Characterization of *Mycobacterium bovis* Persisters in South African Wildlife

by

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Pamela Ncube 08/12/2020

Abstract

Mycobacterium tuberculosis infection in humans can either progress to disease, or in many patients, be contained and lead to a latent infection without evidence of active disease. However, it is unknown whether a similar scenario exists in animals infected with *Mycobacterium bovis.* Knowledge gaps in the pathogenesis and progression of disease in the wide spectrum of livestock and wildlife species that can be infected by *M. bovis* also complicate our understanding of this disease. Since management strategies may differ for latently infected animals, it is important to evaluate whether *M. bovis* has the potential to form persisters, which are believed to underlie latent infection.

In this study, we aimed to characterize *M. bovis* persister formation upon *in vitro* acid stress which mimics the macrophage phagolysosome microenvironment. A total of 23 samples from naturally infected *M. bovis* wildlife species were successfully decontaminated, purified, and genotyped to the strain level. Spoligotyping identified a total of 5 different *M. bovis* spoligotypes, namely SB0121 (16 isolates), SB0130 (4 isolates), SB0140 (1 isolate), SB1474 (1 isolate), and SB1275 (1 isolate). In preparation for persister assay experiments, 22 of the 23 isolates were successfully transformed with the Fluorescent Dilution (FD) reporter plasmid pTiGc. Thereafter, growth curves confirmed that there were no growth defects of the transformants due to the carriage of the reporter plasmid.

Five *M. bovis* strains were selected for acid sensitivity and persister assay experiments, using the acid stress *in vitro* model. This model enriches for persisters, as reflected by a sub-population of viable but non- or slowly replicating (VBNR) bacteria. Laboratory strains, SAMMtb::pTiGc and BCG::pTiGc, had the highest average percentage of VBNR cells at 12.2 ± 1.5% (± SD) and 7.2 ± 0.6%, respectively on day 4. In contrast, the clinical strain with the highest VBNR average percentage was PN18067_1::pTiGc at 1.3 ± 0.1%, while PNMP20_1::pTiGc and PN18062_1::pTiGc had a similar average percentage of 0.2 ± 0.0%. These data suggest that upon acid stress: (i) laboratory strains seem to have a higher propensity to form VBNR populations than three clinical isolates examined, (ii) *M. bovis* may demonstrate VBNR populations following acid stress, although these are very small, (iii) VBNR formation may vary depending on strain genotype. However, it is important to acknowledge that the VBNR populations detected under the conditions employed in this study were very small (0.5 ± 0.6 %), and may not be indicative of significant persister formation. We have, therefore,

not definitively demonstrated persister formation, but note that we only investigated a single stressor, a single time point, and only 3 clinical isolates, leaving this an open question to be further investigated in the future.

This is the first study to use clinical strains coupled with fluorescent reporters to investigate the ability of *M. bovis* to persist and provides some insights into a long-standing question on latent *M. bovis* in animals and describes proof of concept data for future investigation. Future work will need to validate these findings using more complex *in vitro* and *in vivo* models and additional clinical isolates.

Opsomming

Mycobacterium tuberculosis infeksies in mense kan lei tot ‘n siekte toestand gepaard met simptome of onderdruk word en lei tot asimptomatiese latente infeksies. Dis onbekend of dieselfde gebeur in diere met *Mycobacterium bovi*s infeksies. Ons siekte verstandhouding word gekompliseer deur die kennisgaping oor patogenesiteit en siekte progressie van *M. bovis* infeksies in verskeie diere. Die bestuur van latent geïnfekteerde diere kan verskil van dié met ‘n aktiewe siekte dus is dit belangrik om te evaulueer of *M. bovis* die potentiaal het om persister selle te vorm, die onderliggend oorsprong van latente infeksie.

Die doel van die studie was om *in vitro M. bovis* persister vormasie te karakteriseer in suur kondisies wat die natuurlike makrofage omgewing naboots. ‘n Somtotaal van 23 *M. bovis* geïnfekteerde monsters van verskeie wildspesies was suksesvol gedekontamineer, gesuiwer en gegenotipeer. Spoligotipering het 5 verskillende *M. bovis* spoligotipes geïdentifiseer genaamd SB0121 (16 isolate), SB0130 (4 isolate), SB0140 (1 isolaat), SB1474 (1 isolaat), en SB1275 (1 isolaat). Ter voorbereiding van die persister vormings asseserings eksperiment is 22 van die 23 isolate suksesvol getransformeer met die fluoresserende pTiGc plasmied en groeikurwes het die gebrek aan groei defekte bevestig.

Vyf verskillende *M. bovis* stamme was gekies om suur sensitiwiteit en persister vormasie te bestudeer deur die gebruik van ‘n suur-stres *in vitro* model. Die model verryk vir persister vormasie, uitgebeeld as ‘n sub-populasie van lewensvatbare maar geen- of stadig repliserende bakterieë. Beide laboratorium stamme, SAMMtb::pTiGc en BCG::pTiGc, het die hoogste gemiddelde persentasie persister selle gehad na 4 dae met 12.2 ± 1.5% (± SD) en 7.2 ± 0.6%, onderskeidelik. Die kliniese isolaat, PN18067_1::pTiGc, het die hoogste persentasie getoon van al die isolate met 1.3 ± 0.1%, van die hele populasie. Verder het PNMP20_1::pTiGc en PN18062_1::pTiGc beide ‘n gemiddeld van 0.2 ± 0.0% gehad. Die data dui daarop dat in suur gestresde omgewings: i) laboratorium stamme meer geneigd is om persister selle te vorm in vergelyking met kliniese isolate, ii) dat *M. bovis* persister selle kan vorm in suur toestande, iii) en dat persister vormasie verskil tussen stam genotipes. Dit is egter belangrik om in ag te neem dat die persentasie persister populasies onder dié spesifieke studie kondisies baie laag was (0.5 ± 0.6 %) en dalk nie ‘n noemenswaardige aanduiding kan wees van persister vormasie nie. Ons kon dus nie defnitiewe persister vormasie demonstreer nie, maar dis belangrik om in ag te neem dat ons net een stres kondisies bestudeer het by ‘n enekel tydspunt en slegs 3 kliniese

isolate gebruik het. Dit los dus ons vraag van persister vormasie onopgelos en moet verder ondersoek word in die toekoms.

Die studie is die eerste in sy soort wat kliniese isolate gekoppel met fluoresserende gene gebruik om die vermoë van *M. bovis* om persister selle te vorm bestudeer. Verder verskaf dit insig oor latente *M. bovis* infeksies in diere en verskaf bewys-van-konsep data vir toekomstige studies. Bevindings moet egter bekragtig word deur meer komplekse *in vitro* en *in vivo* modelle te gebruik in die toekoms asook addisionele kliniese isolate.

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List of Abbreviations

°C	degrees celsius
µg	microgram
µl	microliter
ng	nanogram
%	percentage
BSL3	Biosafety Level 3
BCG	*Bacillus Calmette-Guérin*
BD	Becton-Dickinson
bTB	bovine TB
CAF	Central Analytical Facility
CO_2	carbon dioxide
D	day
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	deoxyribose nucleic acid
DATIN	dormancy-associated translation inhibitor
dNTPs	deoxyribonucleiotide triphosphates
DR	direct repeat
EDTA	ethylene diamine tetra acetic acid
E. coli	*Escherichia coli*
FCS	forward scatter
FACS	fluorescence-activated cell sorting
FD	fluorescence dilution
GFP	green fluorescence protein
HIV	Human Immunodeficiency Virus
IFN	interferon
IFN-γ	interferon-gamma
IL	interleukin
LTB	latent tuberculosis
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
M. smegmatis	*Mycobacterium smegmatis*
MTC	*Mycobacterium tuberculosis* complex

MGIT	Mycobacteria Growth Indicator Tube
$MgCl_2$	magnesium chloride
*p*mol	picomole
PCR	polymerase chain reaction
min	minutes
ml	milliliter
mM	milimolar
n	number
NO	nitric oxide
OADC	oleic acid–albumin–dextrose– catalase
OD_{600nm}	optical density at 600 nanometers
NaOH-NALC	sodium citrate MycoPrep™ solution
NTM	non-tuberculous mycobacteria
PO_4	phosphate
PBS	phosphate-buffered saline
QFN	QuantiFERON®
RD	Region of Difference
rpm	revolutions per minute
RT	room temperature
SANAC	South African National Aids Council
SAMMtb	Severely Attenuated Mutant of *Mycobacterium tuberculosis*
SB	sodium borate
SDS	sodium dodecyl sulfate
SSC	side scatter
SSPE	sodium chloride-sodium phosphate-EDTA
SOPs	Standard Operating Procedures
SU	Stellenbosch University
TNF-α	tumour necrosis factor-alpha
TST	tuberculin skin test
TB	tuberculosis
Th	T-helper
V	volts
VBNR	viable but non- or slowly replicating
VNTR	variable number of tandem repeats

WGS	whole genome sequencing
WHO	World Health Organization
ZN	Ziehl Neelsen

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Chapter 1

General introduction

1.1. Background

1.1.1. Tuberculosis control: an ongoing challenge

Tuberculosis (TB) control is a worldwide challenge both in humans and animals (WHO 2020). TB is still one of the leading infectious diseases worldwide despite global commitments implemented by the World Health Organisation (WHO) to control, manage, and eradicate it particularly in high burden countries such as South Africa. Latency is considered a major risk factor and a threat to TB control (WHO 2020). A fourth of the world's human population is presumed to be latently infected, and a percentage of these could progress to an active disease state, especially those with compromised immune systems (Houben and Dodd 2016). This has led to research regarding latency and recent support for preventative treatment in humans (Suárez et al. 2019). In animals, TB control is hampered by the constant sources of infections such as the existence of wildlife reservoirs (Fitzgerald and Kaneene 2013). Another added challenge with TB is that it is unclear if latent *M. bovis* exist with animals.

Bacterial species that belong to the *Mycobacterium tuberculosis* complex (MTC) are the aetiological agents for TB in various mammals (Brosch et al. 2002; Du et al. 2011; Corner et al. 2012; Clarke et al. 2016). These species are 99, 95% genetically similar and include but are not limited to *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium caprae,* and *Mycobacterium microti* (Clarke et al. 2016; Villarreal-Ramos et al. 2018). Despite this high genetic similarity, *M. tuberculosis* and *M. bovis* appear to have differential host preferences (Thoen et al. 2010; Bapat et al. 2017). *M. tuberculosis* preferentially infects human hosts, while *M. bovis* is primarily found in animal hosts such as wildlife, livestock, as well as sporadically in humans (Michel et al. 2006). The difference in host preference is not fully understood. However, the predilection of *M. bovis* for animal hosts could be due to (i) physiological differences in these bacilli (Behr and Gordon, 2015; Gonzalo-Asensio et al. 2014), (ii) the presence of three mutations that affect the virulence regulation system PhoPR, or (iii) polymorphisms found in 20 regulatory genes of *M. tuberculosis* and *M. bovis* (Bigi et al. 2016).

1.1.2. *Mycobacterium bovis* in animals and human zoonosis

Mycobacterium bovis is a zoonotic pathogen that can be transmitted between animals as well as from animals to humans and vice versa (Pérez-Lago et al. 2014). This phenomenon is mainly observed in cases where humans work in close contact with animals during feeding or herding (Milián-Suazo et al. 2010), as well as through the consumption of unpasteurized dairy products such as milk (Palmer et al. 2014). The under-recognized prevalence of bovine TB (bTB) in humans is a public health concern (Torres-Gonzalez et al. 2013). With the growing appreciation of the interconnectedness of animals, people, and the environment, the zoonotic nature of *M. bovis* must be considered when implementing TB control strategies.

Given the zoonotic potential of *M. bovis* as well as the significant impact that infections with this organism have on domestic and wild animals, it is important to improve understanding of the biology of this organism. Since animals are also affected by TB, there has been an increase in studies that investigate whether latency exists in animals. *Mycobacterium bovis* is a zoonotic pathogen that can be transmitted from animals to humans and vice versa (Pérez-Lago et al. 2014). Since animals are also affected by TB, there has been a rise in studies that investigate whether latency exists in animals.

Bovine TB was first reported in South Africa (SA) in 1880, while the first wildlife case was reported in 1928 in a greater kudu (*Tragelaphus strepsiceros*) (Paine and Martinaglia 1929). *M. bovis* is believed to have been introduced into SA during colonial times when European settlers came along with their cattle (Renwick et al. 2007). Since then, over 21 different strains have been reported across SA (Michel et al. 2006; Michel et al. 2008; Miller 2015) in 24 wildlife species such as African buffalos, cheetahs, lions, warthogs, leopards, African wild dogs, etc. (Bernitz et al. 2021). The largest wildlife reserves in SA that have been declared endemic for *M. bovis* are Hluhluwe iMfolozi Park (HiP) and the Kruger National Park (KNP), followed by other smaller wildlife reserves (Michel et al. 2006; Hlokwe et al. 2016).

Wildlife reserves are usually situated close to livestock grazing communal lands or separated by fences that are frequently damaged by humans or large animals such as elephants, therefore increasing the risk of spillover of *M. bovis* at human-livestock-wildlife interfaces (Michel et al. 2006; Musoke et al. 2015; Sichewo et al. 2020b). Another transmission risk factor is through animals biting or grooming, or carnivores such as wild dogs and lions feeding on infected carcasses (Viljoen et al. 2015; Higgitt et al. 2019). Transmission of *M. bovis* has been reported from African buffaloes to lions (Michel et al.

2009) and from buffaloes to rural cattle (Musoke et al. 2015), highlighting the complexities for bTB control.

Molecular characterization of *M. bovis* has been applied to understand spatial distribution and transmission between species, from various locations such as KNP in Mpumalanga (Michel et al. 2006), HiP in KwaZulu Natal (KZN) (Hlokwe et al. 2011), and other South African provinces such as Limpopo, Northwest, and Free State (Hlokwe et al. 2014). Typing methods, such as Region of Difference analysis (RD) and spoligotyping, have been used to confirm and differentiate various *M. bovis* strains. RD analysis is a multiplex polymerase chain reaction (PCR) technique that uses "present and absent" primer sequences to target the genomic regions of difference 1, 4, 9, and 12 (Warren et al. 2006). Alternatively, spoligotyping is a form of speciation that uses specific spacer sequences in the Direct Repeat (DR) region of the genome (Kamerbeek et al. 1997). Together these techniques have played a key role in the study of various strains of *M. bovis*.

1.1.3. *Mycobacterium bovis* infection outcomes

Tuberculous pathogens cause infections that can lead to either latent or active TB in humans (Glaziou et al. 2015). Human TB infection outcomes are complex and still being elucidated (Drain et al. 2018). This complexity has led to increased speculation and interest in determining whether latent bTB exists in animals (Demissie et al. 2006; Álvarez et al. 2009; Jones et al. 2011; Alvarez et al. 2017; García et al. 2020). Active disease symptoms in infected people include coughing, night sweats, yellowish purulent sputum, and loss of appetite. Symptomatic individuals can transmit infectious bacilli to others through speaking, coughing, or sneezing. In contrast, latent TB is asymptomatic and individuals are not infectious (Glaziou et al. 2015). During latency, it is thought that the host's immune system combats the pathogen and inhibits it from further proliferation and disease progression. It is noteworthy that latency does not describe the metabolic states of bacteria inside the host but rather is a clinical definition of asymptomatic infection (Parrish et al. 1998; Zhang 2004).

Knowledge gaps in the pathogenesis and progression of disease in the wide spectrum of animal species that can be infected by *M. bovis* also complicate our understanding of this disease. Similar to *M. tuberculosis*, lack of knowledge on *M. bovis* persister formation in the context of latency has also been a drawback in the understanding and management of animal TB. Therefore, it is important to conduct studies that bridge that gap. Findings on the persistence of *M. tuberculosis* (Colangeli et al. 2014) could be used to guide investigations on *M. bovis* persister formation in infected animals. It would be crucial to also compare

differences in conditions for persister formation between *M. bovis* and *M. tuberculosis* to understand whether these are associated with specific mycobacterial characteristics.

1.1.4. Mycobacterial persisters

Bacteria can exist in different metabolic and replicative states during latency. Dormant bacteria are non-replicating and typically metabolically inactive (Hice et al. 2018). Persister bacteria are non- or slowly growing, may exhibit a range of metabolic activity, and can survive over long periods under harsh or stressful conditions, such as antibiotic or macrophage exposure (Balaban et al. 2013; Helaine and Kugelberg 2014; Zhang 2014). While there are various definitions of what persisters are, our working definition is that these are viable, and non- or slowly replicating, drug-tolerant, but not genetically resistant, populations (Balaban et al. 2019; Liu et al. 2020). As a survival strategy, persister bacilli may accumulate lipid bodies and utilize them for energy, hence these lipid bodies are a phenotypic signature of persisters (Deb et al. 2009; Kayigire et al. 2015). Importantly, dormant bacteria and persisters can both play a role in the reactivation of infections (Liu et al. 2020).

Understanding the physiology of persister bacteria may inform strategies on TB control programs in animals. Current programs are based on the paradigm that any infected animal presents a risk for disease spread (Gormley and Corner 2018a). The possibility that animals could develop latent infections without being infectious could inform policy and result in better, targeted management and control strategies tailored to identifying and removing the infectious individual animals. Latently infected animals, especially those individuals with high conservation, economic, or genetic value, would not necessarily be a risk for transmission to other animals and humans. Consequently, latently infected animals might not need to be culled but could rather be managed to prevent progression to active disease. Bridging the existing knowledge gaps in animal latent infections is crucial because it provides potential management changes that could prevent infected animals from progressing to active disease and therefore limit spread (Colangeli et al. 2014; Kondratieva et al. 2014; Parrish et al. 1998). As a first step towards understanding this subject, a literature review has been written and is included in this thesis. The review interrogates the evidence and identifies knowledge gaps regarding latent *M. bovis* infections in animals.

In summary, it is important to determine if latent infections occur in animals and to develop tests that can distinguish between latent and active TB to improve control strategies. The knowledge gained in animals can also inform models to study latency in humans. This

research is significant because it can improve our interpretation of diagnostic tests in animals and humans. Lastly, research on latent TB is important for livestock and wildlife (especially endangered species) to minimize the loss of animals due to unnecessary culling.

1.2. Problem statement

Bovine TB is a chronic, slowly progressive disease in animals and current detection methods cannot distinguish between latent infection, subclinical infection, and active disease. Therefore, we do not have a clear picture as to whether “true” latency occurs, or whether it is a subclinical disease state. Additionally, it is also unclear whether latency can explain inconclusive results such as *M. bovis* culture-positive animals that do not have detectable lesions on gross postmortem and histopathological examinations. Hence, understanding and distinguishing different infection outcomes such as latency, using persisters as a marker for latency, is important for bTB control and animal preservation. It would be crucial to also further compare differences in conditions of persister formation between *M. bovis* and *M. tuberculosis* to understand whether there are differences in bacterial characteristics. As a starting point, it is vital to determine whether *M. bovis* from naturally infected animals can form persister populations as has been observed with *M. tuberculosis.*

1.3. Hypothesis

M. bovis can form persisters, with various strains exhibiting differential persister formation upon treatment with acid stress.

1.4. Aims and objectives

Aim

To determine whether different *M. bovis* strains with different genetic backgrounds and from different animal hosts exhibit differential persister formation.

Objective 1

Isolate and purify clones of *M. bovis* clinical isolates, then genotype using the Region of Difference (RD) analysis and spoligotyping methods.

Objective 2

Transform *M. bovis* strains with the Fluorescence Dilution (FD) reporter plasmid and subsequently assess whether there are any growth defects due to the carriage of the reporter plasmid.

Objective 3

Determine whether different *M. bovis* strains exhibit differential persister formation in response to acid stress.

1.5. Thesis Overview

Chapter 1

Title: General Introduction

This chapter outlines the importance of TB control, the existence of *M. bovis* latent infections, and the implications on transmission at human-animal interfaces. It also highlights the need to study persisters at a single cell level and the potential impact on infections in animals.

Contribution: Wrote and edited various drafts with input from supervisors.

Chapter 2

Literature review: “Latent *Mycobacterium bovis* infections - evidence and knowledge gaps”. Manuscript under review at *Microbiology*. This paper evaluates the evidence for *M. bovis* latent infection in animals, highlights important knowledge gaps in this area, and proposes a way forward to investigate this topic. The paper covers the following themes:

- Progress and knowledge gaps regarding *M. bovis* latent infections.
- Development of control and management strategies for potential latently infected animals.
- Perspectives on developing control and management strategies for potential latently infected animals.
- The importance of the DosR regulon, transcriptional sigma factors, and resuscitation promoting factors that may be responsible for animal latent infections, drawing on *in vitro* and *in vivo* models for human LTB.

Contribution: Performed conceptualization, investigation, writing (original draft, review, and editing) drafts with input from supervisors.

Chapter 3

Title: Purification, genotyping, and transformation of *M. bovis* isolates with FD reporter plasmid

This chapter outlines the methodologies and results of objectives 1 and 2 which include the culture conditions applied, decontamination process, purification of isolates, genotyping processes, the transformation of isolates with the fluorescence dilution reporter plasmid, and growth curves of transformed versus wildtype strains.

Contribution:

- Developed an experimental design and performed the sample decontamination, purification, and Ziehl Neelsen (ZN) staining of the 23 isolates
    - Further contributed to the refinements of the existing *M. bovis* ZN staining Standard Operating Procedures (SOPs).
- Developed experimental design and performed troubleshooting and optimizations of the Region of Difference (RD) analysis of all isolates.
- Performed the purification of the pTiGc plasmid from *Escherichia coli*.
- Developed an experimental design, troubleshooting, and optimization of the transformation of 23 isolates with the pTiGc with the reporter plasmid; this was the first trial in our research group to transform *M. bovis* clinical strains with any plasmid.
    - Further contributed to the development of a new *M. bovis* transformation SOP.
- Synthesized, analyzed results and prepared visual representations thereof.
- Interpreted all results, prepared the discussion, and conclusion sub-sections, and edited various drafts with input from supervisors.

Chapter 4

Title: *Mycobacterium bovis* persister assay following *in vitro* acid stress

This chapter presents the methods and results for objective 3. It describes acid sensitivity and persister assay experiments following exposure to *in vitro* acid stress for *M. bovis* strains carrying the pTiGc plasmid, as compared to laboratory strains SAMMtb and *M. bovis* BCG carrying the pTiGc plasmid.

Contribution:

- Performed experimental design, troubleshooting, and optimizations of the *in vitro* acid stress model using 5 representative strains and controls.
- Synthesized, analyzed results and prepared visual representations thereof.
- Interpreted all results, prepared the discussion, and conclusion sub-sections, and edited various drafts with input from supervisors.

Chapter 5

Title: This chapter presents a general discussion and conclusions, limitations of the study, and recommendations for future research.

Contribution: Wrote and edited various drafts with input from supervisors.

Chapter 6

Title: Reference list

Chapter 7

Title: Appendices

- Supplementary data for chapter 3
    - FD reporter plasmid concentrations and absorbance ratios
    - Colonies observed after transformation of BCG and clinical strains
    - Growth curves
- SOP
- Ethics letters

Chapter 2

Literature Review

Latent *Mycobacterium bovis* infections in animals – evidence and knowledge gaps

This chapter represents a soon-to-be-published review which has been submitted to Microbiology journal (reformatted for the thesis) This paper evaluates the evidence for *M. bovis* latent infection in animals, highlights important knowledge gaps in this area, and proposes a way forward to investigate this topic. The paper covers the following themes:

- Progress and knowledge gaps regarding *M. bovis* latent infections.
- Development of control and management strategies for potential latently infected animals.
- Perspectives on developing control and management strategies for potential latently infected animals.
- The importance of the DosR regulon, transcriptional sigma factors, and resuscitation promoting factors that may be responsible for animal latent infections, drawing on *in vitro* and *in vivo* models for human LTB.

Contribution: Performed conceptualization, investigation, writing (original draft, review, and editing) drafts with input from supervisors.

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2.1. Abstract

Latent tuberculosis (LTB) is an asymptomatic state of infection that has received considerable attention in humans. However, it is unclear if this state occurs in animals that are infected with *Mycobacterium bovis* or *Mycobacterium tuberculosis.* It has been shown that subpopulations of heterogeneous mycobacteria develop during different infection stages in response to host defenses, potentially contributing to different clinical outcomes, which can include LTB. Understanding latent infections in the context of "persister" subpopulations of mycobacteria believed to underlie latency is complex. In this review, we outline key terms such as latency, dormancy, and persisters, and highlight the roles of persisters and dormant bacilli in latency. Furthermore, we offer a perspective on how host immune factors such as host cytokines and chemokines can be exploited as candidate biomarkers for discriminating LTB and active tuberculosis (TB) in animals. We also highlight the importance of the DosR regulon, transcriptional sigma factors (*sigB*, *sigF*, and *sigH*), and resuscitation promoting factors (Rpf) that may be responsible for latent animal infections, drawing on *in vitro* and *in vivo* models for human LTB. We further describe progress and knowledge gaps regarding *M. bovis* latent infections. Lastly, we offer a perspective on developing control and management strategies for potential latently infected animals.

2.2. Introduction

Serious emerging infectious diseases such as COVID-19 (de Sadeleer and Godfroid 2020), Ebola (Rohan and McKay 2020), and H1N1 Swine flu (Jilani and Siddiqui 2018) demonstrate that wildlife can be an important source of zoonotic pathogens for human populations as observed with animal tuberculosis (TB) (Phipps et al. 2019). Animal TB is most commonly caused by *Mycobacterium bovis* (Palmer 2013) or *Mycobacterium tuberculosis* (Landolfi et al. 2015a). Animals with active TB disease can be a source of infection to other animals or humans (Gummow 2003; Sichewo et al. 2019b). The potential zoonotic risk of *M. bovis* has led to an increasing appreciation of the interconnectedness of livestock, wildlife, humans, and their natural environment, which needs to be addressed by multiple scientific disciplines using a "One Health" approach (Kelly et al. 2020). Given the zoonotic potential of *M. bovis* and the detrimental effects that infections may have on domestic animals and wildlife populations, it is important to develop and improve our understanding of the biology of this organism within the context of latent infections.

In humans, *M. tuberculosis* infection can result in a spectrum of clinical outcomes ranging from latency to active TB disease (Barry et al. 2009; Esmail et al. 2012). Latent infections can confound diagnosis and are difficult to completely eradicate with existing chemotherapeutic regimens (Suárez et al. 2019). Latent infections are a global burden and are considered to be a major risk factor for active TB disease, particularly in immune-compromised individuals, and hamper the effective control of TB in humans (Houben and Dodd 2016; WHO 2020). Thus, there is a need to better understand latent *M. tuberculosis* infections in humans as well as determine whether *M. bovis* can cause latent infections in animals. Latent *M. bovis* infections in animals could similarly confound diagnostic approaches and have implications for how control of TB in animals is managed.

M. bovis routes of infection, transmission, and disease outcomes in natural hosts need to be reviewed to understand animal TB latency (Phillips et al. 2003). *M. bovis* may persist in contaminated soil (Barbier et al. 2017), feedstuff (Palmer and Whipple 2006), and shared water sources (Sichewo et al. 2020c); these are all potential sources for new infections. Furthermore, *M. bovis* bacilli can be engulfed by, and persist within, environmental amoeba, thereby presenting an additional risk for infection (Mba Medie et al. 2011). Animals are exposed to multiple potential routes of infection such as respiratory droplets in social species, biting, shared contaminated grazing, browsing, shared water sources, and ingestion of infected prey (Michel et al. 2006; Sichewo et al. 2020c). Harsh environmental conditions, such as drought, may increase the infection risk (Michel et al. 2006). Domesticated animals such as cattle and goats are mostly herded together which increases animal to human and animal to animal transmission of TB (Sichewo et al. 2020b). Furthermore, animal crowding, indoor housing, poor ventilation, or shared feeding are transmission risk factors (Torres-Gonzalez et al. 2013). There is a wide range of wildlife hosts, however, little information regarding their susceptibility to *M. bovis* and pathogenesis is available (Miller and Lyashchenko 2015). Typically, some infected animals do not show any clinical signs of bTB for years, and if they do, it is often only after they have developed advanced disease (Miller 2015; Miller and Lyashchenko 2015). Diagnosis of TB may be an incidental finding while conducting postmortem examinations in animals that have died for reasons other than TB disease, such as co-infections (e.g. canine distemper), injuries, or hunting/poaching. This has led to speculation that is it likely that some animals infected with *M. bovis* may develop different disease states and clinical outcomes, including latent infection (Alvarez et al. 2017).

Despite the progress made in recent years, the detection of latent infection in both humans and animals continues to be a challenge due to a lack of sensitive and specific diagnostic

tools (Bezos et al. 2014; Herrera et al. 2020). Active TB in humans can be diagnosed based on clinical symptoms, immunodiagnostics such as QuantiFERON interferon release assay, molecular diagnostics such as Gene Xpert PCR, and the use of imaging tools such as radiography (Furin et al. 2019). However, it is logistically more difficult to identify active TB in animals especially wildlife (Meiring et al. 2018; Bernitz et al. 2019; Miller et al. 2019). Animal diagnostic tools such as the purified protein derivative (PPD) based tests like the tuberculin skin test (TST) and bovigam PPD assays are still used as the reference tests for animals world-wide (Alvarez et al. 2017). However, responses towards PPDs do not differentiate latent from active infection, and cross-reactivity is observed with certain non-tuberculous mycobacteria and other *M. tuberculosis* complex (MTBC) members (Alvarez et al. 2017). Even after *M. bovis* infection is detected in an animal, there are no accurate tools to differentiate between latent infection and active disease or more importantly, the degree of infectiousness, which is crucial for disease control (Meiring et al. 2018).

Control programs for *M. bovis*, and sometimes *M. tuberculosis*, in animals (Bezos et al. 2014a; Miller 2015; Gormley and Corner 2018a) have historically been developed to protect human health and agricultural interests, rather than animal health. Implementation of these control programs typically involves testing and removal (usually through slaughter) of infected animals. However, the actual enforcement of these programs varies widely between countries, impacting the success of TB control (Gormley and Corner 2018a; Meiring et al. 2018). Moreover, the detection of *M. bovis* in a herd or flock usually results in significant disruption to the animal owner's livelihood due to loss of income associated with culling and quarantine of infected premises (Gormley and Corner 2018a). A further complication for TB control is that some species may be dead-end hosts, while other species, such as African buffaloes (*Syncerus caffer*) and European badgers (*Meles meles*) are reservoir hosts that maintain infections within a population and ecosystem (Palmer 2013; Miller 2015). Reservoir hosts, such as badgers, have incited controversy associated with the culling of test-positive animals, which may or may not be scientifically warranted (Gormley and Corner 2018b). Importantly, the culling of test-positive animals (whether an individual or depopulation of a herd) is often performed without accurate knowledge of the state of disease in the animal. Consequently, this could have significant negative implications especially when culling is applied to wildlife (under human management or free-ranging) and involves threatened or endangered species (Ayele et al. 2004; Meiring et al. 2018). These negative consequences may include financial losses from game sales, hunting, ecotourism, potentially decreased genetic diversity of small or fragmented populations and restrictions on translocations as part of conservation programs (Ayele et al. 2004; Miller 2015).

Cognisant of the potentially significant impact of LTB on animal TB control, this review synthesizes current information on our understanding of “latency”, what is known about LTB in humans, the existing evidence for latency in animals, and examines *in vitro* and *in vivo* model systems for studying latency. This review further aims to identify key knowledge gaps and address potential pathways to improve understanding of latent *M. bovis* infections in animals. Understanding latency in livestock and wildlife could aid in improving management and control strategies of TB, especially in endangered wildlife species or animals with high economical value, protecting food security and livelihoods of livestock owners, especially in developing countries, and mitigating any potential zoonotic outbreaks at livestock-wildlife-human interfaces.

2.3. Definitions

Infection with tuberculous pathogens is complex and can lead to a spectrum of outcomes, from clearance to latent infection to active TB disease in humans, which may also occur in animals (Barry et al. 2009; Furin et al. 2019). The terms latency, dormancy, and persisters are used with variable meanings in different studies due to their relatedness and overlapping features. We, therefore, summarize how these terms are used in this review below.

Latent infection: In humans, LTB infection is an asymptomatic clinical state where individuals are not infectious (Glaziou et al. 2015; Furin et al. 2019). The risk of reactivation throughout a person's lifetime is less than 10% but is known to increase with some risk factors, such as Human Immunodeficiency Virus infection and diabetes (WHO 2020). In animals, it is not clear if latent infections occur, since the criteria and tools used to diagnose this state in humans are often unavailable or not translatable to animal hosts. However, *M. bovis* reactivation in elderly people has been previously demonstrated (Palacios et al. 2016), suggesting that *M. bovis* may have the capacity to persist in a latent state in humans. Therefore, confirmatory studies and investigation of whether latency is driven by the host or the organism (or both), are required.

Active disease*:* Symptoms and signs of active TB in humans include coughing, night sweats, yellowish purulent sputum, loss of appetite, and weight loss. Symptomatic individuals can transmit infectious bacilli in aerosols through speaking, coughing, or sneezing (Glaziou et al. 2015; Furin et al. 2019). Infected animals may be able to shed through various secretions including saliva, aerosolized respiratory droplets, urine, feces, and exudate from fistulated lymph nodes; as well as to predators that consume infected

organs (De Vos et al. 2001; Miller 2015; Sichewo et al. 2020c). In animals, clinical signs of active disease may take months to years to appear and these include weight loss, reproductive failure, decreased milk production, swollen lymph nodes, and cough (De Vos et al. 2001; Miller 2015; Sichewo et al. 2020c). In addition, there are species-specific clinical signs that occur, for example, elbow hygromas, lymphadenopathy, and osteomyelitis in lions (Miller et al. 2015).

***Dormant mycobacteria**:* When mycobacteria encounter certain environmental conditions in the host, the bacilli may enter a dormant state. This dormant state includes a viable but non-culturable population (Chao and Rubin 2010) which is metabolically inactive and non-replicating (Hice et al. 2018) and may contribute to LTB. These dormant mycobacteria can resuscitate or regrow under favorable conditions (Zhang 2004) (Figure 2.1).

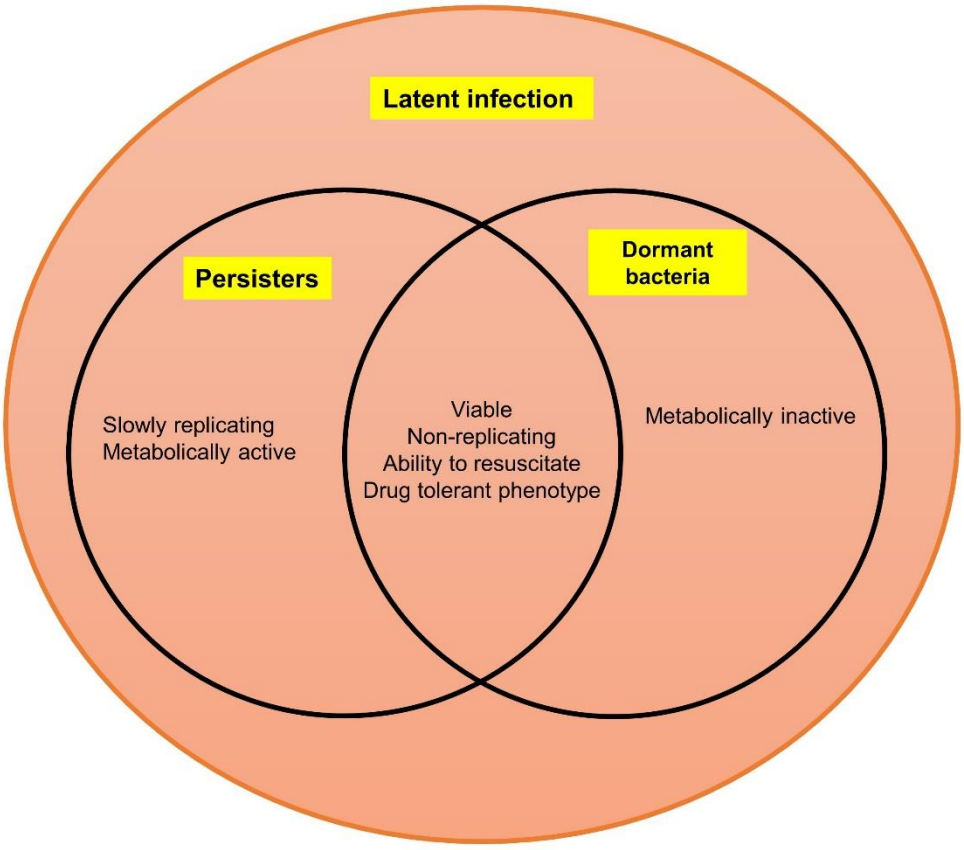


Figure 2.1: An overlap of characteristics of mycobacterial metabolic states involved during latent infection. Persisters and dormant bacteria are believed to underlie the clinical outcome of latent infection. Persisters and dormant bacteria share similar physiological and metabolic characteristics highlighted in the overlapping region. Their slight differences are highlighted in each independent region. (Original image; P. Ncube).

