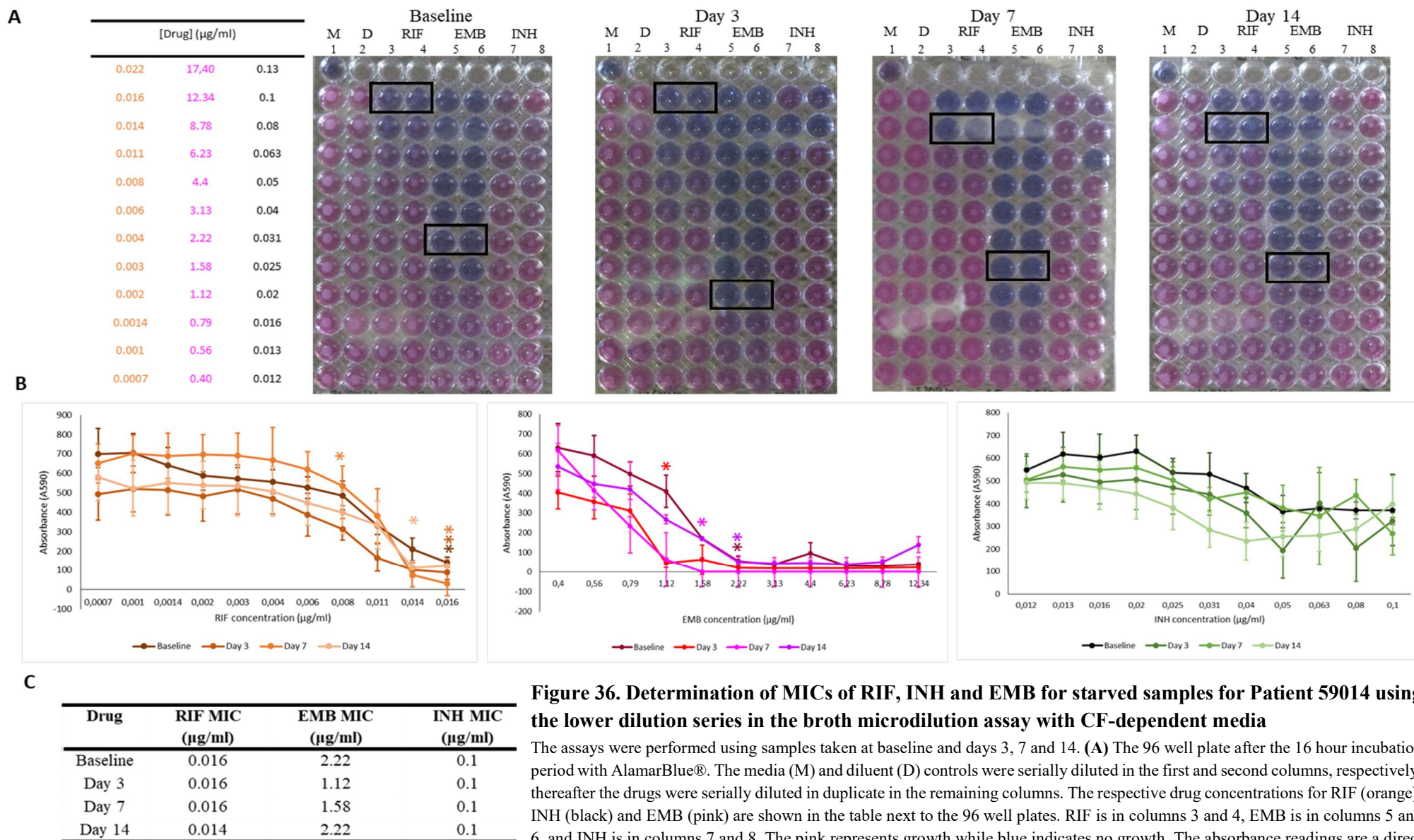
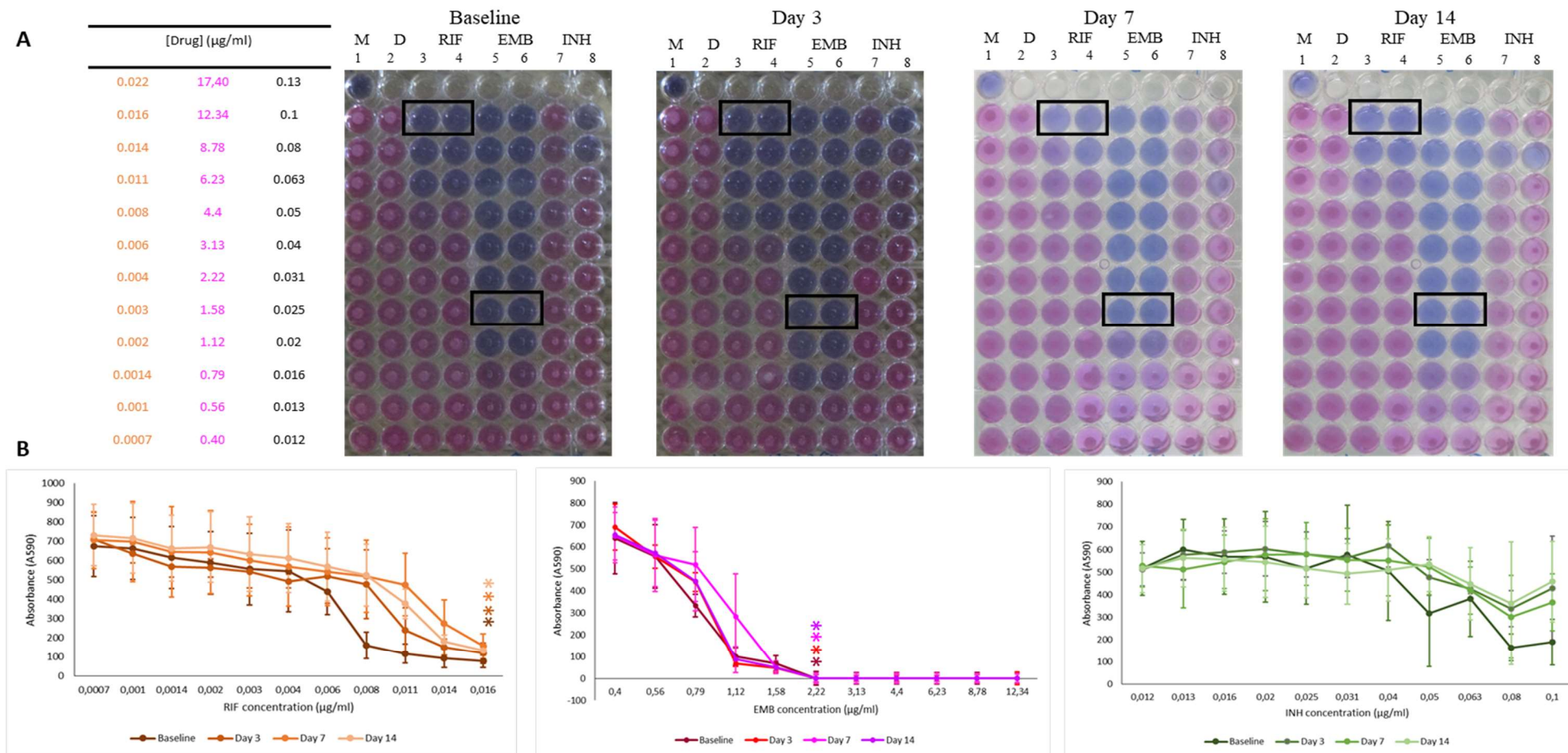


