Novel approaches to the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*)

by

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes 4 original papers published in peer-reviewed journals (Chapters 3-6), 2 chapters of unpublished work (Chapters 7-8) and a review (Chapter 2). The remaining 3 chapters include the general introduction (Chapter 1), general discussion (Chapter 9) and conclusion (Chapter 10). The development and writing of the papers (published and unpublished) were the principal responsibility of myself.

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Summary

Mycobacterium bovis (*M. bovis*) is the pathogen that causes bovine tuberculosis (bTB) in a wide range of host species including livestock and wildlife. Globally, the control of *M. bovis* infection is hindered by the existence of wildlife maintenance hosts. In South Africa, African buffaloes (*Syncerus caffer*) are considered maintenance hosts of bTB, and therefore control in this species will facilitate control in other sympatric wildlife species and livestock. With the limited availability of diagnostic tools and their suboptimal test performances to detect *M. bovis* infection in buffaloes, it is imperative to develop novel approaches to improve the detection of infected buffaloes.

In this study, the QuantiFERON® TB-Gold (QFT) system in combination with the *cattletype*® IFN-gamma ELISA, the QFT interferon gamma (IFN-γ) release assay (IGRA), was shown to have high specificity but poor sensitivity in detecting *M. bovis* infection in buffaloes. The sensitivity of the QFT IGRA was improved by measuring the chemokine IFN-γ-inducible protein-10 (IP-10) in the QFT IP-10 release assay (IPRA). When both cytokines IFN-γ and IP-10 were measured in parallel in the QFT system, sensitivity was further improved and the specificity of the individual assays were maintained. The concentrations of IFN-γ and IP-10 in QFT tubes were used to predict the presence of macroscopic pathology in *M. bovis*-infected buffaloes. Lastly, the immunophenotyping of cattle whole blood identified cellular subsets of bovine leukocytes, however, the production of IP-10 in these cells was not confirmed.

This study has demonstrated that the QFT system is a highly practical stimulation platform to detect *M. bovis* infection in buffaloes with high specificity. The QFT

system and novel *cattletype*® IFN-gamma ELISA is an IGRA with high specificity that can be used to detect *M. bovis* infection in buffalo populations. The cytokine IP-10 is a more sensitive biomarker than IFN-γ and when these two cytokines are measured in parallel in the QFT system, the detection of infected buffaloes is maximised, the specificity is high and the testing procedure is simplified. Finally, the magnitude of IP-10 and IFN-γ concentrations in QFT-processed whole blood can be used as indicators of bTB pathology in *M. bovis*-infected buffaloes.

Opsomming

Die patogeen *Mycobacterium bovis* (*M. bovis*) veroorsaak beestuberkulose (bTB) in ‘n wye reeks gashere wat vee en wild insluit. Wereldwyd word die beheer van *M. bovis* infeksie bemoeilik as gevolg van die teenwoordigheid van nstandhoudingsgashere in die wildsbevolking. Afrikabuffels (*Syncerus caffer*) word as belangrike instandhoudingsgashere van bTB in Suid Afrika beskou met die gevolg dat siektebeheer in hierdie spesie die beheer van bTB in ander simpatriese wildsoorte en vee sal fasiliteer. Met die beperkte beskikbaarheid van diagnostiese tegnieke en gepaardgaande suboptimale toets prestasie, het dit dus noodsaaklik geword om nuwe benaderings wat verbeterde opsporing van *M. bovis* in buffels sal teweegbring, te ontwikkel.

Hierdie studie het getoon dat die QuantiFERON®-TB Gold (QFT) interferon gamma (IFN-γ) vrystellingstoets (IGRA), ’n kombinasie van die QFT sisteem en die *cattletype*® IFN-gamma ELISA, hoë spesifisiteit maar swak sensitiwiteit vir die opsporing van *M. bovis* in buffels het. Die sensitiwiteit van die QFT IGRA kon verhoog word deur die chemokien interferon gamma-geïnduseerde proteïen-10 (IP-10) te meet met die QFT IP-10 vrystellingstoets (IPRA). Deur beide IFN-γ en IP-10 in parallel te meet in die QFT sisteem, is die sensitiwiteit verder verbeter sonder verlies van spesifisiteit van die toets. Die konsentrasies van IFN-γ en IP-10 in QFT buise kon die teenwoordigheid van makroskopiese letsels in *M. bovis* besmette buffels voorspel. Laastens is subgroepe van beesleukosiete deur immunofenotipering van heel beesbloed geidentifiseer, alhoewel die produksie van IP-10 deur hierdie selle nie bevestig kon word nie.

Hierdie studie het gedemonstreer dat die QFT sisteem ‘n uiterse praktiese stimulasieplatform is om *M. bovis* met spesifisiteit te diagnoseer. Die kombinasie van die QFT sisteem met die nuwe *cattletype*® IFN-gamma ELISA lewer ‘n hoogs spesifisiteit IGRA wat gebruik kan word om *M. bovis* infeksie in buffelbevolkings te diagnoseer. Die chemokien IP-10 is ‘n meer sensitiewe biomerker as IFN-γ, maar wanneer hulle in parallel gemeet word in die QFT sisteem, lei dit tot die maksimum opsporing van besmette buffels met ‘n hoë spesifisiteit terwyl die toetstegniek vereenvoudig is. Ter afsluiting, die vlak van IP-10 en IFN-γ konsentrasies wat in heel beesbloed in die QFT sisteem gemeet is, kan as aanwyser dien om siekteletsels in *M. bovis* besmette buffels te voorspel.

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List of abbreviations

[IFN-γ^{Nil}]	IFN-γ concentration in QFT Nil tube
[IFN-γ^{TB}]	IFN-γ concentration in QFT TB antigen tube
[IP-10^{Nil}]	IP-10 concentration in QFT Nil tube
[IP-10^{TB}]	IP-10 concentration in QFT TB antigen tube
ARC	Agricultural Research Council
BAL	bronchoalveolar lavage
bTB	bovine tuberculosis
CFP-10	culture filtrate protein 10 kD
CMI	cell-mediated immunity
CV	coefficient of variation
DNA	deoxyribonucleic acid
DPP® VetTB	Dual Path Platform Vet TB Assay
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secretory antigen target 6 kD
FSC-A	forward scatter area
FSC-H	forward scatter height
HiP	Hluhluwe iMfolozi Park
IFN-γ	interferon gamma
IGRA	interferon gamma release assay
IP-10	interferon gamma-inducible protein-10
IPRA	interferon gamma-inducible protein-10 release assay
GEA	gene expression analysis
GKNP	Greater Kruger National Park
KNP	Kruger National Park

KZN	KwaZulu-Natal
L_1	lesion score 1
L_2	lesions score 2
L_3	lesion score 3
M. africanum	*Mycobacterium africanum*
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
M. canetti	*Mycobacterium canetti*
M. caprae	*Mycobacterium caprae*
MGR	Madikwe Game Reserve
MIRU	mycobacterial interspersed repetitive units
MM	master mix
M. microti	*Mycobacterium microti*
M. mungi	*Mycobacterium mungi*
M. orygis	*Mycobacterium orygis*
M. pinnipedii	*Mycobacterium pinnipedii*
mQFT	modified QuantiFERON®-TB Gold assay
M. suricattae	*Mycobacterium suricattae*
Mtb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NGS	next generation sequencing
NK	natural killer cells
NK-T	natural killer T lymphocytes
NPV	negative predictive value
NVL	no visible lesion

PCR	polymerase chain reaction
PPD	purified protein derivative
PPD_a	*Mycobacterium avium* purified protein derivative
PPD_b	*Mycobacterium bovis* purified protein derivative
PPV	positive predictive value
PWM	pokeweed mitogen
QFT	QuantiFERON®-TB Gold
$QFT^{parallel}$	parallel interpretation of the QFT IGRA and QFT IPRA
QFT-Plus	QuantiFERON®-TB Gold Plus
RD1	region of difference one
RT	room temperature
S/P	sample to positive control ratio
SA	South Africa
SCITT	single comparative intradermal tuberculin test
Se	sensitivity
SFT	skin fold thickness
Sp	specificity
spoligotyping	spacer oligonucleotide typing
SSC-A	side scatter area
TB	tuberculosis
TST	tuberculin skin test
UK	United Kingdom
VPN	Veterinary Procedural Notice
VNTR	variable number of tandem repeats
WGS	whole genome sequencing

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Chapter 1 : General Introduction

This introductory chapter aims to give a literature summary of the host, pathogen and diagnosis of *Mycobacterium bovis* (*M. bovis*) infection. Furthermore, this chapter will highlight the justification for a study investigating novel approaches to detect *M. bovis* infection in African buffaloes (*Syncerus caffer*) as well as setting the study aim and objectives.

Mycobacterium bovis

Mycobacterium bovis (M. bovis) is a member of the *Mycobacterium tuberculosis* complex (MTBC), a group of genetically related mycobacterium species that cause tuberculosis (TB) in a range of mammals (Gagneux, 2018). Of all MTBC members, *M. bovis* has the widest host range and causes bovine tuberculosis (bTB) in domestic animals, livestock, wildlife and humans (Michel et al., 2006). Globally, the eradication of *M. bovis* is hampered by the existence of wildlife reservoirs that serve as constant sources of infection (Fitzgerald and Kaneene, 2013).

M. bovis in South African wildlife

In South Africa (SA), two of the largest wildlife reserves, the Kruger National Park (KNP) and Hluhluwe iMfolozi Park (HiP), as well as a number of smaller wildlife reserves, have been declared endemic for *M. bovis* (Michel et al., 2006; Hlokwe et al., 2016). Many of these wildlife reserves, including KNP and HiP, are adjacent to communal lands where livestock graze freely (de Garine-Wichatitsky et al., 2013; Hlokwe et al., 2014). The wildlife and livestock are only separated by single or double fences, which are frequently damaged by elephants, humans or floods (Jori et al., 2011). Therefore, spillover transmission of *M. bovis* has been documented in South Africa from livestock to wildlife and back, at this livestock-wildlife interface (de Lisle et al., 2002; Musoke et al., 2015).

Mycobacterium bovis infection in African buffaloes

In SA, African buffaloes (*Syncerus caffer*) are the most recognised wildlife maintenance hosts of *M. bovis.* Transmission of *M. bovis* has been documented between buffaloes in the KNP and neighbouring rural cattle (Musoke et al., 2015) as well as in KNP from buffaloes to lions (Renwick et al., 2007; Michel et al., 2009). For this reason, control of *M. bovis* infection

in buffaloes is a key factor in controlling infection in livestock and sympatric wildlife species. Specifically, accurate diagnosis of *M. bovis* infection in buffaloes is required to identify infected animals to remove them from the population to limit the transmission of *M. bovis* (i.e. test-and-cull programmes) and legally move buffaloes within SA to limit the geographic spread of *M. bovis*. However, diagnosis of *M. bovis* infection in buffaloes is limited by the tools available and their suboptimal test performances.

Single comparative intradermal tuberculin test

In buffaloes, the cell-mediated immune response is the primary and earliest response to develop after infection with *M. bovis.* Thus, early diagnosis of *M. bovis* infection relies on using *in vitro* and *in vivo* assays to detect and quantify cell-mediated immunity (CMI) in response to mycobacterial antigens (Goosen et al., 2014a). The *in vitro* single comparative intradermal tuberculin test (SCITT) measures local delayed-type hypersensitivity reaction (after approximately 72 hours) in response to intradermal injection of purified protein derivative (PPD) namely *M. bovis* PPD (PPD_b) and *Mycobacterium avium* PPD (PPD_a), which is included as a comparative antigen (Schiller et al., 2010). The SCITT can be performed with limited infrastructure, does not require the stimulation or transportation of blood samples to accredited laboratories under time and temperature constraints, and results are directly linked to individual buffaloes, limiting the wrong classification of test-positive or negative animals. However, the SCITT requires buffaloes to be chemically immobilised twice and kept confined during this time. Furthermore, the interpretation of assay results may be subjective and the administration of PPDs may affect future test-results (Clarke et al., 2018). Despite these disadvantages, the SCITT remains the only assay approved in SA to diagnose *M. bovis* infection in buffaloes, even though it has not been validated in this species.

Bovigam® IGRA

The standard Bovigam® interferon gamma (IFN-γ) release assay (IGRA) (Prionics AG, Schlieren-Zurich, Switzerland) is the *in vitro* alternative to the SCITT. Whole blood is stimulated overnight with PPDs, like those used in the SCITT, after which the biomarker IFN-γ, a cytokine produced by the activation of sensitised T-lymphocytes, is detected and quantified using an enzyme-linked immunosorbent assay (ELISA) (Goosen et al., 2014b). The specificity (Sp) of the standard Bovigam® IGRA can be improved by the replacement of PPDs with specific mycobacterial antigens. The Bovigam® peptide IGRAs, Bovigam® PC-EC and Bovigam® PC-HP, use peptides simulating early secretory antigen target 6 kD (ESAT-6) and culture filtrate protein 10 kD (CFP-10), and Rv3615 and three additional proprietary mycobacterial antigens, respectively, as stimulating antigens (Goosen et al., 2014b). The use of specific antigens is also more standardised than PPDs, as PPDs may vary between batches and sources (Monaghan et al., 1994; de la Rua-Domenech et al., 2006).