Persisters: LTB may also be driven by persisters (Zhang 2014; Huang et al. 2015). The definition of persisters has led to an ongoing controversy due to the lack of understanding of

their physiology and factors that contribute to their emergence (Chao and Rubin 2010; Balaban et al. 2013; Li et al. 2014), however, there has been progress towards a consensus definition (Balaban et al. 2019). Our working criteria are that persisters are viable, non-, or slowly replicating subpopulations of bacteria that are reversibly drug-tolerant and not genetically drug-resistant (Mouton et al. 2016; Balaban et al. 2019) (Figure 2.1). Persisters have been studied since the 1940s when a study on *Staphylococcus aureus* revealed that a small percentage (1%) of the bacteria was drug-tolerant to penicillin (Bigger 1944). Since then, these viable and non-replicating populations have been investigated and also found in *Escherichia coli* (Spoering et al. 2006) and a wide range of other bacterial species (Balaban et al. 2013) including *M. tuberculosis* (Mouton et al. 2016). However, *M. bovis* persister formation remains understudied.

2.4. Possible *M. bovis* infection outcomes

Latency is one possible outcome of mycobacterial infections (Esmail et al. 2012). Wildlife and livestock are mainly infected with *M. bovis* (Sichewo et al. 2020c) while a few other species such as elephants, rhinoceros, and nonhuman primates can be naturally infected with *M. tuberculosis* (Landolfi et al. 2015a; Miller and Lyashchenko 2015). *M. bovis* and *M. tuberculosis* are 99.95% genetically similar and may have similar patterns of transmission, pathogenesis, and disease progression (Pollock and Neill 2002). However, there are a few genetic and physiological differences between *M. bovis* and *M. tuberculosis* which may play a role in phenotypic differences and subsequent infection outcomes (Bigi et al. 2016; García et al. 2020). Additionally, different animal species may have dissimilar immune responses to *M. tuberculosis* and *M. bovis* (Villarreal-Ramos et al. 2018; Pereira et al. 2020), further complicating our understanding of disease outcomes such as latency. While there is a growing recognition that animals can have a spectrum of infection outcomes (Michel et al. 2017; Gormley and Corner 2018b), there are limited *M. bovis* infection studies that describe “true” latency.

A recent review by Garcia *et al.* (2020) reviewed pathology, protein, and gene expression differences in *M. tuberculosis* and *M. bovis* infections to determine whether latent *M. bovis* occurs in cattle (García et al. 2020). Based on available evidence, they concluded that *M. bovis* infections in cattle were less likely to cause LTB but instead progress more readily to active disease. In this review, we broaden the scope of this question by considering the following: i) *M. bovis* infection outcomes of other animal hosts (besides cattle), such as wildlife, (ii) examining how different *in vitro* and *in vivo* models could be harnessed to

address questions on latent *M. bovis* infection, (iii) highlighting the inter-related states of LTB, dormancy and persister mycobacteria and how these need to be considered in future latent *M. bovis* research, (iv) evaluating how a combination of antemortem and postmortem diagnostic tools could provide insights into LTB in wildlife, (v) proposing host-pathogen biomarkers that may be applied in distinguishing the wide spectrum of different *M. bovis* infection outcomes, and vi) offering a way forward to tackle LTB studies in the future. Filling knowledge gaps on LTB in animals will positively influence management strategies and bTB control.

In recent years, veterinary experience with naturally-infected animals has resulted in speculations that *M. bovis* infected animals may develop latent infections. These speculations have been based on various observations such as positive antigen-specific immunologic test results but no visible gross or histopathologic lesions (Neill et al. 1994; Alvarez et al. 2017). Although cross-reactivity to NTMs may be one explanation for these results (Ramos et al. 2015), improved diagnostic tools that are more specific and sensitive are being developed to discern the various causes, which could include latent infection (Miller et al. 2015; Chileshe et al. 2019; Srinivasan et al. 2019). Therefore, these observations, both in experimentally or naturally infected animals, emphasize the complex dynamics that should be considered when investigating whether “true” latency in animals exists. Although Garcia *et al.* (2020) considered the latency question by using cattle as a model for other animals (García et al. 2020), other factors such as the host-pathogen complexities involved in individual animals and among different species need to be further interrogated (Renwick et al. 2007; Pereira et al. 2020).

Infection outcomes in humans are typically better understood than those in animals. It is more difficult to study natural *M. bovis* infections in animals due to the logistics of handling large or wild animals, lack of clinical signs, the long interval between infection and developing clinical signs, limited ability to identify source and route of transmission as well as a paucity of diagnostic tools (Miller 2015; Miller et al. 2015; Miller and Lyashchenko 2015). In humans, the factors that determine the outcome of infection are still unclear but are likely to involve an interplay between pathogen and host responses (Riska and Carleton 2002; Meier et al. 2018). Both the innate and adaptive immune systems may have variable success in containing the infection leading to a spectrum of TB states including latent infection (Esmail et al. 2012). The pathogenesis and disease outcomes (Table 2.1) observed with *M. bovis* infection are generally similar among taxonomically related host species but can be variable in different groups of animals such as carnivores and herbivores (Barry et al. 2009; Furin et

al. 2019; Pereira et al. 2020). In addition, individual host and pathogen dose-dependent responses result in a wide spectrum of outcomes (Pereira et al. 2020). Although there are speculations that as *M. bovis*

Table 2.1: **Probable stages of *M. bovis* infection, disease progression, and host-pathogen interactions of wildlife and livestock.** The dissemination of these bTB stages may vary for different animal hosts depending on the immune response, *M. bovis* strain that infected the animal, and exposure to risk factors in their natural habitat. Original table; P. Ncube: based on sources found in Pollock and Neill (2002); Riska and Carleton (2002); Álvarez et al. (2009); Drain et al. (2018).

bTB Stage		Host-pathogen interactions
Uninfected		- No immune response, and no viable bacteria
Infected	Cleared infection	- Innate and adaptive immune responses clear the bacteria - The immune memory response may last for a period after the pathogen has been eliminated - No viable bacteria
	Latent infection	- Detectable immune responses present - Viable and slow or non-replicating bacteria present - No clinical signs of infection
	Subclinical infection	- A transition state from latency or initial/early infection to active disease - May have a combination of low or non-replicating and actively replicating bacteria - Microbiological evidence of viable active bacteria - Physical changes start to occur (in humans, signs such as nodules in the lungs are observed, but the no clinical symptoms of the disease)
Diseased	Active disease	- Actively replicating bacteria present - Clinical signs may be present, including coughing, weight loss, swollen lymph nodes, abnormal discharge - The animal may be infectious to others and culturable bacteria may be present in secretions, depending on the location of infection and type of samples used for analysis

strains evolved, they lost their ability to enter a slow or non-replicative state compared to *M. tuberculosis* (Cassidy 2006), further studies are needed to confirm this.

Broad outcomes of infection with *M. bovis* may include: cleared, infected, or diseased (Table 2.1), however, the different stages of infection have been difficult to characterize (Bezos et al. 2014a; Meiring et al. 2018). Drain et al. (2018) postulated four categorical states of human TB which are LTB (person has viable and non- or slowly replicating *M. tuberculosis* bacilli), incipient (*M. tuberculosis* has some metabolic activity to indicate the ongoing or impending progression of the infection), subclinical (radiological abnormalities or microbiological evidence of active, viable *M. tuberculosis*) and active disease (person has symptoms/signs of clinical *M. tuberculosis* disease). On the other hand, stages in infected animals may be categorized as follows: infected (presence of viable bacteria and immune response present, but no clinical signs), and diseased (presence of actively replicating bacteria, immune response, and clinical pathology) (Table 2.1) (Riska and Carleton 2002). However, more research is needed to develop methods that clearly distinguish different infection states.

2.5. Candidate models for *M. bovis* latent infections

There has been considerable progress in understanding the mechanisms leading to latent *M. tuberculosis* infections in humans (Dutta and Karakousis 2014; Kondratieva et al. 2014; Cohen et al. 2019). These studies have exploited *in vitro* and *in vivo* models using *M. tuberculosis* strains (Choreño-Parra et al. 2019), *M. bovis* BCG strains, and (to a lesser extent) *M. bovis* clinical strains to investigate latency, dormancy, and persistence (Boon et al. 2001; Boon and Dick 2002). Commonly used dormancy and persistence models are described in this section.

2.5.1. *In vitro* models

In vitro models are pivotal for simulating the microenvironment that mycobacterial pathogens may encounter in the host, leading to latent infections in human and animal TB (Alnimr 2015; Gibson et al. 2018). These models focus on bacterial growth restriction, metabolic activity shutdown, phenotypic drug tolerance, and upregulation of dormancy genes upon exposure to microenvironmental stresses, including hypoxia (Wayne and Sohaskey 2001), nutrient starvation (Xie et al. 2005), and acid stress (Vandal et al. 2009). These conditions aim to

mimic the microenvironment within the macrophage or granuloma, which are key host defenses.

Hypoxia: The Wayne model for hypoxia is a well-characterized model that mimics the oxygen-restricted microenvironments within the necrotic granuloma environment that arises during the progression of *M. tuberculosis* infections. The Wayne model leads to a metabolic shutdown, growth arrest, the termination of replication and transcription, thickening of the outer cell wall, and drug tolerance of the mycobacteria (Wayne and Hayes 1996). This model has been applied to *M. bovis* BCG and it was found that BCG could survive and adapt to hypoxia through upregulation of DosR (Rv3133c), an essential dormancy response regulator (Boon and Dick 2002). The knowledge gained from the physiologic and metabolic state of *M. tuberculosis* and BCG during hypoxia provides some insights that can be applied to understanding *M. bovis* dormancy in clinical strains (Hutter and Dick 1999; Lim et al. 1999; Boon and Dick 2002). Gideon *et al.* (2010) demonstrated genes from the region of difference (RD) 2 (Rv2659c) and 11 (Rv2658c) were upregulated in *M. tuberculosis* during hypoxia (Garnier et al. 2003). However, since RD11 is absent in all *M. bovis* strains, it would not be expected to be involved in adaptation to hypoxia, whereas RD2 could be involved. RD2 seems to have been lost during the passage of BCG (Garnier et al. 2003), thus suggesting that BCG may use different mechanisms to adapt to hypoxia compared to *M. bovis* and *M. tuberculosis*. Additionally, Rv0188, an enduring hypoxic response antigen was found to be solely associated with low pathologic scores in cattle infected with *M. bovis*, suggesting that it may play a role in latency (Jones et al. 2011), and highlighting it as a potential antigen to explore in future studies.

***Nutrient starvation*:** Nutrient starvation is a model that deprives the pathogen of essential nutrients such as a carbon source (Xie et al. 2005), simulating the microenvironments within the caseous granuloma (Betts et al. 2002). Thus, mycobacteria persisting within a caseous granuloma may have limited carbon sources and amino acids compared to those in the macrophage phagolysosome (which may have access to different nutrients) (Primm et al. 2000). Nutrient starvation has been shown to slow the transcription apparatus, cell division, energy metabolism, and lipid biosynthesis (Betts et al. 2002). Furthermore, nutrient starvation has been demonstrated to induce the stringent response (stress response in reaction to limited essential growth resources) in bacteria (Ramisetty et al. 2016), cause a morphology change to an “L form” (Slavchev et al. 2013), and result in drug-tolerance to isoniazid and para-aminosalicylic acid (Gengenbacher et al. 2010). During *in vitro* nutrient starvation, *M. bovis* BCG can upregulate the Rv3134c/*devR*/*dev*S transcriptional regulator

which is present in *M. tuberculosis* (Rodriguez et al. 2008), suggesting that this model may have the potential for investigating dormancy in *M. bovis* clinical strains.

***Acid stress*:** It is well established that upon infection with pathogens, macrophages acidify their intracellular environment (Vandal et al. 2009). This acid stress model thus aims to mimic the microenvironment encountered by *M. tuberculosis* residing within the macrophage phagolysosome (Vandal et al. 2009). The exposure of *M. tuberculosis* to acid stress leads to growth arrest, dormancy, and phenotypic tolerance to isoniazid (Fisher et al. 2002; Richter and Saviola 2009). *M. bovis* BCG was found to be tolerant to low pH through the adoption of a homeostatic balance between the internal and external pH (Rao et al. 2001). Further, BCG was tolerant to low pH in the presence of glutamates as a carbon source (Gallant et al. 2016), highlighting that in an acidic environment, a subpopulation of *M. bovis* cells that can utilize glutamates as a carbon source may have the ability to persist longer than those that cannot utilize the glutamates. Nevertheless, there are limited studies that have applied this model to *M. bovis*, hence, further investigation is needed.

***Multiple stress models*:** Multiple stress models more closely mimic the complex stresses that the pathogen might encounter in the macrophage or granuloma (Gibson et al. 2018). The widely used multiple stress model for dormancy involves hypoxia (5% O_2 content), high CO_2 (10%), nutrient deprivation (10% Dubos medium), and acidic pH (5.0) to mimic the microenvironment of the macrophage phagolysosome or granuloma (Deb et al. 2009). This model creates various phenotypic changes in *M. tuberculosis* such as growth cessation, lipid body biosynthesis, loss of acid-fast staining, and development of drug tolerance to isoniazid and rifampicin (Rodriguez et al. 2008). Characteristics of mycobacteria in the multi-stress model may be a more physiologically relevant tool for understanding the dormancy/persister response of *M. bovis in vitro.*

***Macrophage models*:** Macrophage models are a form of multi-stress model that recapitulates what happens in the host macrophage phagolysosome (Rao et al. 2001). Macrophages typically applied in TB studies include human monocyte-derived macrophages (Heifets et al. 2000), human alveolar macrophages (Nozaki et al. 1997), murine macrophage cell lines, murine bone marrow-derived macrophages (Gallant et al. 2016; Mouton et al. 2016), human monocyte cell line THP-1 macrophages (Tullius et al. 2003), and bovine alveolar macrophages (Malone et al. 2018). Since *M. tuberculosis* and *M. bovis* strains have previously demonstrated diverse transcriptional responses upon infection of bovine alveolar macrophages or THP-1 macrophages (Li et al. 2017; Malone et al. 2018), it is clear that to

study latent *M. bovis* infection, the selection of a macrophage cell line that will represent animal host responses must be carefully considered.

Single stress *in vitro* models may be applied as proof of concept for novel questions since they resemble some aspect of host response upon infection, but are limited in their ability to reflect the repertoire of host defenses upon infection. On the other hand, multi-stress models may be applied to validate fundamental knowledge gained from single stress models since they better represent the complexity of host responses upon infection. To further validate fundamental information gained using *in vitro* models, *in vivo* models must be employed to gain translational knowledge that may better represent the complexity of host responses, particularly in animals.

2.5.2. *In vivo* models

In vivo models in TB can be exploited to help develop various tools, interventions, improve research on pathogenic mechanisms, determine the bacterial load in various organs, assess pathological changes, and track disease progression (Riska and Carleton 2002; Aichele et al. 2003; Zhan et al. 2017). Furthermore, *in vivo* models more closely emulate the pathogenesis, host-pathogen interactions, and host defense mechanisms in natural infections (Dutta and Karakousis 2014). The species used for *in vivo* models of TB include mice (Kupz et al. 2016), rabbits (Nedeltchev et al. 2009), cattle and goats (Villarreal-Ramos et al. 2018), guinea pigs (Clark et al. 2015), and non-human primates (Lin et al. 2009; Zhang et al. 2018).

Murine models: Murine TB models are widely used due to their feasibility, relatively low cost, and availability of a wide range of immunological reagents (Alnimr 2015). Mice can be used to investigate host immune factors involved in human latent infection, however, they tend to develop high bacillary burdens and pathological changes, leading to early death, which makes them more appropriate for studying active disease (Botha and Ryffel 2002; Zhan et al. 2017). The Cornell mouse model is a “classic” TB latency model, in which infected mice are treated with antibiotics to reduce bacteria to undetectable levels, prior to reactivation (Scanga et al. 1999); this model is technically challenging, and it is not clear how well this reflects LTB in other hosts. Alternative approaches have been developed, in which mice are vaccinated before being infected with *M. tuberculosis* (Lu et al. 2015). These models result in a steady asymptomatic state that resembles human latency and in which bacteria enter a viable and slowly or non-replicating state (Dutta and Karakousis 2014; Zhan et al. 2016). In a recent review, it was suggested that C57BL/6 (Kupz et al. 2016) and

C3HeB/FeJ (Kramnik and Beamer 2016) laboratory mouse strains show promise for investigating LTB after drug and vaccination interventions. These murine strains could potentially be applied to study the mechanisms of latency and relapse in *M. bovis*-infected animals (Shi et al. 2011; Zhan et al. 2017). However, although *M. bovis* mouse models have been used to investigate potential TB vaccines (Logan et al. 2008; Civello et al. 2020), study virulence and immunopathology (León et al. 2009), characterize molecular and cellular features of bTB (Bouté et al. 2017; Cheng et al. 2019), and host-pathogen responses upon infection (Wang et al. 2019), *M. bovis* has not yet been applied in studies of LTB in mice.

Guinea pigs and rabbits: The guinea pig model of TB has been used to study infection and disease progression (Bolin et al. 1997; Clark et al. 2015), *M. bovis* diagnostics (Chambers et al. 2001), vaccine development (Chandran et al. 2019), and as a model of LTB (Kashino et al. 2008). Guinea pigs and rabbits are more susceptible to TB than mice, however, lack of immune reagents limits investigation of the reactivation status of dormant bacteria (Zhan et al. 2017). The guinea pig model has been applied for *M. tuberculosis* persistence (Lenaerts et al. 2007) and latency (Kashino et al. 2008) but is yet to be used for *M. bovis* studies of latency. Nedeltchev *et al.* (2009) showed that rabbits infected with *M. bovis* Ravenel and *M. bovis* AF2122/97 had higher colony-forming units in pulmonary cavity lesions compared to *M. tuberculosis* H37Rv or CDC15151 (Nedeltchev et al. 2009), suggesting that *M. bovis* is likely to progress to more severe stages of acute infection with active mycobacterial replication, while *M. tuberculosis* infections may be arrested at early stages in a slow or non-replicative state (Garcia et al. 2020). However, further validation of this finding is needed, considering the diversity of *M. bovis* clinical strains.

***Non-human primates*:** Common non-human primate TB models use Cynomolgus macaques (*Macaca fasicularis*) and Rhesus macaques (*Macaca mulatta*) (Zhang et al. 2018). These models remain the gold standard for TB translational studies although they are significantly more expensive than other models (Zhang et al. 2018). They are regarded as advanced infection and disease models because they closely recapitulate the clinical and pathological disease spectrum observed in humans (Lin et al. 2009). The Cynomolgus macaque model has been previously employed to study latent *M. tuberculosis* infection and disease states (Lin et al. 2009; Lin et al. 2010). Although the non-human primate model may be the best *in vivo* model to investigate *M. tuberculosis* latency, this model still needs to be used with *M. bovis*.

Cattle and goats: Cattle are the most commonly used species for investigating bTB pathogenesis (Tanner and Michel 1999; Pollock and Neill 2002; Phillips et al. 2003). Cattle and goats experimentally infected with *M. bovis* often develop clinical signs and progression to disease, whereas infections with *M. tuberculosis* H37Rv or other *M. tuberculosis* isolates are reported to be less pathogenic (Bezos et al. 2015; Dong et al. 2017; Villarreal-Ramos et al. 2018; García et al. 2020). Although cattle are a natural host of bTB, they may not reflect infection outcomes in the wide variety of wildlife species infected with *M. bovis* (Fine et al. 2011; Rodríguez-Hernández et al. 2016), hence, further investigation is needed using other recently developed wildlife models such as badgers (Gormley and Corner 2018b).

In vivo models may mimic some features of active or latent human TB, and offer a more diverse host-pathogen repertoire of infection outcomes (Clark et al. 2015; Lu et al. 2015) but variations of the conditions and parameters utilized in different *in vivo* studies do not address all possible disease outcomes (Zhan et al. 2017). Moreover, different *M. bovis* strains may result in different pathological or disease outcomes, hence more validation studies are needed for these models. There may also be host species and bacterial strain differences in *M. bovis* pathogenesis, highlighting the importance of carefully selecting both the animal host and *M. bovis* strains to accurately determine bTB outcomes. There is still uncertainty on whether *M. bovis* is more likely to progress to active disease than *M. tuberculosis* or not, therefore, further investigation is required.

Diagnosis of latency in animals

Accurate diagnostic tests are crucial for controlling the spread of bTB in animals (de la Rua-Domenech et al. 2006; Watrelot-Virieux et al. 2006; Ramos et al. 2015). However, detecting *M. bovis* infection in animals is still a challenge, especially in wildlife. Validated bTB diagnostic tests are limited and often require species-specific approaches (de la Rua-Domenech et al. 2006; Denis et al. 2007; Bezos et al. 2014; Bernitz et al. 2018; Roupie et al. 2018; Bernitz et al. 2019). In addition, current diagnostic tests cannot distinguish between infection, possible latency, and disease (Meiring et al. 2018). Latent infections in humans are diagnosed by lack of clinical symptoms and no visible pathologic changes on thoracic radiologic examination, but positive antigen-specific immunologic responses (Chegou et al. 2009; Drain et al. 2018; Suárez et al. 2019; Furin et al. 2019). However, an *M. bovis*-infected animal may lack clinical signs, but this does not necessarily mean that it has a latent infection (Villarreal-Ramos et al. 2018). Animals with a positive tuberculin skin test or other immunological responses are considered infected but may have a variety of presentations

on postmortem examination, including lack of pathological changes, localized to generalized disease. Hence, a lack of clinical signs in animals cannot be used to diagnose latent infection without other specific criteria to distinguish it from incipient infection, subclinical and active disease (Drain et al. 2018). This is challenging in animals since radiography is often unavailable and it can be difficult to obtain antemortem samples to detect mycobacterial organisms or DNA.

While the *in vitro* and *in vivo* models can be employed to dissect mechanisms involved in *M. bovis* latency at a genomic, transcriptional, proteomic, and phenotypic level, the identification of latent infection in an individual animal will likely require a combination of antemortem and postmortem diagnostic tests. Currently available antemortem tests include the (i) direct detection of viable bacilli by culture of respiratory samples such as bronchoalveolar lavage fluid (BALF), swabs, trunk washes (Miller et al. 2015), and other secretions (urine, milk, feces) (Sweeney et al. 2007; Ereqat et al. 2013; Pereira et al. 2020); (ii) direct detection of mycobacterial DNA from respiratory samples (Miller et al. 2015); (iii) indirect detection of infection using cell-mediated immunological tests including *in vivo* tuberculin skin test TST (Table 2.1) (Parsons et al. 2011; Pai et al. 2014; Alvarez et al. 2017), *in vitro* antigen-specific interferon-γ release assays (IGRAs) (Denis et al. 2007; Alvarez et al. 2017; Clegg et al. 2017; Bernitz et al. 2018); (iv) indirect detection by antigen-specific humoral responses; and (v) detection of circulating host and pathogen markers in whole blood (Du et al. 2011). On postmortem examination, the presence of *M. bovis* in lymph nodes, lungs, and any other organ without evidence of disease would support latency (de la Rua-Domenech et al. 2006; Álvarez et al. 2009; Alvarez et al. 2017; Villarreal-Ramos et al. 2018). Specifically, postmortem tests should include (i) mycobacterial culture of tissue specimens (Katale et al. 2017); (ii) microscopic examination of tissues using conventional histopathology and immunohistology (Nedeltchev et al. 2009; Roupie et al. 2018), and (iii) mycobacterial DNA detection in tissue samples (Lim et al. 2012; Nuñez-Garcia et al. 2018).

A combination of diagnostic criteria has been used in humans to determine latent infection, although in animals limited diagnostic tests and inconclusive results can confound interpretation. In some cases, immunoassays are positive in the absence of evidence of disease; instead of regarding these results as false positives (Hope et al. 2005; Bezos et al. 2014b), there have been speculation that latency may explain (i) animals that had no visible tuberculous lesions but were culture and IGRA positive (Neill et al. 1994); (ii) initially negative TST but positive IGRA results in animals that later became positive on both tests; and (iii) animals that had no signs of disease on histopathological examinations, were culture

negative but TST and IGRA positive (Alvarez et al. 2017) (Table 2.2). Hence, the development of sensitive and specific tests is required to demonstrate the existence of latent infections. In a recent human TB study, *in vitro* antigen-specific stimulation of cytokine IFN-γ, IL-2, IL-5, IL-10, IL-1RA, and monocyte chemoattractant protein-1 (MCP-1/CCL2) production was proposed to be useful for distinguishing active TB from LTB (Suzukawa et al. 2016). Therefore, these cytokines should be investigated in *M. bovis* infected animals.

Table 2.2: **Summary of possible infection stages and associated antemortem and postmortem tests for wildlife and livestock.** (Original table; P. Ncube)

bTB/ TB Stage		Antemortem tests		Post mortem tests	
		TST	IGRA	Tissue Culture	Tissue histopathology consistent with bTB (Yes/No)
Uninfected		Negative	Negative	Negative	No
Infected	Cleared infection – no clinical signs	Negative (innate immune system clearance) Positive (adaptive system immune clearance)	Positive for a short period before waning (adaptive system immune clearance)	Negative	No
	Latent infection – no clinical signs	Positive	Positive	Negative	No
		Negative	Negative	Positive	No
	Subclinical infection – no clinical signs	Positive	Positive	Positive	Yes
Diseased	Active disease – clinical signs present	Positive	Positive	Positive	Yes
References	(Pollock and Neill 2002)	(Parsons et al. 2011; Pai et al. 2014; Alvarez et al. 2017).	(Denis et al. 2007; Alvarez et al. 2017; Clegg et al. 2017).	(Pollock and Neill 2002; Riska and Carleton 2002; Álvarez et al. 2009)	

TST=tuberculin skin test, **IGRA**=interferon-γ release assay

With growing knowledge on the heterogeneity of *M. tuberculosis* physiological states during different infection stages, these data suggest the possibility that *M. bovis* may share the ability to exist as viable but slowly or non-replicating bacilli, which would be necessary to create a latent infection. Therefore, both host and pathogen characteristics need to be explored to determine if latent infection occurs in animals and then identify antemortem and postmortem tests that can distinguish different states of infection and disease.

2.6. Candidate host biomarkers of latency

Currently, there are no specific immune factors solely associated with latency in humans and animals because the majority of host factors in latency overlap with those for active disease (Bapat et al. 2015; Herrera et al. 2020). Some studies have made progress towards identifying host factors indicative of latent infection but identifying host biomarkers that solely distinguish latency and active disease in humans is still a challenge (Bapat et al. 2015; Herrera et al. 2020).

The use of both *in vitro* and *in vivo* models in human TB studies has shed light on possible host factors resulting in latency. Biomarkers that are useful in identifying latent infections in humans are tumor necrosis factor (TNF) α, IFN-γ, and IL-12, based on a quantitative comparison of cytokine levels in the blood of latently infected vs actively diseased patients (Dutta and Karakousis 2014; Alvarez et al. 2017; Herrera et al. 2020). Transcriptomic profiling of immune responses in a Gambian study found that an antiapoptotic gene *BCL2* is a marker that indicates the onset of active disease very early after infection (Sutherland et al. 2011). It was found that B-cell lymphoma 2 (BCL2) was significantly lower in individuals with active disease versus latently infected individuals (Sutherland et al. 2011), making it a potential host candidate biomarker to differentiate between latent infections and active disease. Another study done in The Netherlands found that host factors IL-13 and autoimmune regulator (AIRE) were markers correlating to disease progression (Sloot et al. 2015).

In some human studies, multiple cytokine assays that differentiate active disease and LTB have been applied to diagnose a latent state (Chegou et al. 2009; Kim et al. 2012). Suzukawa *et al.* (2016) found that combined analysis of IFN-γ, IL-2, IL-5, IL-10, IL-1RA, and MCP-1/CCL2 cytokines found in QuantiFERON supernatant, may be useful for distinguishing active TB from LTB in humans (Suzukawa et al. 2016). Furthermore, a recent systematic review, that compared host factors that may be involved in LTB, highlighted that IL-1, IL-6, IL-9, IL-17, and IL-IRA may be promising candidate host factors for identifying latency (Herrera et al. 2020).

Future studies would need to investigate a latent infection cytokine panel that is reliable and reproducible for use in animals. The development of such a panel would need to consider the complexities of TB biology, infection outcomes, and bacterial heterogeneity in an individual animal and across species. Further studies are required to identify host biomarkers to distinguish different disease states in animals.

2.7. Candidate pathogen biomarkers for latency

There are various microbial factors believed to be upregulated during latent *M. tuberculosis* infection. These include factors that mediate energy metabolism, lipid production, stringent response, growth restriction, hypoxia response, and toxin-antitoxin modules (Dutta and Karakousis 2014). Therefore, these should be considered as potential candidate biomarkers for latent infections with *M. bovis*.

DosR regulon: The dormancy survival regulator (DosR) regulon is one of the well-studied major systems that regulate mycobacterial response to anaerobic conditions (Boon et al. 2001; Boon and Dick 2002). The DosR regulon is responsible for the maintenance and upkeep of mycobacterial persister populations believed to underlie latency and is conserved in *M. tuberculosis* (Leistikow et al. 2010; Chao and Rubin 2010; Arroyo et al. 2018). This regulon is a 48 component system regulated by the DosR (Voskuil et al. 2003). DosR genes include *nrdZ*, *narX*, *narK2*, *ctpF*, *otsB1, fdxA*, *pdxA*, *pfkB*, *acr*, *acg*, and *dosR,* which respond to environmental stress conditions leading to mycobacterial dormancy and persistence (O' Toole et al. 2003; Voskuil et al. 2003; Keren et al. 2011). DosR antigen Rv2031c (*acr* or *hsp*X) has previously been found to be involved in latency (Gideon et al. 2010). The DosR antigen Rv2031c findings were supported by studies performed in cattle infected with *M. tuberculosis* H37Rv (Demissie et al. 2006) and *M. bovis* AF 2122/97, demonstrating that this antigen may be a potential candidate to differentiate between active disease and LTB in animals. In another study, it was suggested that the reason *M. bovis* may lack the ability to create a latent infection, compared to *M. tuberculosis,* was the lack of detectable DosR/S/T regulon antigens (Rv2627c, Rv2628, Rv2029c, and Rv1733c) in cattle naturally infected with *M. bovis* (Jones et al. 2011), however, this may need further investigation. A recent study has recognized the potential of DosR and Rpf antigens (Rv2029c (PfkB), Rv2389c (RpfA), Rv0867c (RpfA) and E6-C1) from *M. tuberculosis* to discriminate between active TB and LTB in a TB endemic population in Medellin,

Colombia (Arroyo et al. 2018). This may indicate that these could serve as candidates to discriminate between active infection and LTB in animals. Similar responses were observed in other studies conducted in African, Asian, European, Indian, and Brazilian populations, suggesting immune responses independent of the environmental background, human genetics, and possibly of the circulating *M. tuberculosis* strains, and thus the likelihood of universal immune response to DosR antigens in latent infections.

Transcriptional factors: The main host environmental driver of latency in *M. tuberculosis* infection is believed to be hypoxia, but more recent studies have discovered additional factors that could play a role. Transcription of mRNA sigma factor genes is implicated in various stress responses and may play a role in the adaptation of the tubercle bacilli to stressful host environments (Demaio et al. 1996). Amongst these are the sigma factor F gene (*sigF*) (responsible for the slow growth of bacteria) (Michele and Ko 2010), and *HspX/ Acr*/ Rv2031c genes that encode for the α-crystallin homolog small heat shock protein (sHSP16.3) (Sherman et al. 2001; Jee et al. 2018). *SigF* is upregulated in *M. tuberculosis* persisters, suggesting that this factor could play a crucial role in latency (Keren et al. 2011). Furthermore, the *sigF* gene is responsible for regulating oxidative stress, antibiotic stress, cold shock, and nutrient depletion (Demaio et al. 1996; Keren et al. 2011). Sigma factor B (*sigB*) and Sig H are additional factors induced by heat and oxidative stress (Rodrigue et al. 2006; Sachdeva et al. 2010). Although *sigF* has been demonstrated to be induced in hypoxic conditions and the stationary phase of growth in *M. bovis* BCG (Demaio et al. 1996; Manganelli et al. 1999), there is a need to further validate this finding and investigate which transcriptional factors are specifically involved in *M. bovis* latency in different clinical strains.

The tools and methods to study *M. tuberculosis* persisters have improved in recent years. Mycobacterial candidate genes for latency may be similar in *M. tuberculosis* and *M. bovis* clinical strains due to their genetic similarities, which would make it easier to reproduce models using *M. tuberculosis,* thus accelerating latency research in animals. However, some of the physiological differences between these strains may reveal different candidate genes for latency upon infection and this must be taken into consideration during experimental design.

2.8. Knowledge gaps

Since there is inconclusive evidence on whether “true” latency exists in animals infected with *M. bovis* or *M. tuberculosis,* there are knowledge gaps that include:

- The lack of sensitive and specific diagnostic tools for detection of *M. bovis* infection in livestock and wildlife and lack of tests that can differentiate between latent infection, subclinical, and active TB disease in animals.
- A paucity of studies describing the pathogenesis of *M. bovis* infection in different animal species and a lack of clarity on the stages of infection involved in *M. bovis* or *M. tuberculosis* infection in animals.
- Lack of conclusive evidence to support the existence of LTB in animals and limited research on latent infections in animals.
- Poor understanding of the physiological states of *M. bovis* that could promote persister formation and LTB.

2.9. Future research

There are strong fundamental data from *in vitro* and *in vivo* studies that can be used to improve our understanding of *M. bovis* latent infection in animals, however, future research should focus on:

- Identification of blood-based host and pathogen biomarkers that can differentiate between active TB and various stages of *M. bovis* infection in different animal species.
- Utilizing available tools to study *M. bovis* persisters at a single-cell level to understand their physiological, phenotypic, and molecular features and their involvement in LTB.
- Development and evaluation of appropriate infection and disease detection methods for understanding the pathogenesis and epidemiology of *M. bovis* in different animal species.
- Comparing the pathogenesis of *M. bovis* in various animal species to understand what underlies the chronic asymptomatic state in infected hosts.
- Cross-species studies (*M. bovis* vs *M. tuberculosis*) could highlight similarities and differences in persister formation/latent infections that could help to advance understanding of LTB in humans as well.

2.10. Conclusion

There are still large knowledge gaps regarding whether *M. bovis* latent infections occur in animals. Multifactorial triggers of latency must be considered when examining the host and pathogen mechanisms involved, and especially when extrapolating information from humans to the wide variety of species that can be infected with *M. bovis*. Latent infections in wildlife and livestock may occur and be under-recognized due to the lack of defined criteria and diagnostic tools. Results obtained from antemortem and postmortem examinations have supported the possibility of latency. Although there are promising methods to study human latency, they have yet to be comprehensively applied to *M. bovis* infections in animals.

It is crucial to determine if latent *M. bovis* infection occurs since it will have an impact on management and control strategies. Secondly, *in vitro* and *in vivo* models can be applied to broaden the fundamental and translational research on latent infections in animals. When designing research strategies, it is important to be aware of the complex risk factors that animals encounter in the natural environment to closely mimic these conditions. Thirdly, further investigation of *M. bovis* bacteria under various stress conditions is required to document the physiological changes that occur in persister or dormant bacteria and to understand how these might contribute to LTB.

Future research can also focus on methods to further identify persisters, not only in culture but also directly in animal samples to characterize their phenotypes. Studying *M. bovis* latent infection in animals is important because it helps differentiate individuals that may be infected but not infectious from those with a greater risk of spreading the disease. The capability of identifying different states of infection in animals can inform policy and improve management strategies based on associated risks. The knowledge gained from animal research can also inform models to study latency in humans. Bridging the existing knowledge gaps may also provide potential management changes that could prevent infected animals from progressing to active disease (and therefore spread). Lastly, research on latency is important for livestock as well as wildlife (especially endangered species) to minimize the loss of animals due to unnecessary culling.