**Figure 36. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59014 using the lower dilution series in the broth microdilution assay with CF-dependent media**

The assays were performed using samples taken at baseline and days 3, 7 and 14. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.



**C**

| Drug     | RIF MIC (µg/ml) | EMB MIC (µg/ml) | INH MIC (µg/ml) |
|----------|-----------------|-----------------|-----------------|
| Baseline | 0.016           | 2.22            | 0.1             |
| Day 3    | 0.016           | 2.22            | 0.1             |
| Day 7    | 0.016           | 2.22            | 0.1             |
| Day 14   | 0.016           | 2.22            | 0.1             |

**Figure 37. Determination of MICs of RIF, INH and EMB for starved samples for Patient 57134 using the lower dilution series in the broth microdilution assay with CF-independent media**

The assays were performed using samples taken at baseline and days 3, 7 and 14. (A) The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. (B) The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. (C) Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

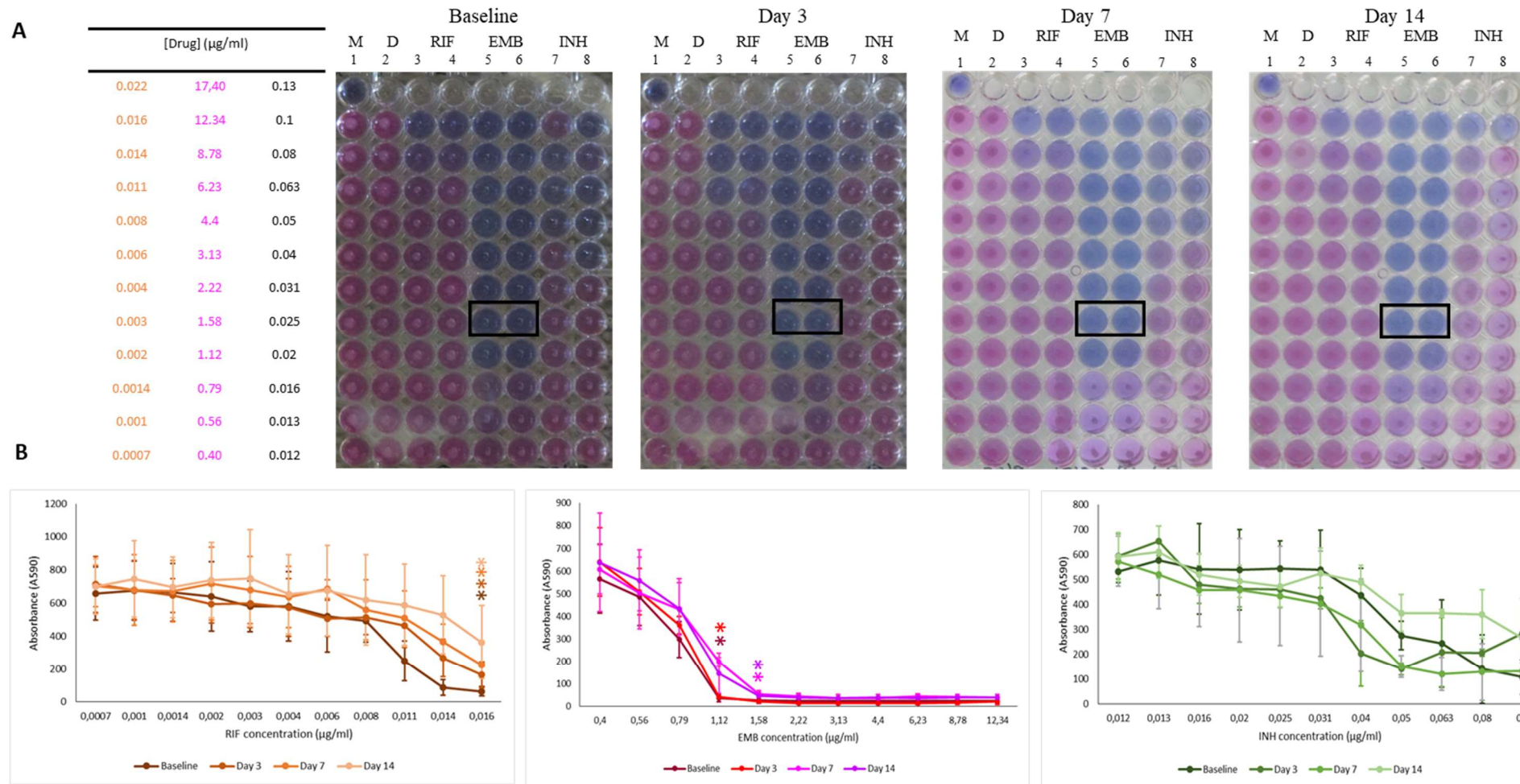


**Figure 38. Determination of MICs of RIF, INH and EMB for starved samples for Patient 57134 using the lower dilution series in the broth microdilution assay with CF-dependent media**

The assays were performed using samples taken at baseline and days 3, 7 and 14. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