QuantiFERON®-TB Gold system

The QuantiFERON®-TB Gold (QFT) system (Qiagen, Venlo, Limburg, Netherlands) is an innovative whole blood stimulation platform using specific mycobacterial antigens in an easy-to-use, field-friendly format. The QFT system comprises of three tubes; i) Nil tube containing saline (unstimulated control), ii) TB antigen tube containing peptides simulating antigens ESAT-6, CFP-10 and TB7.7(p4) (stimulated) and iii) mitogen tube containing phytohemagglutinin (positive control). Whole blood can be collected directly into each tube after which tubes are incubated at 37 °C overnight, plasma harvested and cytokine biomarkers measured by ELISA. The practicality of the tubes together with the use of specific antigenic peptides make this system highly suitable for detecting *M. bovis* infection in buffaloes. Parsons et al. (2011) described the modification of the QFT assay (mQFT) to

detect *M. bovis* infection in buffaloes, an IGRA using QFT stimulation tubes and an in-house bovine-specific IFN-γ ELISA. The manufacturers of the QFT system (Qiagen) have since developed the commercially available *cattletype*® ruminant-specific IFN-gamma ELISA. Furthermore, Qiagen has improved the performance of the QFT system for human application by including an additional antigen tube (TB2) and modifying the antigens in the original TB antigen tube (TB1). The new QFT Plus system is comprised of four tubes; i) Nil tube, ii) TB1 antigen tube containing peptides simulating antigens ESAT-6 and CFP-10, ii) TB2 antigen tube containing the same peptides as those in TB1 plus shorter peptides simulating antigens ESAT-6 and CFP-10, and iii) mitogen tube (Theel et al., 2018).

IP-10 as biomarker of infection

The cytokine IFN-γ is the archetypal biomarker of the cell-mediated immune response but several additional candidate biomarkers have been evaluated to detect *M. bovis* infection in buffaloes (Goosen et al., 2014a). Of the biomarkers assessed, IFN-γ-inducible protein-10 (IP-10) was identified as the chemokine that demonstrated a similar ability as IFN-γ to differentiate between *M. bovis*-infected and uninfected buffaloes (Goosen et al., 2014a). Furthermore, IP-10 was described as a sensitive biomarker of antigen recognition in whole blood stimulation assays, namely the QFT IP-10 release assay (IPRA), to detect *M. bovis* infection in buffaloes (Goosen et al., 2015).

IP-10 as an indicator of pathology

In humans and cattle, the utility of IP-10 as a biomarker of *Mycobacterium tuberculosis* (*Mtb*) and *M. bovis* infection, respectively, is compromised by elevated levels of IP-10 in some unstimulated control tubes (Whittaker et al., 2008; Parsons et al., 2016). Elevated IP-10 in the unstimulated control decreases the differential value of IP-10 between the TB antigen

and unstimulated control and this may cause a false-negative test result. In humans, elevated levels of IP-10 in unstimulated control tubes may be associated with active TB and has been used to distinguish *Mtb*-infected and uninfected patients (Whittaker et al., 2008; Chen et al., 2011). Moreover, IP-10 is included in a serum (*ex vivo*) biomarker signature for the diagnosis of active TB in humans (Hussain et al., 2010; Chegou et al., 2016). These studies suggest that elevated levels of IP-10 in unstimulated buffalo whole blood samples may indicate the extent of disease in animals and therefore, this requires additional investigation. Furthermore, insight into the production of IP-10 in buffaloes may shed light on the mechanisms that cause elevated levels of IP-10 in the unstimulated control.

Immunobiology of IP-10

The immunobiology of IP-10 production in both cattle and buffaloes is unknown, while in humans, IP-10 production has been extensively explored. In humans, the chemokine IP-10 has been reported to be produced by neutrophils (Gasperini et al., 1999), macrophages (Agostini et al., 2001), monocytes (Vargas-Inchaustegui et al., 2010) and B lymphocytes (Hoff et al., 2015). A greater understanding of the cells involved in IP-10 production in bovids is required to explain elevated IP-10 levels observed and may allow the effective use and interpretation of this chemokine as a diagnostic marker of *M. bovis* infection. Since obtaining cattle whole blood samples is easier than obtaining buffalo whole blood samples, a pilot study conducted in cattle using bovid-specific reagents may be useful to develop the methods required to study IP-10 production, which can then be applied to buffaloes.

Justification of study

African buffaloes are maintenance hosts of *M. bovis* in SA and therefore a key species on which to focus control measures, as this will in turn facilitate control of infection in other

wildlife species and livestock (de Vos et al., 2001). However, the detection of *M. bovis* infection in buffaloes is confounded by the suboptimal performances and logistical drawbacks associated with currently available diagnostic tests. New and established CMI-based assays utilised in novel ways, may improve the detection of infection in buffaloes and simplify testing procedures. Evaluating data from each component of the cytokine assays in addition to the final assay result of these tests may also allow additional interpretation with regards to presence of disease, as observed in humans (Whittaker et al., 2008; Chegou et al., 2016). Moreover, calculating the *M. bovis* infection prevalence of a herd based on mycobacterial culture, and subsequently calculating predictive values of the assays will provide greater insight into the test performance of new and established CMI-based assays. Testing different herds with varying infection prevalences and bTB history will allow the performances of assays to be determined in different settings. Furthermore, investigating the immunobiology of IP-10 in cattle, which has not yet been done, will provide a platform for future studies in buffaloes to understand the production of IP-10 and assess IP-10 as a potential biomarker of *M. bovis* infection and bTB disease.

Study aims and objectives

Aim

To improve the detection of *M. bovis* infection in African buffaloes (*Syncerus caffer*) using novel diagnostic approaches.

Objectives

1. To evaluate novel and established assays of CMI, utilised in innovative ways, for detection of *M. bovis* infection in buffaloes

2. To determine the impact of bTB pathology on interpretation of assay results in *M. bovis*-infected buffaloes.
3. To use flow cytometry to investigate IP-10 production in cattle.

Ethical approval for this study was granted by the Stellenbosch University Animal Care and Use committee (SU-ACUD15-00065, SU-ACUD15-00072 and SU-ACUD16-00097)

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Chapter 2 : Wildlife tuberculosis in South Africa

Unpublished review chapter

Abstract

The *Mycobacterium tuberculosis* complex (MTBC) is a genetically related group of mycobacteria that cause tuberculosis in wildlife. The MTBC have a wide mammalian host range including ungulates, carnivores, primates, and small rodents. Wildlife tuberculosis in South Africa threatens management strategies and conservation efforts to protect wildlife. Furthermore, the human/wildlife/livestock interface allows multi-directional transmission events, adding to the complexity of this disease. The epidemiology of MTBC members that infect free-ranging wildlife species in South Africa and the diagnostic tools available to detect infection in these species are reviewed in this chapter.

1. Introduction

Tuberculosis (TB) is an infectious disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC): *Mycobacterium africanum* (*M. africanum*), *Mycobacterium bovis* (*M. bovis*), *Mycobacterium canetti* (*M. canetti*), *Mycobacterium caprae* (*M. caprae*), *Mycobacterium microti* (*M. microti*), *Mycobacterium mungi* (*M. mungi*), *Mycobacterium orygis* (*M. orygis*), *Mycobacterium pinnipedii* (*M. pinnipedii*), *Mycobacterium suricattae* (*M. suricattae*), *Mycobacterium tuberculosis* (*Mtb*), chimpanzee bacillus and dassie bacillus (Gagneux, 2018). Despite exhibiting low deoxyribonucleic acid (DNA) sequence diversity, the MTBC organisms are epidemiologically unique and have a diverse mammalian host range including domestic animals, livestock, wildlife and humans (Hlokwe et al., 2014; Dippenaar et al., 2017).

In South Africa (SA), the existence of extensive human/wildlife/livestock interfaces pose a risk of MTBC transmission between these groups. The risk of spillover of TB from wildlife to domestic livestock may have regulatory consequences and subsequent trade restrictions. In addition, detection of wildlife TB can lead to quarantine of wildlife premises and threaten conservation and tourism, which can have extensive environmental and socio-economic implications for SA (Meiring et al., 2018). Wildlife reservoirs serve as recurrent sources of infection as disease persists in these species, and can be maintained in the absence of new introductions of infection, posing a threat for reinfection of livestock and other sympatric wildlife species (Fitzgerald and Kaneene, 2013).

With the advent of new techniques to detect MTBC infection in wildlife, our understanding of TB continues to evolve. Recent additions to our knowledge of *M. bovis*, *Mtb*, dassie bacillus, *M. mungi*, *M. suricattae* and *M. orygis*, causative agents of TB in free-ranging

wildlife in SA, are reviewed in this chapter with a focus on new developments in epidemiology and diagnostics.

2. Epidemiology of *M. bovis* infection in wildlife in SA

Mycobacterium bovis has the broadest host range of all MTBC organisms and is responsible for the most common form of wildlife TB in SA, bovine TB (bTB). Twenty-four wildlife species have been confirmed to be infected with *M. bovis* in SA (Table 2.1), with some of the new species added being on the IUCN Red List of Threatened Species™ (http://www.iucnredlist.org/). These include cases in the African wild dog (*Lycaon pictus*), black (*Diceros bicornis*) and white rhinoceros (*Ceratotherium simum*), giraffe (*Giraffa camelopardalis*) and African elephant (*Loxodonta africana*) (Miller et al., 2017a, 2018a, unpubl. data, Higgitt et al., 2018; Hlokwe et al., 2019) (Figure 2.1).

Infected wildlife are classified as either maintenance or dead-end hosts, depending on the dynamics of the infection (Renwick et al., 2007). African buffaloes (*Syncerus caffer*) and greater kudu (*Tragelaphus strepsiceros*) are recognised maintenance hosts of *M. bovis*, while warthogs (*Phacochoerus africanus*) may be maintenance hosts under certain conditions, i.e. increased population densities (de Vos et al., 2001; Keet et al., 2001; Roos et al., 2016). Other wildlife species and their roles as hosts in infection and transmission are not yet fully understood.

2.1. Routes of infection and transmission

Although TB is described as a respiratory disease, the outcome of infection varies in different species and also depends on the route of infection. For example, the spillover of *M. bovis* from buffaloes to African lions (*Panthera leo*) resulted from consumption of infected prey

Table 2.1 Free-ranging wildlife species confirmed to be infected with Mycobacterium bovis in South Africa, detailed by the location of the infected species with references. Superscript numbers correspond to species in Figure 2.1.

Species	Common name	KNP[a]	GKNP[b]	HiP[c]	KZN[d]	MGR[e]	SNR[f]	Other locations	Reference/s
Syncerus caffer[1]	African buffalo	√	√	√	√	√	√	Mpumalanga	Rodwell et al., 2001; Hlokwe et al., 2011, 2016
Lycaon pictus[2]	African wild dog	√	-	√	-	-	-	-	Higgitt, 2018; Higgitt et al., 2019
Mungos mungo[3]	Banded mongoose	√	-	-	-	-	-	-	Brüns et al., 2017
Diceros bicornis[4]	Black rhinoceros	√	-	-	-	-	-	-	Miller et al., 2017a
Connochaetes taurinus[5]	Blue wildebeest	-	√	-	-	-	-	-	Hlokwe et al., 2014
Tragelaphus scriptus[6]	Bushbuck	√	-	-	-	-	-	-	Michel et al., 2015
Potamochoerus porcus[7]	Bush pig	-	-	√	-	-	-	-	Michel et al., 2009; Hlokwe et al., 2011
Papio ursinus[8]	Chacma baboon	√	-	√	√	-	-	Limpopo	Keet et al.,1996, 2000; Michel et al., 2009, 2013; Hlokwe et al., 2016
Acinonyx jubatus[9]	Cheetah	√	√	-	-	-	-	-	Keet et al., 1996; Michel et al., 2009
Syvicapra grimmia[10]	Common duiker	-	-	-	-	-	-	Eastern Cape	Paine and Martinaglia, 1929
Taurotragus oryx[11]	Eland	-	√	√	-	-	-	-	Michel et al., 2006, 2009
Loxodonta africana[12]	Elephant	√	-	-	-	-	-	-	Miller unpubl. data
Giraffa camelopardalis[13]	Giraffe	√	-	-	-	-	-	-	Hlokwe et al., 2019
Tragelaphus strepsiceros[14]	Greater kudu	√	√	√	√	-	√	Addo Elephant Park	Paine and Martinaglia, 1929; Bengis et al., 2001; Hlokwe et al., 2016
Mellivora capensis[15]	Honey badger	√	-	√	-	-	-	-	Michel et al., 2009
Aepyceros melampus[16]	Impala	√	-	-	-	-	-	-	Michel et al., 2009
Genetta tigrina[17]	Spotted genet	√	-	√	-	-	-	-	Michel, 2002; Michel et al., 2009
Panthera pardus[18]	Leopard	√	√	√	-	-	√	-	Michel, 2002; Michel et al., 2009
Panthera leo[19]	Lion	√	-	√	√	√	√	-	Bengis et al., 1996; Michel et al., 2009
Tragelaphus angasii[20]	Nyala	-	-	-	-	-	-	Gauteng	Hlokwe et al., 2016
Antidorcas marsupialis[21]	Springbok	-	-	-	-	-	-	-	Michel et al., 2015
Crocuta crocuta[22]	Spotted hyaena	-	√	-	-	-	-	-	Michel et al., 2009
Phacochoerus aethiopicus[23]	Warthog	√	√	√	√	-	-	-	Roos et al., 2016
Ceratotherium simum[24]	White rhinoceros	√	-	-	-	-	-	-	Miller et al., 2018a

[a] Kruger National Park

[b] Greater Kruger National Park

[c] Hluhluwe iMfolozi Park

[d] KwaZulu-Natal

[e] Madikwe Game Reserve

[f] Spioenkop Nature Reserve

(Renwick et al., 2007), which is supported by the location of bTB pathology in the head and mesenteric lymph nodes of carnivores (Maas et al., 2013). Although carnivores are usually considered dead-end hosts, lions may serve as reservoirs of disease in some cases, as respiratory shedding of viable *M. bovis* has been documented in this species (Miller et al., 2015a). Omnivores, namely chacma baboons (*Papio ursinus*), warthogs and honey badgers (*Mellivora capensis*) may also become infected via scavenging on infected carcasses (Michel et al., 2006; Renwick et al., 2007). Therefore, the epidemiology of bTB in SA is complicated by the presence of multiple susceptible hosts and potential routes of infection.