Chapter 3

Purification, genotyping, and transformation of the clinical *M. bovis* isolates with Fluorescence Dilution reporter plasmid

3.1. Introduction

Bovine tuberculosis (bTB) remains a global challenge to manage and control in animals (Meiring et al. 2018). Research has been conducted on *M. bovis* pathogenesis (Phillips et al. 2003), infection dynamics (presence and prevalence) within animal populations (Sichewo et al. 2019a; Sichewo et al. 2020a; Sichewo et al. 2020b), and diagnostic tools for different animal species (Landolfi et al. 2015a; Srinivasan et al. 2019; Chen et al. 2020; Palmer et al. 2020). However, there are still fundamental knowledge gaps in whether latent *Mycobacterium bovis* infections exist.

Understanding *M. bovis* latent infections can positively contribute to the management and control of infected animals, thereby potentially saving endangered species and other valuable individuals from being culled if they are not infectious. The current legislation in many countries worldwide requires that infected animals be culled, without knowledge of the infection stage of the animal (Gormley and Corner 2018a). This has socioeconomic consequences. Animals that have TB can be a source of infection for other animals and people. However, animals with latent infections, may not necessarily present a risk for transmission to other animals and people, which may allow them to be managed without culling.

Currently available diagnostic tests are not able to make a clear distinction between different clinical TB states (Lahuerta-marin et al. 2018), making it difficult to study latency. Existing diagnostic tests for TB in animals are typically host-based and are currently unable to distinguish between latent and active infection. Therefore, understanding latency in animals is crucial to protect wildlife, conserve biodiversity, and protect food security and livelihoods, especially in developing countries. A small population of persistent bacteria is believed to underlie the latent state, however, *M. bovis* persisters are understudied. This study attempts to address this gap by application of a novel dual fluorescence replication reporter system (Mouton et al. 2016) to *M. bovis.*

M. bovis infects a wide host range of wildlife, some of which can be reservoir hosts, which leads to challenges in bTB eradication (Michel et al. 2006; Miller and Lyashchenko 2015). In South Africa, although testing of commercial livestock still occurs, very limited testing of communal herds and wildlife is done due to lack of resources; therefore, infected animals may only be detected when they die or are culled for other reasons (Miller 2015). Postmortem examinations permit evaluation and sample collection from lymph nodes and other organs which can be used to confirm *M. bovis* infection using mycobacterial culture or other techniques (Gormley et al. 2014).

Culture-dependent methods, using solid or liquid media, are considered as the gold standard for identifying mycobacterial infection (Kaneene et al. 2010; Gormley et al. 2014). Rigorous decontamination (Vartoukian et al. 2010) and purification techniques are performed since samples containing mycobacteria can be contaminated with other microorganisms and the mycobacteria grow slowly (Pfyffer and Palicova 2015). Due to these factors, mycobacterial culture may be insensitive and misclassify truly infected animals as *M. bovis* negative. Therefore, although culturing from tissue samples is usually the most desirable method for achieving definitive results, it has limitations. Tissue samples are best for isolating *M. bovis*, however, these are usually only available postmortem. Alternatively, antemortem samples can be used for culture, and are collected as oropharyngeal swabs, bronchoalveolar lavage fluid, or other secretions, but these have been shown to have lower sensitivity for detection of *M. bovis* by culture due to sporadic shedding. However, the advantage of these sample types is that they can be easily collected (de la Rua-Domenech et al. 2006).

Ziehl Neelsen (ZN) analysis is also used in conjunction with mycobacterial culture to identify the presence of mycobacteria. ZN analysis involves staining that allows visualization of the cell wall characteristics of mycobacteria by microscopy (Cook 1997). This method is cheap, provides rapid presumptive diagnosis, however, its sensitivity depends on the site of infection and severity of the disease (Nema 2012). Another limitation is that it cannot distinguish between mycobacterial species. Samples can include respiratory secretions; tissues or bacteria present in the culture media. The presence of acid-fast bacteria indicates that mycobacteria may be present in the sample, as demonstrated in this study.

To speciate and further characterize *M. bovis* isolates, molecular typing techniques are applied to isolates, for example, using Region of Difference (RD) analysis (Warren et al. 2006), which

can distinguish *M. bovis* from *M. tuberculosis*. This technique can be performed directly on DNA extracted from a collected sample or after mycobacterial culture. It uses a polymerase chain reaction (PCR) to amplify genetic regions specific to the pathogen of interest (Warren et al. 2006). This method has been widely used in the diagnosis of bTB in wildlife and can differentiate between non-tuberculous mycobacteria (NTMs) and MTC organisms and between *M. bovis* and other MTC organisms (Michel et al. 2006). Spoligotyping is another PCR-based speciation method that is used to genotype isolates to strain level for further characterization (Kamerbeek et al. 1997). Both RD and spoligotyping methods were applied in this study.

3.2. Methods and Materials

3.2.1. Ethics approval

M. bovis and *M. tuberculosis* clinical isolates, tissues, and laboratory strains that were used in this study have existing ethical approvals and these are as follows:

- Animal Tissue Use Approval Research Ethics Committee: Animal Care and Use (REC: ACU) Reference #: ACU-2019-9086 to expire on 11-04-2024.
- Lion tissue use: (REC: ACU) Protocol number 1489 to expire on 19-10-2022.
- Utilization of a severely attenuated, double auxotrophic of Mycobacterium tuberculosis to (a) rapidly screen anti-mycobacterial compounds and (b) to advance understanding of mycobacterial physiology. Research Ethics Committee: Biosafety and Environmental Ethics (REC: BEE). BEE-2020-18621 to expire on 27-09-21.
- Evolution, biology, and host-pathogen interactions of mycobacteria: Research Ethics Committee: Biosafety and Environmental Ethics (REC: BEE) reference number: BEE-2020-13049 to expire on 11-03-2021.

3.2.2. Source of *M. bovis* infected tissue samples

The samples used in this study were obtained from ongoing studies by the Animal TB research group (Roos et al. 2016; Olivier et al. 2017; Bernitz et al. 2018; Higgitt et al. 2019). The tissue samples were obtained from 6 different locations from public and private game reserves (Figure 3.1;Table 3.1 Table 3.1); Location A (LA; n=5), Location B (LB; n=2), Location C (LC; n= 1), Location D (LD; n=5), Location E (LE; n=1), Location F (LF; n= 9).

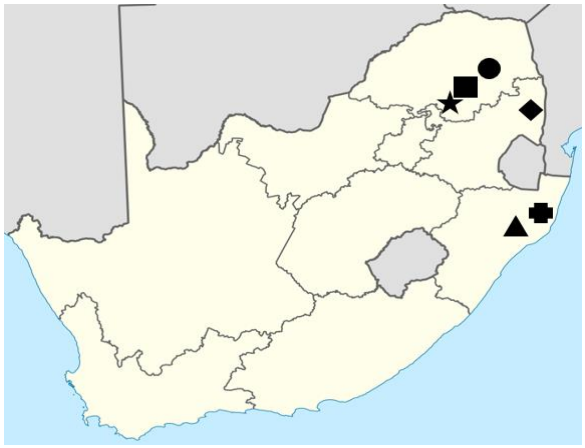


Figure 3.1. Geographical locations in South Africa where the *M. bovis* infected animal samples were obtained. ● Location A (LA), ■ Location B (LB), ★ Location C (LC), ◆ Location D (LD), ✚ Location E (LE), ▲ Location F (LF).

3.2.3. Bacterial strains and plasmids

Experiments with a severely attenuated mutant of *Mycobacterium tuberculosis* H37Rv *ΔleuD ΔpanCD,* (also referred to as SAMMtb) (Sampson et al. 2004; Mouton et al. 2019) and *M. bovis* BCG Pasteur strains were carried out in Biosafety Level 2 laboratory (BSL2), while work with live clinical *M. bovis* was carried out in Biosafety Level 3 facilities (BSL3), within the Division of Molecular Biology and Human Genetics, following established SOPs. Experiments were conducted with three technical and three biological replicates. Twenty-one cryopreserved *M. bovis* culture samples (stocks prepared on beads and glycerol) and four *M. bovis*-infected tissue samples were cultured according to the established SOPs for mycobacterial culture (Figure 3.1). These samples were from different geographical areas which included public and private game reserves (Figure 3.1). The lab strains *M. bovis* Bacillus Calmette-Guérin (BCG) Pasteur and SAMMtb (Sampson et al. 2004) were used as comparator strains. SAMMtb was previously transformed with the replication reporter plasmid (Figure 3.2) and appropriate control plasmids (Mouton et al. 2016). Control plasmids included no reporter, TurboFP635 (red fluorescent protein) only, and GFP (green fluorescent protein) only plasmids (Figure 3.2). *Escherichia coli* pTiGc cryopreserved stock was used for pTiGc plasmid propagation.

Table 3.1: Samples used to investigate *M. bovis* persisters. WTh-warthog, L-lion, B-buffalo, WD-wild dog.

Animal	Culture ID	Type of sample	Geographical Location
WTh	PNMP1A_1	Beads	LD
WTh	PNMP3C_2	Beads	LD
WTh	PNMP7_1	Beads	LD
WTh	PNMP8_1	Beads	LD
WTh	PNMP9_1	Beads	LD
WTh	PNMP20_1	Beads	LD
L	PN18064_3	Tissue (Peripheral lymph node)	LA
L	PN18065_2	Tissue (Abdominal lymph node)	LA
L	PN18066_3	Tissue (Lung)	LF
L	PN18067_1	Tissue (Prescapular lymph node)	LE
B	PN18068_1	Beads	LF
B	PN18069_3	Beads	LF
B	PN18070_3	Beads	LF
B	PN18071_1	Beads	LF
B	PN18072_3	Beads	LF
B	PN18073_2	Beads	LF
B	PN18073_3	Beads	LF
B	PN18062_1	Beads	LC
WD	PN18074	Beads	LA
WD	PN18075	Beads	LA
WD	PN18076	Beads	LA
WD	PN18077	Beads	LB
WD	PN18078	Beads	LB

Location A (LA), Location B (LB), Location C (LC), Location D (LD), Location E (LE), Location F (LF).

Table 3.2: Plasmids and strains used in the current study.

Strain	Description	Source/ Reference
E.coli DH10B carrying pTiGc	*hsp60(ribo)-turboFP635 hsp60-gfp*, Kan^R, episomal	(Mouton et al. 2016)
SAMMtb wild type	Double leucine and pantothenate auxotroph of *M. tuberculosis, ΔleuD, ΔpanCD,* Hyg^R	(Sampson et al. 2004)
SAMMtb::pSTCHARGE3	Double leucine and pantothenate auxotroph of *M. tuberculosis* carrying pSTCHARGE3 plasmid *hsp60(ribo)-turboFP635* (inducible TurboFP635 under control of theophylline-inducible riboswitch), KanR, episomal	(Mouton et al. 2016)
SAMMtb::pST5552	Double leucine and pantothenate auxotroph of *M. tuberculosis* carrying plasmid pST5552 plasmid *hsp60(ribo)-egfp* (inducible EGFP under the control of theophylline-inducible riboswitch), Kan^R, episomal	(pST5552) Addgene plasmid #36255
SAMMtb::pTiGc	Double leucine and pantothenate auxotroph carrying pTiGc plasmid *hsp60(ribo)-turboFP635 hsp60-gfp*, Kan^R, episomal	(pTiGc) (Mouton et al. 2016) Addgene, plasmid #78314
M. bovis BCG	BCG Pasteur	Gift from Veterinary Laboratories Agency Weybridge, UK

Hyg^R, hygromycin-resistant; Kan^R, kanamycin-resistant

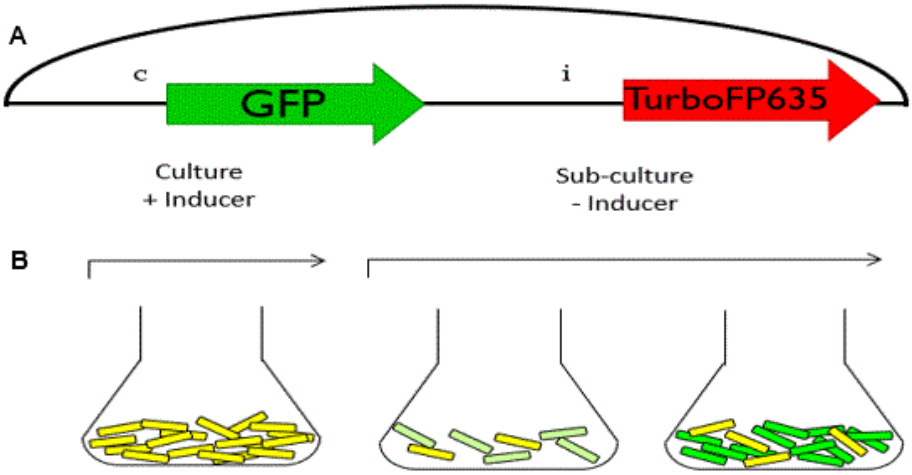


Figure 3.2: Fluorescent Dilution reporter plasmid. A) The pTiGc plasmid carries a constitutive (c) green fluorescent protein (GFP) and an inducible (i) red fluorescent protein (TurboFP635). **B)** The red fluorescent protein is under the control of a theophylline inducible riboswitch-based promoter, which allows for tight regulation and strong induction of the red fluorescent protein. When induced with theophylline, bacteria carrying the pTiGc plasmid will exhibit both red and green fluorescence (culture + inducer). If theophylline is subsequently withdrawn, and bacteria are actively replicating, the level of red fluorescence will be halved with each successive cell division (subculture + inducer), and this can be monitored with flow cytometry or microscopy. Slowly or non-dividing bacteria will retain high levels of red fluorescence (Image modified and adapted from Mouton et al. 2016).

3.2.4. Mycobacterial culture and purification

All experiments were conducted following the Standard Operating Procedures (SOPs) for the Host-Pathogen Mycobactomics and Animal TB research groups. Twenty-three *M. bovis* isolates from naturally infected wildlife [8 African buffaloes (*Syncerus caffer*), 6 warthogs (*Phacochoerus africanus*), 5 wild dogs (*Lycaon pictus*), and 4 lions (*Panthera leo*)] were chosen to reflect diverse hosts and genotypes for subsequent assessment of persister formation (Table 3.1). All the required tissue and culture samples were available as part of ongoing studies by the Animal TB research group. Experiments with samples containing live pathogenic mycobacteria were conducted in the biosafety level 3 (BSL3) laboratory.

3.2.4.1. Tissue homogenization

Approximately 1 cm^3 sections of tissues were cut and added to 2 ml 1x MycoPrep™ phosphate buffer (Becton Dickinson, Franklin Lakes, New Jersey, USA) in a skirted 50 ml tube. The tissues were homogenized for 15 min, speed 12 using sterilized 4 mm steel beads

in a bullet blender (Next Advance, New York, New York, USA). Thereafter the samples were decontaminated.

3.2.4.2. Culturing from bead stocks

The stocks from beads were cultured by adding one bead to the MGIT tube. The MGIT tubes were incubated at 37ºC in the BACTEC™ automated mycobacterial detection MGIT 960™ TB (Becton Dickinson) and monitored weekly for growth up to 56 days before being discarded in the event of no bacterial growth. The MGIT machine uses BD BBL™ MGIT media and patented sensors, which efficiently leverage advanced fluorometric technology to facilitate highly accurate detection of oxygen consumption (Bae et al. 2008; Rodrigues et al. 2009). The system continuously performs automated quality control to ensure precise and reliable operation and provides results as positive/negative and numerical growth units. A positive detection means there is bacterial growth whereas a negative detection means there is no bacterial growth.

3.2.4.3. Decontamination process

The homogenized tissue samples and cultures from bead stocks were added to the phosphate buffer. The mixtures were added to 2 ml of sodium citrate MycoPrep™ solution (NaOH-NALC) (Becton Dickinson) at a ratio of 1:1 (v/v), incubated for 15 min, and vortexed briefly. Samples were vortexed for 15-30 seconds every 5 min during the incubation period, so the contents of the whole tube were exposed to the MycoPrep™. After incubation, 15 ml of 1x MycoPrep™ phosphate buffer was added to neutralize samples, vortexed, and centrifuged at 4 000 rpm for 20 min. The supernatant was discarded, and the pellet was added to a MGIT tube supplemented with 800 µl BBL MGIT PANTA® media containing antibiotic mixture (Becton Dickinson).

3.2.4.4. Purification process

After flagging positive on the MGIT machine, samples were checked for purity by culturing on blood agar media (Biomerieux, Midrand, SA) to detect fastidious bacteria. Each plate was divided into 4 quadrants, 200 µl of each sample dropped into each quadrant, left to briefly dry, and incubated at 37ºC for up to 48 hours. Furthermore, the samples were spread plated on LB agar (Becton, Dickinson) to check for non-fastidious contaminants. Samples found to be

contaminated were subjected to another cycle of decontamination as described above until pure cultures were obtained.

Aliquots from MGITs for both tissue samples and bead stocks were streaked onto 7H11 agar supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC) (Becton Dickinson), 0.1% (v/v) glycerol with x2 Selectatab tablets added (a multi-component freeze-dried tablet containing 26% ticarcillin, 5% polymyxin B, <5% amphotericin B and <5% trimethoprim in an inert carrier) (Davies Diagnostics, Gauteng, South Africa) and incubated at 37ºC until colonies were observed on plates. Growth of cultures was checked weekly, and thereafter single colonies were picked and transferred to 7H9 media supplemented with 10% oleic acid–albumin–dextrose–catalase (OADC) (Becton Dickinson), 0.1% (v/v) glycerol plus, 0.05% (w/v) Tween-80 (7H9-OGT) and incubated at 37ºC for 2 weeks non-shaking. Isolates were sub-cultured into a 50 ml volume, until OD_{600nm} = 1 for 7 days at 37ºC non-shaking. Thereafter, stocks were prepared and cryopreserved at -80ºC.

3.2.5. Ziehl Neelsen (ZN) staining

Ziehl Neelsen (ZN) staining is a mycobacterial staining method used to check for acid-fast bacilli (AFB) and purity of a sample (Farnia et al. 2002). ZN staining was applied to detect and confirm acid-fast bacilli in cultured samples. Aliquots of samples that flagged positively on the MGIT machine were fixed on microscope slides (Lasec, Western Cape, SA) using serum albumin (National Health Laboratory Service, Western Cape, SA) and heat-fixed at 85ºC for 2 hours. To perform ZN staining, microscope slides were placed on a rack and flooded with carbol fuchsin, heated until steaming point with a flame, and then allowed to stand for 5 min ensuring that each slide had enough carbol fuchsin. The slides were rinsed with water, drained, then flooded with 0.5% acid alcohol, and left to stand for 2 min, cleaned with water, and drained. The slides were then flooded with methylene blue and left to stand for 2 min, rinsed, and left to air dry, then examined using a light microscope at 100x using oil immersion. BCG was used as a positive control for AFB staining.

3.2.6. Region of Difference (RD) Analysis

Region of Difference (RD) analysis is a multiplex polymerase chain reaction (PCR) that uses primer sequences to target the genomic regions of difference 1, 4, 9, and 12. The

amplification of these regions in the bacterial genome is used to genotype and confirm species in the *Mycobacterium tuberculosis* complex (MTC) (Warren et al. 2006). Culture sample aliquots of 200 µl were boiled at 99ºC for 30 min before being removed from the BSL3 laboratory. Each PCR reaction contained 2 µl boiled culture (containing DNA template), 5 µl 5X Q-buffer, 2.5 µl 10X buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTPs, 0.5 µl of each primer (50 pmol/µl), 0.125 µl 250 units HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) and was made up to 25 µl with nuclease-free water.

The PCR thermocycling was performed using a T1000™ ThermoCycler (Bio-Rad Laboratories, Portland, Oregon, USA) and the conditions for RD analysis were as follows: the initial step was performed at 95°C for 15 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, elongation at 72°C for 1 min. After the last cycle, the samples were incubated at 72°C for 10 min. Gel electrophoresis was performed using a 1.5% agarose gel (Lonza, Western Cape, SA) in sodium tetraborate (SB) buffer (Sigma Aldrich, St. Louis, Missouri, USA) to identify amplification products. For the preparation of the gel, 5 µl (0.5 µg/ml) of ethidium bromide was added to the agarose and poured into a gel cast with a comb, and allowed to set. The gel was transferred into the gel reservoir and covered with SB buffer. PCR product (5 µl) was mixed with 2 µl of loading buffer and added to their respective wells. The Gene Ruler 100bp Plus DNA ladder (Thermo-Scientific, Waltham, Massachusetts, USA) was loaded in the first well. *M. bovis* and *M. tuberculosis* H37Rv served as positive controls and a no DNA template PCR control plus loading buffer served as a negative control. The gel was run at 120V for 2 hours. Band sizes were visualized using gel documentation system G: BOX EF (SynGene, Cambridge, UK) to confirm that amplicons of the expected size were obtained.

3.2.7. Spoligotyping

Spoligotyping (spacer oligonucleotide typing) is a form of speciation that identifies multiple unique spacer sequences to form distinct patterns for each strain by characterizing the direct repeat (DR) locus of *M. tuberculosis*-complex (Figure 3.3) (Kamerbeek et al. 1997). The method is based on polymorphism of the chromosomal DR locus, which contains a variable number of short direct repeats interspersed with non-repetitive spacers. Primers DRa and DRb, allow amplification of the whole DR region, including the interspersed spacers (Figure

3.3). Because DRa is labeled with biotin, the amplified DNA can be used directly for hybridization to the 43 spacer oligonucleotides, which are covalently bound to a membrane.

M. bovis isolates were spoligotyped for strain differentiation as previously described (Kamerbeek et al. 1997). Each PCR reaction contained 2 µl of DNA from boiled cultures, 5 pmol/µl DRa and DRb primers, and 12.5 µl Kapa Biosystems ready-mix buffer (Sigma Aldrich) and 6.5 µl of water to a total volume of 25 µl. The PCR thermocycling conditions were as follows: the initial step was performed at 95°C for 3 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 30 seconds. After the last cycle, the samples were incubated at 72°C for 10 min. For hybridization, 20 µl of the PCR products were added to 150 µl 2x sodium chloride-sodium phosphate-EDTA (SSPE) (Thermo Fisher Scientific, Merelbeke, Belgium) plus 0.1% sodium dodecyl sulfate (SDS), heat-denatured for 10 min at 99°C and cooled on ice immediately.

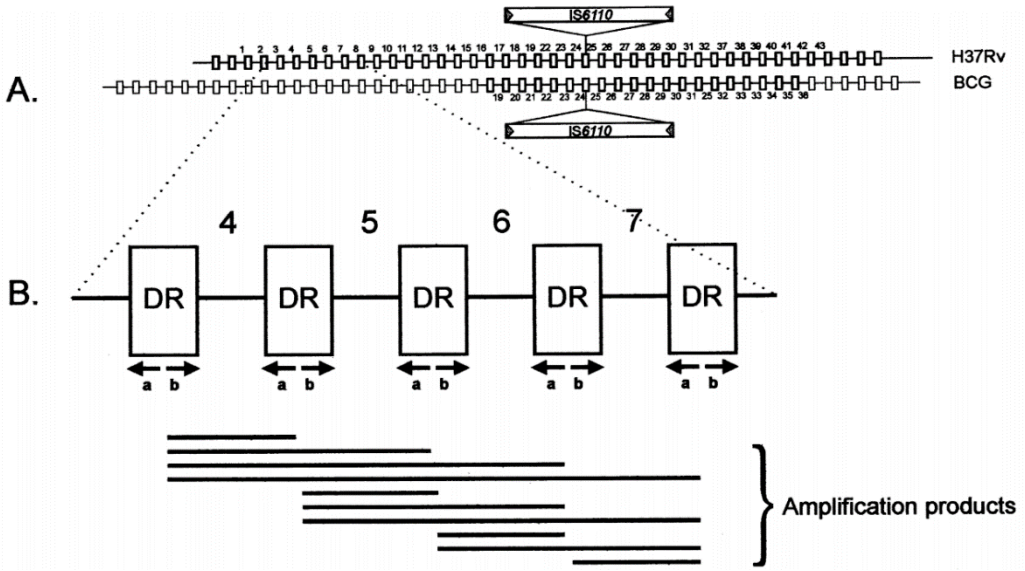


Figure 3.3. Diagram of the principle of spoligotyping by PCR amplification of the DR region. A) The 43 DRs of H37Rv and BCG are usually used as controls in spoligotyping. **B)** Combination amplicons of five possible contiguous DR regions produced using the DRa and DRb primers. (Figure adapted from Kamerbeek et al. 1997).

The membrane was washed at 60°C in 2x SSPE plus 0.1% SDS for 5 min. The membrane was placed on a support cushion in a mini-blotter (slots perpendicular to the line pattern). The residual fluid was removed from the slots by aspiration and the slots were filled with diluted denatured PCR product and hybridized for 60 min at 60°C on a horizontal surface. The

samples were removed from the mini-blotter by aspiration and the membrane was washed twice in 2x SSPE plus 0.5% SDS for 5-10 min at 60°C. Thereafter, 40 ml of 2x SSPE plus 0.5% SDS (preheated to 42°C) was added with 10 µl streptavidin-peroxidase conjugate (500U/ml) (Thermo Fisher Scientific) and incubated at 42°C for 45-60 min. The membrane was washed twice with 2x SSPE plus 0.5% SDS at 42°C for 5-10 min and rinsed with 2x SSPE for 5 min at room temperature. Thereafter, the membrane was incubated in 20 ml (10 ml solution 1 + 10 ml solution 2) enhanced chemiluminescence solution (Thermo Fisher Scientific) mix for 1 minute 30 seconds. The Chemi-Doc Imaging System (Bio-Rad) was used for imaging the band patterns. Thereafter, the spoligopatterns were compared to the *Mycobacterium bovis* spoligotype international database http://www.mbovis.org where the SB number was obtained from matched strains.

3.2.8. Transformation

3.2.8.1. Fluorescence Dilution (FD) reporter plasmid propagation and purification

The FD reporter plasmid was propagated using *E. coli*::pTiGc strain (Table 3.1). The *E. coli* strain carrying the pTiGc plasmid was grown overnight in Luria-Bertani (LB) broth, with 25 µg/ml kanamycin. The plasmid was purified according to the manufacturer's instructions (Promega, Madison, Wisconsin, USA) using the Wizard Plus SV Miniprep DNA Purification System. Thereafter, the pTiGc DNA purity and concentrations were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) (Table S7.1). The DNA was cryopreserved at -80ºC until used.

3.2.8.2. Competent cell preparation and electroporation

Competent cell preparation was performed as previously described with minor adjustments (Parish and David 2015). Ten milliliters of *M. bovis* cultures were inoculated into 7H9-OGT media and incubated without shaking at 37ºC until reaching an OD_{600nm} = 1. To prepare competent cells, cultures were centrifuged at 4000 rpm at 20ºC for 10 min, and the supernatant was discarded. The pellet was resuspended and washed with the original volume (v/v) of 10% pre-warmed glycerol and centrifuged as previously mentioned. For the second wash step, the pellet was resuspended in half the original volume with 10% glycerol.

For the third wash step, the pellet was resuspended in half the previous volume with 10% glycerol. The supernatants were discarded with each round of the wash step.

For transformation, competent cells (400 µl) were transferred into 2 ml tubes. The pTiGc DNA (1 µg) was added to the competent cells. Competent cells (without DNA added) were included as a control for each isolate to rule out the occurrence of any spontaneous resistance-conferring mutations that could give rise to colonies that do not carry the plasmid of interest.

Table 3.3: Time constants of BCG and 23 clinical isolates recorded during electroporation.

Strain	Time constants (ms)	
	No DNA	DNA-cells
BCG	22.8	17.1
PNMP1A_1	22.0	17.2
PNMP3C_2	22.4	17.1
PNMP7_1	22.2	17.1
PNMP8_1	22.1	17.3
PNMP9_1	25.6	17.1
PNMP20_1	16.1	15.0
PN18062_1	22.5	17.8
PN18064_3	19.6	16.0
PN18065_2	21.8	17.1
PN18066_3	21.9	17.1
PN18067_1	22.1	17.3
PN18068_1	23.2	18.0
PN18069_3	22.4	17.3
PN18070_3	22.1	17.3
PN18071_1	21.2	16.9
PN18072_3	22.9	17.7
PN18073_2	23.0	17.9
PN18073_3	23.0	16.0
PN18074	20.2	15.8
PN18075	21.9	16.3
PN18076	22.3	17.3
PN18077	22.5	17.6
PN18078	20.4	15.8

The cells were mixed then transferred to GenePulser 2 mm gap electroporation cuvettes (Lasec), ensuring that there were no air bubbles. Electroporation settings were set at R10, 2.5 kV (Bio-Rad). The cuvettes were secured in the chamber, pulsed, electroporated, and time constants were recorded (Table 3.3).

The transformed cells were recovered by adding 1 ml 7H9-OGT, then incubated non-shaking at 37ºC overnight. The following day, 10^{-1} and 10^{-2} dilutions of cells were performed. The undiluted, 10^{-1}, and 10^{-2} dilutions (200 µl) were plated onto 7H11 plates supplemented with 10% OADC (Becton Dickinson), 0.1% (v/v) glycerol plus 25 µg/ml kanamycin and incubated non-shaking at 37ºC until colonies were observed on plates (Table S7.2). BCG was used as a positive control to check for successful transformation with each round of transformation experiments. If no colonies were observed on the plates after 35 days, plates were discarded.

3.2.8.3. Screening of putative transformants

For each strain, three well-isolated colonies were picked with a sterile tip and grown in 200 µl 7H9-OGT media plus 25 µg/ml kanamycin in Costar 96 well black, opaque clear bottom microplates (Corning, New York, USA), sealed with sterile breathable sealing film (Corning) and incubated non-shaking for 7 days at 37ºC. Fifty (50) µl of cells were sub-cultured into plates containing 150 µl 7H9-OGT plus 25 µg/ml kanamycin with and without 2 mM theophylline to induce the TurboFP635 expression (Mouton et al. 2016). Plates were sealed with sterile breathable sealing film and incubated for 5 days non-shaking at 37ºC. SAMMtb wild type (WT) served to monitor background fluorescence, and SAMMtb::pTiGc, SAMMtb::pTSCHARGE 3, and SAMMtb::pST5552 served as positive controls (Table 3.1).

Five millilitre cultures for controls (SAMMtb::pTiGc, SAMMtb::pTSCHARGE3, and SAMMtb::pST5552) were prepared in 7H9-OGT supplemented with 25 µg/ml kanamycin, 50 µg/ml hygromycin, 24 µg/ml pantothenate, and 50 µg/ml leucine at 37°C, shaking at 180 rpm for 7 days. SAMMtb (WT) was prepared in 7H9-OGT supplemented with 50 µg/ml hygromycin, 24 µg/ml pantothenate, and 50 µg/ml leucine at 37°C, shaking at 180 rpm for 7 days. BCG::pTiGc and *M. bovis::*pTiGc colonies were prepared in 7H9-OGT supplemented with 25 µg/ml kanamycin, at 37°C, shaking at 180 rpm for 7 days. Thereafter, the control cultures were added to the Costar 96 well opaque microplates (Corning) and incubated at 37ºC for 7 days, non-shaking, on the same plates as those with colonies.

After 7 days, the breathable sealing film (Corning) was removed, and the plates were sealed with optically clear film (Corning) and fluorescence readings obtained using FLUOstar Omega 96-well microplate reader (BMG Labtech, Ortenberg, Germany). Optical settings were adjusted using a 485/520 nm and 584/640 nm filter according to GFP and TurboFP635 excitation and emission spectra. The auto-gain adjustment setting was applied to ensure that samples were read across the full dynamic range of a signal by controlling the voltage going to the detector. Therefore, if the signal intensity was low, then the instrument would adjust the voltage to allow detection.

A fold-change was calculated as the fluorescence of the induced cells divided by that of the non-induced cells to confirm successful transformations (data not shown). The fold-change calculation steps were as follows:

Calculation of GFP fluorescence results

- Determine background-adjusted fluorescence reading for GFP by subtracting background green fluorescence reading (Wildtype value) from raw fluorescence data values for each sample.
- Determine fold induction by dividing background-adjusted induced value for each strain by the background-adjusted non-induced value for each strain. The fold-induction for GFP would be expected to be ~1.0, since GFP should be constitutively expressed, at similar levels for all strains, indicating that can accurately track live mycobacteria.

Calculation of TurboFP635 fluorescence results

- Determine background-adjusted fluorescence reading for TurboFP635 by subtracting background red fluorescence reading (Wildtype value) from raw fluorescence data values for each sample.
- Determine fold induction by dividing background-adjusted induced value for each strain by the background-adjusted non-induced value for each strain. The fold-induction for TurboFP635 would be expected to be ≥ 5 which means that the TurboFP635 protein is induced by theophylline, and that it can reliably track live mycobacteria.

Table 3.4: *M. bovis* isolates fold-change results

Culture ID	Fold-change values	
	GFP	**TurboFP635**
SAMMtb ::pTigc	1.00	5.11
SAMMtb ::pSTCHARGE3	N/A	200.21
SAMMtb ::pST5552	1.02	N/A
BCG::pTiGc	0.65	12.53
PNMP1A_1	1.04	519.75
PNMP3C_2	0.96	73.13
PNMP7_1	0.93	101.85
PNMP8_1	1.03	92.85
PNMP9_1	0.88	46.40
PNMP20_1	**1.06**	**23.77**
PN18062_1	**0.96**	**43.12**
PN18064_3	1.30	1253.11
PN18065_2	0.99	64.35
PN18066_3	0.90	130.74
PN18067_1	**0.87**	**67.10**
PN18068_1	0.99	85.55
PN18069_3	1.18	223.15
PN18070_3	0.91	36.31
PN18071_1	0.96	55.58
PN18072_3	0.95	341.50
PN18073_2	1.04	88.79
PN18073_3	**0.94**	**283.37**
PN18074	1.00	54.41
PN18075	1.12	386.54
PN18076	1.00	71.10
PN18077	1.04	594.38
PN18078	**1.00**	**57.77**

Five *M. bovis* isolates selected to characterize persisters (highlighted in red). N/A = non applicable

A high fold-change indicates a high-level induction of the TurboFP635 reporter. This is important to allow for the tracking of several generations upon removal of theophylline. Cells from colonies that had the highest fold-change (≥ 5) (Table 3.4) were transferred into 5 ml 7H9-OGT supplemented with (25 µg/ml) kanamycin and incubated non-shaking at 37ºC. Cultures were sub-cultured into a 50 ml volume and 1 ml (500 µl culture plus 500 µl of 50% glycerol) freezer stocks prepared on beads and cryopreserved at -80°C.

3.2.9. Growth curves

Growth curves were performed to assess whether there were any growth defects due to the carriage of the reporter plasmid. Growth curves were performed in the BSL3 laboratory using established SOPs. Five-milliliter cultures were inoculated into 7H9-OGT supplemented with 25 µg/ml kanamycin and incubated non-shaking at 37ºC for 7 days until they reached an OD_{600nm} = 1. The cultures were briefly sonicated at 37 kHz for 10 min at room temperature in an ultrasonic bath (UC-1D Zeus Automation, South Africa) and filtered through 40 µm cell strainers (Sigma-Aldrich) to minimize clumping. After filtration, OD_{600nm} measurements were performed, and the cultures were adjusted to OD_{600nm} = 0.1 then 200 µl was transferred to Costar 96 well opaque microplates with clear-bottom (Corning) in triplicates, sealed with sterile breathable sealing film, and incubated non-shaking at 37ºC. Only the inner wells of the plate were used for the growth curves to prevent evaporation; the outer ring of wells was filled with water. The sealed plates were transferred to a sealed container before incubation. OD_{600nm} measurements were performed on day 0 and every 2 days thereafter until day 16. Growth curve data analysis was performed on the GraphPad Prism 8.4.0 software (GraphPad Software, Inc., USA). Growth curve data are expressed as the mean ± standard deviation (SD).

3.3. Results

3.3.1. Sample purification, ZN staining, and RD analysis

The first objective was to culture, purify and genotype *M. bovis* isolates in preparation for experiments described in chapter 4. Twenty-five samples from buffaloes, wild dogs, warthogs, and lions were initially selected from 6 locations (referred to as Location A to F) which included both public and private game reserves. However, two of these (buffalo samples from location C) failed to grow and were not further pursued (data not shown). Twenty-three *M. bovis* isolates were MGIT culture-positive. ZN staining successfully confirmed the presence of acid-fast bacilli (AFB) and the absence of non-AFB.