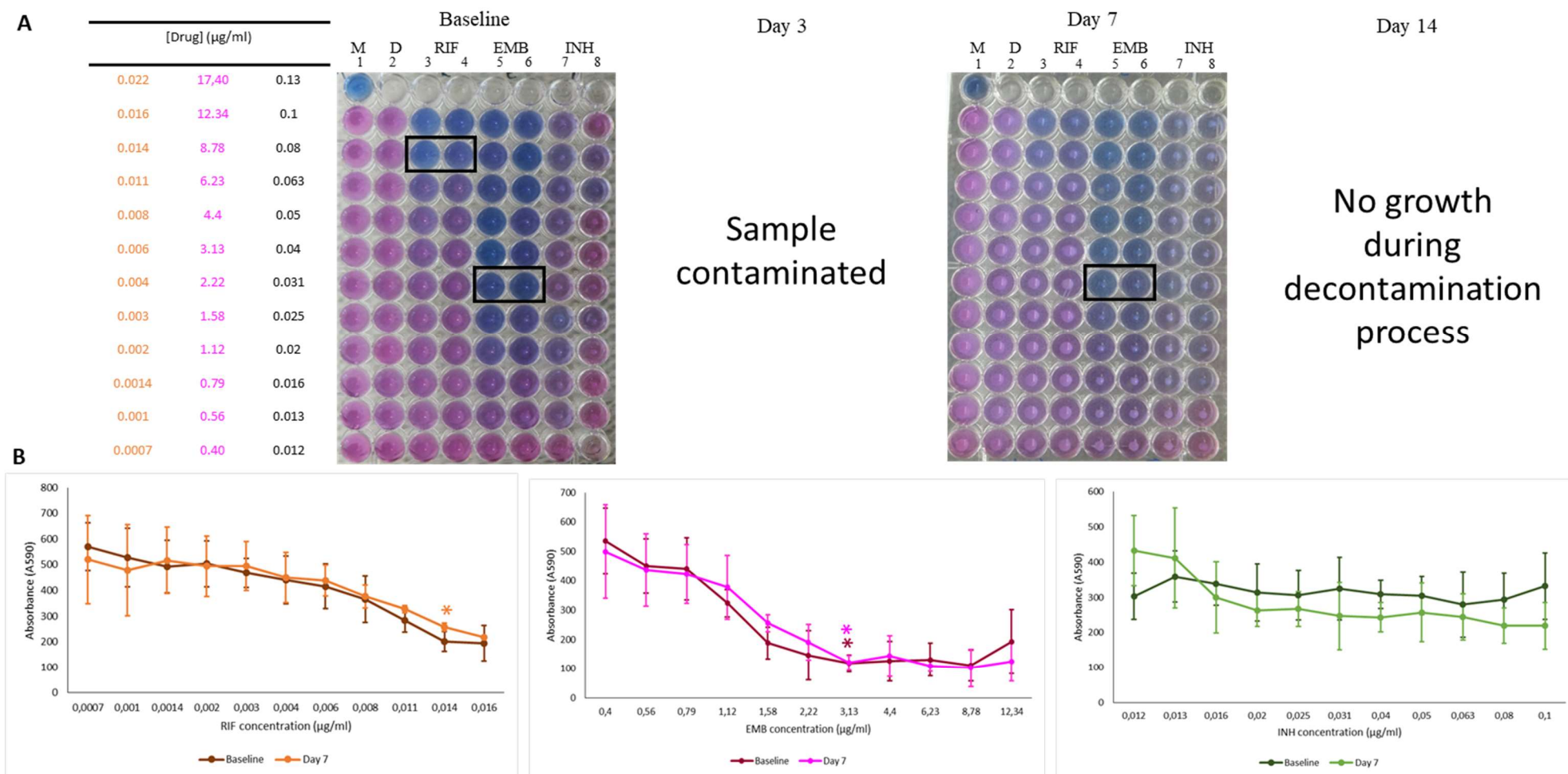
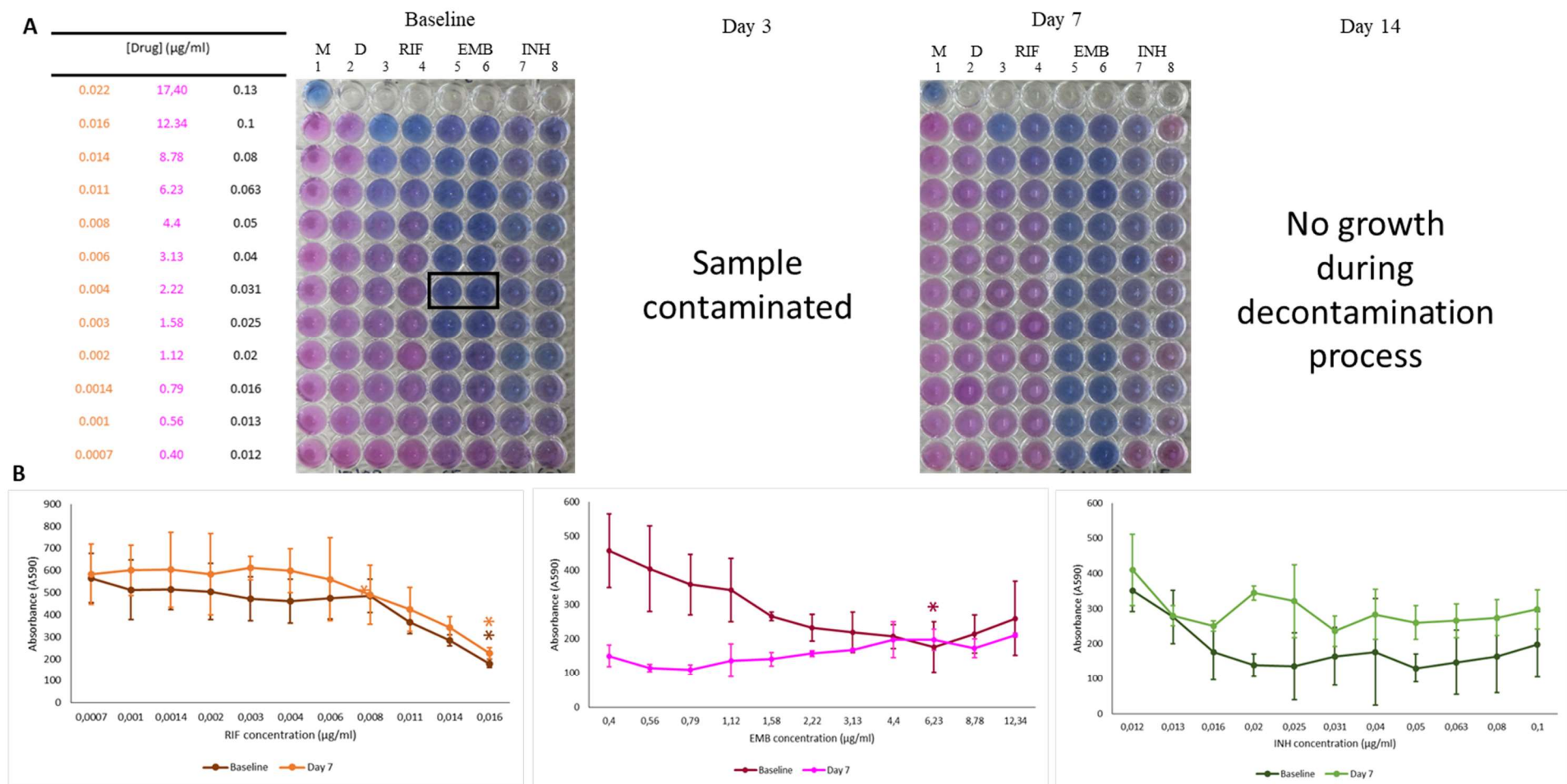


**Figure 39. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59032 using the lower dilution series in the broth microdilution assay with CF-independent media**

The assays were performed using samples taken at baseline and day 7. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.