The behaviour and social structure of different species may influence potential routes of infection and transmission. Social wildlife species namely buffaloes, warthogs, wild dogs and antelope may become infected via direct contact. Wild dogs may transmit bacteria when they regurgitate food for their pups or when they characteristically lick each other's mouths (Woodroffe et al., 1997). This is supported by a study in which *M. bovis* was cultured from wild dog oropharyngeal swabs (Higgitt et al., 2019). Warthogs are communal burrowers and cooperative breeders which may increase their risk of intra and inter-species transmission (Roos, 2018).

In less social species, namely giraffe, cheetah (*Acinonyx jubatus*), and some ungulate species, transmission of *M. bovis* may be indirect and via contaminated environmental sources by sharing grazing and water sources with maintenance hosts. Due to presence of bTB pathology in the lungs of white rhinoceroses, it was proposed that the source of *M. bovis* infection was via aerosolization of mycobacteria from a contaminated environment; however, a study reported rare shedding of bacilli during localised *M. bovis* infection (Michel et al., 2017; Miller et al., 2018a). Environmental contamination was also proposed as the source of

infection in an *M. bovis*-infected black rhinoceros (Miller et al., 2017a). Environmental contamination is believed to be the predominant cause of interspecies transmission of *M. bovis* between cattle and European badgers (*Meles meles*) in the United Kingdom (Drewe et al., 2013) and cattle and European wild boar (*Sus scrofa*) in Spain (Barasona et al., 2014), which is feasible since *M. bovis* is able to persist in the environment for up to 15 months (Sweeney et al., 2007). However, very limited information is available on routes of transmission of *M. bovis* in wildlife and on the role of environmental contamination in the epidemiology of bTB in SA.

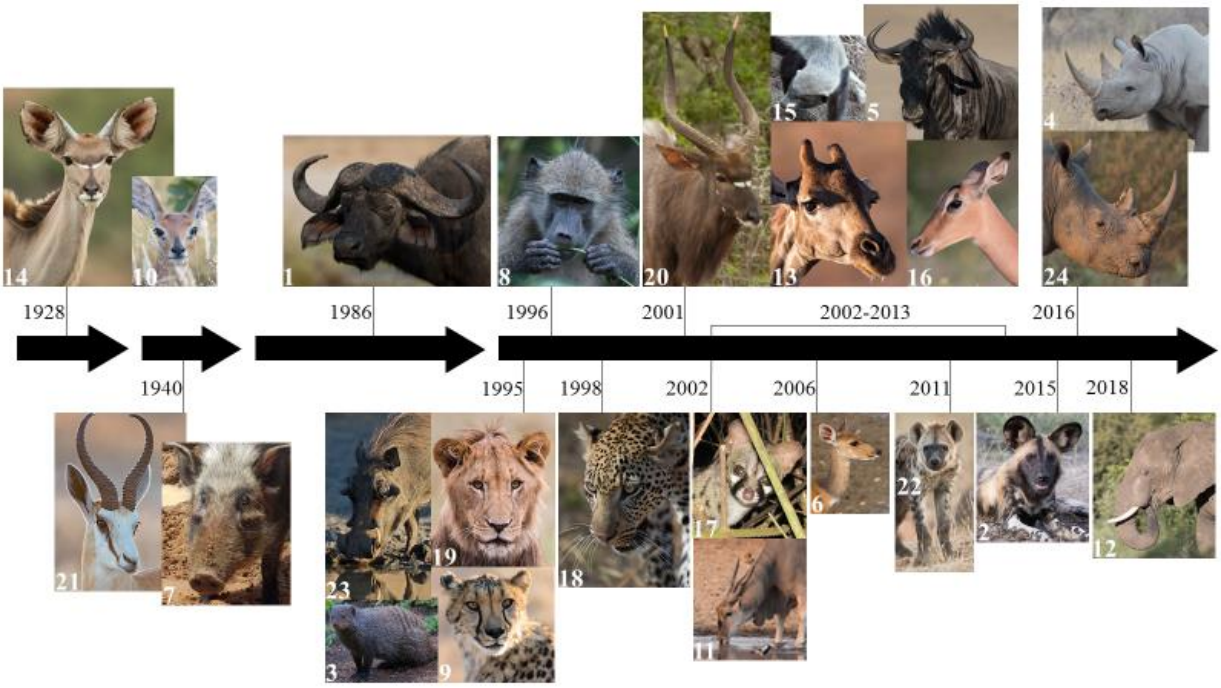

Figure 2.1 Index cases of Mycobacterium bovis infection in free-ranging wildlife species in South Africa over the last century (Dawson, n.d.). Species correspond to the superscript numbers in Table 2.1.

2.2. Geographic distribution and spread

Initially, *M. bovis* in wildlife was restricted to specific geographic regions within SA, but during the last century, infection has been found across much of the country. First reported in

1928 in the Eastern Cape (Paine and Martinaglia, 1929), three of SA's largest wildlife reserves, the Hluhluwe iMfolozi Park (HiP), Kruger National Park (KNP) and Madikwe Game Reserve (MGR) are endemic for *M. bovis* (Bengis et al., 1996; Jolles, 2004; Hlokwe et al., 2016). More recently, a number of smaller public and private reserves in KwaZulu-Natal (KZN) and Greater Kruger National Park (GKNP) have had confirmed cases of *M. bovis* infection in wildlife species (Figure 2.2) (Hlokwe et al., 2016). Movement of infected wildlife is a risk factor in the geographical spread of TB. However, there are few regulatory requirements in SA that address this risk, other than issuing quarantine notices to infected premises. The Veterinary Procedural Notice (VPN), which outlines testing requirements for the legal movement of buffaloes is under revision by Department of Agriculture, Forestry, and Fisheries (DAFF) at the time of this writing (DAFF, 2017). However, there are no other requirements for TB testing of wildlife species in SA prior to translocations.

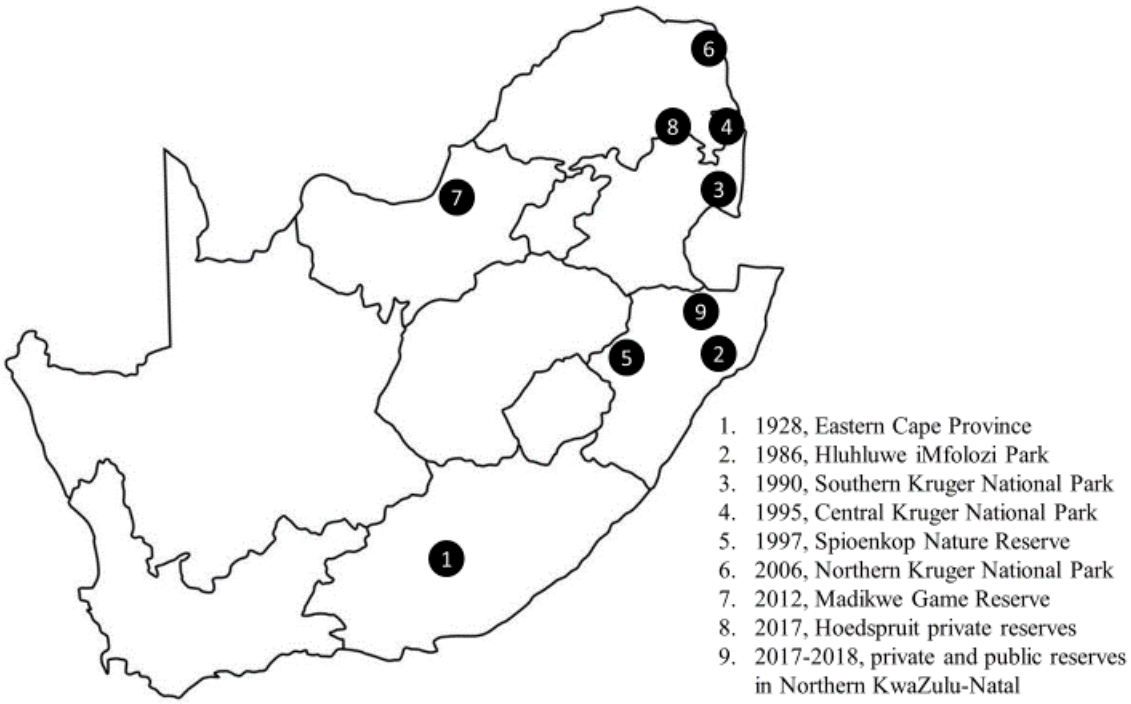

Figure 2.2 The geographical distributions of Mycobacterium bovis during the last century in free-ranging wildlife species in South Africa in chronological order of documentation.

Translocations are key to conservation efforts in SA. For example, predator populations, including wild dog and cheetah, are managed as metapopulations in SA by moving animals between reserves to maintain population growth and genetic diversity (Davies-Mostert et al., 2015; Buk et al., 2018). Efforts to protect rhinoceroses from poaching also require the movement of animals from large populations in KNP and HiP to other reserves (Miller et al., 2018a). However, the presence of bTB restricts movement of animals and lack of validated diagnostic tests confound conservation efforts, although movement of untested animals may contribute to the geographical spread of TB in SA.

2.3. Susceptibility

Susceptibility to *M. bovis* infection and subsequent outcomes of infection and disease differ between wildlife hosts. Ungulates are generally considered susceptible to infection with progression to classical granulomatous disease. Enlarged parotid lymph nodes are a clinical sign of bTB infection in kudu (Keet et al., 2001) while elephants only show clinical signs at advanced stages of disease (Miller et al., 2019). A study by le Roex et al. (2013) identified gene polymorphisms associated with disease susceptibility in African buffaloes.

Susceptibility to infection and disease may differ between rhinoceros species based on extrapolation from historical cases suggesting browser species (black rhinoceroses) may be more likely to acquire and develop disease than grazing species, such as white rhinoceroses (Miller et al., 2017b). The macroscopic pathology of bTB in a black rhinoceros was similar to that reported in captive rhinoceroses (pulmonary disease), while white rhinoceroses appear to be able to limit disease progression, based on the very localised disease in naturally and experimentally infected animals (Michel et al., 2017; Miller et al., 2017a, 2018a).

Carnivores are generally considered to be susceptible to infection but may not develop disease. Immune sensitisation to *M. bovis* has been detected in spotted hyenas (*Crocuta crocuta*) (Higgitt et al., 2017), but there has been no evidence of clinical disease, which suggests that spotted hyenas may exhibit a unique and robust immune system that makes them less susceptible (Harrison et al., 2004). In contrast to hyenas, lions, leopards (*Panthera pardus*), and cheetah are susceptible to *M. bovis* infection as well as bTB disease and can have high morbidity and mortality rates (Keet et al., 1996). This is in agreement with other infectious diseases such as anthrax, where hyenas are not susceptible to disease but wild felids develop clinical signs and death (Lembo et al., 2011).

Susceptibility of small wild mammals is generally unknown. In banded mongooses (*Mungos mungo*), *M. bovis* infection and disease has been reported but in general, insufficient information is available regarding the susceptibility to infection and disease in smaller mammals and requires further investigation (Brüns et al., 2017).