RD analysis was used to further confirm the presence of *M. bovis* and to rule out non-tuberculous mycobacteria (NTMs). RD analysis was successfully used to genotype the 23 isolates and determine if (i) the isolates were part of the MTC complex based on the presence of RD1 with a DNA fragment of 146bp, and (ii) the isolates were *M. bovis,* based on the

absence of RD 4, 9,12 with DNA fragments of 268bp, 108bp, and 306bp, respectively. Initial attempts at RD analysis were hampered by inconsistent PCR results (data not shown). This inconsistency could have been due to the use of crude boiled DNA samples, which meant that some samples may have had a higher concentration that others. Following testing and optimization of PCR (i.e., increasing input DNA volume to 2 µl to accommodate for samples with lower concentrations), multiplex RD PCR was performed on all 23 samples. From this, 10/23 samples yielded all 4 expected bands upon multiplex PCR (representative examples shown in Figure 3.4A). However, 13/23 isolates failed to yield multiple bands when performing multiplex PCR (representative examples are shown in Figure 3.4B), despite repeated attempts and correct amplification of control samples. As an alternative approach, singleplex PCR reactions were performed to identify the RD regions of the remaining samples (representative examples shown in Figure 3.4C). Together, these approaches confirmed that all 23 selected isolates were *M. bovis*.

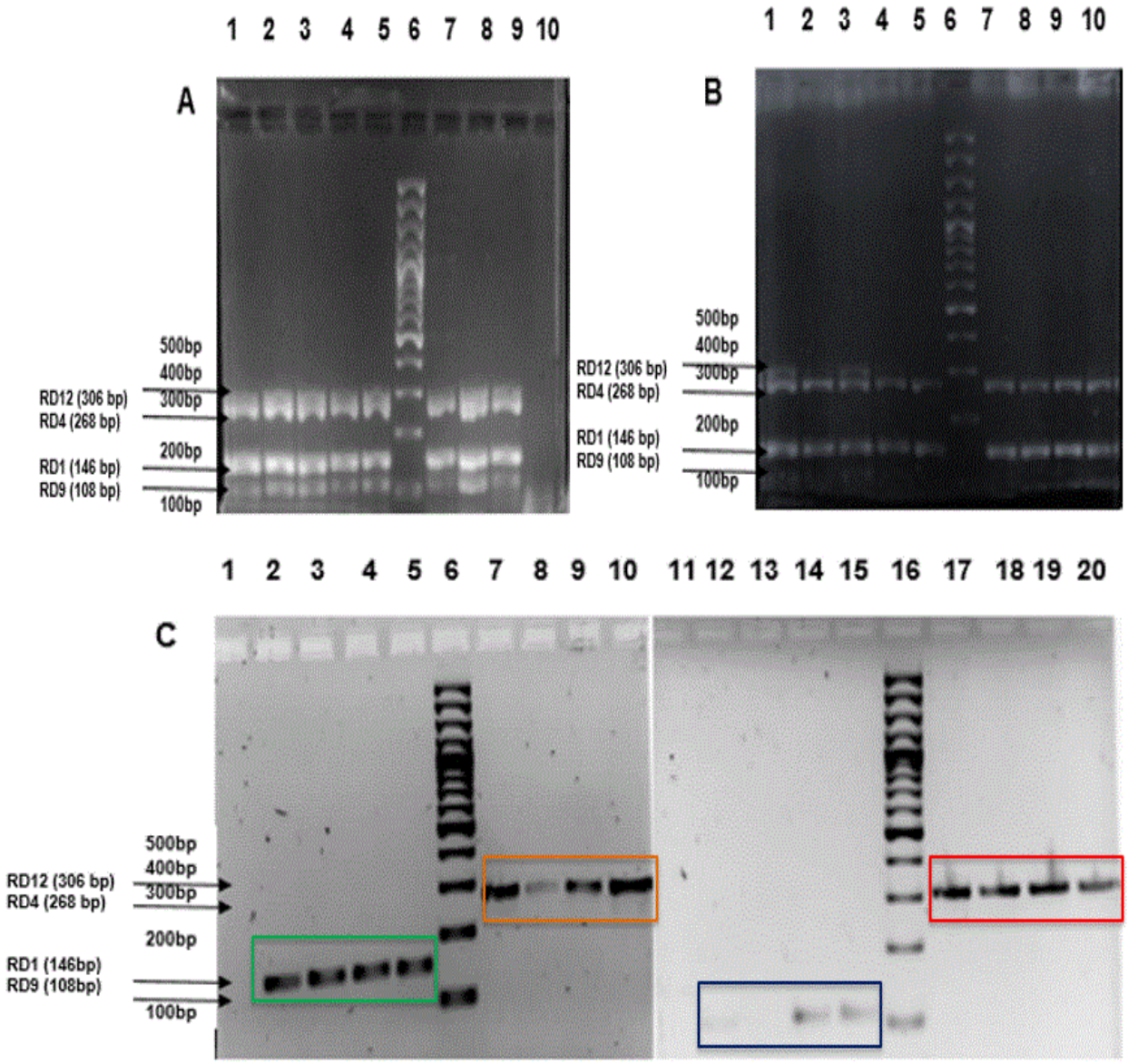


Figure 3.4. Region of Difference (RD) PCR products. A) Representative results where all 4 expected DNA fragments were amplified. Lane 1; *M. bovis* BCG (positive control), Lane 2; PNMP1A_1, Lane 3; PNMP3C_1, Lane 4; PNMP7_1, Lane 5; PNMP8_1, Lane 6; GeneRuler 100bpPlus DNA ladder, Lane 7; PNMP9_1, Lane 8; PNMP20_1, Lane 9; PN18062_1, Lane 10; negative control (no DNA template). **B)** Representative results of multiplex PCR where some of the DNA fragments were amplified. Lane 1; *M. bovis* BCG (positive control), Lane 2; PN18073_2, Lane 3; PN18073_3, Lane 4; PN18074, Lane 5; PN18075, Lane 6; GeneRuler 100bpPlus DNA ladder, Lane 7; PN18076, Lane 8; PN18078, Lane 9; PN18078, and Lane 10; (PN18078). **C)**. Representative results of successful singleplex PCR with individually amplified DNA fragments. **(Lane 2-5; RD 1, green rectangle):** Lane 1; negative control (no DNA template), Lane 2; PN18075, Lane 3; PN18076, Lane 4; PN18077, Lane 5; PN18078, Lane 6; GeneRuler 100bpPlus. **(Lane 7-10; RD 4, orange rectangle):** Lane 7; PN18075, Lane 8; PN18076, Lane 9; PN18077, Lane 10; PN18078, Lane 11; negative control. **(Lane 12-15; RD 9, blue rectangle):** Lane 12; PN18075 Lane 13; PN18076 Lane 14; PN18077, Lane 15; PN18078 Lane 16; GeneRuler 100bpPlus, **(Lane 17-20; RD 12, red rectangle):** Lane 17; PN18075, Lane 18; PN18076, Lane 19; PN18077, and Lane 20; PN18078. PCR = polymerase chain reaction. bp = base pairs.

3.3.2. Spoligotyping

To further genotype the *M. bovis* isolates, spoligotyping was performed. Spoligotyping demonstrated the expected signature for *M. bovis* which lacked direct repeats 3, 9, 16, 21, and 39-43 (Table 3.5), thereby confirming that the isolates were all *M. bovis*. As expected, the presence/absence of certain direct repeats discriminated between different genotypes.

A total of 5 different spoligotypes were found, namely SB0121, SB1275, SB0130, SB1474, and SB0140. The predominant strain was SB0121 (16 isolates), followed by SB0130 (4 isolates). Whereas SB0140, SB1474, and SB1275 were represented by one isolate each. The 16 SB0121 isolates were from 5 warthogs, 6 buffaloes, and 5 wild dogs. These isolates were purified from samples obtained from Location D (LD) in Mpumalanga, Location F (LF) in KZN, Location B (LB) in Limpopo, and Location A (LA) in Limpopo and are common in the geographical areas from which they were isolated (Hlokwe et al. 2014). The 4 isolates with SB0130 spoligotypes, were all from lion samples, obtained from 3 different locations: LA, LF, and Location E (LE).

One isolate from a buffalo sample from LF was the SB0140 number. Interestingly, two isolates (PN18073_2 and PN18073_3) from colonies picked from the same plate during purification steps were found to have different spoligotypes, SB0121 and SB0140, respectively. A sample from buffalo from Location C had spoligotype number SB1474. One isolate from a warthog sample collected at Location D was typed as SB1275. Based on these results, five *M. bovis* isolates with five different spoligotypes were selected; these had TurboFP635 fold-induction values above 23 (Table 3.4, indicated in red); these were selected to have diverse representative strains from various locations and different animal species for further investigation (Table 3.5). The rationale was to select distinct genotypes to determine and characterize persister formation from various strains.

Table 3.5 Spoligotype patterns for *M. bovis* strains isolated from samples collected from (LA), (LB), (LC), (LD), (MP), (LE), and (LF). *M. tuberculosis* H37Rv (MTC control), BCG strain (*M. bovis* control). The spoligopatterns were compared to the *Mycobacterium bovis* spoligotype international database where the SB number was obtained from matched strains.

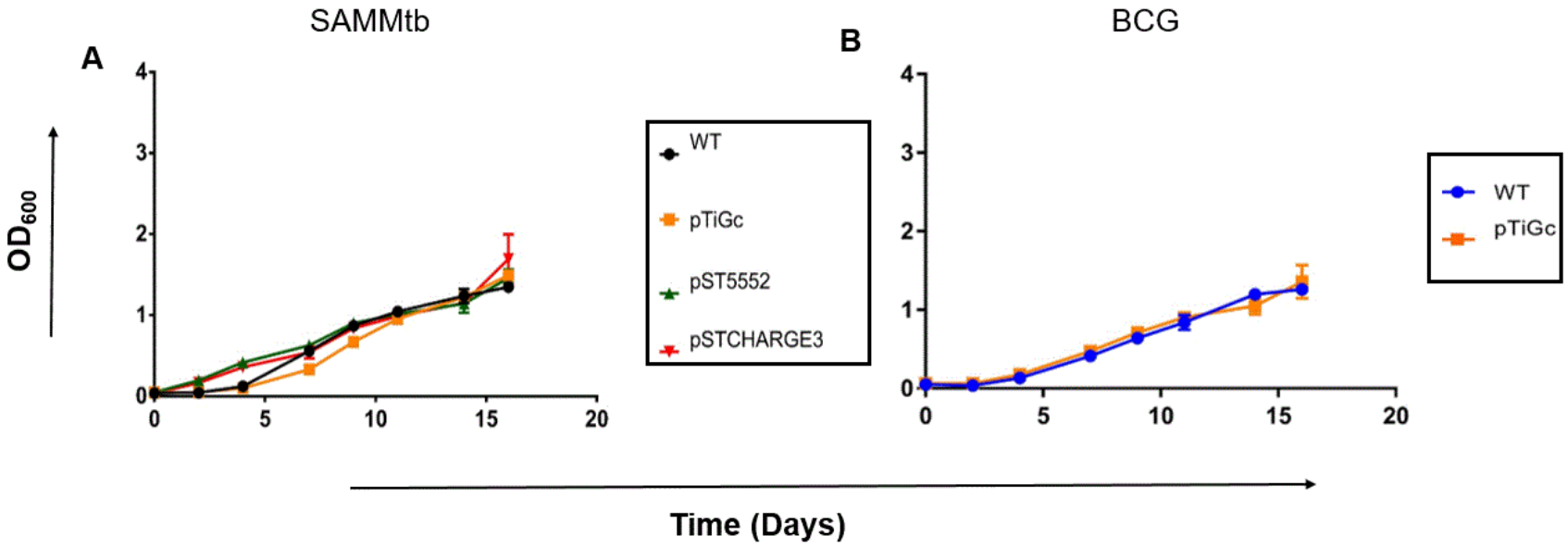
Animal specie	Strain	Spoligopatterns	SB number
n/a	H37Rv MTC control		n/a
n/a	BCG *M. bovis* control		SB0120
Warthog	PNMP1A_1		SB0121
Warthog	PNMP3C_2		SB0121
Warthog	PNMP7_1		SB0121
Warthog	PNMP8_1		SB0121
Warthog	PNMP9_1		SB0121
Buffalo	PN18068_1		SB0121
Buffalo	PN18069_3		SB0121
Buffalo	PN18070_3		SB0121
Buffalo	PN18071_1		SB0121
Buffalo	PN18072_3		SB0121
Buffalo	PN18073_2**		SB0121
Wild dog	PN18074		SB0121
Wild dog	PN18075		SB0121
Wild dog	PN18076		SB0121
Wild dog	PN18077		SB0121
Wild dog	**PN18078**		SB0121
Lion	PN18064_3		SB0130
Lion	PN18065_2		SB0130
Lion	PN18066_3		SB0130
Lion	**PN18067_1**		SB0130
Buffalo	**PN18073_3****		SB0140
Buffalo	**PN18062_1**		SB1474
Warthog	**PNMP20_1**		SB1275

Location A (LA), Location B (LB), Location C (LC), Location D (LD), Location E (LE), Location F (LF). Five *M. bovis* isolates with different spoligotypes were selected to characterize persisters (highlighted in red). **** = two isolates from colonies picked from the same plate found to be different spoligotypes.

3.3.3. Transformation and growth curves

Transformation of 23 *M. bovis* strains with the pTiGc reporter plasmid was performed to be able to subsequently track the viability and replication dynamics of persisters at a single-cell level using flow cytometry. Twenty-two of 23 isolates were successfully transformed with the pTiGc plasmid (data not shown). However, one isolate (PN18076) was not successfully transformed, despite four separate attempts. After electroporation and plating of this isolate on agar plates, no colonies grew on solid media after 4 weeks.

Growth curves of SAMMtb (Figure 3.5A), BCG (Figure 3.5B), and all 22 successfully transformed *M. bovis* clinical isolates (wild type vs transformants) were performed (Figure 3.5; Table S7.1). Figure 3.5 depicts growth curve data for SAMMtb, BCG, and the five clinical strains selected for subsequent experiments. The majority of the strains showed identical growth curves for wild type vs transformants (Figure 3.5C, F & G) and (Figure S7. 1A, F, H, I, J, L, M, N & O), demonstrating that there were no growth defects in the transformants due to the carriage of the reporter plasmid. On the other hand, some growth curves for wild type vs transformants showed similar for the first 7-10 days (Figure 3.5C, D & E) and (Figure S7. 1B, C, D, E, G, K, P & R), with notable differences appearing from day 10 to 16 (attributed to clumping). In the majority of cases, the pTiGc variant growing a bit appeared to grow slightly faster at later time points than the WT, with the exception of PN18077 where the WT grew faster than pTiGc transformed isolate. Notably, the WT and pTiGc variants for each strain all showed identical growth kinetics days 0-4, corresponding to the time frame of subsequent experiments.



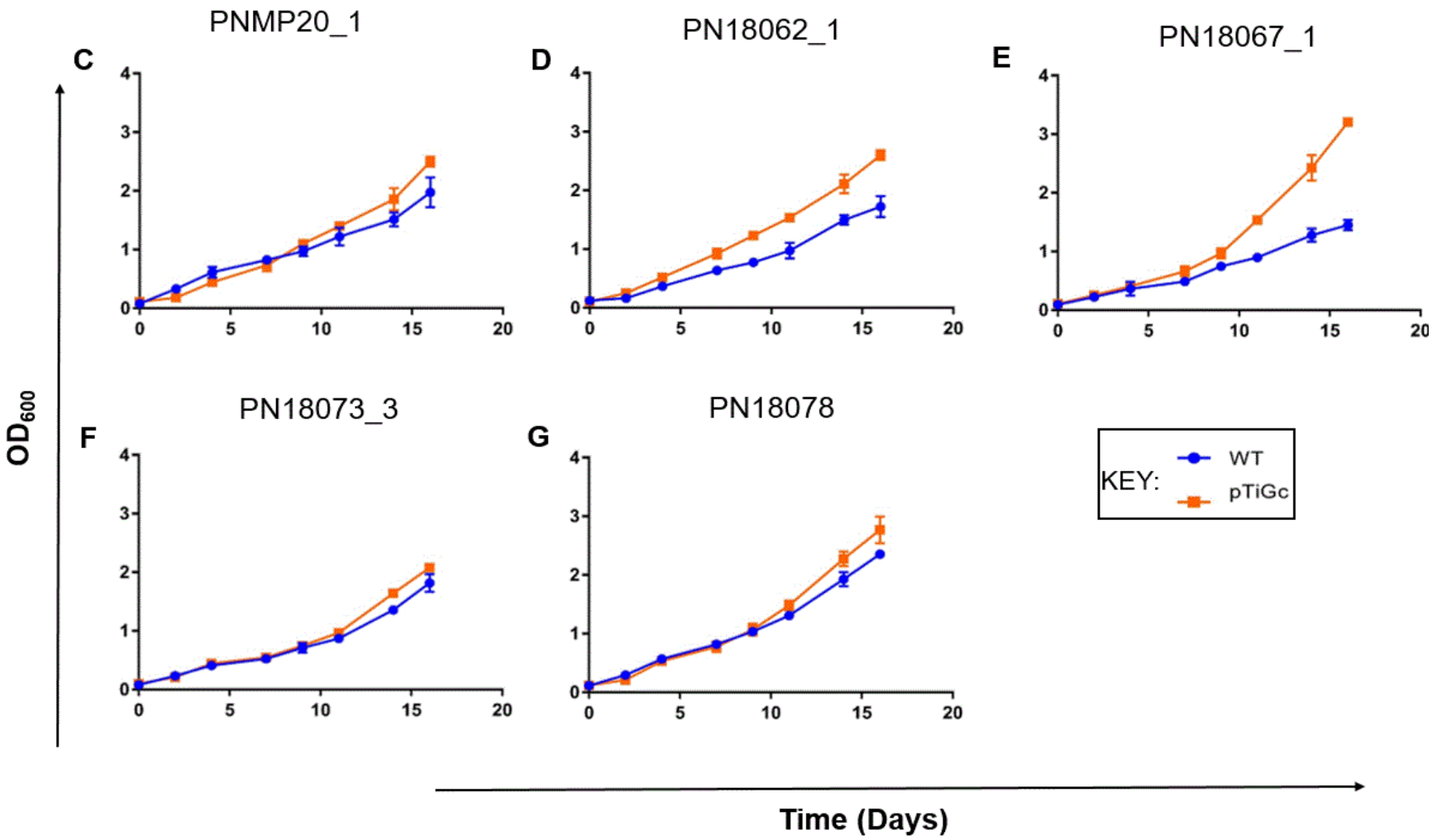


Figure 3.5: Wildtype vs pTiGc transformed growth curves of lab strains and clinical isolates. A) SAMMtb growth curve; wild type (WT), pTiGc (red and green), pST5552 (green only), pSTCHARGE3 (red only). **B)** BCG growth curve; wild type (WT), pTiGc (red and green). **C)** PNMP20_1 growth curve; wild type (WT), pTiGc (red and green). **D)** PN18062_1 growth curve; wild type (WT), pTiGc (red and green). **E)** PN18067_1 growth curve; wild type (WT). **F)** PN18073_3 growth curve; wild type (WT), pTiGc (red and green). **G)** PN18078 growth curve; wild type (WT), pTiGc (red and green). Two independent experiments were performed (each experiment performed with technical triplicates). Data are means ± standard deviation from two biological replicates.

3.4. Discussion

The main objectives of the work described in this chapter were to purify and genotype the 25 clinical *M. bovis* isolates acquired from buffaloes, warthogs, lions, and wild dogs, and to subsequently transform these isolates with the pTiGc fluorescence dilution reporter plasmid.

3.4.1. Sample purification, mycobacterial culture, and ZN staining

Mycobacterial culture was completed for all 25 samples to confirm *M. bovis* infections (Gormley et al. 2014). Twenty-three of 25 samples were successfully cultured, decontaminated, purified, and genotyped. However, two samples showed no growth after the decontamination process. The decontamination process with NaOH-NALC coupled with homogenization and centrifugation of samples is a reliable method for decontamination (Farnia et al. 2002) and was largely successfully applied in our study. However, the downside of this technique is that it can affect the viability and reduce bacterial count (Mtafya et al. 2019). Therefore, this process must be carefully monitored and checked to ensure that cells remain viable after decontamination. We speculate that the two tissue samples that did not grow on solid media might have had lower initial bacterial counts and lost their viability (Mtafya et al. 2019). Although various strains were found, the decontamination treatment and multiple culture steps may have decreased the detection of multiple genotypes (Mtafya et al. 2019).

Furthermore, as expected, ZN staining confirmed the presence of acid-fast bacilli (AFB) and the absence of non-AFB in all samples (Cook 1997; Farnia et al. 2002). Although the ZN technique identifies all mycobacteria, it cannot assign isolates to a species level (Watrelot-Virieux et al. 2006; Varello et al. 2008), Therefore, RD analysis, which is specific for the detection of MTC species, and can discriminate *M. bovis* from other species (Warren et al. 2006), was performed. As expected, RD analysis confirmed all isolates as *M. bovis*.

3.4.2. Spoligotyping

To further discriminate the *M. bovis* isolates, spoligotyping was effectively applied (Table 3.5). Five different spoligotypes were established among the 23 *M. bovis* isolates, namely SB0121, SB0130, SB0140, SB1275, and SB1474.

In this study, a wide variety of animal species were found to be infected with spoligotype SB0121. Spoligotype SB0121 has been previously reported in a range of animals such as

cattle, cheetah, greater kudu, leopards, bushbuck, baboons, warthogs, lions, and buffaloes (Musoke et al. 2015). Furthermore, it has been reported in various locations such as Location A (LA) (where it is the predominant strain), Location D (LD) in Mpumalanga, and Gonarezhou National Park (south-eastern Zimbabwe) (Michel et al. 2008; Hlokwe et al. 2013; Hlokwe et al. 2014). In France, SB0121 is considered the third most common spoligotype after SB0120 and SB0134. Taken together, this indicates that this strain is geographically widespread within SA and on other continents (Hauer et al. 2015).

M. bovis strains with the SB0130 spoligotype were isolated from lion samples from 3 different locations (LA, LE, LF), suggesting that this spoligotype is also geographically widely distributed (Hlokwe et al. 2013; Hlokwe et al. 2014; Sichewo et al. 2019a). In addition to being found in lion samples in this study, this spoligotype has been reported in cattle and buffaloes in Location F (LF) (KZN), North West, and Mpumalanga provinces (Hlokwe et al. 2013; Hlokwe et al. 2014; Sichewo et al. 2019a).

Our study also identified spoligotype SB0140 from a buffalo sample from location LF (KZN). This is consistent with a previous study that reported SB0140 in buffaloes at Spioenkop reserve (KZN), indicating its prevalence in KZN (Dippenaar et al. 2017). Moreover, SB0140 has been reported in buffaloes in Mpumalanga and Limpopo provinces in both livestock and wildlife (Hlokwe et al. 2014). Spoligopattern SB1474 was found in one buffalo sample from LC. Spoligopattern SB1474 has previously been reported in buffalo from LA and LF (Hlokwe et al. 2011; Hlokwe et al. 2013; Hlokwe et al. 2014). Interestingly, SB1474 is believed to have evolved from SB0140 (Hlokwe et al. 2011). Spoligotype SB1275 from a warthog isolate has been recently reported in this species (Roos et al. 2016).

Interestingly, two isolates (PN18073_2 and PN18073_3) from colonies picked from the same plate during purification steps were found to exhibit different spoligotypes, SB0121 and SB0140. This finding suggested the possibility that an animal may be co-infected by two different *M. bovis* strains. Previous studies have shown that animals can be co-infected by two or more genotypes prevalent on the same farm or game reserves (Hlokwe et al. 2011; Hlokwe et al. 2014). Another explanation could be that in-host evolution occurred, which has been reported in *M. tuberculosis* studies (Morales-Arce et al. 2019), however, the differences in the spoligotype patterns do not support that. In the future, whole-genome sequencing (WGS) could be used to examine this further.

In this study, it was found that there is a possibility of co-infections of animals in natural infections. Previous studies have highlighted that co-infections are common in animals, particularly wildlife (Hlokwe et al. 2011; Hlokwe et al. 2014). Therefore, future studies may investigate how this co-infection contributes to infection outcomes in animals. This would be assessed by colonies either by picking all colonies on each plate or performing deep sequencing where all conies are scrapped and sequenced (Wada et al. 2015). Such a strategy would indicate and fully represent which samples had multiple strains.

Furthermore, *M. bovis* co-infections may be missed during mycobacterial isolation from animal samples. Therefore, missing *M. bovis* co-infections in individual animals could skew interpretation regarding the transmission of *M. bovis* infections using strain identification. This finding also raises further questions on whether co-infections with other MTC members like *Mycobacterium orygis*, non-tuberculous mycobacteria (NTMs) (Roos et al. 2016), viruses (Oma et al. 2016; Burimuah et al. 2020), or fungi (Kuria and Gathogo 2013) impact wildlife and what the possible implications of such co-infections could be at an individual or systems level, as well as how this could affect latent infection outcomes in wildlife.

Spoligotyping was chosen as a genotyping method because it is affordable, well-established, is well standardized and easily comparable across different laboratories method. However, spoligotyping is a relatively crude strain typing tool and has limited resolution since it only investigates a partial region of the genome (Kamerbeek et al. 1997). In addition to spoligotyping, other sensitive techniques fingerprinting methods, such as the restriction fragment length polymorphism (RFLP) markers IS*6110*, polymorphic GC-rich sequence (PGRS), and variable number of tandem repeats (VNTR) (Hlokwe et al. 2013), have been applied to attain better resolution (Michel et al. 2008; Hlokwe et al. 2013). These methods have been effective due to their high levels of genetic diversity and reproducibility. Whole-genome sequences (WGS) is the most sensitive typing method that would offer greater discriminatory power and is expected to offer more detailed genotyping (Broeckl et al. 2017; Otchere et al. 2019; Abdelaal et al. 2019). WGS has been previously applied in mycobacterial studies and may be useful in the future to evaluate genomic differences in these strains (Black et al. 2015; Guimaraes and Zimpel 2020).

3.4.3. Transformation and growth curves

Transformation is one of the key techniques in molecular biological research (Jacobs et al. 1991; Parish and David 2015). This technique has been successfully applied in many mycobacterial studies (Michele and Ko 2010; Jain et al. 2016; Mouton et al. 2016).

Transformation of the *M. bovis* isolates with the reporter plasmid and growth curves were performed (for the first time) in preparation for studying persister formation, which is discussed in chapter 4. Twenty-two of 23 *M. bovis* strains were successfully transformed, indicating that clinical *M. bovis* strains are capable of incorporating the pTiGc plasmid. Conversely, one strain isolated from a wild dog, PN18076 (SB0121), was refractory to transformation, despite multiple attempts, and the basis for this is unknown. Surprisingly, the PN18076 strain is the same spoligotype as other strains of 15 isolates which were transformed; more detailed genotyping (such as WGS) may provide some insights on why it was refractory to transformation. It is speculated that the host from which the strains were isolated could play a role in influencing intra-strain phenotypic heterogeneity and physiological differences which could make some strains more robust to environmental changes (such as introducing foreign DNA into cells) (Parbhoo et al. 2020). However, it is unlikely that host-related phenotypic heterogeneity would be maintained after several rounds of *in vitro* culture (Pereira et al. 2020).

All clinical WT vs transformants strains grew similarly from day 0 to 10 days, although; a few strains had slight differences after day 10 of culture; this could be attributed to clumping of cells at later time points which would not be considered a growth defect. It is noteworthy that the persister assays were performed at early time points (day 0 to 4), where growth curves of all 22 strains were similar. Notably, the transformed strains were not induced with theophylline when performing these growth curves. Induction with theophylline could impose a fitness cost to transformed strains compared to the wildtype during persister assay experiments due to the production of the TurboFP635 fluorescence protein. However, to discount possible fitness cost, experiments before acid stress and theophylline was withdrawn.

TurboFP635 does not result in any growth defects (Mouton et al. 2016), suggesting that this would not influence the *M. bovis* transformants. Growth curves of WT vs transformants may highlight growth defects or enhancements. However, some studies have revealed that *M. tuberculosis* tends to be hypersensitive to stress and undergo physiological changes that may

cause differences between the wildtype vs transformed/mutated strains (Vandal et al. 2009). A recent study performed using the Nile red and ethidium bromide accumulation assay found that *M. smegmatis* mutants had undergone cell wall changes compared to the WT (Ruan et al. 2020), thus affecting the uptake of nutrients, which highlights additional assays may be required to reveal defects/enhancements between the WT and transformants. Therefore, additional phenotypic assays such as Nile red and ethidium bromide accumulation experiments may be added in the future to study both growth kinetics and cellular changes of the wild type versus the transformants to get a full picture and ensure there are no growth defects. This would be important since the study of persisters is tracked by the plasmid, however, the incorporation of the plasmid should not enhance/inhibit the growth of transformed strains. This finding could interfere with the detection of “VBNR” bacteria.

3.5. Summary

Of the 25 samples initially selected, 23 samples were successfully cultured, decontaminated, purified, and genotyped. Twenty-two were subsequently successfully transformed with the pTiGc reporter plasmid. Growth curves of the WT and transformants were similar and therefore no alteration in growth characteristics were introduced. This objective was performed in preparation for persister assay experiments discussed in chapter 4.

Chapter 4

Assessing *Mycobacterium bovis* persister formation following *in vitro* acid stress

4.1. Introduction

M. tuberculosis has been extensively studied and shown to cause latent infections in humans (Parrish et al. 1998; Wakamoto et al. 2013; Dutta and Karakousis 2014; Drain et al. 2018; Cohen et al. 2019). However, it is not understood if latent *M. bovis* infections exist in animal hosts. *In vitro* and *in vivo* latency models have been proposed over the past years for human latent infections (Zanella et al. 2008; Dutta and Karakousis 2014; Vernon and Bishai 2020), but have not been applied in the context of *M. bovis* in animals. Researchers have speculated about latency in animals based on antemortem and postmortem results (Bezos et al. 2014b; Alvarez et al. 2017). While some studies have offered insights on whether "true" latency exists in animals (Nuñez-Garcia et al. 2018; García et al. 2020), it remains controversial, inconclusive, and requires new approaches to address this fundamental question.

Latency is believed to be driven by "persisters" that exist in a viable but non- or slowly replicating state with a drug-tolerant phenotype (Zhang 2014; Huang et al. 2015; Van den Bergh et al. 2017). Persisters have been studied since the 1940s when a study on *Staphylococcus aureus* revealed that a small percentage (1%) of bacteria was drug-tolerant to penicillin (Bigger 1944). Since then, persisters have been reported in *Escherichia coli* (Spoering et al. 2006) and a wide range of other bacterial species (Kussell et al. 2005; Balaban et al. 2013; Helaine et al. 2014; Helaine and Kugelberg 2014), including mycobacteria (Mouton et al. 2016). Furthermore, it is believed that within the persister subpopulation, there is a spectrum of bacteria that could be viable and slowly replicating (Wood et al. 2013; Zimmermann et al. 2015), viable and non-replicating (Gengenbacher et al. 2010), or viable but non-culturable (Gample et al. 2019; Zhang et al. 2020), and these may vary or be in different proportions with different infection states.

Mycobacterial persister populations are also believed to contribute to TB drug tolerance, recurrence, and latency in human TB (Wakamoto et al. 2013; Huang et al. 2015; Malherbe et al. 2016; Moreira et al. 2016). For many years there was a challenge with studying persisters due to their small numbers, however, recent studies conducted in *Salmonella typhimurium* described

a tool to investigate bacterial replication dynamics at a single-cell level through the use of a dual fluorescence reporter system (Helaine et al. 2010; Helaine and Kugelberg 2014). Subsequently, a novel Fluorescence Dilution (FD) replication reporter system for mycobacteria was developed to study various questions (Mouton et al. 2016). This tool allows the monitoring of bacterial population replication dynamics at the single-cell level. Therefore, this study also applied this tool, coupled with *in vitro* acid stress, to evaluate and characterize *M. bovis* persister formation.

In vitro acid stress has previously been applied in mycobacterial studies (Portaels and Pattyn 1982; Vandal et al. 2009; Lin and Xin 2020) and to study various latency and dormancy questions (Deb et al. 2009; Mouton et al. 2016; García et al. 2018; Mesatywa, 2019, unpublished). The *in vitro* acid stress model mimics the macrophage phagolysosome microenvironment (Vandal et al. 2009; Adugna et al. 2014), and microniches that may be encountered by *M. bovis* upon infecting the host. The macrophage and granuloma environments are stressful environments for pathogens; these may be microaerophilic to hypoxic, acidic, and high in CO_2 (Palmer, 2013). It was found that *M. bovis* and *M. tuberculosis* optimally grew between pH 5.8 and pH 6.5, with conditions outside this range negatively affecting the growth of these pathogens (Portaels and Pattyn 1982). Another study found that *M. tuberculosis* growth was halted at pH 4.5 (Vandal et al. 2009). Recent studies have applied acid stress at pH 4.5 to *M. smegmatis* (Apiyo et al. 2020, under review), SAMMtb (Mouton et al. 2016; Mesatywa 2019, unpublished; Mouton et al. 2019), and *M. bovis* BCG (Gallant et al. 2016), and found that acid stress negatively affected the growth of these mycobacteria. Although some multi-stress models included pH stress (Deb et al. 2009), it is still important to study the impact of pH as a single stress to provide proof of concept before attempting multiple stress models. Therefore, this study aimed to evaluate the impact of acid stress on *M. bovis* clinical strains to generate proof of concept data and insights on whether *M. bovis* may have the ability to persist under harsh environments.

Phosphoproteomic analysis of SAMMtb revealed molecular insights into the strain's response to acid stress at pH 4.5 and identified 289 phosphopeptides mapping to 202 proteins after 45 minutes, and 9 upregulated proteins (involved in entering a non-replicating state through altering cellular and metabolic process) after 24 hours of exposure to acid stress (Mesatywa 2019, unpublished), highlighting the importance of the acid stress model in persister studies. Characterization of *M. bovis* persister subpopulations, at the single-cell level, is pivotal to bridging existing knowledge gaps on whether "true" latency exists in animals or it is a stage of subclinical infection.

In this study, objective three was to evaluate and characterize *M. bovis* persisters and elucidate replication dynamics following *in vitro* acid stress through flow cytometry. Flow cytometry is a powerful method that can monitor cells at the single-cell level and has been applied in TB research to study mycobacterial replication dynamics (Mouton et al. 2016; Mouton et al. 2019; Parbhoo et al. 2020).

4.2. Materials and Methods

4.2.1. Bacterial strains and plasmids

The lab strains *M. bovis* bacillus Calmette-Guérin (BCG) Pasteur and the Severely Attenuated Mutant of *M. tuberculosis* (SAMMtb) were used as comparison strains. SAMMtb had previously been transformed with the replication reporter plasmid. Additional control plasmids included no reporter, red only, and green only plasmids which served as flow cytometry controls (Section 3.2.3; Table 3.2). Five representatives of *M. bovis* pTiGc strains were used for this experiment as a foundation for the persister assay. All work with live pathogenic mycobacteria were carried out in Biosafety Level 3 facilities (BSL3), while the non-pathogenic mycobacteria were cultured in a Biosafety Level 2 lab (BSL2) within the Division of Molecular Biology and Human Genetics, following established SOPs. Isolates from beads and tissues were cultured according to the established SOPs for mycobacterial culture.

Table 4.1: Plasmids and strains used in this study.