**C**

| Drug     | RIF MIC (µg/ml) | EMB MIC (µg/ml) | INH MIC (µg/ml) |
|----------|-----------------|-----------------|-----------------|
| Baseline | 0.16            | 6.23            | 0.1             |
| Day 3    | -               | -               | -               |
| Day 7    | 0.16            | NO MIC          | 0.1             |
| Day 14   | -               | -               | -               |

The dash (-) means that the sample was not successfully purified.

**Figure 40. Determination of MICs of RIF, INH and EMB for starved samples for Patient 59032 using the lower dilution series in the broth microdilution assay with CF-dependent media**

The assays were performed using samples taken at baseline and day 7. **(A)** The 96 well plate after the 16 hour incubation period with AlamarBlue®. The media (M) and diluent (D) controls were serially diluted in the first and second columns, respectively, thereafter the drugs were serially diluted in duplicate in the remaining columns. The respective drug concentrations for RIF (orange), INH (black) and EMB (pink) are shown in the table next to the 96 well plates. RIF is in columns 3 and 4, EMB is in columns 5 and 6, and INH is in columns 7 and 8. The pink represents growth while blue indicates no growth. The absorbance readings are a direct correlation of the amount of live cells present in each well. **(B)** The MIC was determined by plotting the absorbance readings versus the drug concentrations for each drug. The black boxes on the plates represent the MIC and the stars indicate the corresponding MIC on the graphs. **(C)** Summary of the obtained MIC values. The experiment was performed in triplicate and the error bars represent the standard deviation between experiments.

## 4. Discussion

### 4.1 Decontamination outcomes for patient samples

Several studies have found that sputum from TB patients with active disease contains a high proportion of non-replicating bacteria undetectable by standard culture methods such as colony-forming units (CFU) and MGIT assays (Garton et al 2008; Mukamolova et al 2009; Chengalroyen et al 2016; Dartois et al 2016). The study by Mukamolova et al. (2009) identified a non-replicating bacterial population in sputum samples from 25 drug-susceptible, treatment-naïve patients that could only be detected in a liquid-based limiting dilutions assay, called the most probable number (MPN) assay. The samples utilized in this study were harvested and stored from previously performed MPN assays in the BMG study using limiting dilutions of patient sputa (Peters et al Manuscript in preparation). The BMG study was aimed at assessing the treatment response of DCTB using the liquid-based MPN assay compared to routine culturing methods (CFU enumeration and MGIT assays). Four treatment response groups were identified in the principal study, namely: early non-responders, paradoxical worsening, classic bi-phasic and non-responders. A total of 112 stored samples from 16 patients were included in this study – 46 samples from 7 patients in the early non-responders group, 43 samples from 5 patients in the paradoxical worsening group and 23 samples from 4 patients in the classic biphasic group. For the early non-responders and paradoxical worsening groups, 76.1% and 60.5% of samples stored from the MPN assay were successfully decontaminated with 1X PANTA in liquid media as per Section 2.3. A low number of samples (17.4%) were successfully decontaminated in the classic bi-phasic group. Samples that could not be purified were due to the lack of growth of the bacteria or contamination. The samples that struggled to grow in liquid or solid media most likely did not have a significant initial bacterial load. In addition, sputa also contain contaminating bacteria from the lung environment. Although the sputum samples analyzed in the BMG study were decontaminated before carrying out the MPN assay and despite subsequent treatment of the stored MPN sample with PANTA in this study, the contaminants could not be eradicated suggesting that these bacteria are resistant to the antibiotics that make up PANTA, which are polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin.

## **4.2 Comparison of the 2-fold dilution and lower dilution series in determining the drug susceptibility of the MPN samples**

The drug susceptibility of the purified MPN samples was assessed by broth microdilution assay to determine the minimum inhibitory concentration (MIC) of select drugs (RIF, INH, EMB and PZA) required to kill the *M. tuberculosis*. The MIC is a time-dependent endpoint that identifies the lowest drug concentration required to inhibit the growth of *M. tuberculosis*. The more conventional 2-fold dilution of a pre-determined drug concentration is usually used to assess MICs however, the difference between the 2-fold differences between the fold changes can sometimes not be accurate in determining small changes in drug susceptibility. To overcome this, a lower dilution series was tested based on a study conducted by Colangeli et al. (2018) which utilized a 1.41 dilution to determine MICs for RIF and 1.26 dilution series to determine MICs for INH. The 1.41 dilution adds a value between the fold changes of a conventional 2-fold dilution, whereas the 1.26 dilution adds 2 values. These dilution series were chosen based on the expected MICs and the starting drug concentrations required for the select drugs. The expected MIC for RIF (0.003-0.006 µg/ml) is lower than the expected MIC for INH (0.025 µg/ml) and the starting drug concentrations were 0.062 µg/ml and 0.8 µg/ml, respectively. If the 1.26 dilution were used for RIF, the starting drug concentration would be lower than that of the 1.41 dilution, requiring less of the drug to make up the starting drug concentration, potentially resulting in errors due to micropipette limitations. Additionally, the 1.41 dilution series was independently chosen to perform the broth microdilution assay using EMB as the 1.26 dilution series resulted in a starting drug concentration that was too minute to accurately pipette. The expected MIC for RIF for H37Rv was 0.003-0.006 µg/ml, our results show a MIC of 0.003 µg/ml was obtained using the 2-fold dilution series and a more precise MIC of 0.004 µg/ml was obtained using the 1.41-fold dilution series. The smaller dilution series for INH using H37Rv resulted in a MIC (0.032 µg/ml) closer to the expected value (0.025 µg/ml) rather than the 2-fold dilution series (0.05 µg/ml). In both cases, the lower dilution series resulted in a MIC closer to the expected MICs showing that the additional increments in antibiotic concentrations in the lower dilution series provide a more accurate MIC using the broth microdilution assay. These small differences in MICs relative to the clinical breakpoint, which is the concentration at which bacteria are susceptible to successful treatment with an antibiotic, may result in inaccurate dosage levels which may result in inadequate treatment of bacterial infections.