3. Epidemiology of *Mtb* infection in wildlife in SA

Mycobacterium tuberculosis is the primary pathogen that causes TB in humans, although globally there are numerous reports of *Mtb* infection and disease in captive animals (Michel et al., 2003; Miller et al., 2018b). In SA, reports of *Mtb* infection in free-ranging wildlife species are limited to an African elephant and chacma baboons, which may be primarily due to indirect transmission (Table 2.2).

In 2016, the first fatal case of TB in a free-ranging African elephant due to *Mtb* was discovered in the KNP (Miller et al., 2019). Extensive disease was present upon necropsy while the source of the *Mtb* strain F11, commonly found in people in SA, remains unknown.

The source of infection may have been indirect contact through human-derived contaminated food or infectious biological discharge, or inadequate treatment of waste water, human waste from visitors, staff, or human settlements along the reserve's boundary fences. Alternatively, the elephant may have had direct contact with an infected animal, although unlikely (Miller et al., 2019). Baboons are also known to go through human waste at picnic sites and houses, which may be the source of *Mtb* infection in this species (Drewe et al., 2012). In SA, as the human population increases and conservation areas become more fragmented, wildlife habitats are being encroached upon and together with the high burden of *Mtb* in the country, increased transmission of *Mtb* at human/wildlife interfaces can be expected (Michel et al., 2013). Globally, wildlife conservation is threatened by the increased transmission of diseases between humans and wildlife (Jones et al., 2008).

4. Epidemiology of Dassie bacillus, *M. mungi*, *M. suricattae*, and *M. orygis* infection in wildlife in SA

In SA, three members of the MTBC have been confirmed in only a single species; dassie bacillus in rock hyraxes (*Procavia capensis), M. mungi* in banded mongooses, and *M. suricattae* in meerkats (*Suricata suricattae*) (Table 2.2). These three MTBC members evolved from a common ancestor, *M. africanum*, individually within different hosts (Clarke et al., 2016a). Originating from the same common ancestor, *M. orygis* has only been identified in buffaloes in SA (Gey van Pittius et al., 2012) (Table 2.2), although *M. orygis* has been reported in captive antelope and humans elsewhere (van Soolingen et al., 1994; van Ingen et al., 2012).

4.1. Dassie bacillus

In 1954, dassie bacillus was isolated in rock hyraxes for the first time in the Eastern Cape in SA (Wagner et al., 1958). Since then, numerous cases of TB in rock hyraxes have been documented around SA, suggesting the widespread distribution of this organism (Clarke et al., 2016a). Dassie bacillus appears to be transmitted via the respiratory tract in rock hyraxes as pathology is primarily observed in the lungs (Parsons et al., 2008). Susceptibility to disease may vary in rock hyraxes as some animals with severe disease exhibit clinical signs, while others remain asymptomatic (Cousins et al., 1994; Parsons et al., 2008).

Table 2.2 Mycobacterium tuberculosis complex (MTBC) index cases in free-ranging wildlife in South Africa, detailed by the year and location of the affected species with references.

MTBC	Species	Year	Location	Reference/s
Mtb[a]	*Papio ursinus*	1998	Western Cape	Parsons et al., 2009
	Loxodonta africana	2018	Kruger National Park	Miller et al., 2019
Dassie bacillus	*Procavia capensis*	1954	Eastern and Western Cape	Wagner et al., 1958; Parsons et al., 2008
M. mungi[c]	*Mungos mungo*	1999	Botswana and Zimbabwe	Alexander et al., 2010; Fitzermann, 2017
M. surricattae[b]	*Suricata suricattae*	1990s	Northern Cape	Drewe, 2010; Parsons et al., 2013
M. orygis[d]	*Syncerus caffer*	2007	KwaZulu-Natal	Gey van Pittius et al., 2012

[a] *Mycobacterium tuberculosis*

[b] *Mycobacterium suricattae*

[c] *Mycobacterium mungi*

[d] *Mycobacterium orygis*

4.2. *M. mungi*

In 1999, TB due to *M. mungi* was reported in banded mongooses in the Chobe National Park in Botswana, on the northern border of SA, and between 1999 and 2010 additional outbreaks, causing rapidly progressive disease in mongooses, were documented in this region as well as adjacent areas in Zimbabwe (Alexander et al., 2010). Although this pathogen has not yet been

documented in SA, spillover from Zimbabwe to SA is expected. Due to erosion of the nasal planum in infected mongooses, the likely route of entry is the nasal cavity (Alexander et al., 2016). However, shedding of *M. mungi* from anal gland secretions suggests an additional route of transmission in this species. Furthermore, a study demonstrated an association between mongooses foraging in garbage and increased risk of acquiring TB when bacteria enter broken skin incurred through injuries (Flint et al., 2016).

4.3. *M. suricattae*

The first confirmatory diagnosis of TB in meerkats in SA was in 2001, in a long-term study population, the Kalahari Meerkat Project in the Northern Cape (Drewe et al., 2009; Drewe, 2010). The causative organism was later identified as *M. suricattae* (Parsons et al., 2013). There have been numerous additional reports of TB fatalities in this population of meerkats (Clarke et al., 2016b). Several sources of *M. suricattae* infection and transmission routes have been suggested in meerkats. Being a strongly social species, respiratory infection may occur when in close contact while transmission via wounds during grooming or fighting has also been proposed (Drewe, 2010; Drewe et al., 2011). Meerkats appear to be very susceptible to disease and present with typical granulomas in the lungs and other organs, with rapid disease progression to death (Alexander et al., 2002). A study reported older animals may be at a greater risk of disease than younger animals and suggested group and individual level risk factors may exist for developing disease in meerkats (Patterson et al., 2017). As clinical signs associated with TB are more often seen in meerkats and banded mongooses than rock hyraxes, the virulence of *M. suricattae* and *M. mungi* is suggested to be higher than for the dassie bacillus (Fitzermann, 2017).

4.4. *M. orygis*

In 2007, *M. orygis*, then classified as oryx bacillus, was identified in a buffalo with typical TB lesions on a private wildlife reserve in KZN (Gey van Pittius et al., 2012). Since then, no additional cases of *M. orygis* infection in buffaloes or other wildlife species have been reported in SA, although the limited genetic speciation of isolated MTBC members may contribute to this. Limited information is available regarding the susceptibility of wildlife to *M. orygis* infection and disease.

5. Diagnostic tools for TB in wildlife in SA

The validation of diagnostic tests for wildlife is limited by the access to and number of high-quality samples from confirmed infected and uninfected species (Chileshe et al., 2019). Due to logistical challenges, it can be difficult to confirm MTBC infection, especially from suspected cases using ante mortem samples. In this section, we review recent advances in techniques for detecting MTBC infection in wildlife in SA.

5.1. Mycobacterial culture

In recent years, there have been advances in techniques for improving direct detection of MTBC organisms; however, most techniques still require growing the organism to detectable levels using different mycobacterial culture techniques. Due to the inherent slow growth of mycobacteria, the development of improved culture techniques using special media was initiated. The BACTEC™ MGIT™ (Becton Dickinson, Franklin Lakes, NJ, USA) is an automated mycobacterial growth detection system that has been used to culture MTBC organisms from post mortem tissue samples as well as ante mortem bronchoalveolar lavage (BAL), trunk wash fluid and oropharyngeal swab samples from wildlife. Application of these techniques has permitted ante mortem diagnosis of *M. bovis* infection in lion, wild dog and white rhinoceros (Miller et al., 2015a; Michel et al., 2017; Higgitt et al., 2019). Moreover,

TiKa (TiKa Diagnostics, UK) is a novel specialised culture medium, used together with the BACTEC™ MGIT™ system, with the unique ability to stimulate MTBC growth, and improve sensitivity (Se) of mycobacterial culture, even from samples with low bacterial loads or high dilution. TiKa increases mycobacterial recoverability, improves the Se of detection and decreases the time required to determine a result compared to standard culture methods (Bull et al., 2017). A pilot study has shown TiKa improves MTBC growth and time to a positive result in a number of wildlife species including buffaloes, elephants and rhinoceroses (Goosen, unpubl. data).

5.2. Direct detection

Various polymerase chain reaction (PCR)-based methods have been developed and adapted to identify specific MTBC organisms, based on detecting the presence of mycobacterial DNA, from either cultured clinical samples or directly from clinical samples (Michel et al., 2009; Goosen, unpubl. data). Three of the most common molecular typing tools used to genetically differentiate MTBC members are: i) spacer oligonucleotide typing (spoligotyping), ii) variable number of tandem repeats (VNTR) typing of mycobacterial interspersed repetitive units (MIRU), and iii) region of difference (RD) analysis (Kamerbeek et al., 1997; Brudey et al., 2004; Supply et al., 2006; Warren et al., 2006). Spoligotyping is most commonly used to detect and genotype the MTBC isolates to determine the phylogenetic relationships to organisms from specific geographical regions and sources (Table 2.3). Next generation sequencing (NGS) is a novel tool with increased resolution and discriminatory power compared to the other three genotyping methods. The generation of whole genome sequences (WGS) allows distinct genetic profiles to be identified at a nucleotide level, and MTBC molecular epidemiology and genetic diversity can be investigated with finer resolution (Dippenaar et al., 2017). In warthogs, NGS permitted the

differentiation of MTBC isolates unable to be differentiated using spoligotyping (Roos, 2018). Therefore, increased application of WGS will improve molecular epidemiology of TB outbreaks in wildlife and at interfaces.

Table 2.3 The SB numbers of Mycobacterium bovis isolates identified in free-ranging wildlife species in South Africa as determined by spoligotyping, by the location of the infected species and including references.

SB #	KNP[a]	GKNP[b]	HiP[c]	KZN[d]	MGR[e]	References
SB0120	-	√	-	-	-	Hlokwe et al., 2014; Roos, 2018
SB0121	√	√	√	√	-	Hlokwe et al., 2011; Dippenaar et al., 2017 Miller et al., 2018a; Roos, 2018
SB0130	-	√	√	√	√	Hlokwe et al., 2011, 2014; Michel et al., 2009; Dippenaar et al., 2017
SB0140	-	-	√	√	√	Hlokwe et al., 2016; Dippenaar et al., 2017
SB1275	-	√	-	-		Roos, 2018
SB1388	-	√	-	-	-	Roos, 2018
SB1474	-	-	√	√	-	Hlokwe et al., 2011
SB2200	-	√	-	-	-	Hlokwe et al., 2014
SB0294	-	√	-	-	-	Hlokwe et al., 2019

[a] Greater Kruger National Park

[b] Kruger National Park

[c] Hluhluwe iMfolozi Park

[d] KwaZulu-Natal

[e] Madikwe Game Reserve

Automation of PCR-based methods allows procedures to be simplified, standardised and is useful for high throughput data. The GeneXpert® (Cephied, Sunnyvale, CA, USA) is a cartridge-based automated screening test endorsed by the World Health Organisation

designed to diagnose *Mtb* infection in humans via PCR. It is a rapid, simple-to-use, automated platform that is being optimised to detect *Mtb* and *M. bovis* from wildlife samples including homogenised post mortem tissue samples and ante mortem BAL fluids and swabs (Goosen, unpubl. data).

5.3. Indirect detection

Despite improvements in identifying MTBC directly, detection of host immune responses remains the principal method used to diagnose infection in wildlife. Indirect methods rely on measuring the anamnestic cell-mediated or humoral immune responses to MTBC antigens in host species (Table 2.4). The presentation of clinical disease may also be used as an indirect method of detecting TB but this is insensitive as clinical disease is typically only observed in advanced stages (Renwick et al., 2007).

5.3.1. Cell-mediated immunity

The detection of cell-mediated immune responses relies on stimulating a memory response, *in vivo* or *in vitro*, with MTBC antigens and then quantifying that response as a delayed-type hypersensitivity reaction or production of cytokine biomarkers of immune activation, respectively. The development of specific stimulating antigens instead of tuberculin, which varies according to batches and sources (Monaghan et al., 1994), has improved the detection of cell-mediated immune responses to MTBC organisms (Goosen et al., 2014). The QuantiFERON® TB-Gold (QFT) system is an easy-to-use stimulation platform, with standardised antigenic peptides and controls, which when used with specific cytokine enzyme-linked immunosorbent assays (ELISAs), can detect *Mtb* and *M. bovis* infection in a number of wildlife species (Parsons et al., 2009; Olivier et al., 2017; Higgitt et al., 2017, 2018; Roos et al., 2018; Bernitz et al., 2019). Gene expression analysis (GEA), in which a

changing gene expression pattern reflects a biological response, has shown utility in the diagnosis of *M. bovis* infection in lions and buffaloes by measuring the expression of CXCL9 mRNA and interferon gamma (IFN-γ) mRNA, respectively (Parsons et al., 2012; Olivier et al., 2017). Furthermore, GEA has been used to detect immune sensitization to *M. bovis* in spotted hyena, measuring the expression of CXCL9 and CXCL11 mRNA (Higgitt et al., 2017).