Strain	Description	Source
SAMMtb::pTiGc	*M. tuberculosis* reference strain carrying pTiGc (hsp60(ribo)-turboFP635 hsp60-gfp, Kan^R, episomal)	(Mouton et al. 2016)
BCG::pTiGc	*M. bovis* reference strain carrying pTiGc (hsp60(ribo)-turboFP635 hsp60-gfp, Kan^R, episomal)	This study
PNMP20_1::pTiGc	*M. bovis* reporter strains	
PN18062_1:: pTiGc		
PN18067_1:: pTiGc		
PN18073_3:: pTiGc		
PN18078:: pTiGc		

Hyg^R, hygromycin resistant; Kan^R, kanamycin resistant

4.2.2. Persister assay

4.2.2.1. Bacterial culture and induction with theophylline

Five-milliliter cultures of controls (SAMMtb::pTiGc, SAMMtb::pTSCHARGE3, and SAMMtb::pST5552) were prepared in 7H9-OGT supplemented with 25 µg/ml kanamycin, 50 µg/ml hygromycin, 24 µg/ml pantothenate, and 50 µg/ml leucine at 37°C, shaking at 180 rpm for 7 days. While, SAMMtb (WT) was prepared in 7H9-OGT supplemented with 24 µg/ml pantothenate, and 50 µg/ml leucine at 37°C, shaking at 180 rpm for 7 days. BCG::pTiGc and *M. bovis::* pTiGc cultures were prepared in 7H9-OGT supplemented with 25 µg/ml kanamycin, at 37°C, shaking at 180 rpm for 7 days. The five clinical *M. bovis* strains that had been transformed with pTiGc (Figure 4.1) were cultured in 5 ml in 7H9-OGT supplemented with 25 µg/ml kanamycin at 37ºC in a non-shaking incubator for 7 days. On day 7, the OD of each strain was measured and cultures were adjusted to OD_{600nm} = 0.2.

SAMMtb::pTiGc, SAMMtb::pTSCHARGE3, and SAMMtb::pST5552 were pelleted for 5 min at 4000 rpm, and sub-cultured in 10 ml 7H9-OGT supplemented with 25 µg/ml kanamycin, 50 µg/ml hygromycin, 24 µg/ml pantothenate, 50 µg/ml leucine and containing theophylline (2 mM or 4 mM) to induce the expression of TurboFP635 at 37°C, shaking at 180 rpm for 7 days. SAMMtb (WT) was pelleted for 5 min at 4000 rpm and prepared in 7H9-OGT supplemented with 24 µg/ml pantothenate, 50 µg/ml leucine at 37°C, and containing theophylline (2 mM or 4 mM) shaking at 180 rpm for 7 days. BCG::pTiGc and *M. bovis::* pTiGc cultures were pelleted for 5 min at 4000 rpm and prepared in 7H9-OGT supplemented with 25 µg/ml kanamycin, and containing theophylline (2 mM or 4 mM) to induce the expression of TurboFP635 at 37°C, non-shaking for 7 days.

On day 7, a 100 µl aliquot of each culture was transferred to a Costar 96 well opaque (black) clear bottom microplates (Corning, New York, USA). Fluorescence measurements were performed on a plate reader to ensure that the induction process was successful. The green fluorescent protein (GFP) fluorescence intensity was captured by excitation at 485nm and emission at 520nm, while the TurboFP635 fluorescence intensity was captured by excitation at 580nm and emission at 640nm, as previously described (Mouton et al. 2016). The OD_{600nm} measurements were performed on a plate reader. Thereafter the cultures were washed with an equal volume of PBS for 5 min at 4000 rpm, transferred to 2 ml screw cap tubes, adjusted to an OD_{600nm} = 0.2, pelleted, and the supernatant removed before the acid stress step.

4.2.2.2. In vitro acid stress, sampling, and flow cytometry preparation

Isolates adjusted to OD_{600nm} = 0.2 in a final volume of 10 ml were cultured in 7H9-OGT supplemented with 25 µg/ml kanamycin and adjusted to pH 4.5, 5.0, or 5.5 using hydrochloric acid. The control or non-treated set was sub-cultured in 10 ml 7H9-OGT supplemented with 25 µg/ml kanamycin (adjusted to pH 6.5 using hydrochloric acid). The cultures were incubated at 37ºC for non-shaking for 4 days. Samples (1 ml) were transferred into 2 ml tubes, sonicated for 10 min at room temperature, and filtered. Thereafter, the cultures were centrifuged for 5 min at 14 000 rpm and the supernatant was discarded. The cells were fixed by adding 200 µl of 4% formaldehyde+PBS+0.05% Tween-80 to the pellet, mixed, and incubated at room temperature for 30 min. Afterwards, cells were centrifuged for 5 min at 14 000 rpm and the supernatant was removed. The cells were washed twice by adding 300 µl PBS+0.05% Tween and centrifuging for 5 min at 14 000 rpm. The supernatant was discarded, and samples were resuspended in 200 µl of PBS+Tween-80 and stored at 4ºC in the dark until flow cytometric analysis. Before flow cytometry was run, the samples were pelleted and resuspended in PBS. The sampling time points were days 0, 2, and 4.

4.2.3. Flow cytometry

Bacterial flow cytometry is a well-established technique in the host-pathogen laboratory and was carried out according to protocols under the auspices of Stellenbosch University (SU) Central Analytical Facility (CAF) flow cytometry core. The *M. bovis* pTiGc transformants and control strains that were exposed to acid stress or control conditions were fixed as described above and then assessed with the use of the BD FACSJazz™ flow cytometer (Becton Dickinson). The GFP fluorescence intensity was captured by excitation at 488nm, using a 530/30 filter, and TurboFP635 fluorescence intensity was captured by excitation at 561 nm, using a 610/20 filter as previously described (Mouton et al. 2016). For each sample, 30,000 events were captured. Data were captured using the Sortware version 1.1.0.84 and analyzed using FlowJo software (FlowJo, LLC, Ashland, Oregon, USA). Flow cytometry data will be deposited in the publicly accessible FLOW repository database at www.flowrepository.org. Experiments were conducted in two biological and three technical replicates.

4.2.4. Flow cytometry data analysis gating strategy

The gating strategy was performed as described in Mouton et al. (2016). Briefly, SAMMtb (WT, pTiGc, pST5552, pSTCHARGE3) controls were used to set up the gating template in the

FlowJo™ workspace (Figure 4.1A). The primary gate was selected for bacterial cells based on the Side Scatter (SSC) vs Forward Scatter (FSC) properties (Figure 4.1B), followed by gating for live cells on the TurboFP635 vs GFP plots (Figure 4.1C). The wildtype is shown in quadrant (Q4), the SAMMtb::pSTCHARGE3 (red only) in quadrant (Q3), SAMMtb::pTiGc (both red and green) quadrant (Q2), SAMMtb::pST5552 (green only) quadrant (Q1). Histogram plots of bacterial numbers (%Max) versus TurboFP635 (Figure 4.1D) were generated and the geometric mean fluorescence intensity of the live cells was determined. Furthermore, a gate for the quantification of viable but non- or slowly replicating (VBNR) was set up (Figure 4.1E). The principle of this gating is that if cells cultured in media at pH 6.5 were predominately actively replicating cells and fell within or below the lower 50% of the day 0 histogram plot, then the day 4 cells treated with acid stress (pH 4.5 or pH 5) that fell in the upper 50% day 0 plot showed a slowed or arrested growth and were therefore considered the “VBNR” cells. Therefore, the percentage of the day 4 cells treated with acid stress (grey population) that fell within the gate (indicated by the black dotted rectangle) was determined gated to quantify the percentage of the VBNR cells. The VBNR population is assumed to be enriched for “persister” cells believed to underlie latency. The gating was first performed on one sample, then checked against controls and selected experimental samples to ensure an optimal gating setup. Thereafter, the optimized gating strategy was consistently applied to all samples. The graphs were prepared on the GraphPad Prism 8.4.0 software (GraphPad Software, Inc., USA).

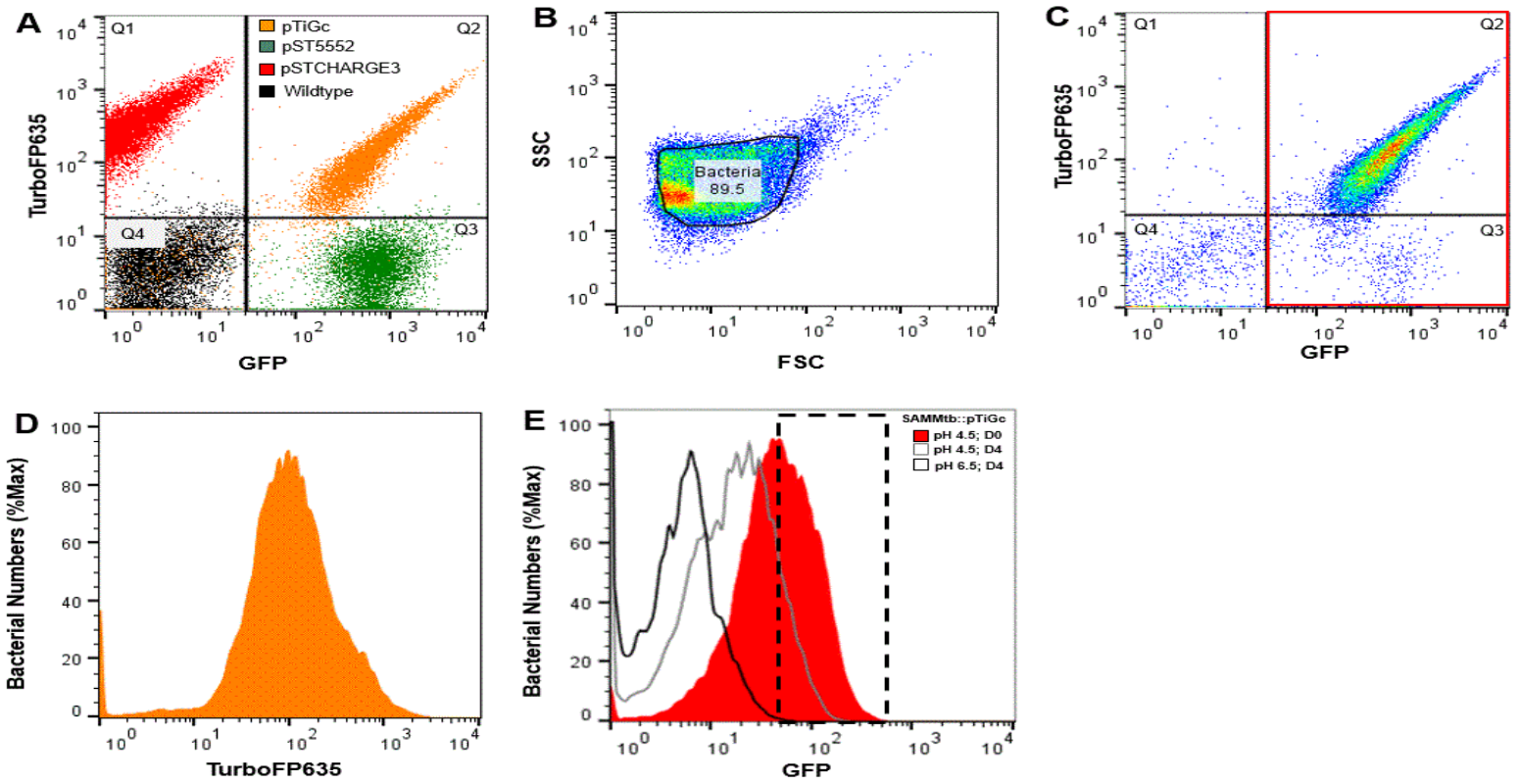


Figure 4.1: Gating strategy. A) TurboFP635 versus GFP plots of control strains were overlayed and applied to gate for the samples in each experiment; Wildtype (black), pSTCHARGE3 (red), pTiGc (orange), and pST5552 (green). **B)** To capture the bacterial population, a gate in a side scatter (SSC) vs. forward scatter (FSC) plot was applied. **C)** A rectangular gate was set up within the bacterial population to select all live (GFP positive) bacteria (highlighted in red rectangle). **D)** The histogram plot of bacterial numbers (%Max) versus the TurboFP635. **E)** A gate for the quantification of viable but non- or slowly replicating (VBNR) cells was set up (highlighted in the black dashed rectangle); the pH 6.5 cultures were predominately actively replicating cells that fell within the lower 50% of the day zero (D0) histogram plot while day 4 (D4) cells that fell in the upper 50% D0 plot exhibited a slowed or arrested growth. Using the gate indicated in the black dotted rectangle, calculations of what percentage of the grey population falls within the gate were performed as a proxy for VBNR. The above plots are example plots that show the principle of gating strategy**.** D=Day. GFP=green fluorescent protein.

4.3. Results

4.3.1. Impact of pH 4.5 on viability

An assessment of the susceptibility to acid stress of the control and clinical strains was performed, since this stress condition would be used in the subsequent persister assays. SAMMtb::pTiGc, BCG::pTiGc, and five clinical *M. bovis* strains carrying the pTiGc reporter were evaluated for response to acid stress, initially at pH 4.5 (selected based on previous work) (Mouton et al. 2019), with control cells cultured in media adjusted to pH 6.5. During data analysis, dot plot overlays were assessed to determine the differential sensitivity of cells exposed to pH 4.5. In these plots, bacterial cells that retained green fluorescence were considered to be live, while the loss of green fluorescence was indicative of cell death.

4.3.1.1. SAMMtb::pTiGc and BCG::pTiGc strains

SAMMtb::pTiGc and BCG::pTiGc were used as control and comparator strains. As expected, the control cells at normal pH 6.5 had the highest mean percentages of viable cells, > 98.4 ± 1.5% (Figure 4.2). SAMMtb::pTiGc and BCG::pTiGc cells cultured in normal media had a mean percentage of 99.2 ± 0.3% (± SD) (see representative dot plot in Figure 4.3A) and 98.4 ± 0.3% live GFP positive cells (see representative dot plot in Figure 4.3C), respectively on day 4. In contrast, the mean percentages of live cells of SAMMtb::pTiGc and BCG::pTiGc cells treated at pH 4.5 were 44.5 ± 32.5% (see representative dot plot in Figure 4.3B) and 6.5 ± 1.1% (see representative dot plot in Figure 4.3D), respectively on day 4.

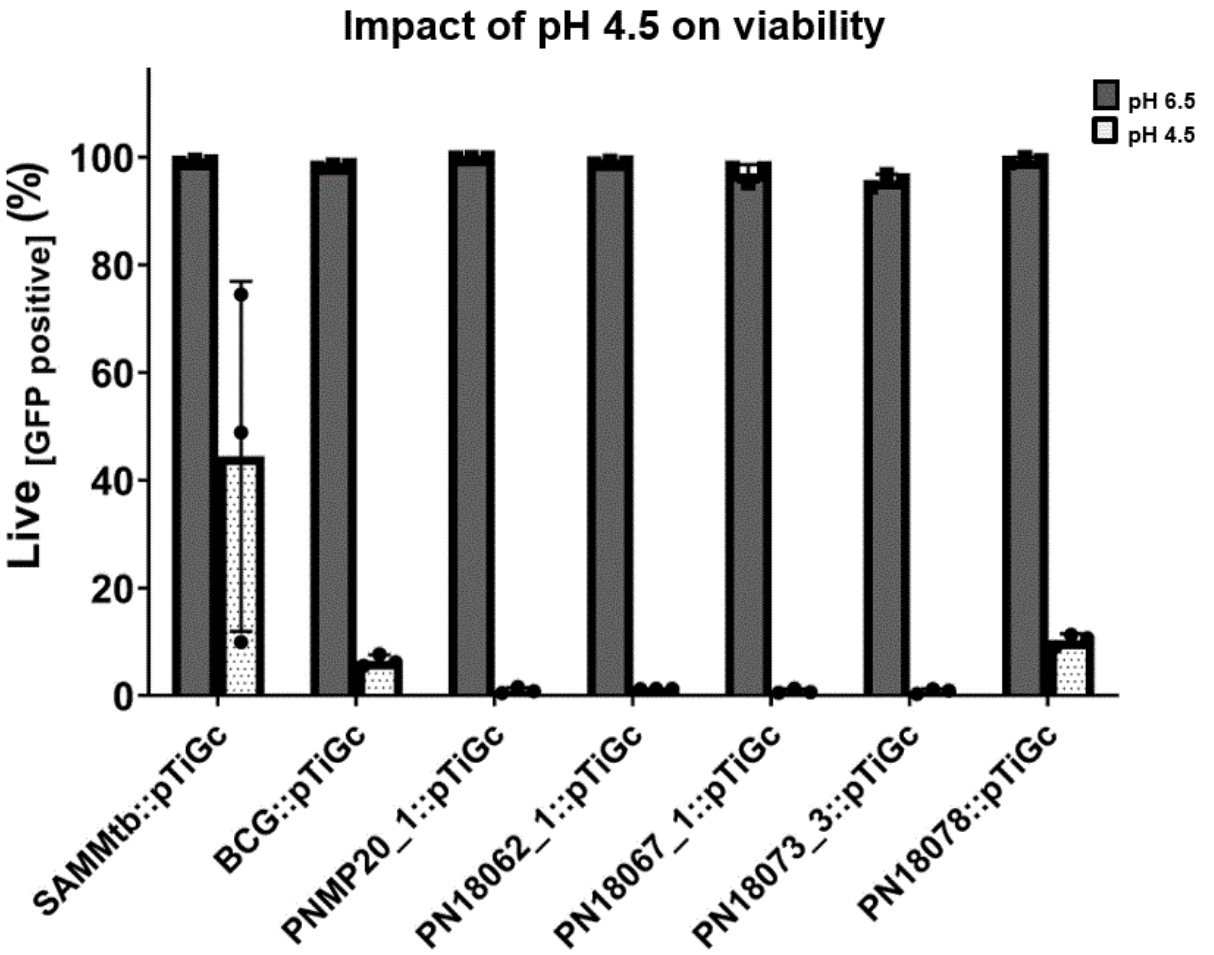
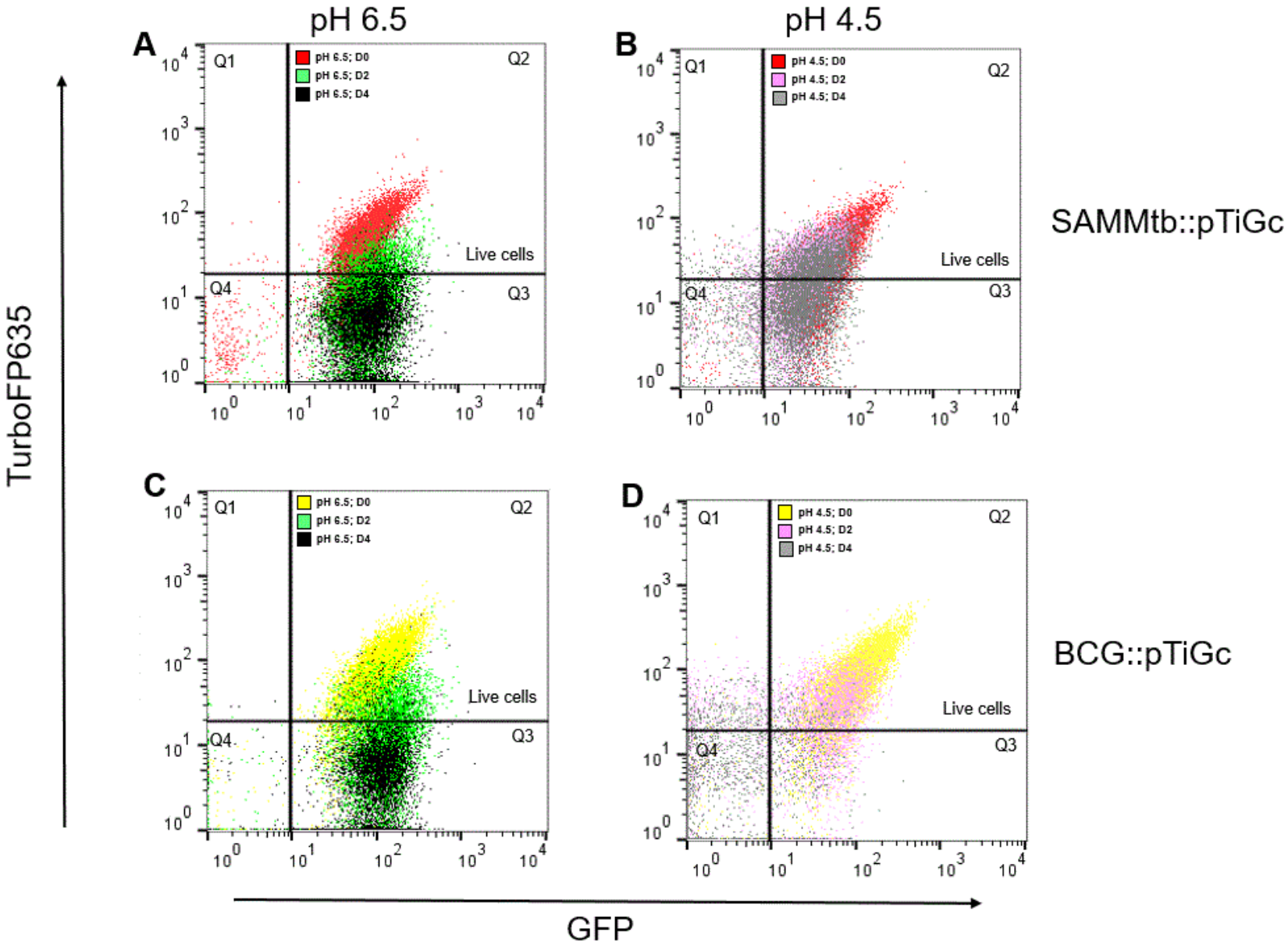
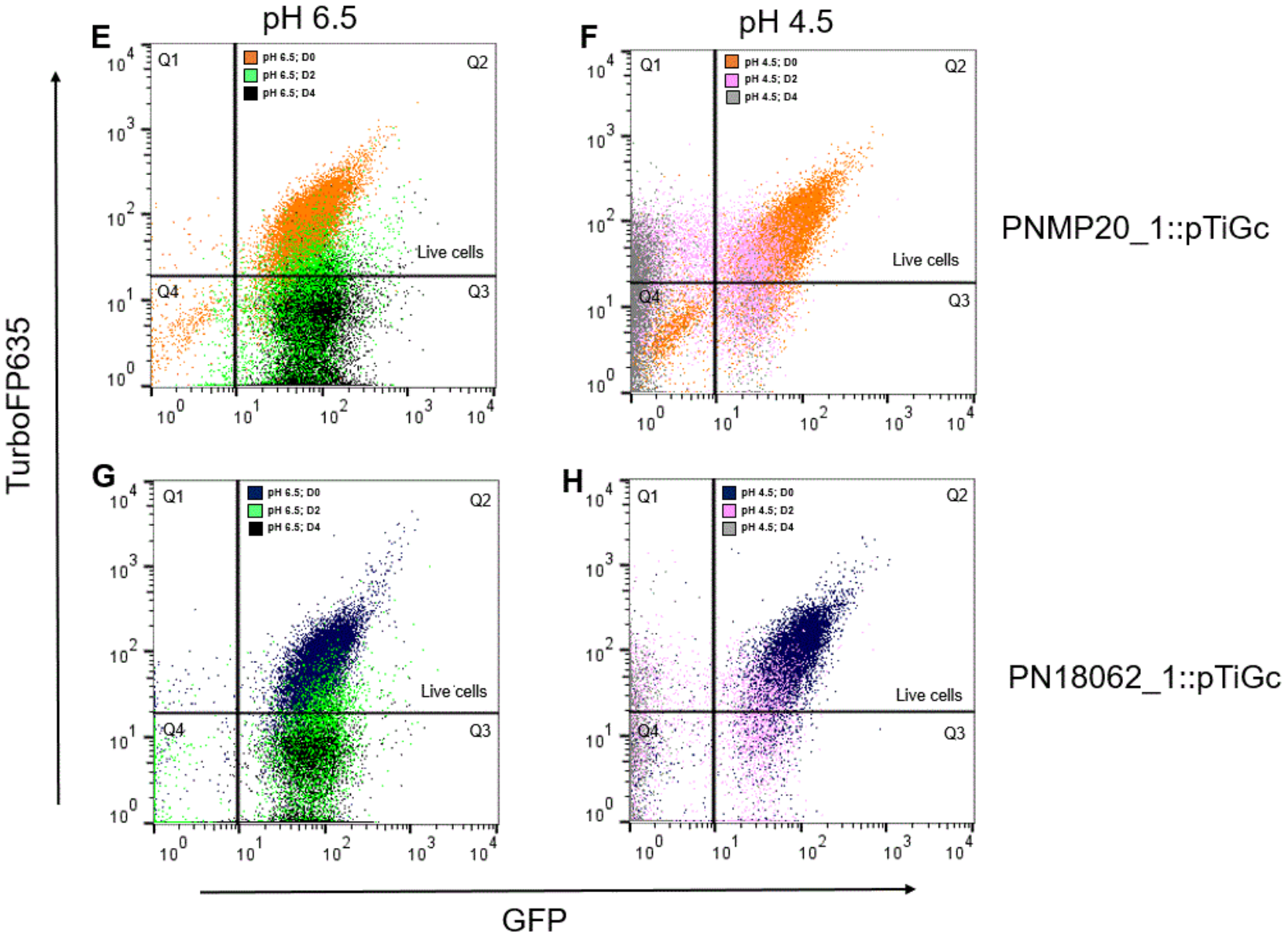
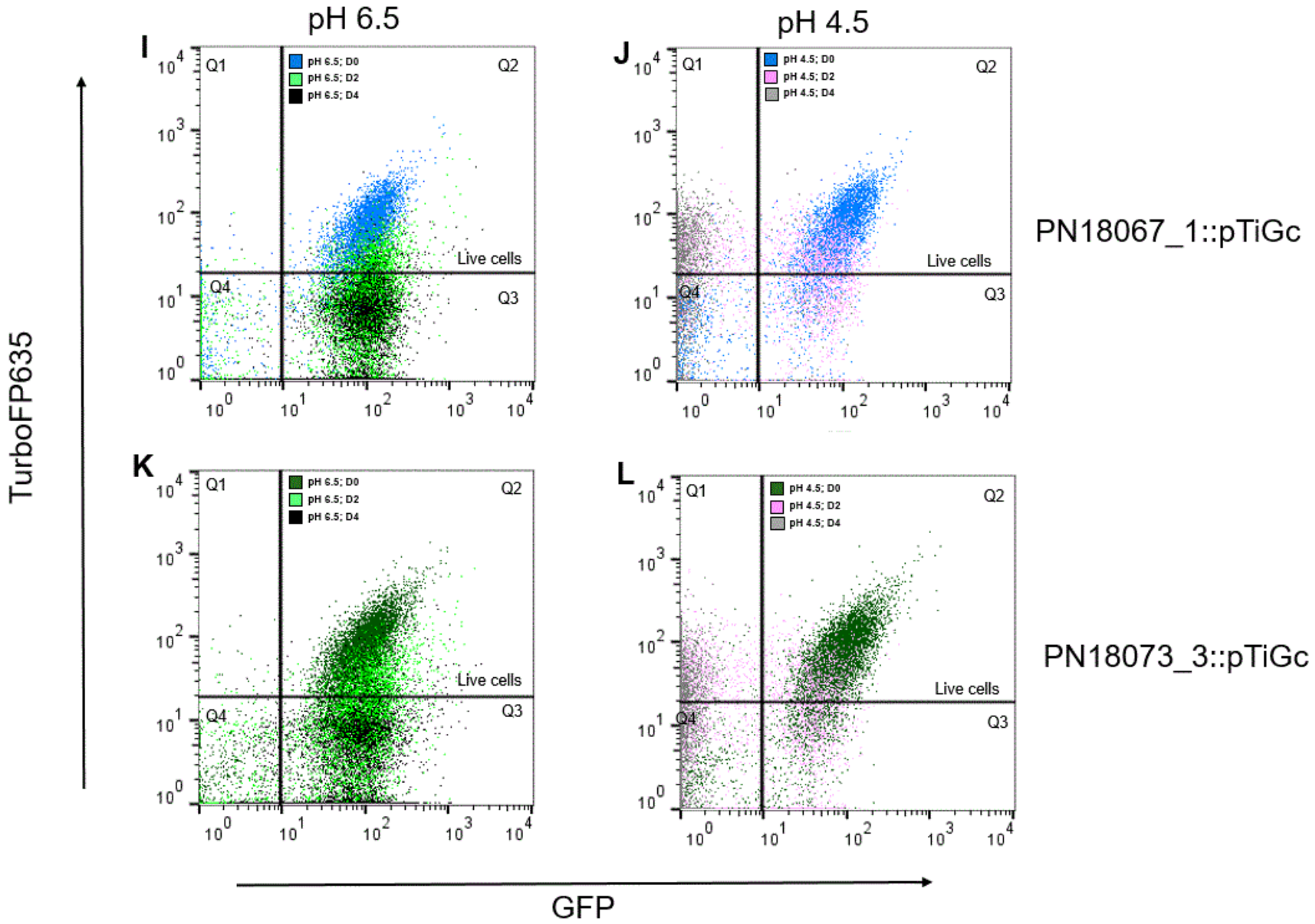


Figure 4.2: Impact of pH 4.5 on viability. Percentage of viable cells upon treatment at pH 4.5 compared to control with 2 mM pH 6.5 on day 4. The plots show percentages of mean ± standard deviation (SD) values of one biological replicate (performed in three technical replicates).







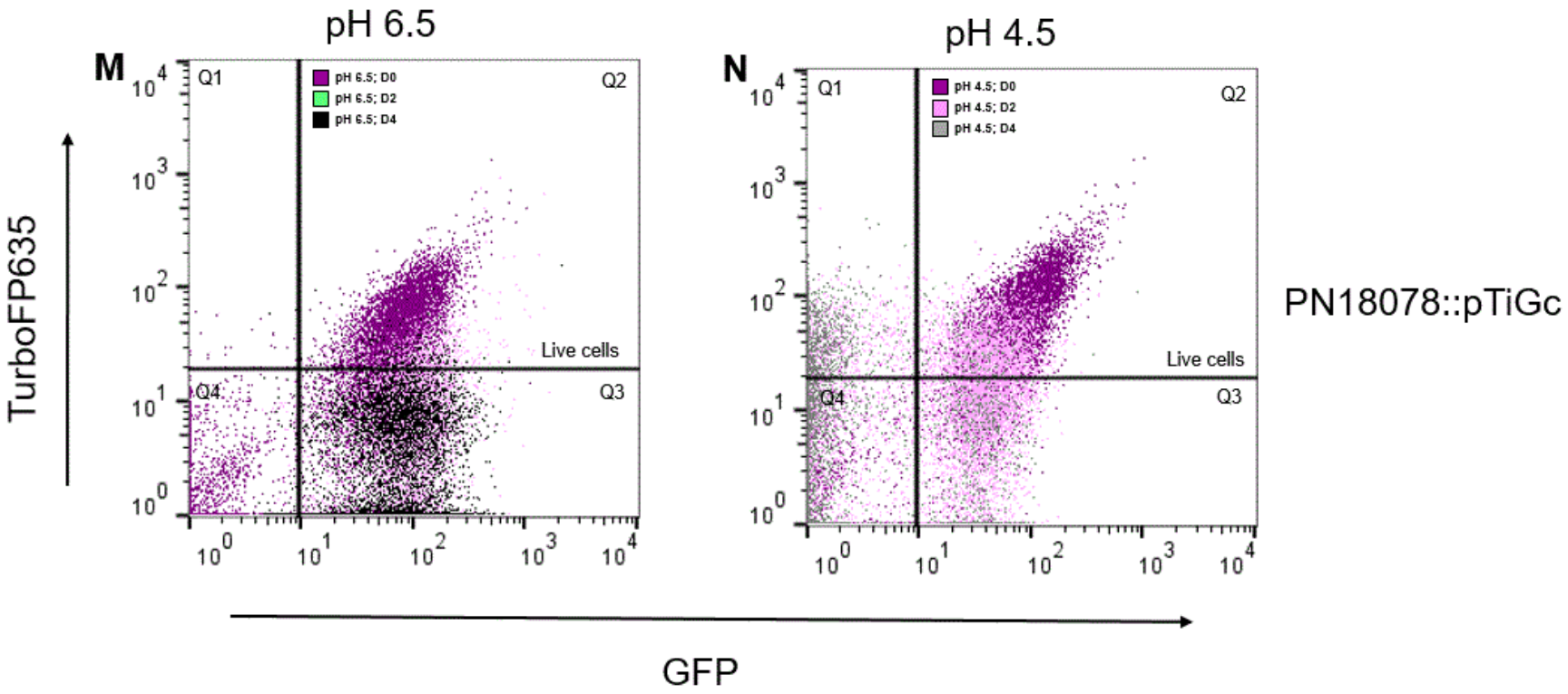


Figure 4.3: Acid stress sensitivity of SAMMtb::pTiGc, BCG::pTiGc strains, and five clinical strains of *M. bovis* following treatment at pH 4.5. A, C, E, G, I, K, M). An overlay of dot plots of SAMMtb::pTiGc, BCG::pTiGc PNMP20_1::pTiGc PN18062_1::pTiGc, PN18067_1::pTiGc, PN18073_3::pTiGc and PN18078::pTiGc cells cultured in normal media at pH 6.5 on D0, 2, and 4. **B, D, F, H, J, L & N)** An overlay of dot plots of SAMMtb::pTiGc cells, BCG::pTiGc, PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc, PN18073_3::pTiGc and PN18078::pTiGc cells in acidic media pH 4.5 on D0, 2, and 4. Representative plots of one independent biological repeat are shown (performed with technical triplicates). GFP = green fluorescent protein. D = day. pH 6.5 = control for actively replicating bacteria. pH 4.5 = cells treated with acid stress to evaluate cell viability and differential killing. Q 1-4 = quadrant 1 to 4. Q2 & 3= GFP positive or live cells. Q1 & 4 = GFP negative or dead cells.

4.3.1.2. Clinical *M. bovis* strains

The mean percentage of live cells (GFP positive) of clinical strains that were cultured in normal media on day 4, were very similar to the control strains SAMMtb::pTiGc and BCG::pTiGc (Figure 4.2). Specifically, there were 99.9 ± 0.1%, 99.4 ± 0.6%, 99.0 ± 0.3% and 97.0 ± 1.6% (± SD) live cells for strains PNMP20_1::pTiGc (Figure 4.3E), PN18062_1::pTiGc (Figure 4.3G), PN18078::pTiGc (Figure 4.3M), and PN18067_1::pTiGc (Figure 4.3I), respectively. One exception was PN18073_3::pTiGc (Figure 4.3K) which presented a slightly lower mean percentage of 95.6 ± 1.2% live cells (Figure 4.3E) on day 4. The mean percentages of live cells at pH 4.5 were 1.3 ± 0.0%, 1.0 ± 0.5%, 0.9 ± 0.5%, and 0.9 ± 0.4%, for strains PN18062_1::pTiGc (Figure 4.3H), PNMP20_1::pTiGc (Figure 4.3F), PN18073_3::pTiGc (Figure 4.3L), and PN18067_1::pTiGc (Figure 4.3J), respectively. One exception was PN18078::pTiGc (Figure 4.3N), which showed a slightly higher mean percentage of live cells of 10.3 ± 1.2% (Figure 4.2).

4.3.2. Impact of pH 5 on viability

The results described above suggested that cultures grown in media pH 4.5, had substantial sensitivity to pH 4.5 which led to a loss of viability. This high level of cell death, rather than growth arrest, would be likely to compromise the qualitative and quantitative assessment of subsequent persister assays. Therefore, following further optimization, it was concluded that pH 4.5 was not adequate to be applied for quantification of persisters, and pH 5 was selected for quantification of persisters in 3 selected clinical isolates. Prior to persister quantification, dot plots were prepared to check the viability of cells treated with pH 5.

4.3.2.1. SAMMtb::pTiGc and BCG::pTiGc strains

As expected, and in agreement with the results described in section 4.3.1, the mean percentages (lab strains) of live cells cultured on normal media at pH 6.5 on day 4 were high (> 99 ± 1.1%) (Figure 4.4). The mean percentages of live cells of SAMMtb::pTiGc and BCG::pTiGc cells cultured in normal media on day 4 were 99.4 ± 0.2% (± SD) (see representative dot plot in Figure 4.5A) and 97.0 ± 0.4% (see representative dot plot in Figure 4.5C), respectively (Figure 4.4). In contrast, the mean percentages of live cells of SAMMtb::pTiGc and BCG::pTiGc cells treated with pH 5 on day 4 were 72.7 ± 4.8% (see representative dot plot in Figure 4.5B) and 7.2 ± 1.2% (see representative dot plot in Figure 4.5D), respectively.