### 4.3 The drug susceptibility of purified MPN samples

The DCTB population was not completely eradicated in any of the response groups by day 14 of treatment, as the MPN assay identified a  $\pm 1$ -2 log higher bacterial count than the CFU assay indicating that the first-line drugs are unable to eliminate the DCTB (Peters et al Manuscript in preparation). Hence, determining whether these MPN samples are drug-tolerant or drug-resistant will aid in understanding the underlying mechanisms these DCTB use to adapt and evolve resistance to the drugs. Therefore, the drug susceptibility of the purified MPN samples was determined by broth microdilution assay using the four first-line drugs- RIF, INH, EMB and PZA. The established lower dilution series (1.41 and 1.26) was used for RIF, INH and EMB whilst the conventional 2-fold dilution was used for PZA.

The result of each broth microdilution assay, however, is dependent on multiple factors such as different culture dilutions, incubation temperature and time and the individual performing the assay (Mouton et al 2018). To account for this variability a standard was set by ISO (ISO 20776-2) which states that a MIC deviation of two dilution factors is acceptable (Mouton et al 2018). Therefore, after the MICs were established for the H37Rv for RIF, INH, EMB and PZA, antibiotic concentrations of two dilution factors higher than those MICs were set to account for this variability. The standard was not set for RIF because the BMG study from which these samples were derived excluded patients diagnosed with RIF resistance, therefore any samples which have MICs for RIF higher than that of H37Rv were analyzed further. Our results showed that samples from patient 59041 from the early non-responders group, patients 59014 and 57134 from the paradoxical worsening group, and patient 59032 from the classic bi-phasic group had resistance to one or more of the four-first line drugs. Samples from patient 59041 showed resistance to all four antibiotics, patient 59014 showed resistance to RIF, INH and PZA, patient 57134 showed resistance to only RIF and patient 59032 showed resistance to RIF, EMB, and PZA. Using these results patient 57134 would be considered to have RIF-resistant TB and patients 59041, 59014 and 59032 would be considered to have multi-drug resistant TB. Additionally, patients 59014, 57134 and 59032 were infected with Beijing strains whilst patient 59041 was infected with the Central Asian strain (CAS) as shown in Appendix B (Peters et al Manuscript in preparation). There is no correlation between the response groups and the resistance patterns observed, indicating that the drug response of the patients, in this study, to antibiotic treatment did not correspond to the patient's drug susceptibility profile but rather to their strain type.

#### 4.4 Sequencing analysis on select drug-resistant samples

To better understand how these DCTB adapt to promote their survival during antibiotic treatment, the resistance conferring regions for RIF, INH and EMB were amplified for the samples displaying resistance to these drugs to identify whether this observed resistance is genotypic or phenotypic. Additionally, the resistance conferring regions for the drugs were amplified for the wildtype laboratory strain, H37Rv to use as reference sequences. The RRDR (rifampicin resistance determining region) was amplified for RIF as approximately 95% of the mutations associated with rifampicin-resistant tuberculosis are located within this region in the *rpoB* gene (Zaw et al 2018). The *katG* gene and the promoter *ahpC* promoter region were amplified for INH as mutations occur more frequently in *katG* (42-95% of isolates) than in *ahpC* (6-43% of isolates), whilst 10% have mutations in both regions (Bollela et al 2016). Lastly, the *embCAB* operon was amplified for EMB, as the majority of the mutations commonly occur in *embB* and occasionally in *embC* (Xu et al 2015). The results showed no mutational alterations in the respective regions for each of the drugs except for *ahpC*. There was a single point mutation found in the baseline and day 7 samples of patient 59041 from the early non-responders group. This point mutation (G → A), at position 46 upstream of the transcriptional start site of *ahpC* (*ahpC* -46A), is the most common in both INH resistant and susceptible isolates but plays no role in drug resistance or phenotypic tolerance (Sreevatsan et al 1996; Zhang et al 1996; Kiepiela et al 2000; Baker et al 2005; Hosek et al 2006). Immunoblot analysis by Sreevatsan et al (1996) showed that isolates containing this mutation had no detectable alkyl hydroperoxidase synthesis, indicating that this polymorphism is not a compensatory mutation affecting gene regulation (Sreevatsan et al 1996). Additionally, the *ahpC* -46A mutation is not associated with INH resistance but is rather a phylogenetic marker associated with CAS and Delhi strains (Baker et al 2005; Lavender et al 2005). The strain typing for the patient samples used in this study was performed in the BMG study conducted by Peters et al (Manuscript in preparation) and the spoligotyping pattern identified samples obtained from patient 59041 as CAS. However, the day 3 sample for patient 59041 did not display the -46A mutation. Our results show that the day 3 sample for patient 59041 has a 2-fold higher MIC for INH and EMB compared to the baseline and day 7 samples (Figures 14 and 15). Additionally, this day 3 sample did not produce DCBT in CF-independent conditions whilst the baseline and day 7 samples did (Figure 29). This indicates that the day 3 sample may be a different strain and the patient may have a mixed strain infection.

The Beijing strain is the most virulent and successful strain which is associated with drug resistance and a high bacterial load (Yimer et al 2015). The spoligotyping data generated in the BMG study showed that the samples from patients 59014, 57134 and 59032 were all Beijing strains (Peters et al Manuscript in preparation) (Appendix B). Our sequencing data for patients 59014, 57134 and 59032 showed no mutational alterations in the respective regions for each of the drugs and as such the resistance observed through the drug susceptibility testing in Section 3.4 was a result of the strain type and not genotypic alterations.

#### **4.5 Successful generation of DCTB state and drug susceptibility testing**

Several studies have shown that when *M. tuberculosis* encounter stressful conditions such as hypoxia, nutrient deprivation, change in pH, acidification, starvation, and exposure to reactive oxygen species and nitrogen intermediates the bacteria are driven into a low metabolic state that is non-replicating and drug-tolerant in order to survive (Zhang et al 2012; Smith et al 2013; Nguyen 2016; Gold and Nathan 2017). The resuscitation of the bacteria from patient sputa in the MPN assays from the BMG study may have resulted in a loss of the DCTB phenotype, thus, the carbon starvation assay was performed in order to drive the resuscitated bacteria back into a reversible non-replicating, drug-tolerant DCTB state (Betts et al 2002; Voskuil et al 2003; Voskuil et al 2004; Rustad et al 2008). The samples of select patients (59041, 59014, 57134, and 59032) were starved for a week with PBS to produce DCTB and to perform broth microdilution assays for RIF, INH and EMB using the starved samples. Our results show that DCTB was successfully produced (resuscitation index greater than 1) for all patient samples except the day 3 sample of patient 59041 in the early non-responders group for the CF-independent condition (Figure 29). This indicates that the day 3 sample required culture filtrate (CF) to assist in the resuscitation process whereas the remaining samples did not need CF. Additionally, the lab strain (H37Rv) was subjected to the starvation process but growth was only observed until the  $10^5$  dilution compared to the starved cultures which had growth until the  $10^7$  dilution. This indicates that the samples had resuscitated bacteria before starvation compared to the lab strain. Furthermore, the MICs obtained for RIF, INH and EMB using the starved cells of the samples of select patients showed that the bacteria adapted during the starvation process to promote their survival and evade elimination by the antimicrobial drugs (Figures 30-32). The average MICs obtained for RIF and INH after starvation were 0.0158 µg/ml and 0.1 µg/ml, respectively. The broth microdilution assay has a pre-determined endpoint thus, the MICs obtained for RIF and INH may have been higher if higher drug concentrations of the respective drugs were tested. The MICs obtained for EMB were