The cytokine IFN-γ is the most commonly used biomarker to quantify *in vitro* cell-mediated immune responses in wildlife. Commercially available ELISAs have been evaluated and optimised to detect cytokine production in specific wildlife species; the ruminant *cattletype*® IFN-gamma ELISA (Qiagen, Venlo, Limburg, Netherlands) detects buffalo IFN-γ (Bernitz et al., 2018), the Quantikine canine IFN-γ ELISA (R&D Systems, Inc., MN, USA) detects wild dog IFN-γ (Higgitt et al., 2017) and the equine IFN-γ ELISA^{PRO} (Mabtech, Nacka Strand, Sweden) detects white rhinoceros IFN-γ (Chileshe et al., 2019). Moreover, alternative biomarkers to IFN-γ have been investigated with the chemokine IFN-γ-inducible protein-10 (IP-10), which has been shown to be a sensitive biomarker of immune activation in buffalo, meerkats and warthogs (Clarke et al., 2016b; Roos et al., 2018; Bernitz et al., 2019). Additionally, the parallel measurement of IFN-γ and IP-10 has been shown to maximise the detection of *M. bovis*-infected buffaloes while maintaining specificity (Sp) (Bernitz et al., 2019).

5.3.2. Humoral immunity

Serological assays have also been used to detect infection by MTBC organisms in multiple wildlife species and results may be obtained faster than cell-mediated immunity (CMI)-based

Table 2.4 Indirect diagnostic tools to detect infection with members of the Mycobacterium tuberculosis complex in free-ranging wildlife species in South Africa.

Species	**TST/SCITT**[a]	**IGRA**[b]	**CRA**[c]	**Serology**	**GEA**[d]	**References**
Syncerus caffer	√	√	√	√	√	Jolles et al., 2005; Parsons et al., 2012; van der Heijden et al., 2016; Bernitz et al., 2019
Papio ursinus	√	√	-	-	-	Keet et al., 2000; Parsons et al., 2009
Panthera leo	√	√	-	√	√	Keet et al., 2010; Miller et al., 2012; Olivier et al., 2017; Viljoen et al., 2018
Panthera pardus	√	-	-	-	-	Kerr unpubl. data
Crocuta crocuta	-	-	-	-	√	Higgitt et al., 2017
Acinonyx jubatus	√	-	-	√	√	Kerr unpubl. data
Lycaon pictus	√	√	-	√	-	Higgitt et al., 2018
Phacochoerus aethiopicus	√	-	√	√	-	Roos et al., 2016, 2018
Ceratotherium simum	-	√	-	-	-	Chileshe et al., 2019
Diceros bicornis	-	√	-	√	-	Morar et al., 2013; Miller et al., 2015b
Loxodonta africana	-	√	-	√	-	Kerr et al., 2019
Suricata suricattae	-	-	√	√	-	Drewe et al., 2009; Clarke et al., 2016b

[a] tuberculin skin test/single comparative intradermal tuberculin test

[b] interferon gamma release assay

[c] cytokine release assay

[d] gene expression assay

assays as whole blood stimulation is not required. However, CMI-based assays have shown greater Se in many species (Maas et al., 2013) and detect mycobacterial infections earlier than serological assays, as the humoral immune response requires the *in vivo* production of antibodies, which may only occur later in infection. Circulating antibodies can be detected via ELISA as demonstrated in warthogs where an indirect in-house purified protein derivative (PPD) ELISA and the TB ELISA-VK® kit (Vacunek, Bizkaia, Spain) were able to differentiate between *M. bovis* culture-positive and negative animals (Roos et al., 2016). Alternatively, circulating antibodies can be detected using rapid cartridge-based systems. The Dual Path Platform Vet TB Assay (DPP® VetTB) for Cervids® (Chembio Diagnostic Systems, Inc., Medford, New York, USA) has been used to diagnose *M. bovis* infection in warthogs (Roos et al., 2016) and the Elephant TB STAT-PAK® Assay (Chembio) and the DPP® VetTB for Elephants (Chembio) was used to determine the seroprevalence of *M. bovis* infection in elephants in KNP (Kerr et al., 2019). The development and optimisation of diagnostic tools to identify MTBC infection is the gateway to identify affected species or individuals to manage transmission and disease.

6. Discussion

Multi-host pathogen systems are intrinsically complex and an ecosystem level approach to effective disease management is required. *Mycobacterium bovis* and *Mtb* are multi-host pathogens and thus, the population dynamics of each host species and multidirectional transmission at interfaces makes control difficult. The epidemiology of the pathogen in each host needs to be understood at an individual and population level as information from one species cannot be easily extrapolated to the next species. The acquisition of infection varies between hosts and this may influence

susceptibility, disease progression and transmission. This may define the role of the host in the ecosystem, i.e. a maintenance or dead-end. Lions become infected with *M. bovis* via ingesting infected meat and this may cause generalised disease. Systemic infection results in relatively rapid mortality in lions (Keet et al., 1996) and for this reason, lions are considered dead-end hosts. Alternatively, buffaloes become infected via inhalation of aerosolised bacteria and this causes localised disease in the lungs and lymph nodes (Michel et al., 2007). Morbidity and mortality due to *M. bovis* infection may take months or years, so infected buffaloes are constant sources of infection and for this reason are considered maintenance hosts. Moreover, an array of factors such as environmental conditions, like droughts, may impact the risk and outcome of infection.

Experimental infection of wildlife is not always be possible, but allows an in-depth understanding of the infection, immune responses, pathological changes, clinical signs and transmission of wildlife TB (Michel et al., 2017). Moreover, a powerful tool to perform in depth epidemiological analyses is NGS. In New Zealand, NGS was used to investigate transmission of *M. bovis* between cattle and brushtail possums (*Trichosurus vulpecula*) with the possibility of incorporating this method of typing into routine testing programmes (Crispell et al., 2017). As the cost of NGS decreases and accessibility increases, this method may become the standard tool for investigating TB outbreaks. Moreover, NGS can be used to determine if the reoccurrence of bTB in populations is residual infection or reintroduction, which improves the understanding of the infection risks, transmission and maintenance hosts (Biek et al., 2012; Walker et al., 2013; Crispell et al., 2017).

The increase in geographical distribution of TB in SA highlights the need to identify infected species by enforcing testing and preventing the movement of test-positive animals (Hlokwe et al., 2014). The human/wildlife/livestock interface is increasing with highly fragmented conservation areas being encroached upon by humans (Davies-Mostert et al., 2015; Hunter, 2018). Moreover, poorly maintained biosecurity (fences) within SA, and between SA and bordering countries, increase the risk of geographical spread of TB (Hlokwe et al., 2014). However, physical barriers such as biosecurity measures may not be effective in containing warthogs or kudus, which are two potential maintenance hosts of *M. bovis* in SA (Keet et al., 2001; Roos et al., 2018).

In SA over the past decade, MTBC members have been discovered in new wildlife species. It is unknown whether this is due to an advancement in diagnostic tools, increased inter-species transmission, or a combination of both (Hlokwe et al., 2014). Moreover, the true prevalence and incidence of wildlife TB is unknown, even though this is important for understanding the epidemiology and management of this disease. Neglecting subclinical infections may lead to misleading conclusions regarding the source, maintenance, and transmission of TB within or between populations.

Current tests to detect TB infection in wildlife usually require animals to be captured and chemically immobilised. In addition to the cost and logistical issues, the intermittent availability of tuberculin and immobilisation drugs in SA can limit testing. The development of tools with improved performance that require non-invasive sampling methods, such as faecal sampling, would greatly improve screening options (Roug et al., 2014). For endangered/threatened hosts, conventional methods

of bTB control, such as test-and-cull programmes, cannot be ethically implemented, although may be a regulatory requirement. To increase the detection of infected species in SA, surveillance needs to be increased, which is currently limited by the diagnostic tools available**.** A pilot surveillance project in KNP, using retrospective serological testing (DPP® VetTB) and prospective culture of BAL samples, has been implemented to detect *M. bovis* and *Mtb* infection in elephants to assess the potential risk in this species (Miller et al., 2019). However, there are other opportunities for surveillance, including examination of hunted animals, those found dead due to poaching or other events, animals culled for population management, during immobilization for movement, and at the abattoir (Miller et al., 2017a). These may be less costly and provide a broader sampling of animals to increase surveillance.

Although the need for control of TB in wildlife in SA is recognised, similar to many developing countries worldwide, limited resources confound control measures. Wildlife TB threatens management and conservation efforts to protect wildlife and joint cooperation by government, communal and commercial farmers, conservation organizations, veterinarians and industry is required to effectively control the disease. The conservation of all susceptible species relies on accurate tools to identify and remove infected animals from the population or limit the movement of infected animals to areas that remain uninfected.

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Chapter 3 : Detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) tubes and the Qiagen *cattletype*® IFN-gamma ELISA

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Abstract

African buffaloes (*Syncerus caffer*) are wildlife maintenance hosts of *Mycobacterium bovis*, the cause of bovine tuberculosis. Consequently, *M. bovis*-infected buffaloes pose a transmission risk for cattle and other wildlife species. Previously, a modification to the Qiagen QuantiFERON®-TB Gold (QFT) system, using QFT tubes and an in-house bovine interferon-gamma (IFN-γ) ELISA, was evaluated for the detection of *M. bovis* infection in buffaloes. Subsequently, Qiagen has developed a commercially available *cattletype*® IFN-gamma ELISA for the detection of antigen-specific IFN-γ release in ruminants. The aim of this study was to investigate the use of QFT tubes and the *cattletype*® IFN-gamma ELISA, in a QFT IFN-γ release assay (IGRA), to detect *M. bovis* infection in African buffaloes. The test agreements between the QFT IGRA, single comparative intradermal skin test (SCITT) and Bovigam® IGRA in two *M. bovis*-exposed buffalo populations (n = 134 and n = 92) were calculated and κ coefficients ranged from 0.65 (95% CI 0.48-0.82%) to 0.86 (95% CI 0.72-0.99%). Increasing the QFT incubation time in one *M. bovis*-exposed buffalo cohort (n = 92), from 20 to 40 h, had no effect on the QFT IGRA test results. Inter-assay and intra-assay reproducibility determination for the QFT IGRA produced coefficient of variations (CV) < 9.1% and < 1.7%, respectively. A total of 21/21 known *M. bovis*-unexposed buffaloes tested QFT IGRA-negative. Moreover, the QFT IGRA test result values were significantly greater for 13 *M. bovis* culture-positive buffaloes compared with 14 *M. bovis*-exposed culture-negative ($p < 0.01$) and 21 *M. bovis*-unexposed ($p < 0.001$) buffaloes, respectively. These findings suggest that the combination of QFT tubes and the *cattletype*® IFN-gamma ELISA is a promising new diagnostic assay for the detection of *M. bovis*-infection in African buffaloes.

However, further research is needed to evaluate the sensitivity and specificity of the assay in larger African buffalo populations.

1. Introduction

Mycobacterium bovis (*M. bovis*) infection causes bovine tuberculosis (bTB), a zoonotic disease affecting a broad range of hosts (Michel et al., 2006). Two of South Africa's (SA) largest wildlife reserves, the Kruger National Park (KNP) and Hluhluwe iMfolozi Park (HiP), are endemic for *M. bovis*. Within these reserves, African buffaloes (*Syncerus caffer*) are one of the most important maintenance hosts of *M. bovis* (de Vos et al., 2001). More recently, buffaloes in smaller reserves like Madikwe Game Reserve (MGR) and privately-owned farms in SA have been diagnosed with bTB (Hlokwe et al., 2016). *M. bovis*-infected buffaloes do not only pose a transmission risk to other wildlife species, particularly large carnivores such as African lions (*Panthera leo*) (Olivier et al., 2017), but also to economically important livestock such as domestic cattle populations (de Vos et al., 2001; Musoke et al., 2015). Early detection of *M. bovis* infection in buffaloes is necessary to reduce the risk of transmission to other animals. Furthermore, prompt identification of these animals will reduce the risk of infection in buffaloes through translocation and improve the success of disease management strategies.

The early diagnosis of *M. bovis* infection relies primarily on the measurement of pathogen-specific cell-mediated immune responses (Vordermeier et al., 2000). In South Africa, the only such test approved for buffaloes is the single comparative intradermal tuberculin test (SCITT) that detects a delayed-type hypersensitivity response to *M. bovis* purified protein derivative (PPD_b). However, this test has numerous drawbacks: i) the use of PPD_b, a cocktail of *M. bovis* antigens, may result in cross-reactive immune responses to other non-virulent mycobacteria (Michel, 2008); ii) repeating the SCITT may lead to the desensitization of infected animals

(Coad et al., 2010) and iii) the interpretation of the SCITT response can be subjective and may vary between operators (Pers. Comm. A McCall). Furthermore, administering and reading the test in wildlife poses logistical challenges as animals need to be confined for three days and immobilised twice during this period.