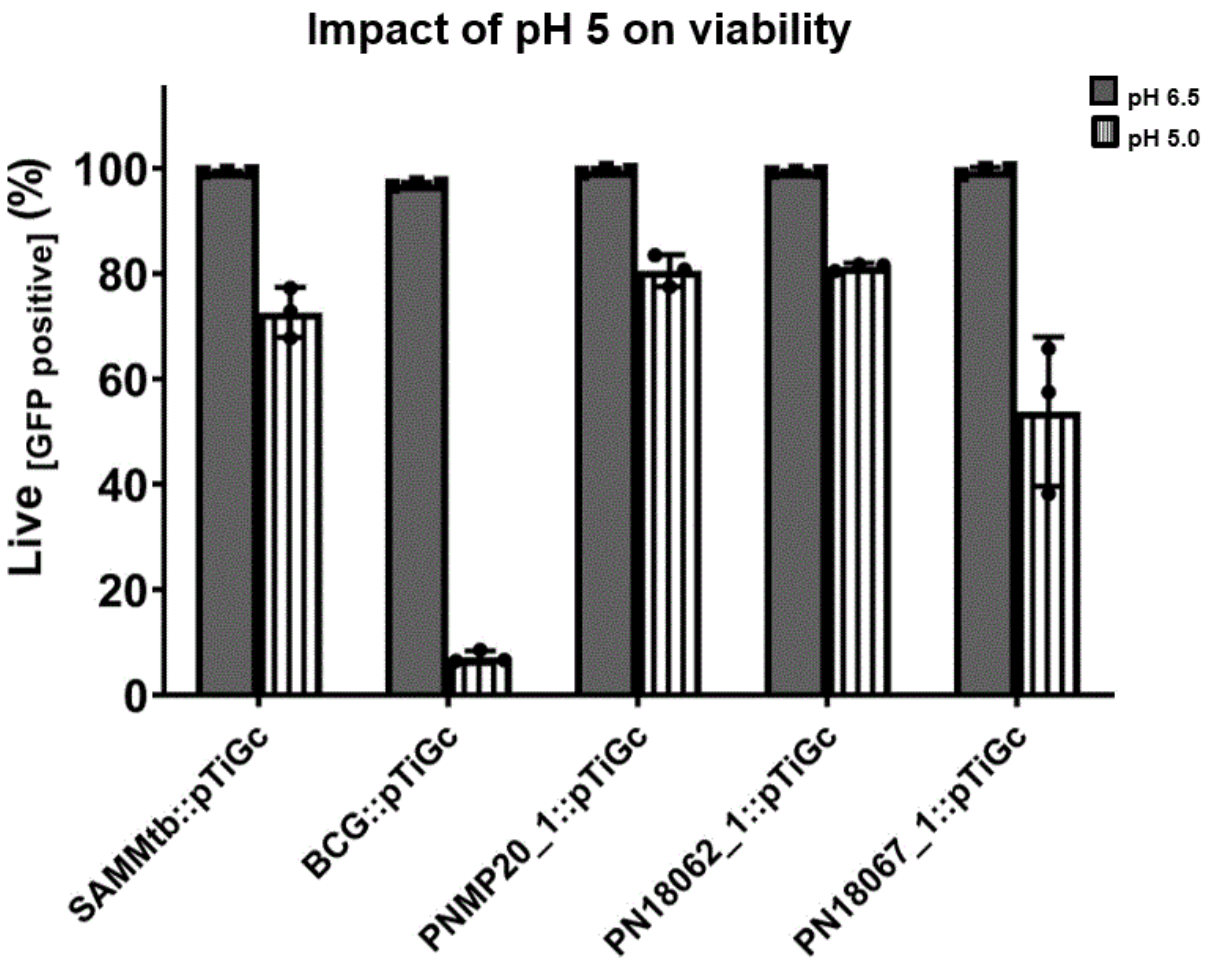
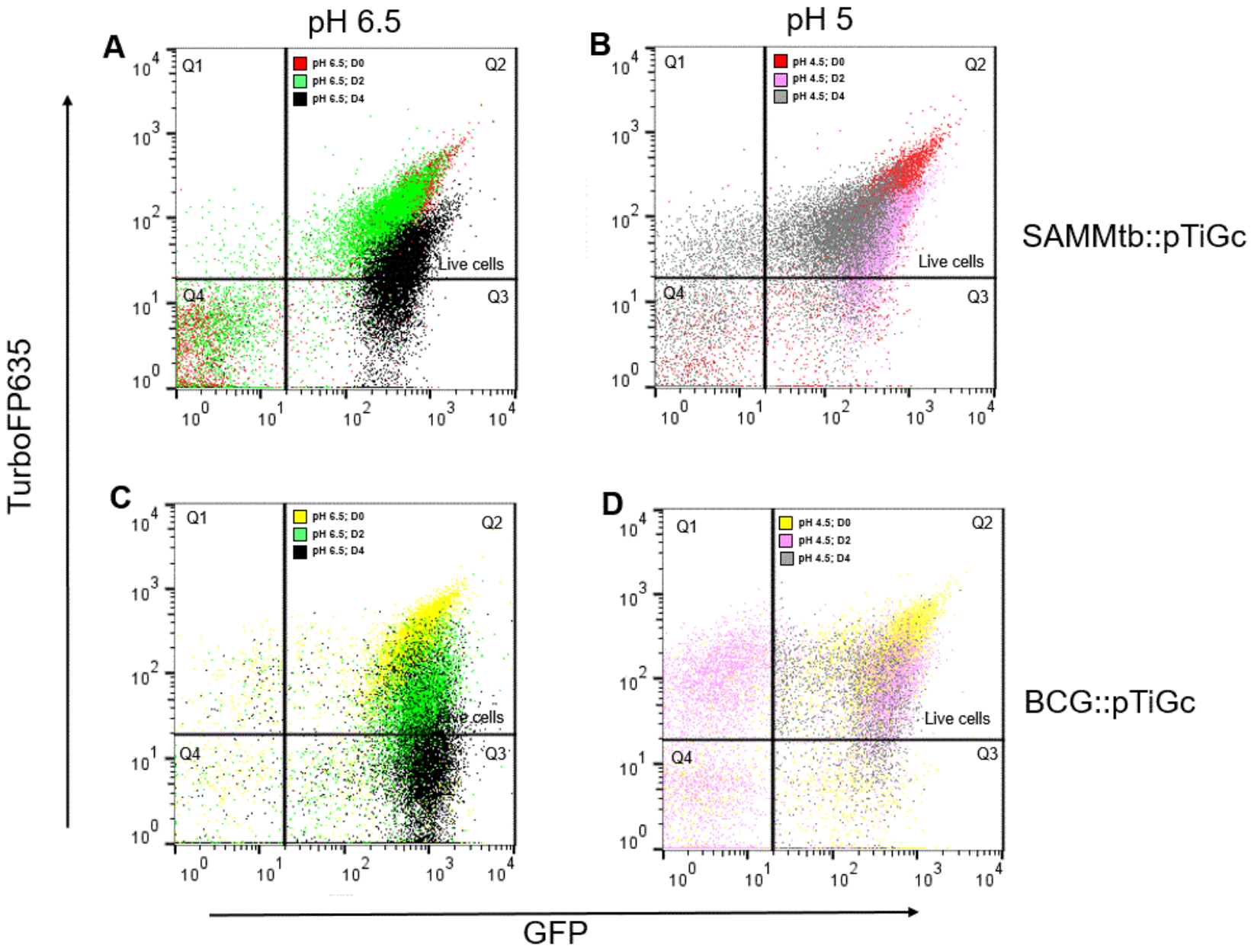
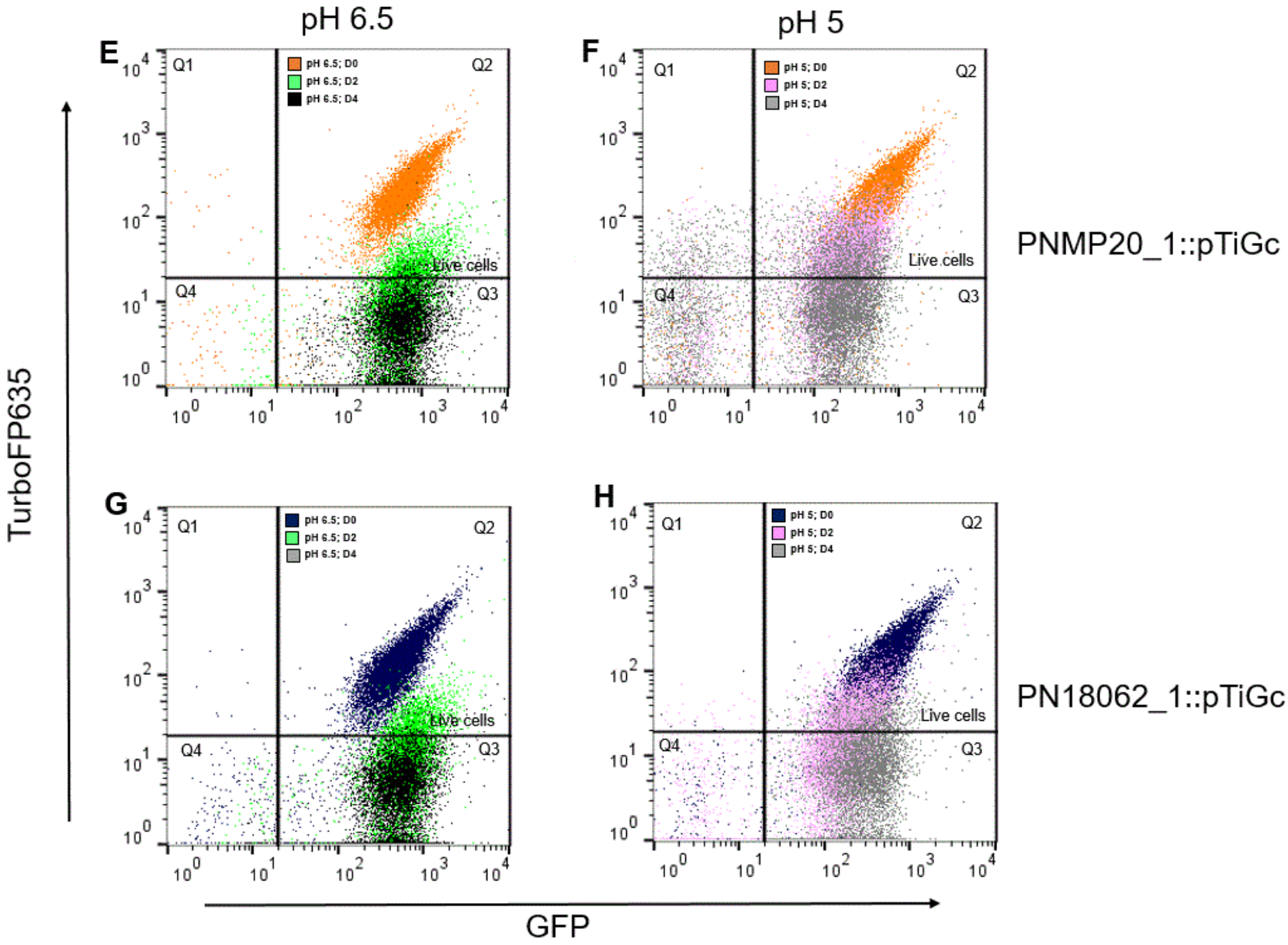


Figure 4.4: Impact of pH 5 on viability. Percentage of viable cells upon treatment at pH 5 compared to control pH 6.5 with 4 mM induction on day 4. The plots show percentages of mean ± standard deviation (SD) values of one biological replicate (performed in three technical replicates).

4.3.2.2. Clinical *M. bovis* strains.

The mean percentage of live cells of clinical strains that were cultured in normal media on day 4 was found to be similar to SAMMtb::pTiGc (Figure 4.5C). The mean percentage values for the clinical isolates were 99.6 ± 0.5%, 99.4 ± 0.2%, and 99.6% ± 0.7 for strains PNMP20_1::pTiGc (Figure 4.5E), PN18062_1::pTiGc (Figure 4.5G), and PN18067_1::pTiGc (Figure 4.5I), respectively. In contrast, mean percentage of live cells on day 4 following acid stress at pH 5, were 80.6 ± 3.0%, and 81.3 ± 0.7%, for strains PNMP20_1::pTiGc (Figure 4.5F), and PN18062_1::pTiGc (Figure 4.5H), respectively. One exception was strain PN18067_1::pTiGc which had a slightly lower mean percentage of live cells, 53.8 ± 14.2% (Figure 4.5J).





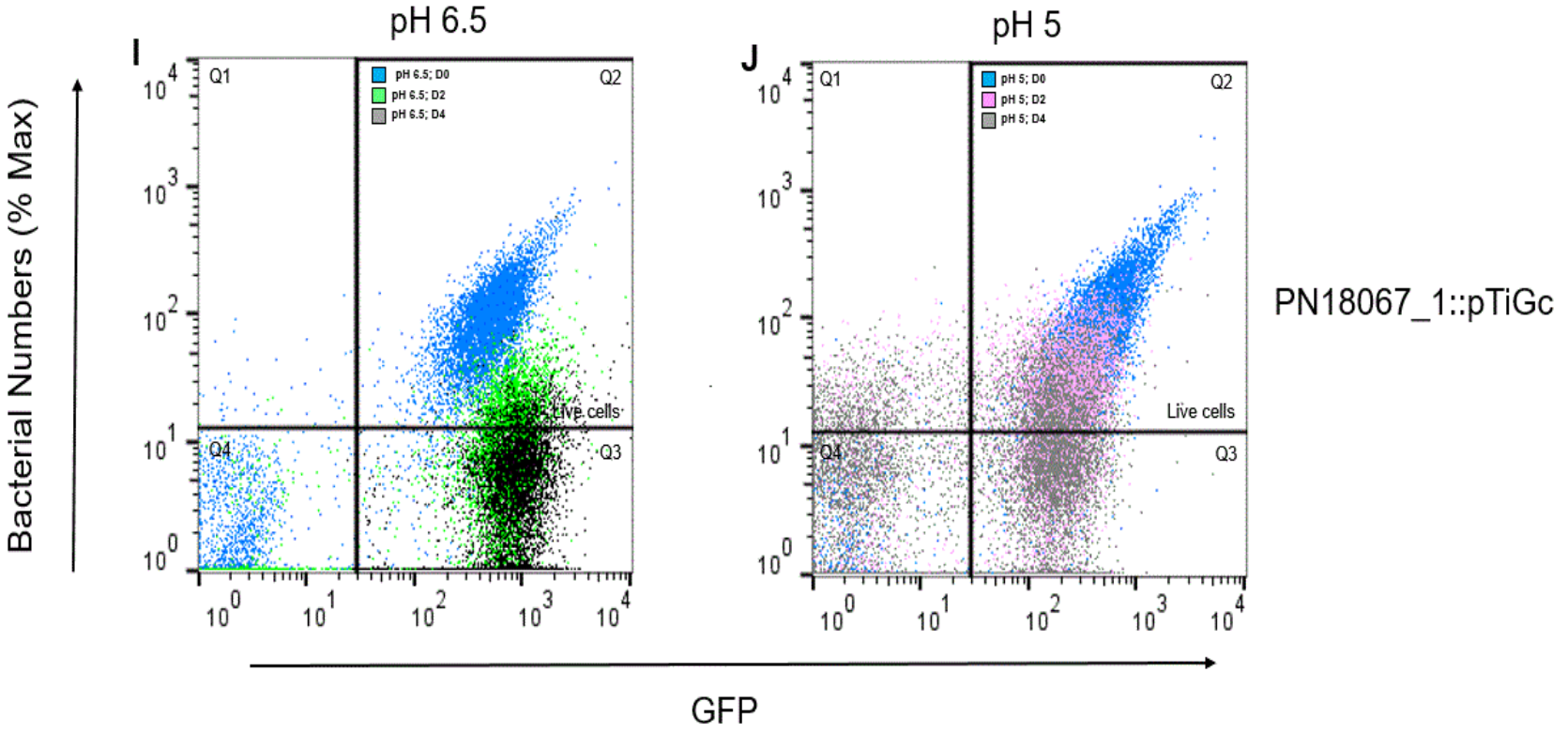
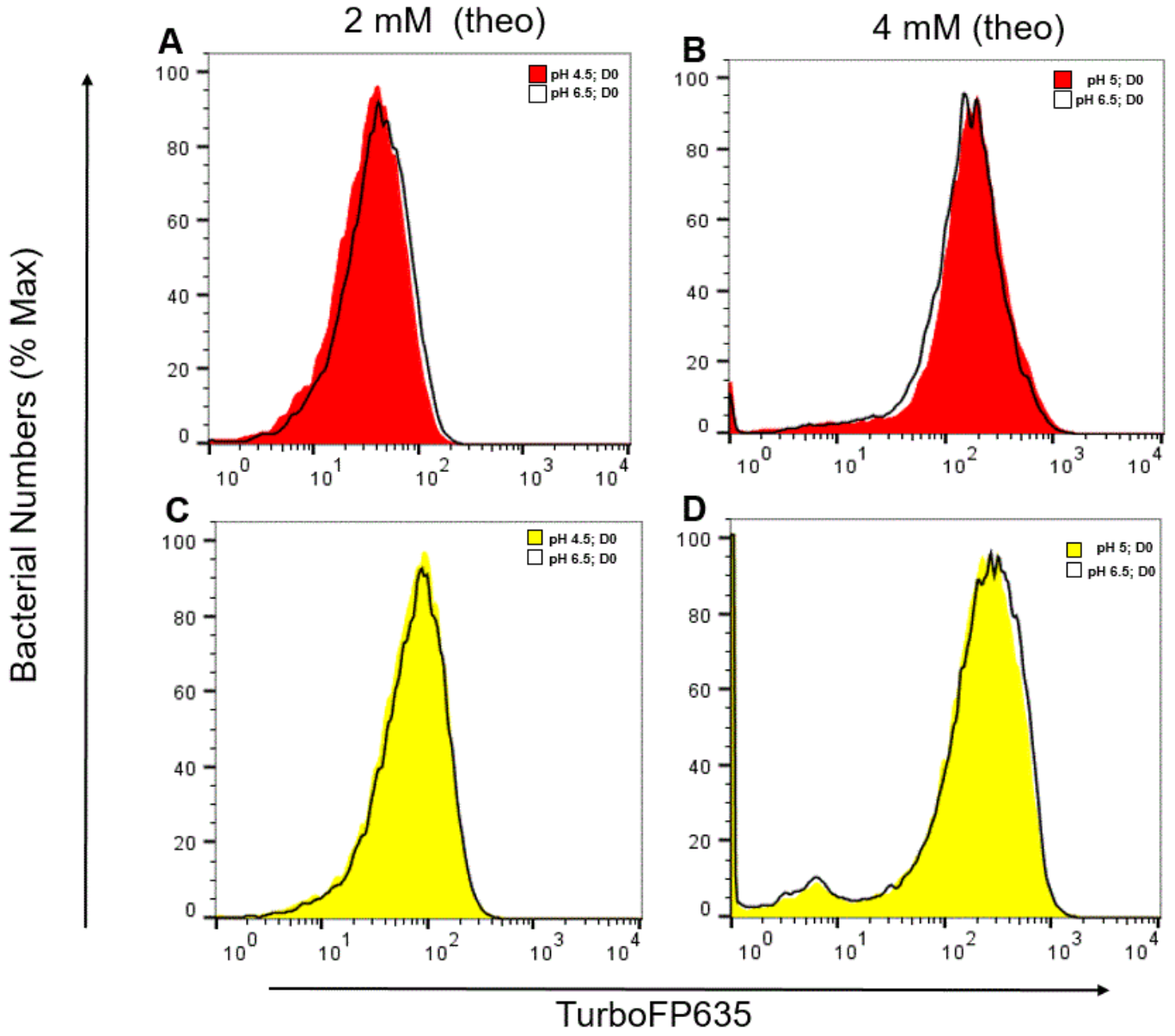
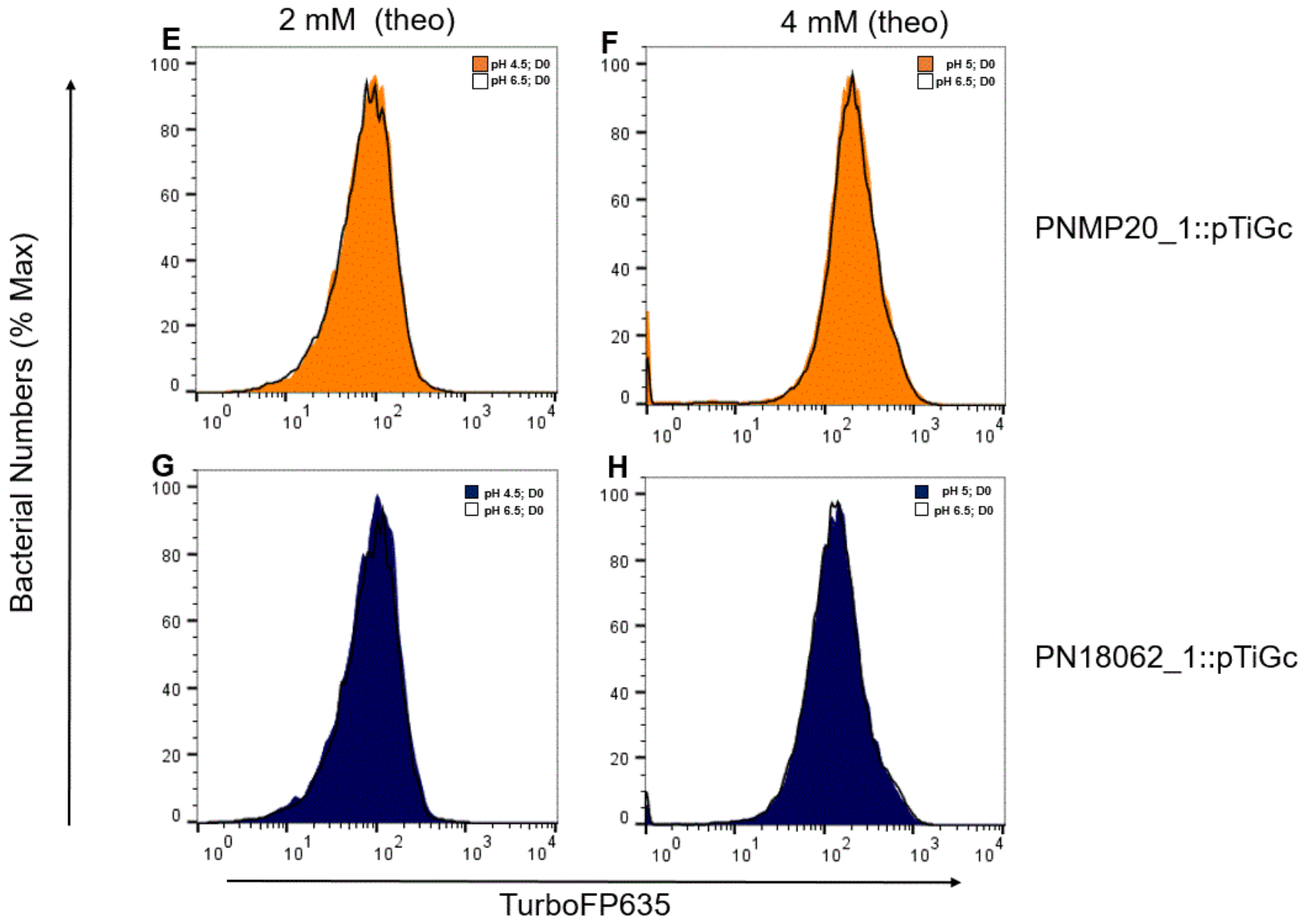


Figure 4.5: Acid stress sensitivity of SAMMtb::pTiGc, BCG::pTiGc strains, and three clinical strains following treatment with pH 5. A, C, E, G, & I) An overlay of dot plots of SAMMtb::pTiGc, BCG::pTiGc PNMP20_1::pTiGc PN18062_1::pTiGc and PN18067_1::pTiGc cells in normal media at pH 6.5 induced with 4mM (theo) on D0. **B, D, F, H, & J)** An overlay of dots plots of SAMMtb::pTiGc cells, BCG::pTiGc, PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc in acidic media at pH 5, induced with 4mM (theo) on D0. TurboFP635 = red fluorescent protein. D = day. Representative plots of one independent biological repeat are shown (performed with technical triplicates). theo = theophylline. pH 6.5 = control for actively replicating bacteria. pH 5 = cells treated with acid stress.

4.3.4. Optimal theophylline induction

To assess whether optimal theophylline induction was achieved, day 0 histogram overlays of cultures induced with 2 mM versus 4 mM were prepared. Optimal theophylline induction would be expected to give a MFI of ≥ 100. Notably, 2 mM theophylline successfully induced the cells of all strains but was not sufficient (the induction MFIs were < 100) for both lab strains SAMMtb::pTiGc (Figure 4.6A), BCG::pTiGc (Figure 4.6C) and the clinical strains carrying the pTiGc plasmid (Figure 4.6E, G, and I). Similar MFIs were observed for all pH conditions on day 0 (Figure 4.6). Due to sub-optimal induction levels observed with 2 mM theophylline, theophylline concentration was increased to 4 mM and improved inductions (MFI ≥ 100) were observed (Figure 4.6B, D, F, H, and J) for lab and clinical strains.





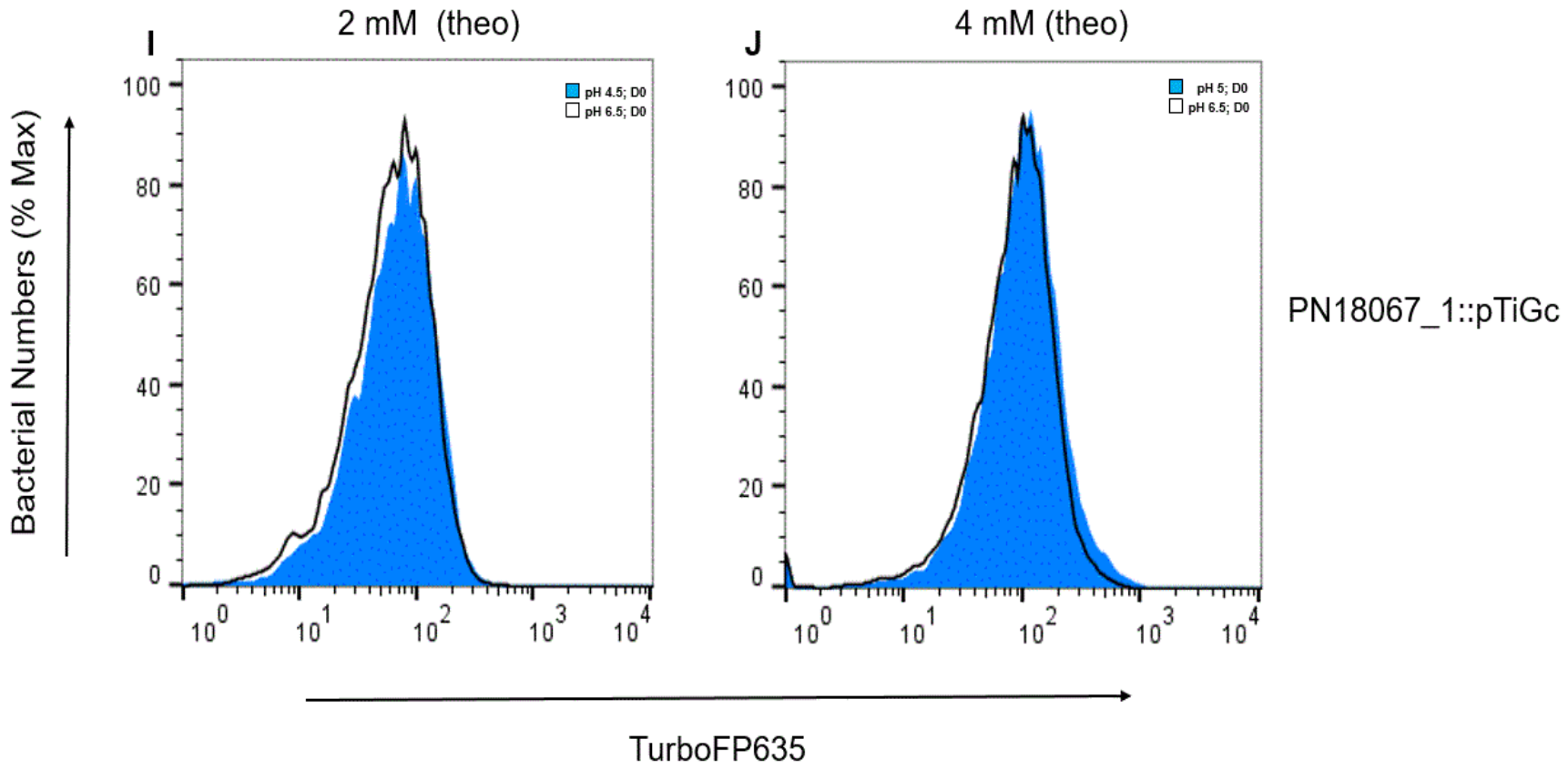
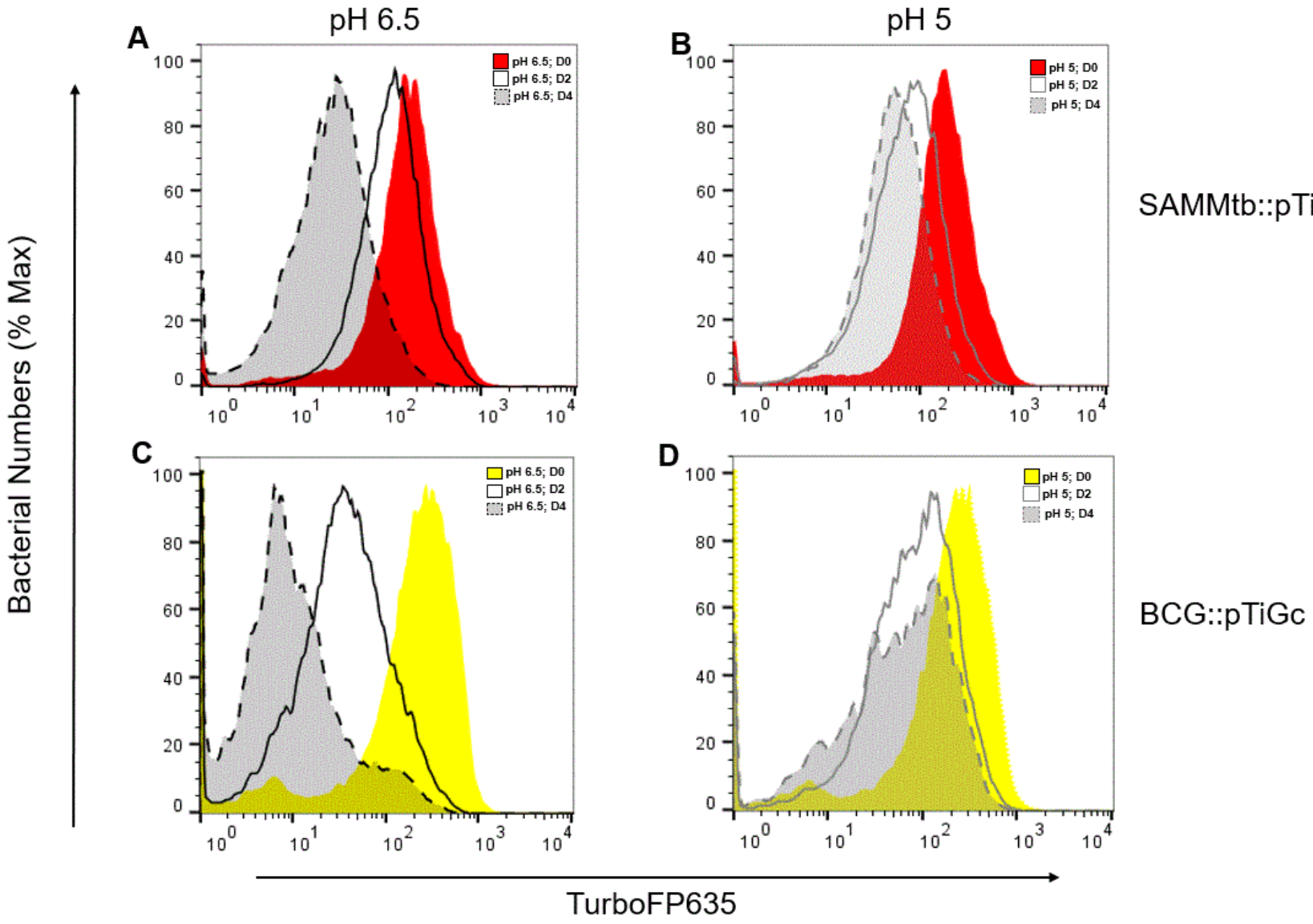


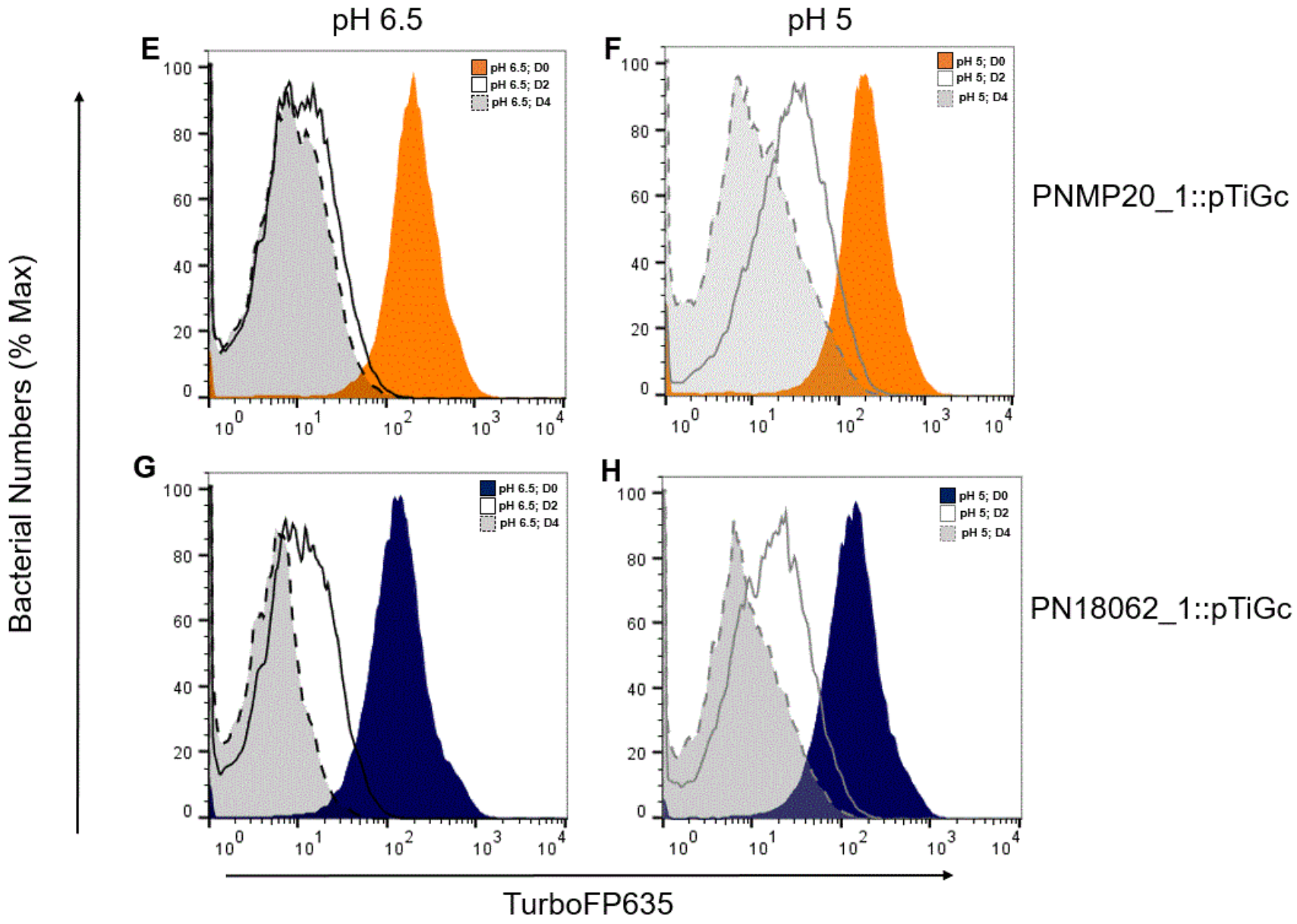
Figure 4.6: Theophylline induction with 2 mM versus 4 mM, respectively. A, C, E, G, & I) An overlay of histogram plots of SAMMtb::pTiGc, BCG::pTiGc PNMP20_1::pTiGc PN18062_1::pTiGc PN18067_1::pTiGc cells in normal media at pH 6.5 and acidic media at pH 4.5 induced with 2mM (theo) on D0. **B, D, F, H, & J)** An overlay of histogram plots of SAMMtb::pTiGc cells, BCG::pTiGc, PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc in normal media at pH 6.5 and acidic media at pH 5, induced with 4mM (theo) on D0. TurboFP635 = red fluorescent protein. D = day. Representative plots of one independent biological repeat are shown (performed with technical triplicates). theo = theophylline. pH 6.5 = control for actively replicating bacteria. pH 5 = cells treated with acid stress.