approximately 2-4 fold higher than the MICs obtained for the unstarved counterpart. The starved cultures were more susceptible to EMB compared to RIF and INH suggesting that the metabolic changes adapted by the bacteria during starvation do not compensate enough for the antimicrobial effects of EMB. The increase in drug tolerance of the samples after the starvation process and the lack of genetic mutations present in the resistance conferring regions of RIF, INH and EMB suggest that these DCTB phenotypically adapt to withstand the effect of the drugs.

A study conducted by Gupta et al. (2015) identified 14 differentially expressed proteins during the resuscitation and non-culturable ‘persister’ phases of the wild-type strain, H37Rv. The proteins identified play a role in virulence (Rv2031c, *hspX*), detoxification (Rv317c, 60kDa chaperone (*groEL1*)), adaption pathways (Rv3418c, *groES*), regulatory pathways (Rv1479, *moxR*), information pathways (Rv0685, *tuf*; Rv0009, *ppiA* and Rv3015c), metabolism (Rv2667; *clpC2*) and respiration (Rv1308, *atpA*) (Gupta et al 2015). Rv2744c (35 kDa chaperon) is upregulated at high temperatures, whilst Rv2623, Rv2626c and Rv3786c are induced when the bacteria are exposed to hypoxic and nitrosative stress conditions (Gupta et al 2015). The protein expression levels were high during the non-culturable phase then decreased during the resuscitation phase and were only marginally expressed during the replicating phase. The findings suggest that the difference in protein expression between the non-culturable and resuscitation phases is not fully lost, meaning that when these DCTB are resuscitated, the bacteria can still withstand the antimicrobial effects of the drug albeit at a reduced level (Gupta et al 2015). Our data shows that the DCTB phenotype is reduced but not completely lost in these strains since the MICs obtained for patients 59014, 57134 and 59032 before starvation shows some degree of resistance to one or more of the drugs (Figures 14-16).

## 5. Limitations and future studies

The drug susceptibility of the samples utilized in this study was determined using the *in vitro* broth microdilution assay which is not an accurate representation of *in vivo* drug concentrations found during treatment. Additionally, patients are treated with a combination of drugs when undergoing TB treatment rather than a single drug as tested in the broth microdilution assay. Future studies can include testing multiple drugs to better imitate *in vivo* conditions. Only resistance-conferring regions to RIF, EMB and INH were analyzed, however, mutations can occur in other regions of the genome. Thus, whole genome sequencing should be performed on these select patient samples in the future. In addition, future research could incorporate gene

expression studies such as microarrays, which is a technique used to detect expression levels of multiple genes at a given time, to identify mechanisms behind the observed phenotypic tolerance of the DCTB.

## **6. Conclusion**

This study showed that these DCTB present in patient sputum samples after 14 days of treatment evade the antimicrobial effects of rifampicin, isoniazid and ethambutol by employing phenotypic modifications rather than possessing genotypic alterations. It is still unknown as to the exact mechanisms adopted by these DCTB to promote bacterial heterogeneity and persistence.

## 7. Appendix

### 7.1 Appendix A - List of reagents

**Table A1. Preparation of liquid and solid media.**

| Media | Components for 1 L                                                                                              | Description                                                                                                                      |
|-------|-----------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| 7H9   | 4.7 g Difco™ Middlebrook 7H9 broth (BD), 2 ml glycerol (SIGMA®), 100 ml OADC and 2 ml of 25 % Tween 80 (SIGMA®) | Dissolve 7H9 powder and glycerol in 900 ml of distilled water (dH <sub>2</sub> O). Autoclave, cool and then add OADC and Tween80 |
| 7H11  | 10.5 g Middlebrook 7H10 agar base (SIGMA®), 5 ml glycerol and 100 ml OADC                                       | Dissolve 7H11 powder and glycerol in 900 ml of dH <sub>2</sub> O. Autoclave, cool to 55°C and then add OADC and Tween80          |

**Table A2. Preparation of TAE buffer.**

| Reagent          | Components for 1 L                                         | Description                                                                     |
|------------------|------------------------------------------------------------|---------------------------------------------------------------------------------|
| TAE buffer (50X) | 242 g Tris, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA | Dissolve Tris and acetic acid in EDTA and make up to 1 L with dH <sub>2</sub> O |
| TAE buffer (1X)  | 20 ml 50X TAE                                              | 20 ml of TAE made up to 1 L with dH <sub>2</sub> O. Adjust the pH to 8.0        |

**Table A3. Preparation of PBS.**

| Reagent | Components for 1 L   | Description                                                           |
|---------|----------------------|-----------------------------------------------------------------------|
| PBS     | PBS tablets (SIGMA®) | Dissolve 4 tablets in 1 L of dH <sub>2</sub> O. Adjust the pH to 7.4. |

## 7.2 Appendix B - Sample demographics, HIV status and strain type

**Table A4. Demographics, HIV status and strain type for the patient samples in each response group.**

| <b>Response Group</b> | <b>Patient ID</b> | <b>Gender</b> | <b>Age</b> | <b>HIV status</b> | <b>Strain type</b> |
|-----------------------|-------------------|---------------|------------|-------------------|--------------------|
| Early non-responsive  | 59006             | Male          | 27         | Negative          | Fam36              |
|                       | 59052             | Male          | 25         | Negative          | X3                 |
|                       | 59041             | Male          | 27         | Positive          | CAS                |
|                       | 59028             | Male          | 39         | Positive          | Lam9               |
|                       | 57163             | Male          | 32         | Negative          | S-fam              |
|                       | 57158             | Male          | 31         | Positive          | T1                 |
|                       | 57162             | Male          | 31         | Positive          | T1                 |
| Paradoxical worsening | 59014             | Female        | 27         | Negative          | Beijing            |
|                       | 59035             | Male          | 48         | Negative          | X3                 |
|                       | 59127             | Male          | 52         | Negative          | T1                 |
|                       | 59015             | Female        | 20         | Negative          | T1                 |
|                       | 57134             | Female        | 43         | Positive          | Beijing            |
| Classic bi-phasic     | 59007             | -             | -          | Positive          | Lam3               |
|                       | 59019             | Female        | 22         | Positive          | T4                 |
|                       | 59023             | Male          | 32         | Negative          | X2                 |
|                       | 59032             | Female        | 35         | Positive          | Beijing            |

- Demographic data was not available.