An alternative test, the interferon-gamma (IFN-γ) release assay (IGRA) is an *in vitro* whole blood assay that is more suitable than the SCITT for use in wildlife since it only requires the collection of a single blood sample (Grobler et al., 2002). The Bovigam® IGRA, utilizing both PPD_b and *Mycobacterium avium* PPD (PPD_a) as stimulating antigens, is a commercially available IGRA used for the diagnosis of *M. bovis* infection in domestic cattle (de la Rua-Domenech et al., 2006) and has shown great promise for use in buffaloes (Grobler et al., 2002). The inclusion of PPDs as antigens in this IGRA, as for the SCITT, may also result in a cross-reactive immune response to non-virulent mycobacteria; however, the specificity (Sp) of IGRAs can be improved by using specific mycobacterial peptides as stimulating antigens (Vordermeier et al., 2001; Bass et al., 2013).

The test performance of a modified QuantiFERON®-TB Gold (QFT) assay (mQFT), using commercially available QFT tubes and an in-house bovine-specific IFN-γ ELISA, has previously been reported for African buffaloes (Parsons et al., 2011). The QFT system uses three specific antigenic peptides, early secretory antigen target 6 kD (ESAT-6), culture filtrate protein 10 kD (CFP-10) and TB7.7(p4), encoded in the region of difference one (RD1) that is not present in most nontuberculous mycobacteria and *M. bovis* Bacille Calmette-Guèrin (Vordermeier et al., 2001, Warren et al., 2006). Parsons et al. (2011) showed the relative sensitivity (Se) and Sp

of the mQFT to be comparable to that of the SCITT. Additionally, the relative Se of the mQFT could be improved by increasing the whole blood antigen incubation time (Goosen et al., 2014).

Qiagen has developed a new commercially available *cattletype*® IFN-gamma ELISA, suitable to detect antigen-induced IFN-γ release, with a specified cutoff for determining positive and negative responses. In this study, we aimed to investigate the use of QFT tubes in conjunction with the *cattletype*® IFN-gamma ELISA in a novel IGRA. We aimed to: i) compare the performance of the QFT IGRA with the SCITT and Bovigam® IGRA; ii) investigate the effect of increased duration of blood incubation on the assay result; iii) determine the reproducibility of the assay and iv) evaluate the assay in *M. bovis*-unexposed buffaloes.

2. Materials and methods

2.1. *M. bovis*-exposed buffaloes in HiP

During July 2016, 403 randomly selected free-ranging buffaloes were mass captured during the park's 18th annual bTB test-and-cull programme. All buffaloes were immobilised, lithium heparin whole blood was collected via jugular venipuncture and the SCITT were performed as described below. All suspect and positive SCITT buffaloes (n = 22) were culled by gunshot. In addition to the 22 buffaloes, an additional five SCITT-negative buffaloes were culled based on suspicion of bTB. Of all the SCITT-negative buffaloes (n = 381), 70 animals were randomly selected and used together with all culled buffaloes for this study.

2.2. *M. bovis*-exposed buffaloes in MGR

One-hundred and thirty-four buffaloes were opportunistically sampled from the MGR during bTB prevalence testing from May to July 2016. Free-ranging buffaloes were individually captured in MGR and immobilised, after which lithium heparin whole blood was collected via jugular venipuncture and SCITTs performed as described below. Bovigam® IGRAs were performed at the Tuberculosis Laboratory of the Agricultural Research Council (ARC)-Onderstepoort Veterinary Institute in Pretoria, South Africa as described previously (van der Heijden et al., 2017). All suspect and positive SCITT buffaloes and positive Bovigam® IGRA buffaloes (n = 34) were culled by gunshot. All buffaloes sampled (n = 134) were used in this study.

2.3. *M. bovis*-unexposed and uninfected buffaloes

Twenty-one buffaloes were opportunistically sampled from *M. bovis*-free farms (based on previous herd history) in South Africa during 2016 and were used as *M. bovis*-uninfected controls.

2.4. SCITT

All SCITTs were performed in HiP and MGR as previously described (Parsons et al., 2011). Briefly, an area on either side of the buffalo's neck was shaved and the baseline skin fold thickness (SFT) measured. Intradermal injections of 0.1 ml of PPD_b (3000 IU) and 0.1 ml of PPD_a (2500 IU) were administered on the left and right side of the animal's neck, respectively. After three days, buffaloes were immobilised and their SFT measured. A differential SFT measurement of $\geq$ 2 mm between the PPD_b injection site compared with the PPD_a injection site was defined as SCITT-positive. Subjective criteria were used to classify animals with differential inflammatory

reactions of between 0 and 2 mm as SCITT-suspect (Parsons et al., 2011).

2.5. Bovigam® IGRA in HiP

Whole blood aliquots (250 µl) from all 403 buffaloes sampled were co-incubated with i) 25 µl phosphate buffered saline (Nil) ii) 25 µl PPD_a (2750 IU/ml) (Onderstepoort Biological Products, Pretoria, South Africa) iii) 25 µl PPD_b (3300 IU/ml) (Onderstepoort Biological Products) and iv) 25 µl pokeweed mitogen (PWM), 5 µg/ml final concentration (Sigma-Aldrich, St. Louis, MO, USA) in 2 ml safe-cap microcentrifuge tubes. All samples were inverted three times, incubated at 37 °C for 20 h, centrifuged at 2550 x *g* for 10 min after which the plasma fraction was harvested and stored at -4 °C for later detection of IFN-γ using the Bovigam® ELISA (Prionics AG, Schlieren-Zurich, Switzerland) per manufacturer's protocols. Due to the unavailability of Bovigam® ELISA plates at the time of testing in HiP, the Bovigam® ELISA was conducted retrospectively on samples from selected animals.

2.6. QFT IGRA

For all HiP (*n* = 403) and MGR (*n* = 134) buffaloes, 1 ml aliquots of heparinised whole blood were transferred to QFT tubes (Qiagen, Venlo, Limburg, Netherlands) comprising of: i) Nil tube containing saline; ii) TB antigen tube containing antigenic peptides and iii) mitogen tube, as previously described (Parsons et al., 2011). All tubes were inverted ten times and incubated at 37 °C for 20 h. For samples collected in MGR, the plasma fraction was harvested following centrifugation at 3000 × *g* for 10 min. For samples collected in HiP, 150 µl of plasma was harvested from the tubes and these were incubated for an additional 20 h. The remaining plasma was then harvested as above. The *cattletype*® IFN-gamma ELISA (Qiagen) was performed per

manufacturer's protocols using all plasma samples harvested after 20 h and plasma harvested from the Nil and TB antigen tubes after 40 h from the HiP buffaloes. Results are presented as sample to positive ratio (S/P): (OD of QFT TB antigen – OD of QFT Nil)/ (OD *cattletype*® IFN-gamma ELISA positive control – OD *cattletype*® IFN-gamma ELISA negative control) x 100 as per manufacturer's protocol. A positive test result was defined as: S/P ≥ 35%.

2.7. Reproducibility of the QFT IGRA

Heparinised whole blood was collected post-SCITT from two QFT IGRA/SCITT-positive animals and one QFT IGRA/SCITT-negative animal. For each animal, blood was incubated at 37 °C for 20 h in three separate sets of QFT Nil and TB antigen tubes, and one QFT Mitogen tube. Plasma was harvested following centrifugation and three separate *cattletype*® IFN-gamma ELISAs were performed on the same day using the replicate samples.

2.8. Post mortem examination and mycobacterial culture

Post mortem examinations were performed on all culled buffaloes from HiP (n = 22). All lymph nodes, lungs and bTB-like lesions were sampled carefully for mycobacterial culture. All culture-positive samples were genetically speciated to confirm *M. bovis* infection (Warren et al., 2006). Mycobacterial culture and genetic speciation were performed at Stellenbosch University as previously described (Goosen et al., 2014). Samples collected during post mortems in MGR were not available as part of this study and were therefore not included in the analysis.

2.9. Data analyses

Test agreements between the QFT IGRA, SCITT and Bovigam® IGRA were calculated as Cohen's κ coefficient using the agreement calculator on the GraphPad Software website (http://graphpad.com/quickcalcs/kappa1/). The coefficient of variation (CV) for each stimulation condition, was calculated to determine the inter- and intra-assay reproducibility of the QFT IGRA. Test results for 21 *M. bovis*-unexposed buffaloes were compared with 14 *M. bovis*-exposed culture-negative and 13 *M. bovis* culture-positive buffaloes, using the Kruskal-Wallis H test with a Dunn's Multiple Comparison Test [H (2); $p < 0.05$] available on the GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

In HiP, 9/92 (16%), 19/92 (20%) and 20/92 (21%) buffaloes were QFT IGRA, SCITT and Bovigam® test-positive, respectively and in MGR, 24/134 (18%), 30/134 (22%) and 22/134 (16.5%) of buffaloes were test-positive for the aforementioned tests, respectively. All 21 *M. bovis*-unexposed buffaloes were QFT IGRA test-negative.

In the combined *M. bovis*-exposed populations ($n = 226$), test agreements between the QFT IGRA and SCITT, and the two IGRAs had κ coefficients of 0.69 and greater (Table 3.1). In HiP, test agreement between the QFT IGRA and SCITT was calculated as $\kappa = 0.86$ (95% CI 0.72-0.99%); test agreement between the two IGRAs was calculated as $\kappa = 0.75$ (95% CI 0.58-0.93%) (Table 3.2). Similar test agreements were observed in MGR; agreement between the QFT IGRA and SCITT was calculated as $\kappa = 0.72$ (95% CI 0.58-0.87%), and agreement between the two IGRAs calculated as $\kappa = 0.65$ (95% CI 0.48-0.82%) (Table 3.3).

Table 3.1 Cohen's kappa coefficient (κ) and 95% confidence interval estimates of agreement between the QFT IGRA, SCITT and Bovigam® IGRA for the detection of Mycobacterium bovis infection in African buffaloes in two M. bovis-exposed populations (n = 226).

	QFT IGRA[a]	**SCITT[b]**	**Bovigam® IGRA**
QFT IGRA	1	0.78 (0.67-0.88)	0.69 (0.57-0.81)
SCITT	0.78 (0.67-0.88)	1	0.70 (0.58-0.82)
Bovigam® IGRA	0.69 (0.57-0.81)	0.70 (0.58-0.82)	1

[a] interferon gamma release assay

[b] single comparative intradermal tuberculin test

Table 3.2 Cohen's kappa coefficient (κ) and 95% confidence interval estimates of agreement between the QFT IGRA, SCITT and Bovigam® IGRA for the detection of Mycobacterium bovis infection in African buffaloes in Hluhluwe iMfolozi Park, South Africa (n = 92).

	QFT IGRA[a]	**SCITT[b]**	**Bovigam® IGRA**
QFT IGRA	1	0.86 (0.72-0.99)	0.75 (0.58-0.93)
SCITT	0.86 (0.72-0.99)	1	0.77 (0.61-0.93)
Bovigam® IGRA	0.75 (0.58-0.93)	0.77 (0.61-0.93)	1

[a] interferon gamma release assay

[b] single comparative intradermal tuberculin test

Table 3.3 Cohen's kappa coefficient (κ) and 95% confidence interval estimates of agreement between the QFT IGRA, SCITT and Bovigam® IGRA for the detection of Mycobacterium bovis infection in African buffaloes from Madikwe Game Reserve, South Africa (n = 134).

	QFT IGRA[a]	**SCITT[b]**	**Bovigam® IGRA**
QFT IGRA	1	0.72 (0.58-0.87)	0.65 (0.48-0.82)
SCITT	0.72 (0.58-0.87)	1	0.65 (0.49-0.81)
Bovigam® IGRA	0.65 (0.48-0.82)	0.65 (0.49-0.81)	1

[a] interferon gamma release assay

[b] single comparative intradermal tuberculin test

The effect of lengthening the antigen incubation time of the QFT IGRA, from 20 to 40 h, had no effect on the QFT IGRA test results in HiP (n = 92); the test results for all buffaloes that tested either positive or negative at 20 h remained the same at 40 h (data not shown).

The two QFT IGRA-/SCITT-positive buffaloes selected for the reproducibility study were retrospectively confirmed to be Bovigam® IGRA-/*M. bovis* culture-positive and the one QFT IGRA-/SCITT-negative buffalo selected was confirmed to be Bovigam® IGRA-negative. For the QFT IGRA, the inter-assay (test variability) CVs were < 9.1% and the intra-assay (test precision) CVs were < 1.7% (Tables 3.4 and 3.5). The S/P values remained unchanged for each replicate assay (data not shown).

All *M. bovis*-unexposed buffaloes (n = 21) from historical *M. bovis*-free farms were QFT IGRA test-negative with S/P values < 0.9% (data not shown).

Table 3.4 Inter-assay variability of the QFT IGRA to detect Mycobacterium bovis infection in African buffaloes as determined by the coefficient of variation (CV) using buffaloes from Hluhluwe iMfolozi Park, South Africa.