4.3.5. Differential replication at different pH

Having established pH 5.0 and 4 mM theophylline as preferred stress and induction conditions, these were applied in subsequent assays. To assess replication dynamics under different pH conditions, day 0, 2, 4 histogram plot overlays of cells cultured at pH 6.5 (differential replication dynamics at “normal” pH) versus pH 5 (differential replication at acidic pH), were prepared. The cells cultured in normal media at pH 6.5 were used as a control for actively replicating cells. As expected, SAMMtb::pTiGc, BCG::pTiGc, PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc showed active replication in normal media (Figure 4.7A, C, E, G, and I, respectively). However, the replication differentially varied between the lab strains and clinical strains. SAMMtb::pTiGc demonstrated the smallest fold-reduction in MFI from day 0 to 2, 2 to 4 (Figure 4.7A). BCG::pTiGc had a larger fold-reduction in MFI over time than SAMMtb::pTiGc (Figure 4.7C). The 3 clinical strains demonstrated the largest fold-reduction in MFI from day 0 to 2 (Figure 4.7E, G, and I). This observation is in agreement with growth curve data which show that the lab strains carrying the pTiGc plasmid had a slower replication compared to the pTiGc transformed clinical strains (Figure S7.2).

In contrast to observations of growth in normal media, acid stressed SAMMtb::pTiGc, BCG::pTiGc cells treated at pH 5 showed a reduced decrease in MFI, indicative of a slowing or halt in replication (Figure 4.7B, D). Acid stressed PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc (Figure 4.7F, H, J) also showed a reduced decrease in MFI, relative to cultures in normal media. Differences were observed between the lab and clinical strains. Specifically, SAMMtb::pTiGc and BCG::pTiGc cells had a clear halt in replication (as evidenced by a comparison of day 2 and day 4 plots, Figure 4.7B, D). However, for the clinical strains, a large proportion of the population demonstrated a decrease in MFI from day 2 to day 4.





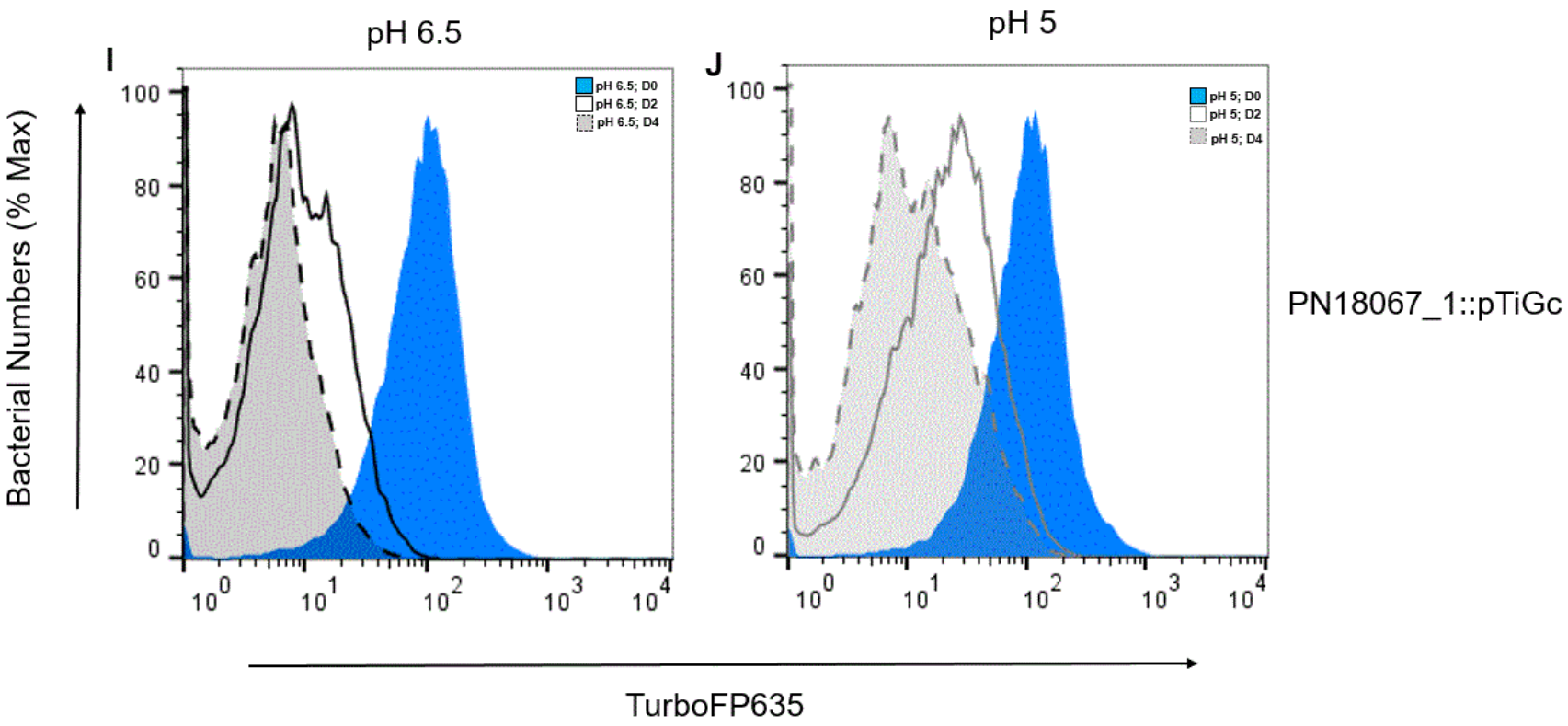


Figure 4.7: Differential replication of SAMMtb::pTiGc, BCG::pTiGc strains, and three clinical strains of *M. bovis* following treatment at pH 5. A, C, E, G, & I) An overlay of histogram plots of SAMMtb::pTiGc, BCG::pTiGc PNMP20_1::pTiGc PN18062_1::pTiGc PN18067_1::pTiGc cells in normal media at pH 6.5 on D0, 2, and 4. **B, D, F, H, & J)** An overlay of histogram plots of SAMMtb::pTiGc cells, BCG::pTiGc, PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc cells in acidic media at pH 5 on D0, 2, and 4. Fluorescence dilution demonstrated differential replication of SAMMtb::pTiGc, BCG::pTiGc, and *M. bovis* clinical strains in normal media at pH 6.5. Mycobacterial strains carrying the pTiGc plasmid were cultured in the presence of 4 mM theophylline, before removal of theophylline and exposure to pH 5 and pH 6.5 until day 4, prior to analyses by flow cytometry. Flow cytometry histograms demonstrated retained TurboFP635 fluorescence intensity in acidic media (pH 5, grey dotted histograms), compared to normal media (pH 6.5, black dotted histograms). The y-axis shows bacterial numbers (%Max), and the x-axis is the TurboFP635 mean fluorescent intensity (MFI). TurboFP635 = red fluorescent protein. D = day. Representative plots of two independent biological repeats are shown (each experiment performed with technical triplicates. pH 6.5 = control for actively replicating bacteria. pH 5 = cells treated with acid stress.

4.3.6. Quantification of persisters

SAMMtb::pTiGc was used as a control strain for the mycobacterial persister assay, as previously described (Mouton et al. 2016). BCG::pTiGc was used as a control for the *M. bovis* persister assay and a comparator to the *M. bovis* clinical strains. Three clinical strains (PNMP20_1::pTiGc, PN18062_1::pTiGc, and PN18067_1::pTiGc) were used to assess and quantify persisters at pH 5. The quantification of “VBNR” cells was performed using day 4 cultures, where some slowing/arrest of growth was observed. The “VBNR” cells were gated as described in section 4.2.4. This gating was based on the assumption that day 4 cells that fell in the upper 50% of the day 0 plot showed a slowed or arrested growth; these cells showed retention of high red fluorescence indicative of slowed/halted replication, and this subpopulation is likely enriched for “VBNR” cells. The quantification of the VBNR population that retained TurboFP635 fluorescent signal above day 0 MFI was calculated as the mean percentage ± SD.

SAMMtb::pTiGc had the highest subpopulation of the “VBNR” population with a mean percentage of 12.2 ± 1.5% (± SD) (Figure 4.9A). The second highest was BCG::pTiGc cells treated with pH 5, 7.2 ± 0.6% (Figure 4.9B). Amongst the clinical strains, PN18067_1::pTiGc had the highest VBNR subpopulation with a mean percentage of 1.3 ± 0.1% (Figure 4.9E; Figure 4.8). On the other hand, strains PNMP20_1::pTiGc (Figure 4.9C), and PN18062_1::pTiGc (Figure 4.9D) treated at pH 5 had similar subpopulations of VBNR cells with a mean percentage of 0.2 ± 0.0% and 0.2 ± 0.0%, respectively (Figure 4.8).

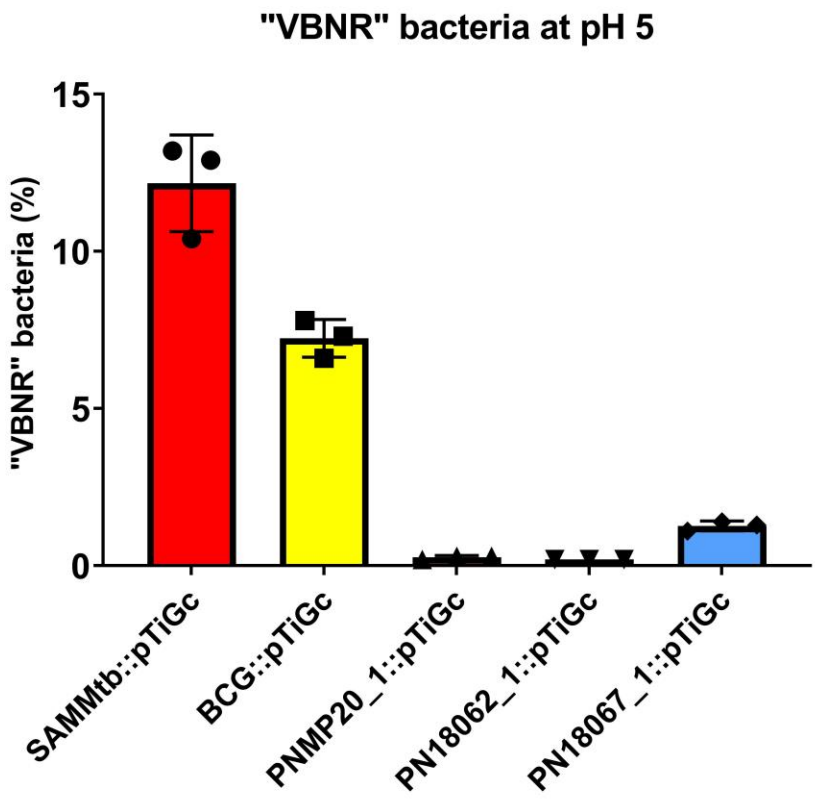
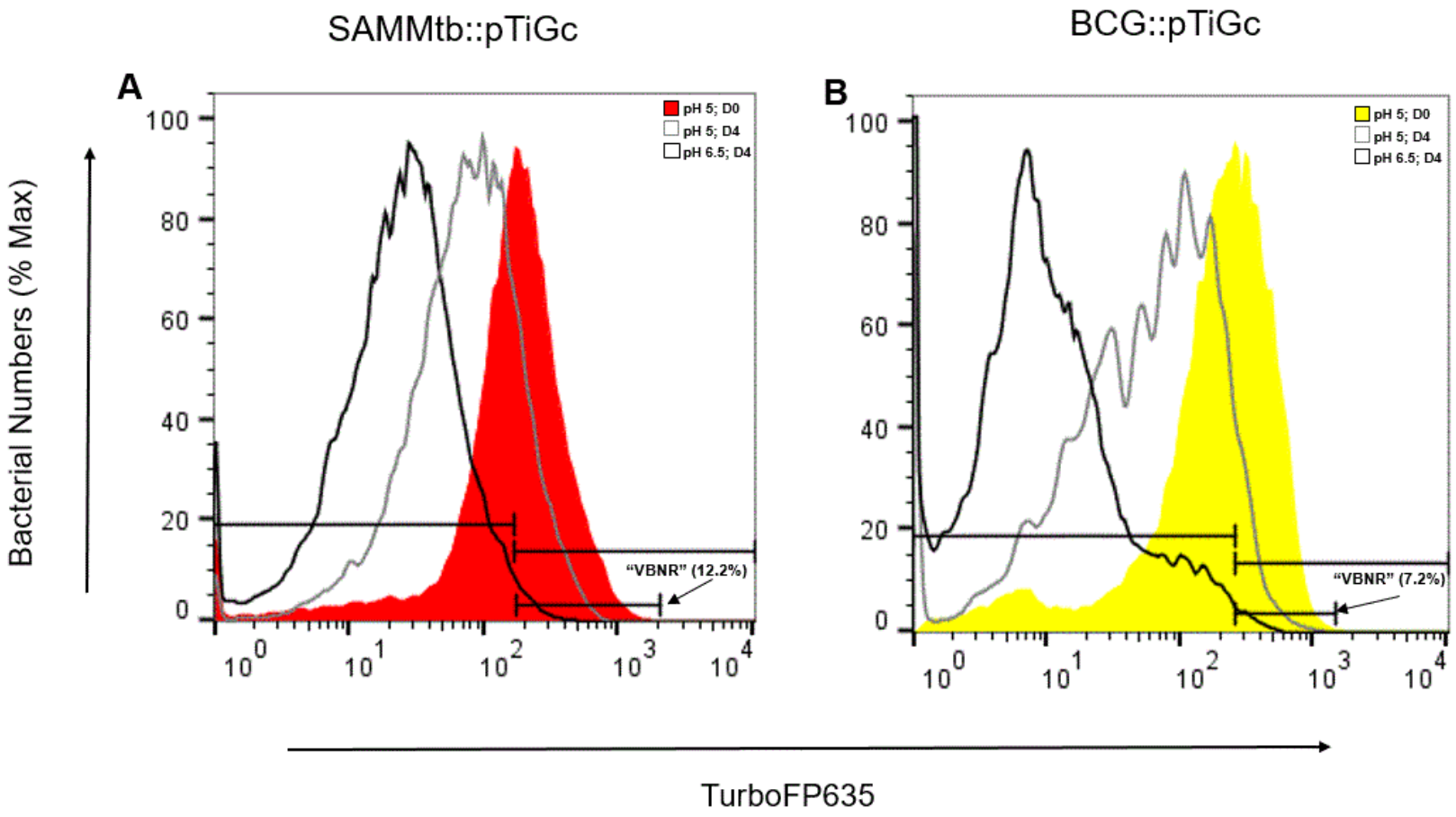
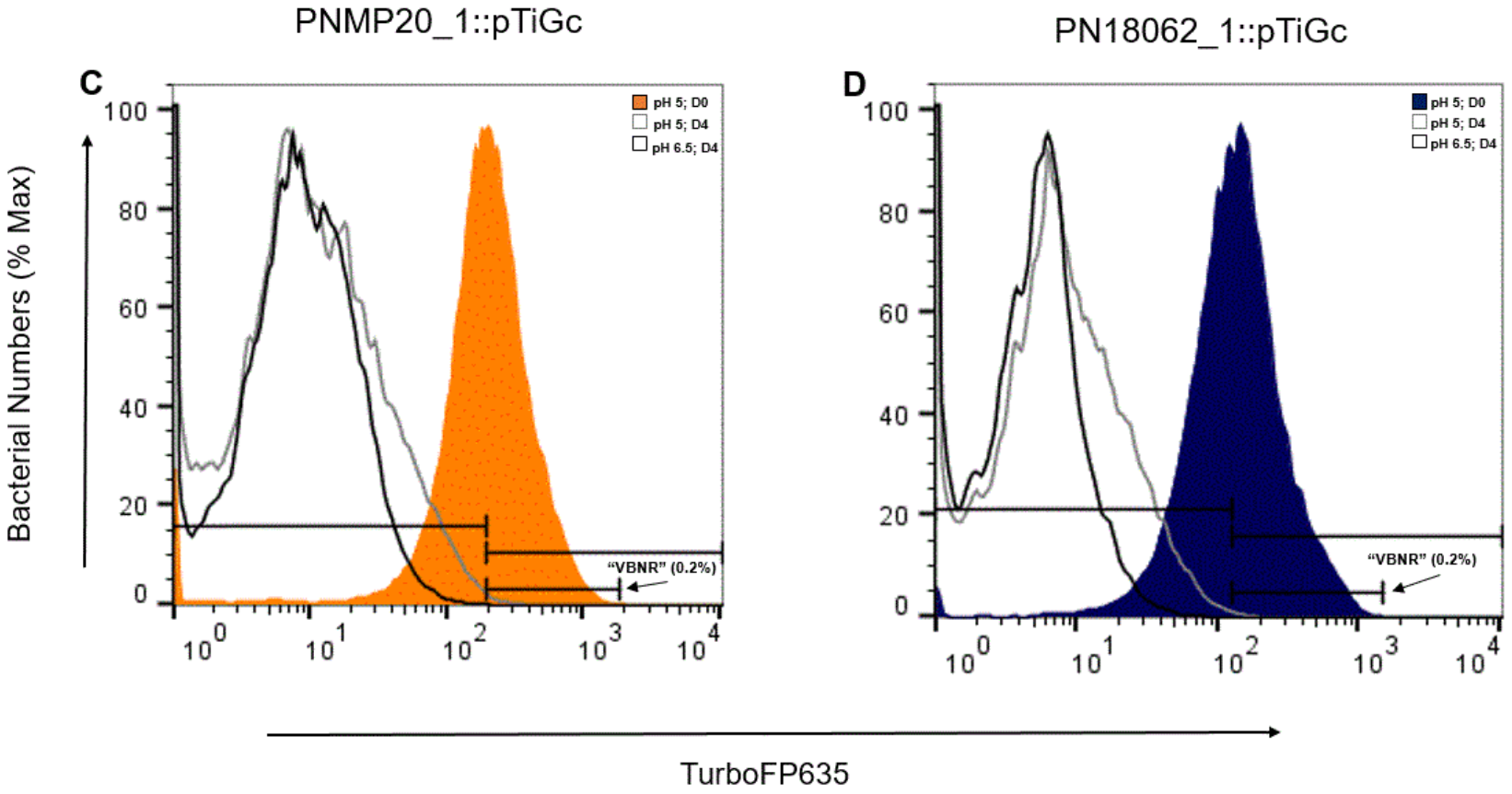


Figure 4.8: The "VBNR" percentages of SAMMtb::pTiGc, BCG::pTiGc strains, and three clinical strains of *M. bovis* induced with 4 mM theophylline following treatment with pH 5 on day 4. "VBNR" = viable but non- or slowly replicating bacteria.





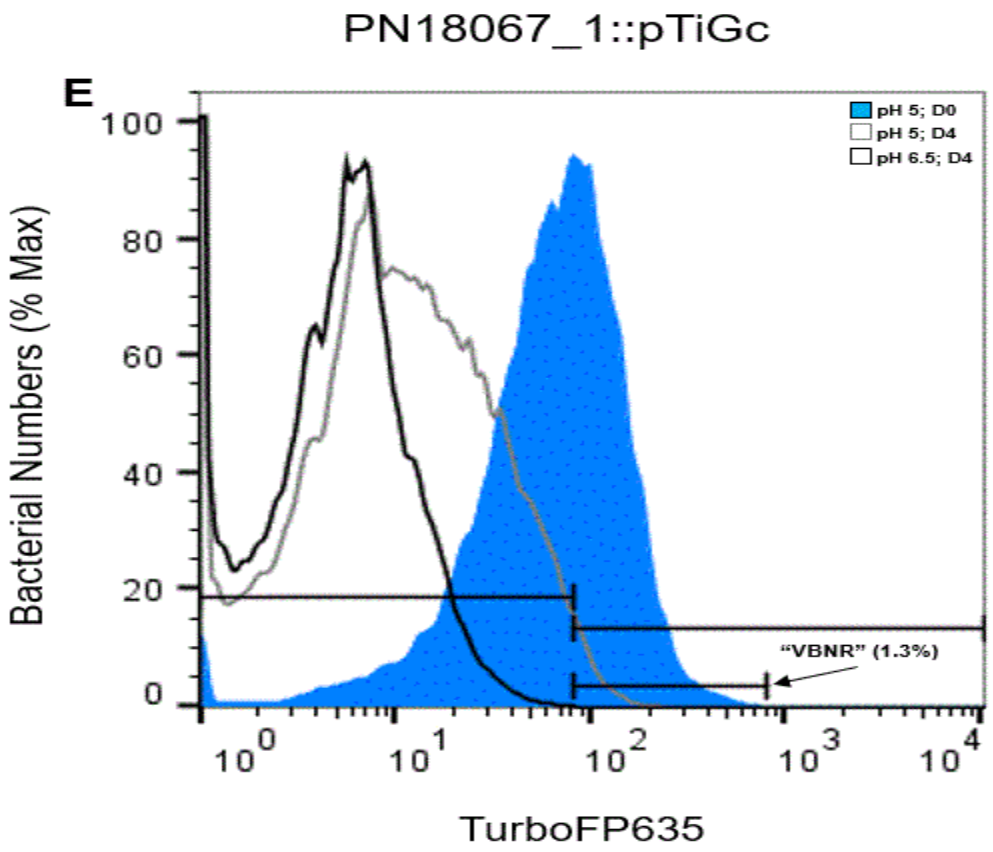


Figure 4.9: Quantification of "VBNR" cells of lab strains and three clinical strains following acid stress at pH 5. A) SAMMtb::pTiGc. **B)** BCG::pTiGc. **C)** PNMP20_1::pTiGc. **D)** PN18062_1::pTiGc. **E)** PN18067_1::pTiGc. Fluorescence dilution demonstrates differential persister formation of SAMMtb::pTiGc, BCG::pTiGc, and three *M. bovis* clinical strains under acid stress. *M. bovis* strains containing pTiGc was cultured in the presence of 4 mM theophylline, before removal of theophylline and exposure to pH 5 and pH 6.5 until day 4, respectively, before analyses by flow cytometry. Flow cytometry histograms demonstrate retained TurboFP635 fluorescence intensity in acidic media (pH 5, grey), compared to control media (pH 6.5, black). The y-axis shows bacterial numbers (%Max); the x-axis is the TurboFP635 mean fluorescent intensity (MFI). D = Day.

4.4. Discussion

The objective of this component of the study was to phenotypically characterize *M. bovis* persister formation in isolates from South African wildlife. Specifically, we characterized whether *M. bovis* isolates had the potential to form viable, but non-replicating subpopulations thought to harbour persister bacteria. This is a pre-requisite to gain insights into whether "true" latent *M. bovis* infections in animals exist as previously observed with *M. tuberculosis* in humans (Dutta and Karakousis 2014). Five representative *M. bovis* strains carrying the pTiGc reporter plasmid were utilized in this work. An *in vitro* acid stress model was applied and used as proof of concept to determine whether *M. bovis* exhibited persister formation. The *in vitro* acid stress model has previously been applied by others (Portaels and Pattyn 1982; Vandal et al. 2009; García et al. 2018) and in our research group and has been shown to enrich for viable but slowly or non-replicating ("VBNR") subpopulations of *M. tuberculosis* (Mouton et al. 2016; Mesatywa 2019, unpublished; Mouton et al. 2019). Furthermore, the acid stress model has been shown to mimic the macrophage phagolysosome microenvironment encountered by pathogenic mycobacteria upon infecting the host (Ehrt and Schnappinger 2009). In this study, *M. bovis* strains carrying the pTiGc were cultured, induced with theophylline, treated with acid stress, and investigated for persister formation as well as replication dynamics through flow cytometry. Flow cytometry is a powerful tool that can measure the viability and replication dynamics of bacterial populations at the single-cell level and has been extensively applied to study persisters and mycobacterial replication dynamics (Parbhoo et al. 2020).

4.4.1. Application of dual fluorescent replication reporter system in *M. bovis*

The FD reporter system is a reliable tool that quantifies and measures mycobacterial replication (Mouton et al. 2016). This reporter system relies on two reporters; GFP as a marker for live bacteria and TurboFP635 as a marker for bacterial replication (Mouton et al. 2016). This study showed that the TurboFP635 reporter was sufficiently bright, tightly regulated, and stable under acidic conditions, demonstrating that it is suitable to be a reliable marker of *M. bovis* quantification and replication dynamics. Furthermore, the FD reporter provided a reliable quantitative measure of *M. bovis* replication comparable to commonly used methods such as OD measurements which is in agreement with a previous mycobacterial study (Mouton et al. 2016). Work reported in this thesis describes the first use of the dual fluorescence replication reporter system in *M. bovis*. Due to time constraints, our phenotypic investigations focused on

only a subset of the recombinant strains generated, but we now have a library of 22 *M. bovis* reporter strains that could be exploited in future studies.

4.4.2. Differential susceptibility to low pH

It has been well described that *M. tuberculosis* can survive stressful host environments (Wayne and Sohaskey 2001). Despite the various stressors exerted on the pathogen, it can adapt and survive through the upregulation of numerous mechanisms such as the DosR regulon (Chao and Rubin 2010). Acid stress is one of the key stresses that the pathogen encounters as a part of the host defense in the macrophage phagolysosome microenvironment and has been shown to favor persister formation (Richter and Saviola 2009). In this study, the effect of low pH on *M. bovis* was assessed prior to the quantification of persisters.

4.4.2.1. *M. bovis* is sensitive to a pH of 4.5

As expected, this study found that lab and clinical *M. bovis* strains cultured on normal media at pH 6.5 had high mean percentages of live cells (Figure 4.2; Figure 4.4), and were actively replicating over 4 days of culture (Figure 4.9). Consistent with this finding, optimal growth between pH 5.8 and pH 6.5 has previously been documented for *M. bovis* and *M. tuberculosis* (Portaels and Pattyn 1982). In contrast, lab and clinical *M. bovis* strains cultured at pH 4.5 on day 4 had differential susceptibilities to acid stress (Figure 4.2), with substantial cell death observed for both lab and clinical strains, demonstrating that low pH has a negative effect on the growth and viability of these mycobacteria (Portaels and Pattyn 1982).

Notably, SAMMtb::pTiGc appeared more tolerant to acid stress than BCG::pTiGc and clinical strains of *M. bovis* (Figure 4.2). This observation is consistent with previous studies that found *M. tuberculosis* was acid stress-tolerant (Vandal et al. 2009; Mesatywa 2019, unpublished; Mouton et al. 2019). However, these studies did not perform a comparison with BCG or clinical *M. bovis* strains, making this study the first to do such a comparison. Moreover, this finding may also partially explain the apparent differences in *M. bovis* and *M. tuberculosis* virulence in animals compared to that of humans (Bigi et al. 2016). Additionally, proteomic results previously revealed that SAMMtb seems to have a heightened baseline stress response (Mouton et al. 2019), further exacerbating the stress response added to acidic pH 4.5. Furthermore, upon exposure to acid stress of pH 4.5, it was demonstrated that at day 2 SAMMtb had a halt in replication compared to *M. tuberculosis* H37Rv (Mouton et al. 2019), confirming its ability to

readily enter a VBNR state under unfavorable conditions. Another study found that the growth of SAMMtb was inhibited at pH 4.5 over 18 days, suggesting its ability to enter a non-replicating state (Mesatywa, unpublished 2019).

BCG::pTiGc was found to be more susceptible to pH 4.5 compared to SAMMtb::pTiGc (Figure 4.2). Although SAMMtb and BCG are both attenuated lab strains (Sampson et al. 2004; Mouton et al. 2019), these strains may respond to stress differently due to their genomic differences. Furthermore, immune responses to BCG may be altered in comparison to *M. tuberculosis* because this model organism encodes the known virulence factors such as factors early secreted antigenic target-6 kDa (Behr et al. 1999), culture filtrate protein-10 kDa (Fortune et al. 2005), and contains a natural RD 1 deletion (Behr et al. 1999). Bigi et al. (2016) reported polymorphisms in 20 regulatory proteins and identified differential expression of genes related to hypoxia between *M. bovis* and *M. tuberculosis*. These differences further highlight that *M. bovis* and *M. tuberculosis* may have different mechanisms that make them differentially respond to stress. A previous study found that wildtype BCG was sensitive to pH 4.5 (Gallant et al. 2016).

M. bovis clinical strains were more sensitive to pH of 4.5 than the two lab strains. BCG can adopt intracellular pH homeostasis which is key to survival at acidic pH (Rao et al. 2001), which may suggest its tolerance to acid stress compared to *M. bovis* clinical strains. Moreover, differential susceptibilities of different *M. bovis* strains to acid stress were observed with PN18078::pTiGc being the least sensitive to stress (Figure 4.2). These differential susceptibilities to pH 4.5 could be attributed to strain differences, with some strains being phenotypically fit to survive acid stress. However, few studies have applied an *in vitro* acid stress model to evaluate how it impacts the survival of pathogenic mycobacteria (Portaels and Pattyn 1982; Mouton et al. 2019). Therefore, studies must be conducted to assess why *M. bovis* clinical strains are differentially sensitive to pH 4.5.

4.4.2.2. *M. bovis* is tolerant to a pH 5

SAMMtb::pTiGc, PNMP20_1::pTiGc, and PN18062_1::pTiGc seemed to be similarly tolerant to pH 5, with PN18067_1 being the least tolerant amongst the clinical isolates (Figure 4.4). This finding is in contrast to the pH 4.5 observations whereby clinical strains substantially lose viability (Figure 4.2), thus indicating the lethal effects of pH 4.5 compared to pH 5. Surprisingly, BCG::pTiGc was more sensitive to pH 5 compared to all clinical strains (Figure 4.4). This observation contradicts those made at pH 4.5 where BCG seemed to be more tolerant to acid stress compared to clinical strains (Figure 4.2). It could be speculated that since there are

several regions deleted from BCG, one of these could have a protective role to pH 5 (Garnier et al. 2003), however, these observations need further investigation.

4.4.3. Optimal theophylline induction

Theophylline is a drug used to treat numerous respiratory conditions such as chronic obstructive pulmonary disease and asthma (Barnes 2006). It has also been reported to treat infant apnea and anosmia (Barnes 2010). Theophylline is derived from a purine derivative methylxanthine that can stimulate the central nervous system, cardiac, diuretic, and bronchial dilation activities (Barnes 2006; Barnes 2010). It is also naturally present in small amounts of cocoa beans and tea (Jilani et al. 2020). The concentrations applied in persister assay experiments are relatively low and are not expected to have a negative effect on the growth of bacteria (Mouton et al. 2016; Mouton et al. 2019). Furthermore, theophylline was washed from the cultures before acid stress experiments, thus minimizing the chance of an additional fitness cost to the bacteria during acid stress. However, in the future, studies can be conducted to confirm this.

This study initially applied 2 mM concentrations to induce the TurboFP635; however, this was not optimal for induction of both lab and clinical strains. Although in a previous study a 2 mM concentration was applied for *M. smegmatis* and SAMMtb (Mouton et al. 2016), it did not yield a similar result in our study. One possible explanation for this is that the previous study induced in shaking cultures, while the current study had to use static cultures for logistical reasons. To improve the level of induction, we tested 4 mM theophylline, which was found to increase TurboFP635 expression for lab and clinical strains.

4.4.4. Replication dynamics of *M. bovis* upon acid stress reveal population heterogeneity

The FD reporter system is a reliable tool for the detection of mycobacterial replication dynamics at the single-cell level (Mouton et al. 2016; Parbhoo 2017, unpublished; Mouton et al. 2019). In this study, we applied this reporter system to assess *M. bovis* replication dynamics upon acid stress. Our results revealed differential replication dynamics of different *M. bovis* strains at different pH conditions, as previously shown in other mycobacterial studies (Mouton et al. 2016). Our findings showed that heterogeneous populations emerged upon acid stress as revealed by a shift from symmetrically distributed histogram plots, to more asymmetric populations with a mixture of actively replicating (indicated by lower Turbo MFI) and non- or slowly replicating populations (indicated by a retained high Turbo MFI) from day 0 to day 4 (Figure 4.7). This

observation is consistent with a previous study performed on *M. smegmatis* and *M. tuberculosis* (Mouton et al. 2016; Parbhoo 2017, unpublished) where bacterial cells recovered from infected macrophages had heterogonous mixtures of actively and non-replicating cells.

4.4.5. Persister formation and adaptation to low pH

Upon infection with a pathogen, the host immune responses either clear the pathogen or result in a spectrum of disease outcomes (Dutta and Karakousis 2014; Drain et al. 2018). Latent *M. tuberculosis* has been documented where the pathogen can persist and adapt to the host immune response without causing clinical symptoms (Barry et al. 2009; Dutta and Karakousis 2014), however, it is not clear whether *M. bovis* has a similar ability.

Bacteria are likely to exist in heterogeneous phenotypic states *in vivo*, including differentially replicating populations. This heterogeneity is promoted by exposure to stressful conditions encountered upon infection and used as a survival strategy in the host (Manina et al. 2015). As the host fights off the infection, it is believed that there is the development of heterogeneous bacterial populations. These may include differentially replicating bacterial populations, from actively replicating bacteria to non- or slowly replicating subpopulations (VBNR), which are thought to be enriched for persister mycobacteria and may underlie latent TB infections (Mouton et al. 2016).

Expectedly, this study found that both lab strains and clinical strains had mixtures of differentially replicating bacteria from actively replicating bacteria to halted bacteria (as an indication of adaptation or tolerance to acid stress). This finding is consistent with previous studies that applied the FD reporter system to *M. smegmatis* and SAMMtb which found that there was a mixture of actively replicating and VBNR bacteria upon macrophage infection (Mouton et al. 2016) and upon acid stress on *M. tuberculosis* H37Rv and SAMMtb (Mouton et al. 2019). However, this is the first exploration of persister formation in *M. bovis* using the pTiGc reporter and paves the way for future studies.

Furthermore, it was found that SAMMtb::pTiGc was the most tolerant to acid stress compared to BCG::pTiGc and clinical strains at pH 5 on day 4 (Figure 4.9), with the largest mean subpopulation. This may be attributed to its auxotrophic nature and genomic differences between *M. bovis* and *M. tuberculosis* which may include capabilities to compensate for multiple stressors in addition to acid stress (Mouton et al. 2019), leading to more cells being tolerant to stress than BCG and clinical strains. Moreover, the tolerance of SAMMtb::pTiGc to acid stress

may be due to its lipid-rich cell wall that maintains a homeostatic balance between the internal and external environments of the cells (Vandal et al. 2009). This cell wall acts as the main barrier to prevent photons from entering the cells, protecting them against external environmental stressors such as acidic pH (Tran et al. 2005), antibiotics, osmotic lysis and allows survival in macrophages. Furthermore, it has been shown that, upon harsh conditions, *M. tuberculosis* growth is arrested and there is an upregulation of lipid biosynthesis genes which alter cell wall structure (Deb et al. 2009; Hussain et al. 2009), further allowing it to persist.

BCG::pTiGc cells had the second highest VBNR mean percentage, demonstrating that BCG may exploit different stress response pathways that confer acid tolerance compared to SAMMtb::pTiGc and the clinical strains (Rodriguez et al. 2008; Gideon et al. 2010). Amongst the clinical isolates of *M. bovis,* PN18067_1::pTiGc had the highest VBNR subpopulation, while PNMP20_1::pTiGc and PN18062_1::pTiGc had similar, very small, subpopulations of VBNR cells (Figure 4.8), demonstrating that *M. bovis* clinical strains may have the differential abilities to form “VBNR” subpopulations. This finding may suggest why some animals may clear the infection, while some could be latently infected, and while others may readily progress to active disease depending on the strains that infected the animals.

In summary, these data suggest that upon acid stress: (i) laboratory strains seem to have a higher predisposition to form VBNR populations than the three clinical strains investigated, (ii) *M. bovis* may exhibit VBNR populations following acid stress, although these are in very low percentages, (iii) VBNR formation may vary depending on strain genotype. Nevertheless, it is important to acknowledge that the VBNR populations detected under the selected conditions in this study were very small (0.5 ± 0.6 %), and may not be indicative of significant persister formation. Therefore, this study has not conclusively demonstrated *M. bovis* persister formation, but it is noted that a single stressor, a single time point, and only 3 clinical isolates were investigated, leaving this an open question for further investigation.