### 7.3 Appendix C - Growth outcomes for each patient in the three response groups.

**Table A5. Growth outcomes for each sample in the early non-responsive group after one cycle of growth in PANTA and two cycles of growth without PANTA.**

| Early non-responsive group |        |     |                |               |                            |
|----------------------------|--------|-----|----------------|---------------|----------------------------|
| Patient ID                 | Strain | Day | Type of sample | Sample number | Outcome                    |
| 59006                      | Fam36  | 0   | Overnight      | 2218          | Growth                     |
|                            |        |     | Spot           | 2575          | No growth                  |
|                            |        |     | Spot           | 2576          | No growth                  |
|                            |        | 3   | Overnight      | 2284          | Growth                     |
|                            |        |     | Spot           | 2290          | Contamination              |
|                            |        | 7   | Overnight      | 2494          | Growth                     |
|                            |        |     | Spot           | 2479          | Growth                     |
|                            |        | 14  | Overnight      | 2940          | Growth                     |
|                            |        |     | Spot           | 2932          | Growth                     |
| 59052                      | X3     | 0   | Overnight      | 4221          | Growth                     |
|                            |        |     | Spot           | *             | *                          |
|                            |        | 3   | Overnight      | 4278          | No growth                  |
|                            |        |     | Spot           | 4272          | Growth                     |
|                            |        | 7   | Overnight      | 4339          | Growth                     |
|                            |        |     | Spot           | 4353          | Growth                     |
|                            |        | 14  | Overnight      | 4450          | No growth                  |
|                            |        |     | Spot           | 4451          | No growth                  |
|                            |        |     | Spot           | *             | *                          |
| 59041                      | CAS    | 0   | Overnight      | 3298          | Growth                     |
|                            |        |     | Spot           | 3719          | Growth                     |
|                            |        | 3   | Overnight      | *             | *                          |
|                            |        |     | Spot           | 3875          | Contamination <sup>x</sup> |
|                            |        | 7   | Overnight      | 3876          | No growth                  |
|                            |        |     | Spot           | *             | *                          |
|                            |        | 14  | Spot           | 4095          | Growth                     |
|                            |        |     | Overnight      | *             | *                          |
|                            |        |     | Spot           | *             | *                          |
| 59028                      | LAM9   | 0   | Overnight      | 2885          | Growth                     |
|                            |        |     | Spot           | 2883          | Growth                     |
|                            |        |     | Spot           | *             | *                          |
|                            |        | 3   | Overnight      | *             | *                          |
|                            |        |     | Spot           | 3277          | Contamination              |
|                            |        | 7   | Spot           | 3278          | Growth                     |
|                            |        |     | Overnight      | *             | *                          |
|                            |        | 14  | Spot           | 3749          | Contamination              |
|                            |        |     | Spot           | 3750          | Growth                     |
|                            |        |     | Overnight      | 3520          | Contamination              |
| 57163                      | S-fam  | 0   | Spot           | 3485          | Growth                     |
|                            |        |     | Overnight      | *             | *                          |
|                            |        | 3   | Spot           | 10571         | Growth                     |
|                            |        |     | Overnight      | 10927         | No growth                  |
|                            |        | 7   | Spot           | 10891         | Growth                     |
|                            |        |     | Overnight      | *             | *                          |
|                            |        | 14  | Spot           | 11087         | No growth                  |
|                            |        |     | Overnight      | 11180         | Growth                     |
|                            |        |     | Spot           | 11178         | Growth                     |
| 57158                      | T1     | 0   | Overnight      | 10531         | Growth                     |

|       |    |    |           |       |               |
|-------|----|----|-----------|-------|---------------|
|       |    |    | Spot      | *     | *             |
|       |    | 3  | Overnight | 10547 | No growth     |
|       |    |    | Spot      | *     | *             |
|       |    | 7  | Overnight | 10917 | Contamination |
|       |    |    | Spot      | 11001 | Growth        |
|       |    | 14 | Overnight | 11146 | Contamination |
|       |    |    | Spot      | 11144 | Growth        |
| 57162 | T1 | 0  | Overnight | 10565 | Growth        |
|       |    |    | Spot      | 10913 | No growth     |
|       |    | 3  | Overnight | 10922 | Growth        |
|       |    |    | Spot      | 11006 | Growth        |
|       |    | 7  | Overnight | *     | Growth        |
|       |    |    | Spot      | *     | Growth        |
|       |    | 14 | Overnight | 11201 | No growth     |
|       |    |    | Spot      | *     | *             |

\* The patient sample was not available for that time point. \* There was contamination in the first decontamination step, however, *M. tuberculosis* colonies were recovered from the solid plates.

**Table A6. Growth outcomes for each sample in the paradoxical worsening group after one cycle of growth in PANTA and two cycles of growth without PANTA.**