Buffalo	**Sample**	**Mean of means[a]**	**SD of means[b]**	**CV of means [c] (%)**
Positive #1[d]	Nil	0.06	0.00	4.73
	TB antigen	2.24	0.17	7.48
Positive #2[d]	Nil	0.06	0.00	4.12
	TB antigen	2.14	0.15	6.81
Negative[e]	Nil	0.00	0.00	0.00
	TB antigen	0.06	0.01	9.08

[a] mean of means of triplicates

[b] standard deviation of means of triplicates

[c] coefficient of variation of means of triplicates

[d] QFT IGRA-/SCITT-/Bovigam® IGRA-/*M. bovis* culture-positive

[e] QFT IGRA-/SCITT-/Bovigam® IGRA-negative

The median S/P values of 13 *M. bovis* culture-positive buffaloes were significantly greater compared with the median S/P values of 21 *M. bovis*-unexposed (H = 28.5; $p < 0.001$) and 14 *M. bovis*-exposed culture-negative buffaloes (H = 15.9; $p < 0.01$) (Figure 3.1). The S/P values from the *M. bovis*-exposed culture-negative buffaloes were also significantly greater than the *M. bovis*-unexposed buffaloes (H = 12.62; $p < 0.05$), although the majority (10/13; 77%) of the *M. bovis*-exposed culture-negative

buffaloes had S/P values < 35% and were therefore QFT IGRA test-negative (Figure 3.1).

Table 3.5 Intra-assay variability of the QFT IGRA to detect Mycobacterium bovis infection in African buffaloes as determined by the coefficient of variation (CV%) using buffaloes from Hluhluwe iMfolozi Park, South Africa.

Buffalo	**Sample**	**Mean[a]**	**SD[b]**	**CV[c] (%)**
Positive #1[d]	Nil	0.06	0.00	0.63
	TB antigen	2.04	0.02	1.20
Positive #2[d]	Nil	0.05	0.00	1.68
	TB antigen	2.02	0.01	0.64
Negative[e]	Nil	0.00	0.00	0.00
	TB antigen	0.06	0.00	1.63

[a] mean of triplicates

[b] standard deviation of triplicates

[c] coefficient of variation of triplicates

[d] QFT IGRA-/SCITT-/Bovigam® IGRA/*M. bovis* culture-positive

[e] QFT IGRA-/SCITT-/Bovigam® IGRA-negative

4. Discussion

It has been previously reported that the Sp of IGRAs may be improved by using specific mycobacterial peptides (Parsons et al., 2011), with a possible decrease in Se (Goosen et al., 2014). In this study, we have shown the test performance of the QFT IGRA, comprised of QFT tubes containing ESAT-6, CFP-10, and TB7.7(p4)

stimulating antigenic peptides and the commercially available ruminant-specific *cattletype*® IFN-gamma ELISA, to be similar to that of the currently available bovine-specific assays that use PPDs *in vivo* (SCITT) or *in vitro* (Bovigam® IGRA) for the detection of *M. bovis* infection in African buffaloes.

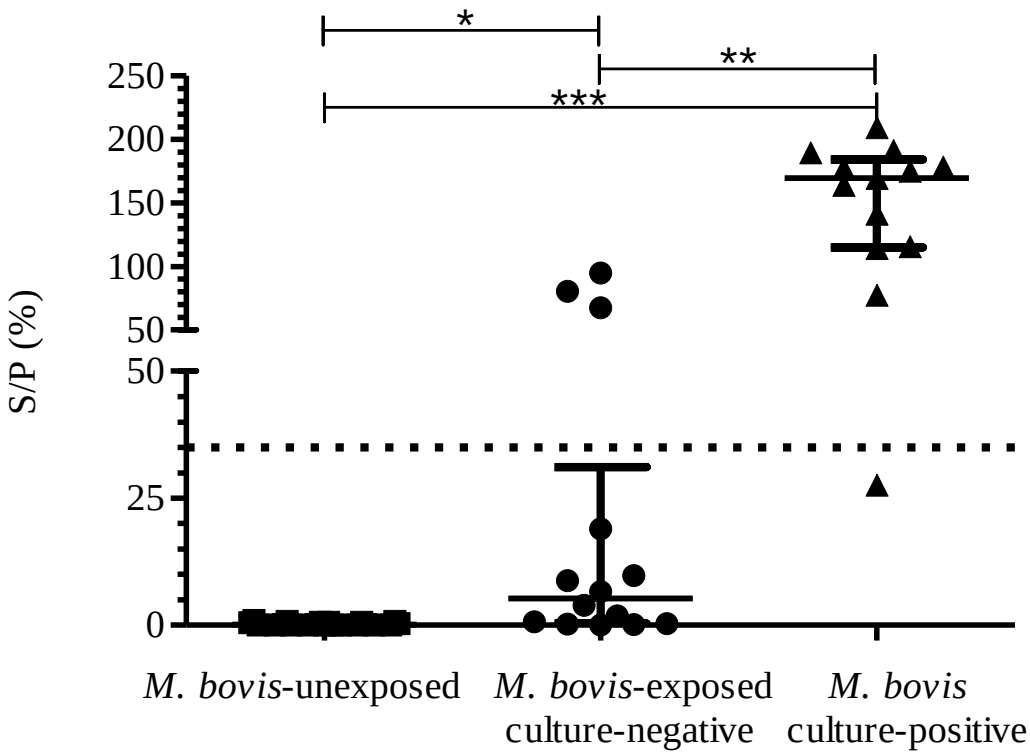

*Figure 3.1 QFT IGRA results (S/P values) for Mycobacterium bovis-unexposed buffaloes (n = 21), M. bovis-exposed culture-negative buffaloes (n = 14) and M. bovis culture-positive buffaloes (n = 13). The manufacturer's prescribed cutoff for the cattletype® IFN-gamma ELISA is indicated by the dotted line (35%). Horizontal bars represent median and interquartile ranges. S/P values were significantly greater in M. bovis culture-positive animals. *, p < 0.05, **, p < 0.01 and ***, p < 0.001*

Test agreements between the QFT IGRA and accepted diagnostic immunoassays for *M. bovis* detection in buffaloes were calculated. Test agreement between the QFT IGRA, SCITT and Bovigam® IGRA in both *M. bovis*-exposed buffalo populations had high κ coefficients, indicating concordance of results (McHugh, 2012) which supports the potential use of the QFT IGRA for the diagnosis of *M. bovis* infection in buffaloes.

Our group previously demonstrated that the Se of the mQFT may be improved by increasing the whole blood incubation time albeit with a possible loss of Sp (Goosen et al., 2014). In the current study, we investigated whether an increased incubation time, from 20 to 40 h, would increase the detection of *M. bovis*-infected buffaloes by the QFT IGRA. Results demonstrated that although a lengthened incubation time did not detect additional QFT IGRA-positive animals, no additional Bovigam® IGRA or SCITT-negative buffaloes were test positive, suggesting no loss of Sp.

The reproducibility of the QFT IGRA was confirmed by calculating the inter- and intra-assay variability. The acceptable CV percentage for inter- and intra-assay reproducibility is < 12% and 10%, respectively. The CV percentages from this study demonstrate the reproducibility of the QFT IGRA and supports the potential use of this assay as a diagnostic tool to detect *M. bovis* infection in buffaloes. The QFT IGRA test results (S/P values) remained unchanged across the replicates which further supports the reproducibility of this assay.

The QFT IGRA was also tested using buffaloes from historical *M. bovis*-unexposed populations that have never tested positive on the SCITT nor Bovigam® IGRA. The

use of known *M. bovis*-unexposed buffalo herds to determine the Sp of an assay is a more reliable approach than using *M. bovis*-exposed culture-negative buffaloes. This is in part due to the high Se of current *M. bovis* assays and the fact that mycobacterial culture is regarded as an imperfect gold standard (de la Rua-Domenech et al., 2006). The QFT IGRA correctly classified all 21 *M. bovis*-unexposed buffaloes as test-negative.

Furthermore, our findings showed the median QFT IGRA S/P values to be significantly greater in *M. bovis* culture-positive buffaloes compared with *M. bovis*-exposed culture-negative buffaloes and *M. bovis*-unexposed buffaloes, respectively, further demonstrating the potential diagnostic use of this IGRA (Figure 3.1). Median S/P values from *M. bovis*-exposed culture-negative buffaloes were significantly greater than *M. bovis*-unexposed buffaloes (Figure 3.1); although, it is known that culture is an imperfect gold standard. Therefore, the three *M. bovis*-exposed culture-negative buffaloes with S/P values above 35% and positive QFT IGRA test results, may be truly infected.

Furthermore, one culture-confirmed *M. bovis*-infected buffalo was QFT IGRA test-negative with an S/P = 27%, just below the manufacturers prescribed cutoff of 35% (Figure 3.1). However, the QFT IGRA has not been validated for buffaloes and a species-specific cutoff may need to be calculated. As previously discussed, culture is an imperfect gold standard and therefore a buffalo-specific cutoff for the QFT IGRA may detect additional *M. bovis*-exposed culture-negative buffaloes that are truly infected. The advantage of a species-specific cutoff value to increase the Se of an assay compared with an increased antigen incubation time, is that the assay procedure

can be standardised across several species with the only difference in the interpretation of the results using the specific cutoff. A larger sample size, where the infection status of all buffaloes is known, is required to confirm whether a species-specific cutoff value or an increased antigen incubation time should be used to optimise the QFT IGRA to detect *M. bovis* infection in buffaloes.

This study reports that the test performance of the QFT IGRA, using QFT tubes and the *cattletype*® IFN-gamma ELISA, is comparable to previously reported diagnostic assays for the detection of *M. bovis* infection in African buffaloes (Parsons et al., 2011; Goosen et al., 2014; van der Heijden et al., 2016). Furthermore, we have shown that a lengthened antigen incubation time had no apparent effect on the QFT IGRA test results. The QFT IGRA was also shown to be highly reproducible and all *M. bovis*-unexposed buffaloes tested negative. Greater IFN-γ release was observed in *M. bovis* culture-positive compared with *M. bovis*-exposed culture-negative buffaloes, and measuring IFN-γ using the *cattletype*® IFN-gamma ELISA's cutoff, we could distinguish between infected and uninfected buffaloes. In conclusion, this study suggests that the use of the QFT IGRA is a promising diagnostic test for the detection of *M. bovis* infection in African buffaloes. However, further research is warranted to determine the Se and Sp of this assay using a larger randomly selected sample population.

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Chapter 4 : Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*)

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Abstract

The diagnosis of *Mycobacterium bovis* (*M. bovis*) infection in African buffaloes (*Syncerus caffer*) relies on detection of the cell-mediated immune response to *M. bovis* antigens using the single comparative intradermal tuberculin test (SCITT) or interferon gamma release assays (IGRAs). The aim of the present study was to determine whether parallel testing with the SCITT and an IGRA increases the number of *M. bovis*-infected buffaloes detected by these assays. Culture-confirmed animals (*n* = 71) tested during routine bovine tuberculosis (bTB) control programmes in Hluhluwe iMfolozi Park and Madikwe Game Reserve in South Africa, were used in this study. Results from 35 buffaloes tested using the SCITT and three Bovigam® IGRAs (cohort A) and 36 buffaloes tested using the SCITT, Bovigam® IGRA and QuantiFERON®-TB Gold IGRA (cohort B) were analysed. The parallel use of the SCITT with selected IGRAs was able to identify all animals in both cohorts. These findings are in agreement with cattle studies supporting the use of the SCITT and IGRAs in parallel to identify the greatest number of *M. bovis*-infected animals. The suggested parallel testing algorithm should be strategically applied to maximise detection of *M. bovis* infection in bTB-positive buffalo herds.

1. Introduction

Mycobacterium bovis (M. bovis) infection causes bovine tuberculosis (bTB), a disease affecting a diverse range of hosts including domestic pets, livestock, wildlife and humans (Michel et al., 2006). African buffaloes (*Syncerus caffer*) are sylvatic reservoirs of *M. bovis* and spillover from buffaloes to wildlife, such as lions, and from buffaloes to livestock, such as cattle, has been demonstrated by molecular characterisation of mycobacterial strains (Hlokwe et al., 2014; Musoke et al., 2015). The first accounts of *M. bovis* infection in buffaloes in South Africa were documented in Hluhluwe iMfolozi Park (HiP) in 1986 (Jolles, 2004) and in Kruger National Park (KNP) in 1990 (Bengis et al., 1996). *M. bovis* infection has since been confirmed in numerous other wildlife species within these reserves, as well as more recently, in buffaloes in Madikwe Game Reserve (MGR) (Hlokwe et al., 2016) and privately-owned farms in South Africa (SA).