4.5. Conclusion

M. bovis and other pathogenic mycobacteria often find themselves in adverse pH conditions and need to adapt to and combat these stressors to survive. The answer on whether *M. bovis* latent infections exist, as observed in *M. tuberculosis,* is elusive, hence this study aimed to characterize *M. bovis* persisters as a proxy to get insights on whether true latency exists in animals. Although it has previously been a challenge to study persisters, the FD reporter system

has made it easier to study the replication dynamics and characterize the phenotypic nature of mycobacteria that encounter stressful environments. This is the first study to investigate *M. bovis* persister formation upon *in vitro* acid stress using flow cytometry. Our data suggest that acid stress of different mycobacterial strains results in differential sensitivity as well as differential persister formation, which could be due to the difference in genomic backgrounds of the mycobacteria.

This study demonstrates the first successful application of the novel dual fluorescence replication reporter system in *M. bovis.* This provides a valuable resource for future use together with *in vitro* or *in vivo* models to offer insights into the development and physiology of persisters. It is important to understand the physiological states of *M. bovis* persisters and their role in latent infection which may have important implications for TB diagnosis and management in animals and humans.

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Chapter 5

General discussion, limitations, future recommendations

5.1. General discussion

Animal TB is a worldwide challenge that is continuously being addressed (Gormley and Corner 2018a) and has numerous negative impacts on animal welfare, financial loss to animal owners, agricultural sectors, and the economy (Ayele et al. 2004; Carruth et al. 2016). Animal TB is primarily caused by *M. bovis* (Kaneene et al. 2010) and in a few cases by *M. tuberculosis* (Landolfi et al. 2015). *M. tuberculosis* has the ability to persist in a latent state and can also become antibiotic tolerant and lead to disease recurrence through specific physiological and metabolic changes (Rifat et al. 2009; El-Baky 2016; Pai et al. 2016; Gold and Nathan 2017). However, there is uncertainty if *M. bovis* can persist in a latent state (Capparelli et al. 2013). Few studies have investigated latency in animals but rather focused on improving diagnostic tools for different animal species and transmission at herd or ecosystem levels (Miller et al. 2015; Pereira et al. 2020).

Transmission of *M. bovis* has been observed between animal species (Sichewo et al. 2020b), from animals to humans (Cleaveland et al. 2007; Olea-Popelka et al. 2017; van den Brom et al. 2020), as well as being found in the environment (Fine et al. 2011; Adams et al. 2013; Ghodbane et al. 2014; Rodríguez-Hernández et al. 2016). As such, wildlife and livestock are constantly in contact with each other and their environments, thus increasing TB transmission risk (Hang'ombe et al. 2012; Sichewo et al. 2020c; Sichewo et al. 2020b). This has led to a greater appreciation of the interconnectedness of animals, humans, and the environment, emphasizing the importance of studying TB using the multidisciplinary "One Health" approach at animal-human-environment interfaces (Zumla et al. 2020; Kelly et al. 2020). Although extensive research has been previously generated on TB in humans (both active disease and latency) (Drain et al. 2018), there are still key knowledge gaps that exist in animal TB, particularly on the existence of latency.

Challenges that exist in animal TB include: (i) that animals that test positive for TB are usually culled without knowledge of the infection state or the infectiousness of the individual animal, (ii) *M. bovis* latent infections are understudied but rather speculated on, and (iii) lack of diagnostic

tests that can distinguish between infection states (Meiring et al. 2018; Wang et al. 2019). Furthermore, animals such as wild dogs are considered endangered species (Woodroffe et al. 2007; Miller et al. 2012; Miller 2015) and there are ethical and conservation priorities that may conflict with disease management programs, which usually involve culling of infected animals. Therefore, it's important to study latent infections in animals and the risk of reactivation and transmission in individuals that may be found to be latently infected. As with differing management of latent and actively infected humans, better targeted management and control programs can be devised to control the spread of TB, save endangered animals, and reduce negative economic consequences for animal owners with an improved understanding of *M. bovis* pathogenesis.

Previous studies have suggested that *M. bovis* may have the ability to cause recurrence in humans due to *M. bovis* infections (Dankner et al. 1993; Palacios et al. 2016; de Aguiar et al. 2020), however, these findings are inconclusive and do not address latency. Also, *M. bovis* infections in humans are understudied due to potential misdiagnosis as *M. tuberculosis,* since these two strains are closely related and present similar symptoms in humans (Adugna et al. 2014). On the other hand, animals such as elephants and mongooses also get infected with *M. tuberculosis* (Alexander et al. 2002; Landolfi et al. 2015), however, there have not been latency studies reported in these animals. In our study, *M. bovis* clinical isolates were obtained from different animal species, highlighting a layer of complexity when dealing with animal TB, with a wide range of hosts compared to a single species in humans, in which most studies have been performed. Therefore, in the future, studies would need to investigate how *M. tuberculosis* infections and pathogenesis in animals and humans differ from those of *M. bovis* to get insights on host responses, infection states, and infection outcomes.

In a recent review, Garcia et al. 2020 discussed the latency question in animals and concluded that *M. bovis* was unlikely to cause latent infections compared to *M. tuberculosis* by using cattle as the model for other animals. In our review that is soon to be published (chapter 2), we broadened the scope of this question by interrogating the following: i) *M. bovis* infection outcomes of other animal hosts (besides cattle), such as wildlife, (ii) examining how different *in vitro* and *in vivo* models could be harnessed to address questions on latent *M. bovis* infection, (iii) highlighting the inter-related states of LTB, dormancy and persister mycobacteria and how these need to be considered in future latent *M. bovis* research, (iv) evaluating how a combination of antemortem and postmortem diagnostic tools could provide insights into LTB in wildlife, (v) proposing host-pathogen biomarkers that may be applied in distinguishing the wide

spectrum of different *M. bovis* infection outcomes, and vi) offering a way forward to tackle LTB studies in the future. Filling knowledge gaps on LTB in animals will positively influence management strategies and bTB control. Due to large knowledge gaps that exist on this topic than evidence, no conclusions were drawn on whether latency exists with animals but rather recommendations for future studies were made.

To build a foundation for this topic, this study aimed to further examine latent *M. bovis* in animals, particularly in wildlife. This was achieved by characterization of *M. bovis* persisters from samples collected from a variety of wildlife species (wild dogs, warthogs, buffaloes, and lions) from various South African public and private game parks. In preparation for persister assay experiments, samples were decontaminated, purified, genotyped to strain level, transformed with the FD reporter plasmid, and thereafter assessed for persisters. Five *M. bovis* strains were identified through spoligotyping and these genotypes were successfully transformed with the FD reported plasmid, suggesting that strain differences did not hamper transformation with the FD reporter system. The FD reporter plasmid was effectively applied and provides a valuable tool to quantify persisters and examine replication dynamics of *M. bovis* under different conditions. The collection of 22 well-characterized reporter strains of *M. bovis* generated in this study have been stored in a host-pathogen laboratory biobank and will provide a useful resource for several future studies.

In this study, we focused on a single stress condition, namely acid stress. Upon performing acid stress sensitivity experiments, this study found that *M. bovis* clinical isolates appeared to be more acid-stress sensitive than lab-adapted strains at pH 4.5. This may be due to genomic differences (Bigi et al. 2016; Dong et al. 2017) and may explain what is observed clinically with *M. bovis* infection whereby infections seem to be either cleared or progress to active disease. This finding may further infer that if an individual animal has a lethal acidic macrophage host response of pH 4.5, it may more easily clear *M. bovis* infection more readily than with *M. tuberculosis,* however, this may also depend on the animal's immune competency.

In addition to differential acid stress sensitivity, persister assay experiments indicated that there was differential persister formation amongst lab strains, between the lab and clinical strains, and amongst the clinical strains at pH 5. SAMMtb::pTiGc and BCG::pTiGc strains had the highest "VBNR" percentages of 12.2 ± 1.5% and 7.2 ± 0.6%, respectively. Previous studies have found that SAMMtb::pTiGc has a more pronounced baseline stress response than *M. tuberculosis* H37Rv, and is more likely to halt replication (i.e. form VBNR cells) upon exposure to acid stress.

SAMMtb may therefore be more likely to enter a persister state upon exposure to stress conditions than other strains/species (Mouton et al. 2019).

It also appeared that different *M. bovis* strains may have differential abilities to form a "VBNR" subpopulation of bacteria upon *in vitro* acid stress model at pH 5, while some may have similar responses. VBNR differences between the lab strains and clinical strains were observed. Two strains with similar VBNR were PNMP20_1::pTiGc (strain from warthog sample), and PN18062_1::pTiGc (strain from buffalo sample). While PN18067_1::pTiGc (strain from lion sample) had the highest VBNR mean percentage. However, the persister percentages for clinical isolates were very low (mean percentage of (0.5 ± 0.6 %) and precluded interpretation that *M. bovis* has the ability to form persisters, leaving this question open-ended for future investigation. Since the acid stress model is a single stress model, it does not represent the multiple stresses that the pathogen encounter upon infection. Therefore, there is a possibility that persister formation may occur more readily when multi-stress models such as the macrophage infection model are applied. Multi-stress models that recapitulate the host immune responses may therefore offer clarity on this question. Three clinical *M. bovis* strains were used to quantify persisters, so in the future, more strains with more biological repeats should be investigated to confirm findings from this study. Furthermore, BCG and clinical strains were incubated non-shaking during the persister assay experiments and it would be interesting to investigate how shaking conditions may affect VBNR formation.

In summary, this study found evidence that different *M. bovis* strains may have differential capacities to form VBNR bacteria. However, due to the low VBNR percentages observed, evidence of persister formation was not concretely determined. This is a novel finding that leaves open-ended questions that require further investigation. While the *in vitro* acid stress model may not recapitulate what happens in natural infection, it gives some insights on how *M. bovis* may respond to harsh environments such as those encountered during the host immune response.

5.2. Limitations

Due to the COVID-19 pandemic, there was a significant delay in laboratory work and the time available for completing experiments was reduced, thus reducing planned outputs. Therefore, ideally, the *M. bovis* persister assay experiments could have been performed in three biological replicates, however, due to COVID-19 challenges, only one biological replicate per strain was

performed. Hence, in the future, additional biological repetitions are needed to corroborate our findings. It was also planned that five strains were to be used to quantify persisters, however only three of the five were used, and so, in the future, at least the additional two strains can be included for quantification.

The BSL3 facility did not have a sufficiently large shaking incubator to accommodate the number of samples that were included in each experiment, thus for logistical reasons, SAMMtb, BCG and clinical strains were cultured non-shaking during growth curve and persister assays experiments. Since, *M. bovis* is an obligate aerobe that requires oxygen for survival, the impact of culturing the strains non-shaking versus shaking is unknown and may need further investigation.

5.3. Future studies

Future studies need to investigate the following:

- Multi-stress *in vitro* models such as a macrophage infection model or *in vivo* models such as murine models may need to be applied in the future since they may more closely mimic what happens to *M. bovis* upon infecting the host.
- Whole-genome sequencing (WGS) may be applied to investigate *M. bovis* strain differences to get additional insights on whether differential acid stress sensitivity or VBNR formation could be due to strain variation or not.
- Investigate direct detection of VBNR cells by methods such as *ex vivo* staining for lipid bodies (a signature of persisters) as a way to provide better detection and differentiate *M. bovis* persisters in different infection states.
- Develop blood-based biomarkers to investigate *M. bovis* infection states.
- Determine if animals with latent infection can progress to active disease like humans.
- Determine the infectiousness of animals with latent or sub-clinical disease.

Chapter 6

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Chapter 7

Appendices

7.1. Fluorescence Dilution reporter plasmid concentrations and absorbance ratios

Table S7.1: Fluorescence Dilution reporter plasmid concentrations and absorbance ratios.

Culture	pTiGc	Absorbance	
	Concentration (ng/µl)	A260/280	A260/230
1	253.6	1.82	1.12
2	230.8	1.81	1.10
3	205.5	1.60	0.66
4	184.0	1.73	0.91
5	165.3	1.72	0.66
6	137.3	1.77	0.89
7	136.2	1.71	0.77
8	126.1	1.70	0.81
9	110.8	1.66	0.74

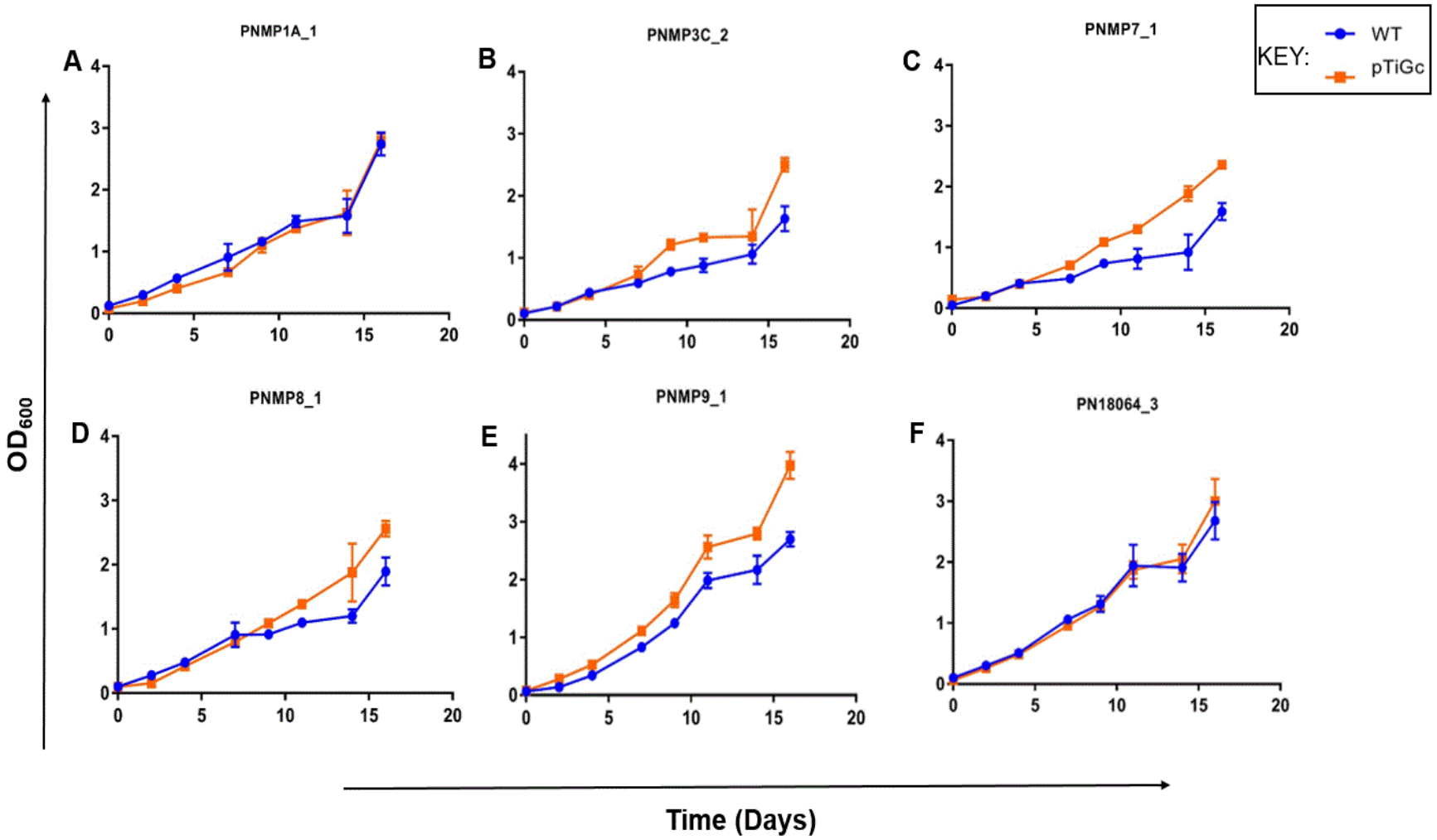
7.2. Colonies observed after transformation of BCG and clinical strains

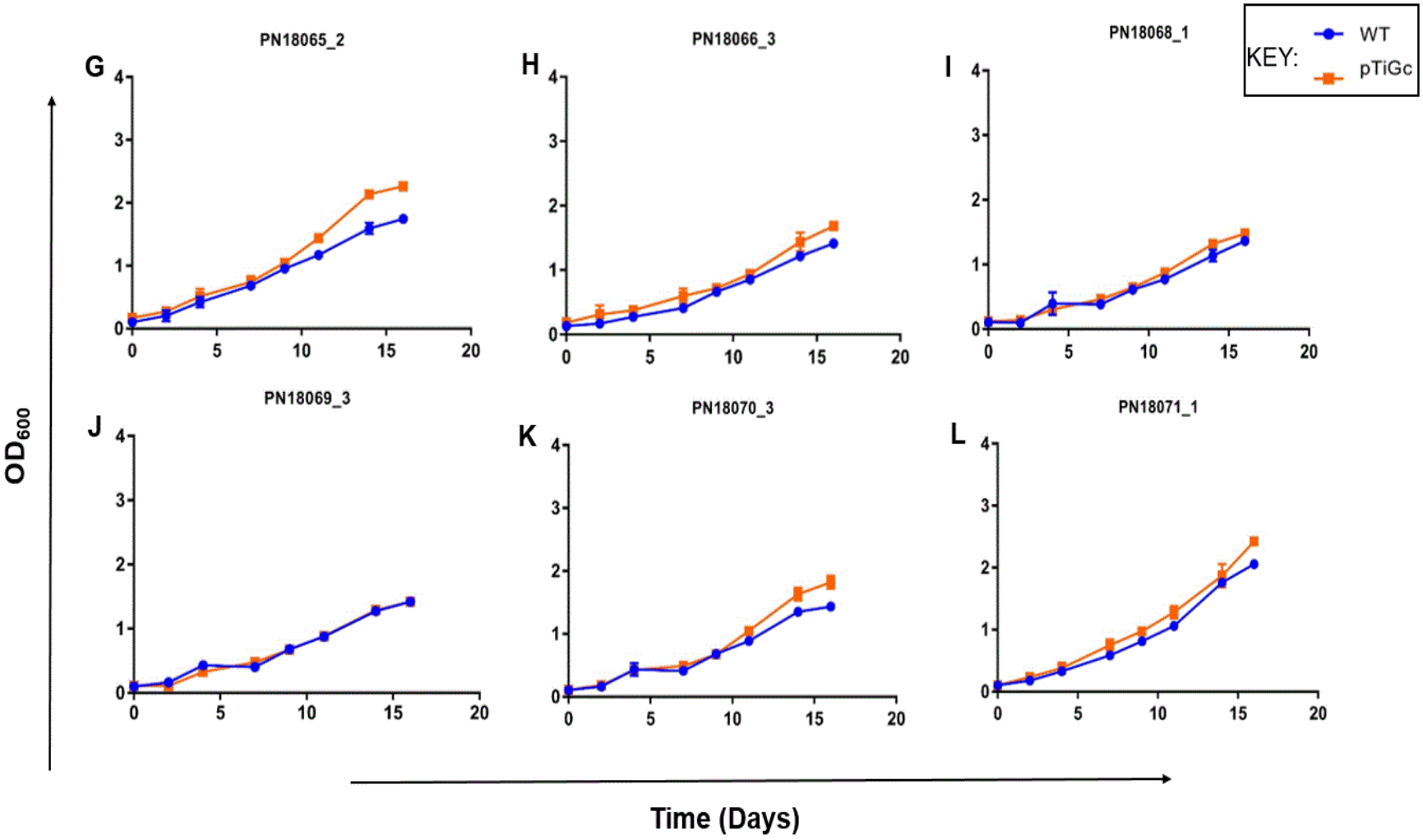
Table S7.2: Colonies observed after transformation of BCG and clinical strains.

Culture ID	Control (No DNA cells)	Undiluted
BCG	TNTC	10
PNMP1A_1	None	6
PNMP3C_2	1	16
PNMP7_1	None	4
PNMP8_1	None	3
PNMP9_1	None	10
PNMP20_1	None	9
PN18062_1	None	30
PN18064_3	None	2
PN18065_2	None	1
PN18066_3	None	7
PN18067_1	None	15
PN18068_1	1	68
PN18069_3	1	19
PN18070_3	None	54
PN18071_1	None	250
PN18072_3	None	20
PN18073_2	None	TNTC
PN18073_3	None	9
PN18075	3	6
PN18076	None	None
PN18077	None	12

TNTC = too numerous to count. Undiluted = 200 µl

7.3. Wildtype vs pTiGc transformed growth curves of clinical *M. bovis* strains





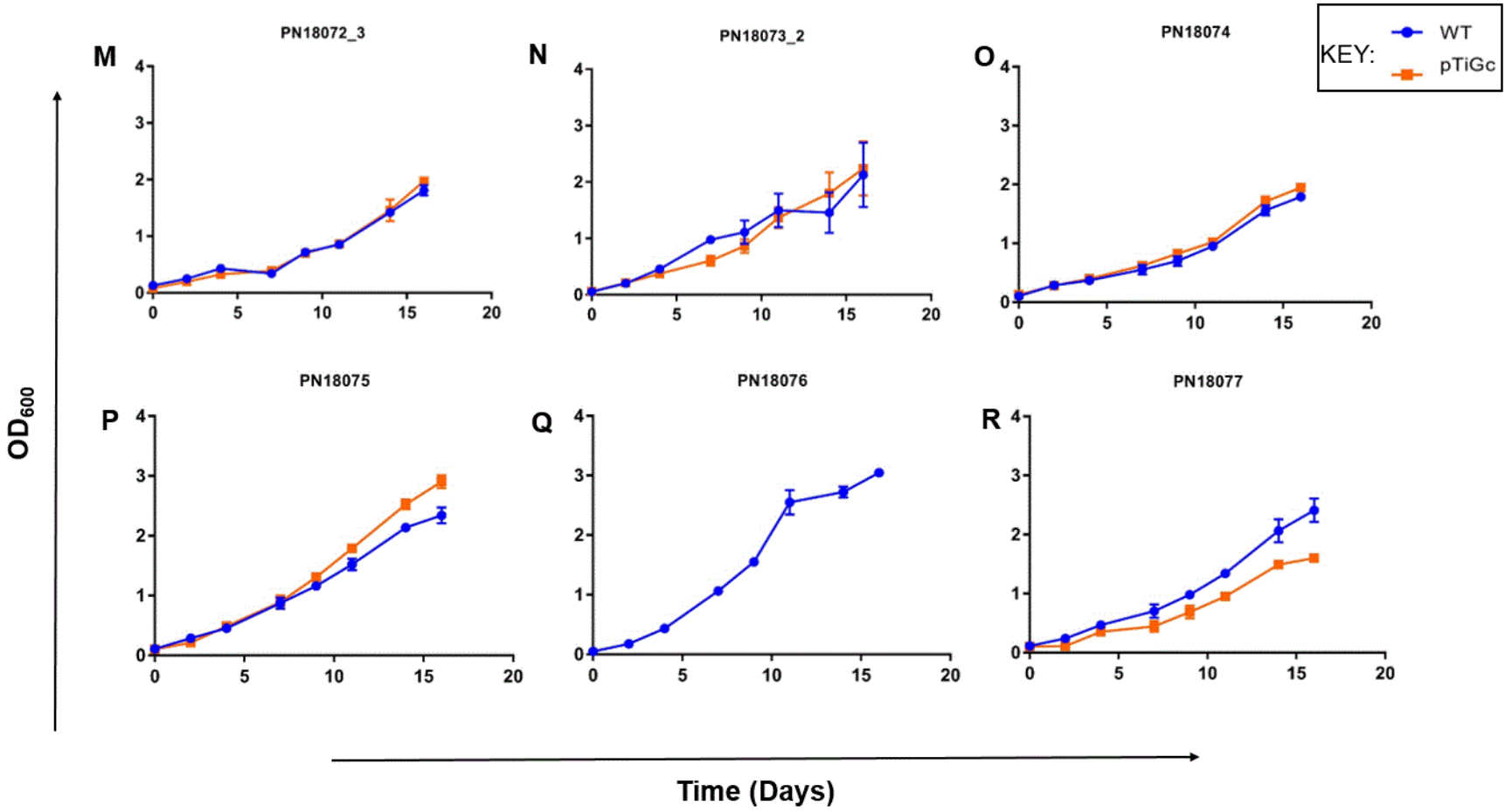


Figure S7. 1: Wildtype vs pTiGc transformed growth curves of clinical *M. bovis* strains. A) PNMP1A_1. **B)** PNMP3C_1. **C)** PNMP7_1. **D)** PNMP8_1. **E)** PNMP9_1. **F)** PN18064_3. **G)** PN18065_2. **H)** PN18066_3. **I)** PN18068_1. **J)** PN18069_3. **K)** PN18070_3. **L)** PN18071_1. **M)** PN18072_3. **N)** PN18073_2. **O)** PN18074. **P)** PN18075. **Q)** PN18076. **R)** PN18077. One independent experiment was performed (performed in technical triplicates). Data are means ± standard deviation (SD) from three technical replicates.

7.4. Growth curves of SAMMtb::pTiGc, BCG::pTiGc and five clinical *M. bovis* pTiGc strains

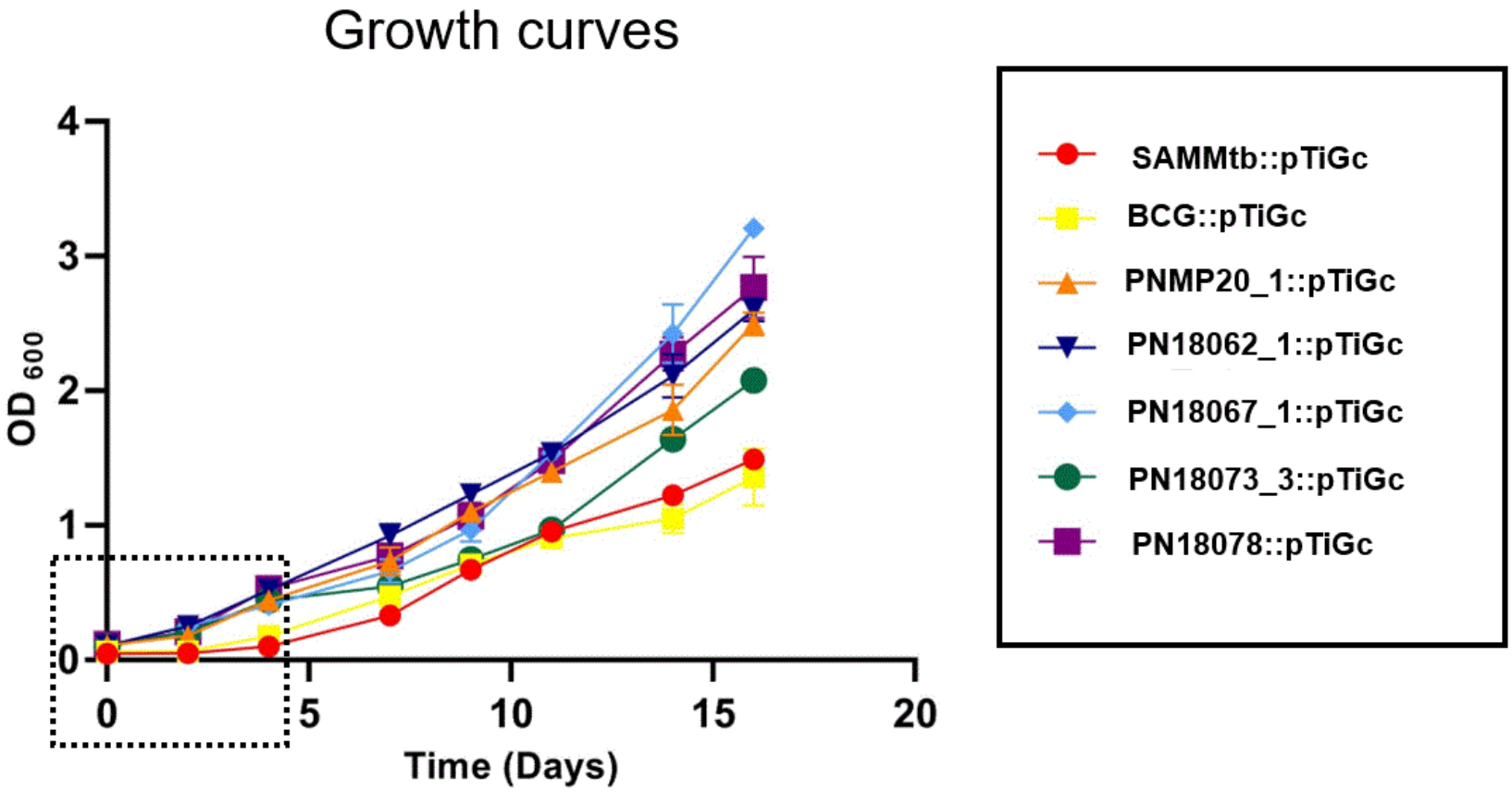


Figure S7.2: Growth curves of SAMMtb::pTiGc, BCG::pTiGc and five clinical *M. bovis* pTiGc strains. Dotted rectangle shows time points of day 0 to 4 where differences are observed between the lab (slower replication) and clinical strains (faster replication). One independent experiment was performed (performed in technical triplicates). Data are means ± standard deviation (SD) from three technical replicates.

7.5. Standard Operating Procedures (SOPs)

UNIVERSITEIT STELLENBOSCH UNIVERSITY

HOST PATHOGEN mycobactomics

HOST-PATHOGEN MYCOBACTOMICS RESEARCH GROUP
Division Molecular Biology and Human Genetics
Laboratory Standard Operating Procedures

MYCOBACTERIUM TUBERCULOSIS AND MYCOBACTERIUM BOVIS COMPETENT CELLS PREPARATION AND TRANSFORMATION			
SOP#: 5b	**Version #:** 1.0 *(Supersedes version x.x)*	**Effective Date:** 5 November 2019 *(This document will be reviewed in two years from effective date)*	Page **1** of **7**

Author:	**Reviewers:** *(Signature confirms that the reviewers agree with the content of the document)*	**Approved by Principal Investigator:** *(Signature confirms final approval)*
(Signature & Date)	(Signature & Date)	(Signature & Date)
Pamela Ncube Msc student Host-Pathogen Mycobactomics	**Samantha Sampson** PI Host-Pathogen Mycobactomics	**Prof Samantha Sampson** Principal Investigator Host-Pathogen Mycobactomics **Approved by QA:** *(Signature confirms document ready for distribution and training)* (Signature & Date) **N. Kriel** Quality Assurance Officer Host-Pathogen Mycobactomics

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HOST PATHOGEN mycobactomics

HOST-PATHOGEN MYCOBACTOMICS RESEARCH GROUP

Division Molecular Biology and Human Genetics
Laboratory Standard Operating Procedures

ZIEHL-NEELSEN STAINING OF MYCOBACTERIA

SOP#: 13a	Version #: 1.0 *(Supersedes version x.x)*	Effective Date: 5/11/19 *(This document will be reviewed in two years from effective date)*	Page 1 of 7

Author:	Reviewers: *(Signature confirms that the reviewers agree with the content of the document)*	Approved by Principal Investigator: *(Signature confirms final approval)*
(Signature & Date)	(Signature & Date)	(Signature & Date)
Pamela Ncube Designation Host-Pathogen Mycobactomics	**Samantha Sampson** Designation Host-Pathogen Mycobactomics	**Prof Samantha Sampson** Principal Investigator Host-Pathogen Mycobactomics
		Approved by QA: *(Signature confirms document ready for distribution and training)* (Signature & Date) **Nastassja Kriel** Quality Assurance Officer Host-Pathogen Mycobactomics

7.6. Ethics Approval Letters

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approval with Stipulations

28 September 2020

PI: Prof Samantha Sampson

REC: BEE Reference #: BEE-2020-18621

Title: Utilization of a severely attenuated, double auxotrophic of Mycobacterium tuberculosis to (a) rapidly screen anti-mycobacterial compounds and (b) to advance understanding of mycobacterial physiology.

Dear Prof Samantha Sampson

Your New Application, with BEE-2020-18621 was reviewed on 21 September 2020 by the Research Ethics Committee: Biosafety and Environmental Ethics via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. Transport SOP (Nanoparticles): UWC to Tygerberg - Some clarity required. It is noted that samples are packaged appropriately at UWC and the SOP explains how the samples should be packaged etc.
1.1 Who manages the primary packaging?
1.2 Is this procedure conducted by the student collecting the samples and more importantly is it done in a BSC?
1.3 Throughout the application, description of all methods done at Tygerberg are performed in a class 2 BSC. Unless, the procedure is describing rules for the student to follow for secondary + outer packaging containment.

Please note that the application for approval and registration of this project will be cancelled automatically if no feedback is received from you within 6 (six) months of the date of this letter.

Please remember to use your REC: BEE reference number: # BEE-2020-18621 on any documents or correspondence with the REC: BEE concerning your research protocol.

If you have any questions or need further help, please contact the REC: BEE office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: BEE policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Biosafety)

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Approval with Stipulations

12 March 2020

PI: Prof Samantha Sampson

REC: BEE Reference #: BEE-2020-13049

Title: Evolution, biology and host-pathogen interactions of mycobacteria

Dear Prof Samantha Sampson

Your New Application, with BEE-2020-13049 was reviewed by the Research Ethics Committee: Biosafety and Environmental Ethics via committee review procedures and was approved on condition that the following stipulations are adhered to:

1. The applicant is requested to submit proof of GMO facility registration as soon as it becomes available.

Please note that the application for approval and registration of this project will be cancelled automatically if no feedback is received from you within 6 (six) months of the date of this letter.

Please remember to use your REC: BEE reference number: # BEE-2020-13049 on any documents or correspondence with the REC: BEE concerning your research protocol.

If you have any questions or need further help, please contact the REC: BEE office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: BEE policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Biosafety)

UNIVERSITEIT
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UNIVERSITY

Animal Tissue Use Approval

11 April 2019

PI: Dr Wynand Goosen

REC: ACU Reference #: ACU-2019-9086

Title: Opportunistic tuberculosis screening of South African wildlife species..

Dear Dr Wynand Goosen

Your Notification, was reviewed on 19 March 2019 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your REC: ACU reference number: # ACU-2019-9086 on any documents or correspondence with the REC: ACU concerning your research protocol.

If you have any questions or need further help, please contact the REC: ACU office at 021 808 9003.

Visit the Division for Research Developments website www.sun.ac.za/research for documentation on REC: ACU policy and procedures.

Sincerely,

Mr Winston Beukes

Coordinator: Research Ethics (Animal Care and Use)

UNIVERSITEIT
STELLENBOSCH
UNIVERSITY

Animal Tissue Use Approval

Date: 19 October 2017

PI Name: Dr Tanya Kerr

Protocol #: 1489

Title: Characterizing Mycobacterium bovis and Feline Immunodeficiency Virus (FIV) infection and disease in African lions (Pathera leo)

Dear Tanya Kerr

The Animal Tissue Notification #1489 , was reviewed on 17 October 2017 by the Research Ethics Committee: Animal Care and Use via committee review procedures and was approved. Please note that this clearance is valid for a period of five years. A new application must be submitted when the source of the material changes.

Applicants are reminded that they are expected to comply with accepted standards for the use of animals in research and teaching as reflected in the South African National Standards 10386: 2008. The SANS 10386: 2008 document is available on the Division for Research Developments website www.sun.ac.za/research.

As provided for in the Veterinary and Para-Veterinary Professions Act, 1982. It is the principal investigator's responsibility to ensure that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of a SAVC-registered veterinary professional or SAVC-registered para-veterinary professional, who are acting within the scope of practice for their profession.

Please remember to use your protocol number, 1489 on any documents or correspondence with the REC: ACU concerning your research protocol.

Please note that the REC: ACU has the prerogative and authority to ask further questions, seek additional information, require further modifications or monitor the conduct of your research.

Any event not consistent with routine expected outcomes that results in any unexpected animal welfare issue (death, disease, or prolonged distress) or human health risks (zoonotic disease or exposure, injuries) must be reported to the committee, by creating an Adverse Event submission within the system.

We wish you the best as you conduct your research.

If you have any questions or need further help, please contact the REC: ACU secretariat at wabeukes@sun.ac.za or 021 808 9008.

Sincerely,

Winston Beukes

REC: ACU Secretariat

Research Ethics Committee: Animal Care and Use