| Paradoxical worsening group |         |     |                |               |               |
|-----------------------------|---------|-----|----------------|---------------|---------------|
| Patient ID                  | Strain  | Day | Type of sample | Sample number | Outcome       |
| 59014                       | Beijing | 0   | Overnight      | 2899          | Growth        |
|                             |         |     | Spot           | 2961          | Growth        |
|                             |         | 3   | Overnight      | 2767          | Growth        |
|                             |         |     | Spot           | 2763          | Growth        |
|                             |         | 7   | Overnight      | 2509          | No growth     |
|                             |         |     |                | 2510          | Growth        |
|                             |         |     | Spot           | *             | *             |
|                             |         | 14  | Overnight      | 3225          | Growth        |
|                             |         |     | Spot           | *             | *             |
|                             |         |     |                |               |               |
| 59035                       | X3      | 0   | Overnight      | 3433          | Growth        |
|                             |         |     | Spot           | 3218          | Growth        |
|                             |         | 3   | Overnight      | 3459          | Growth        |
|                             |         |     | Spot           | *             | *             |
|                             |         | 7   | Overnight      | 3604          | Growth        |
|                             |         |     | Spot           | 3699          | Growth        |
|                             |         | 14  | Overnight      | 3852          | Growth        |
|                             |         |     | Spot           | 3841          | Growth        |
|                             |         |     |                |               |               |
|                             |         |     |                |               |               |
| 59127                       | T1      | 0   | Overnight      | 8928          | No growth     |
|                             |         |     | Spot           | 8922          | Growth        |
|                             |         | 3   | Overnight      | 8946          | Growth        |
|                             |         |     | Spot           | 9031          | No growth     |
|                             |         | 7   | Overnight      | 9327          | Growth        |
|                             |         |     | Spot           | 9333          | Contamination |
|                             |         | 14  | Overnight      | 9476          | Growth        |
|                             |         |     | Spot           | 9508          | No growth     |
|                             |         |     |                |               |               |
|                             |         |     |                |               |               |
| 59015                       | T1      | 0   | Overnight      | 3384          | No growth     |
|                             |         |     | Spot           | 2462          | No growth     |
|                             |         | 3   | Overnight      | 2772          | No growth     |
|                             |         |     |                | 2773          | No growth     |
|                             |         | 7   | Spot           | 2758          | No growth     |
|                             |         |     |                | 2759          | No growth     |
|                             |         |     | Overnight      | 2789          | No growth     |
|                             |         |     |                | 2790          | No growth     |
|                             |         |     | Spot           | 2515          | Growth        |
|                             |         |     |                | 2516          | No growth     |
|                             |         | 14  | Overnight      | 3268          | No growth     |
|                             |         |     |                | 3269          | Growth        |
|                             |         |     | Spot           | 3640          | No growth     |
|                             |         |     |                | 3641          | Growth        |
|                             |         | 0   | Overnight      | 9962          | Growth        |
|                             |         |     | Spot           | 9514          | No growth     |
|                             |         | 3   | Overnight      | 9973          | Growth        |
|                             |         |     | Spot           | *             | *             |
|                             |         | 7   | Overnight      | 10104         | Growth        |
|                             |         |     | Spot           | 10110         | Growth        |
|                             |         | 14  | Overnight      | 10296         | Growth        |
|                             |         |     | Spot           | 10291         | No growth     |

\* The patient sample was not available for that time point.

**Table A7. Growth outcomes for each sample in the classic bi-phasic group after one cycle of growth in PANTA and two cycles of growth without PANTA.**

| Classic bi-phasic |         |     |                |               |               |
|-------------------|---------|-----|----------------|---------------|---------------|
| Patient ID        | Strain  | Day | Type of sample | Sample number | Outcome       |
| 59007             | Lam3    | 0   | Overnight      | 2489          | No growth     |
|                   |         |     | Spot           | *             | *             |
|                   |         | 3   | Overnight      | 2358          | No growth     |
|                   |         |     | Spot           | 2346          | No growth     |
|                   |         | 7   | Overnight      | 2806          | Contamination |
|                   |         |     | Spot           | 2504          | Contamination |
|                   |         | 14  | Overnight      | 2818          | No growth     |
|                   |         |     | Spot           | *             | *             |
| 59019             | T4      | 0   | Overnight      | 3183          | Contamination |
|                   |         |     | Spot           | 2783          | No growth     |
|                   |         | 3   | Overnight      | 3172          | No growth     |
|                   |         |     | Spot           | 3200          | No growth     |
|                   |         | 7   | Overnight      | 3256          | No growth     |
|                   |         |     | Spot           | 3262          | No growth     |
|                   |         | 14  | Overnight      | *             | *             |
|                   |         |     | Spot           | 3351          | Contamination |
| 59023             | X2      | 0   | Overnight      | *             | *             |
|                   |         |     | Spot           | 3036          | No growth     |
|                   |         | 3   | Overnight      | *             | *             |
|                   |         |     | Spot           | 2901          | No growth     |
|                   |         | 7   | Overnight      | 3028          | No growth     |
|                   |         |     | Spot           | *             | *             |
|                   |         | 14  | Overnight      | *             | *             |
|                   |         |     | Spot           | 3458          | Growth        |
| 59032             | Beijing | 0   | Overnight      | *             | *             |
|                   |         |     | Spot           | 3397          | Growth        |
|                   |         | 3   | Overnight      | 3403          | Contamination |
|                   |         |     | Spot           | 3648          | Contamination |
|                   |         | 7   | Overnight      | 3614          | Growth        |
|                   |         |     | Spot           | 3491          | Growth        |
|                   |         | 14  | Overnight      | *             | *             |
|                   |         |     | Spot           | 3533          | No growth     |

\* The patient sample was not available for that time point.

## 7.4 Appendix D - MIC outcomes for each patient in the three response groups.

**Table A8. MIC outcome for each sample (baseline and days 3, 7 and 14) for each response group.**

| Response Group        | Patient | RIF  | INH  | EMB  | PZA  |
|-----------------------|---------|------|------|------|------|
| Early non-responders  | 59006   | SSSS | SSSS | SSSS | **** |
|                       | 59052   | SSS- | SSS- | SSS- | SSS- |
|                       | 59041   | RRS- | SRS  | SSS- | RRS  |
|                       | 59028   | SSSS | SSSS | SSSS | --SS |
| Paradoxical worsening | 59014   | RRSR | RRRR | SSSS | SSSS |
|                       | 59035   | SSSS | SSSS | SSSS | SSSS |
|                       | 59127   | *SSS | SSSS | *SSS | SSSR |
|                       | 57134   | SSRR | SSSS | SSSS | SSSS |
| Classic bi-phasic     | 59032   | S-S- | S-S- | S-S- | R-R- |

\*No MIC produced. – The sample was not available to perform the assay. R stands for resistant. S stands for susceptible.

## 7.5 Appendix E - Clearance certificate to further analyze the samples harvested and stored from the BMG study



R14/49 Miss Natasha Singh

### HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

#### CLEARANCE CERTIFICATE NO. M210448

**NAME:** Miss Natasha Singh  
**(Principal Investigator)**  
**DEPARTMENT:** DST/NRF Centre of Excellence for Biomedical Tuberculosis Research  
National Health Laboratory Services

**PROJECT TITLE:** Assessing the drug susceptibility of resuscitated differentially culturable tubercle bacilli (DCTB) after tuberculosis treatment

**DATE CONSIDERED:** 30/04/2021

**DECISION:** Approved unconditionally

**CONDITIONS:** Approval issued for use of data collected under M200105 and M140265

**SUPERVISOR:** Dr B. Gordhan

**APPROVED BY:**   
Dr CB Penny, Chairperson, HREC (Medical)

**DATE OF APPROVAL:** 04/10/2021

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

#### DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

## 7.6 Appendix F - Turnitin Report

Assessing the drug susceptibility of resuscitated differentially culturable bacilli after tuberculosis treatment

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