Bovine tuberculosis is a chronic disease and progression is characteristically slow. Current methods to detect *M. bovis* infection rely on tests quantifying cell-mediated immune responses to mycobacterial antigens (Vordermeier et al., 2000). Such tests include the single comparative intradermal tuberculin test (SCITT) and interferon gamma (IFN-γ) release assays (IGRAs). The SCITT detects a delayed-type hypersensitivity response to intradermal injection of purified protein derivative (PPD), namely *Mycobacterium avium* PPD (PPD_a) and *M. bovis* PPD (PPD_b). The SCITT is currently the only test approved for the diagnosis of bTB in buffaloes in SA (Schiller et al., 2010).

The IGRA is an *in vitro* alternative to the SCITT and measures the release of IFN-γ in response to *M. bovis* antigens, using an enzyme-linked immunosorbent assay (ELISA) (Grobler et al., 2002). The Bovigam® IGRA is a commercially available test used for the diagnosis of bTB in cattle in which PPDs, like those used in the SCITT, are employed as stimulating antigens (de la Rua-Domenech et al., 2006). The use of a crude mix of mycobacterial antigens, such as PPD, has been shown to compromise IGRA specificity (Sp) in buffaloes (van der Heijden et al., 2016) and as a result, a second generation of IGRAs have been developed using specific peptides as stimulating antigens to improve Sp (Parsons et al., 2011). The Bovigam® peptide IGRAs, Bovigam® PC-EC and Bovigam® PC-HP IGRA, use early secretory antigen target 6 kD (ESAT-6) in combination with culture filtrate protein 10 kD (CFP-10), and Rv3615 in combination with three additional proprietary mycobacterial antigens, respectively, as stimulating antigens (Goosen et al., 2014). The QuantiFERON®-TB Gold (QFT) IGRA is a novel IGRA validated in buffaloes and makes use of the QFT tube stimulation system developed for humans containing ESAT-6 and CFP-10 stimulating antigens (Bernitz et al., 2018). The Bovigam® peptide IGRAs and QFT IGRA have demonstrated high test-Sp in buffaloes (Goosen et al., 2014; Bernitz et al., 2018).

This study aimed to determine if parallel testing of buffaloes in high bTB prevalence herds, using the SCITT and IGRAs, namely the Bovigam®, Bovigam® PC-EC, Bovigam® PC-HP and QFT IGRA, increased the number of *M. bovis*-infected African buffaloes detected.

2. Materials and methods

2.1. Animals

Buffalo populations in HiP and MGR were screened for *M. bovis* infection as part of bTB testing programmes. Buffaloes were immobilised for the SCITT and blood collection, as previously described (Parsons et al., 2011). Buffaloes that tested positive on one or more tests were culled for post mortem examination. Lymph nodes, lungs and bTB-like lesions were examined and sampled for mycobacterial culture as previously described (Goosen et al., 2014). Culture-positive samples were genetically speciated using polymerase chain reaction (PCR) to confirm *M. bovis* infection (Warren et al., 2006).

Table 4.1 Tests performed in Hluhluwe iMfolozi Park in 2015, 2016 and 2017 and Madikwe Game Reserve in 2016 to detect Mycobacterium bovis infection in African buffaloes.

Population	**Year tested**	**Cohort (*n*)**	**SCITT[a]**	**IGRA[b]**			
				Bovigam®	**Bovigam® PC-EC**	**Bovigam® PC-HP**	**QFT IGRA**
	2015	A (35)	√	√	√	√	-
HiP[c]	2016		√	√	-	-	√
	2017	B (36)	√	√	-	-	√
MGR[d]	2016		√	√	-	-	√

[a] single comparative intradermal tuberculin test

[b] interferon gamma release assay

[c] Hluhluwe iMfolozi Park

[d] Madikwe Game Reserve

During 2015, 283 HiP buffaloes were tested using the SCITT and three Bovigam® IGRAs. Of these, 63 buffaloes were culled and 35 were culture-confirmed to be infected with *M. bovis* (cohort A) (Table 4.1). During 2016 and 2017, a total of 688 buffaloes from HiP and MGR were tested using the SCITT, Bovigam® and QFT IGRA. Of these, 94 were culled and 36 were culture-confirmed to be infected with *M. bovis* (cohort B) (Table 4.1). In total, 971 buffaloes were tested over a three-year period in HiP and MGR using the SCITT and a combination of IGRAs. Based on these results, 71 *M. bovis* culture-confirmed buffaloes were selected for inclusion in this study based on mycobacterial culture as the gold standard of *M. bovis* infection.

2.2. Bovigam® IGRAs

In HiP, the Bovigam® IGRA was performed in a temporary field laboratory as previously described (Bernitz et al., 2018). Briefly, aliquots of heparinised whole blood were incubated in microcentrifuge tubes with i) phosphate buffered saline, ii) 2750 IU/ml PPD_a (Onderstepoort Biological Products, South Africa), iii) 3300 IU/ml PPD_b (Onderstepoort Biological Products), and iv) 5 µg/ml pokeweed mitogen (PWM) (Sigma-Aldrich, St. Louis, MO, USA). Samples were incubated at 37 °C for 20 h, the plasma fraction harvested, IFN-γ measured using the Bovigam® ELISA and results interpreted per manufacturer's instructions (Prionics AG, Schlieren-Zurich, Switzerland). The Bovigam® peptide IGRAs, i.e. Bovigam® PC-EC and Bovigam® PC-HP IGRAs, were performed as per the Bovigam® IGRA; however, 0.1 mg/ml PC-EC and 0.1 mg/ml PC-HP peptide cocktails (Prionics AG) were used as stimulating antigens, respectively. For MGR buffaloes, the Bovigam® IGRA was performed at the Tuberculosis Laboratory of the Agricultural Research Council (ARC)-Onderstepoort Veterinary Institute in Pretoria, South Africa with the following modifications to the

Bovigam® protocol (van der Heijden et al., 2017). Aliquots of heparinised whole blood were incubated in 24-well cell-culture plates with i) 1000 IU/ml PPD_a (Prionics AG), ii) 600 IU/ml PPD_b (Prionics AG), and iii) 5 µg/ml pokeweed mitogen (PWM) (Sigma-Aldrich). Aliquots of blood with no added antigen served as unstimulated controls.

2.3. QFT IGRA

The QFT IGRA was performed as previously described (Bernitz et al., 2018). Aliquots of heparinised whole blood were aseptically transferred to tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands) and then incubated at 37 °C for 20 h. The plasma fraction was harvested following centrifugation and the *cattletype*® IFN-gamma ELISA (Qiagen) was performed per manufacturer's instructions.

2.4. SCITT

The SCITT was performed on all buffaloes as previously described (Parsons et al., 2011). An area on either side of the buffalo's neck was shaved and the skin fold thickness (SFT) measured. The SCITT was performed by injecting 2500 IU PPD_a and 3000 IU PPD_b (Onderstepoort Biological Products) intradermally on either side of the buffalo's neck. After three days, buffaloes were immobilised again and the SFT at each injection site was measured. Buffaloes were considered test positive if the increase in SFT at the PPD_b site was 2 mm or greater than that at the PPD_a site.

2.5. Data analyses

For each test and combinations of tests, the proportion of test-positive *M. bovis*-infected buffaloes were compared using a two-tailed z-test where z-scores and *p*-

values were calculated (http://www.socscistatistics.com/tests/ztest/Default.aspx). *P*-values were considered statistically significant if $p < 0.05$.

3. Results and Discussion

In cohort A ($n = 35$), the Bovigam® PC-EC IGRA identified the fewest *M. bovis*-infected buffaloes (28/35), the SCITT and Bovigam® PC-HP IGRA both identified 30/35 buffaloes while the Bovigam® IGRA identified the greatest number (32/35). In cohort B ($n = 36$), the two IGRAs (Bovigam® and QFT IGRA) both identified 32/36 *M. bovis*-infected buffaloes and the SCITT identified 33/36. No single test identified all *M. bovis*-infected buffaloes in either of the cohorts, in agreement with a previous study reporting imperfect sensitivity (Se) of the SCITT and IGRAs in cattle (de la Rua-Domenech et al., 2006). In contrast, parallel testing using the SCITT and an IGRA identified all infected animals; while no two blood-based assays, used in parallel, identified all infected buffaloes.

In cohort A, the SCITT in combination with either of the Bovigam® peptide IGRAs identified all *M. bovis*-infected buffaloes (35/35) and were the only two-test combinations to achieve this in cohort A. Furthermore, using these tests in parallel identified a significantly greater number of infected buffaloes than were identified by any of the individual tests ($p < 0.05$) (Supplementary Table 4.1). The SCITT and Bovigam® IGRA in parallel identified 34/35 buffaloes. This proportion was not significantly different from the 35/35 buffaloes identified by the SCITT and either of the Bovigam® peptide IGRAs. The Bovigam® IGRA used in parallel with either of the Bovigam® peptide IGRAs identified 33/35 *M. bovis*-infected buffaloes while the Bovigam® peptide IGRAs used in parallel identified 30/35 animals (Figure 4.1). In

cohort B, parallel testing using the two blood-based assays identified 35/36 infected buffaloes, while the SCITT and Bovigam® IGRA used in parallel identified all infected buffaloes (36/36), a significantly greater number than detected by the Bovigam® IGRA alone ($p < 0.05$) (Figure 4.2, Supplementary Table 4.2).

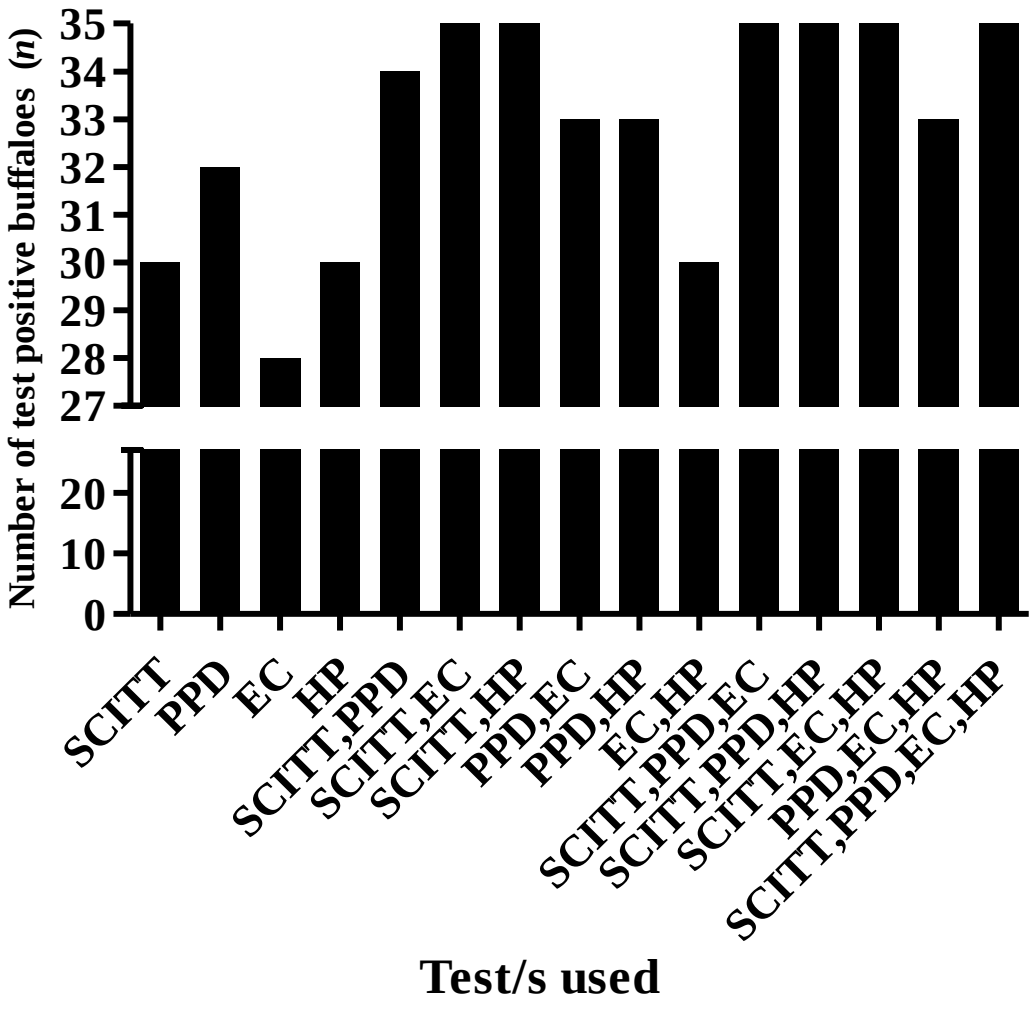

Figure 4.1 Thirty-five Mycobacterium bovis culture-confirmed buffaloes (Cohort A) were tested using four bovine tuberculosis tests [single comparative tuberculin skin test (SCITT), Bovigam® IGRA (PPD), Bovigam® PC-EC IGRA (EC) and Bovigam® PC-HP IGRA (HP)] singly and in combination. The number of test-positive buffaloes for each test and various combinations are shown. Statistical analyses were performed and represented in Supplementary Table 4.1.

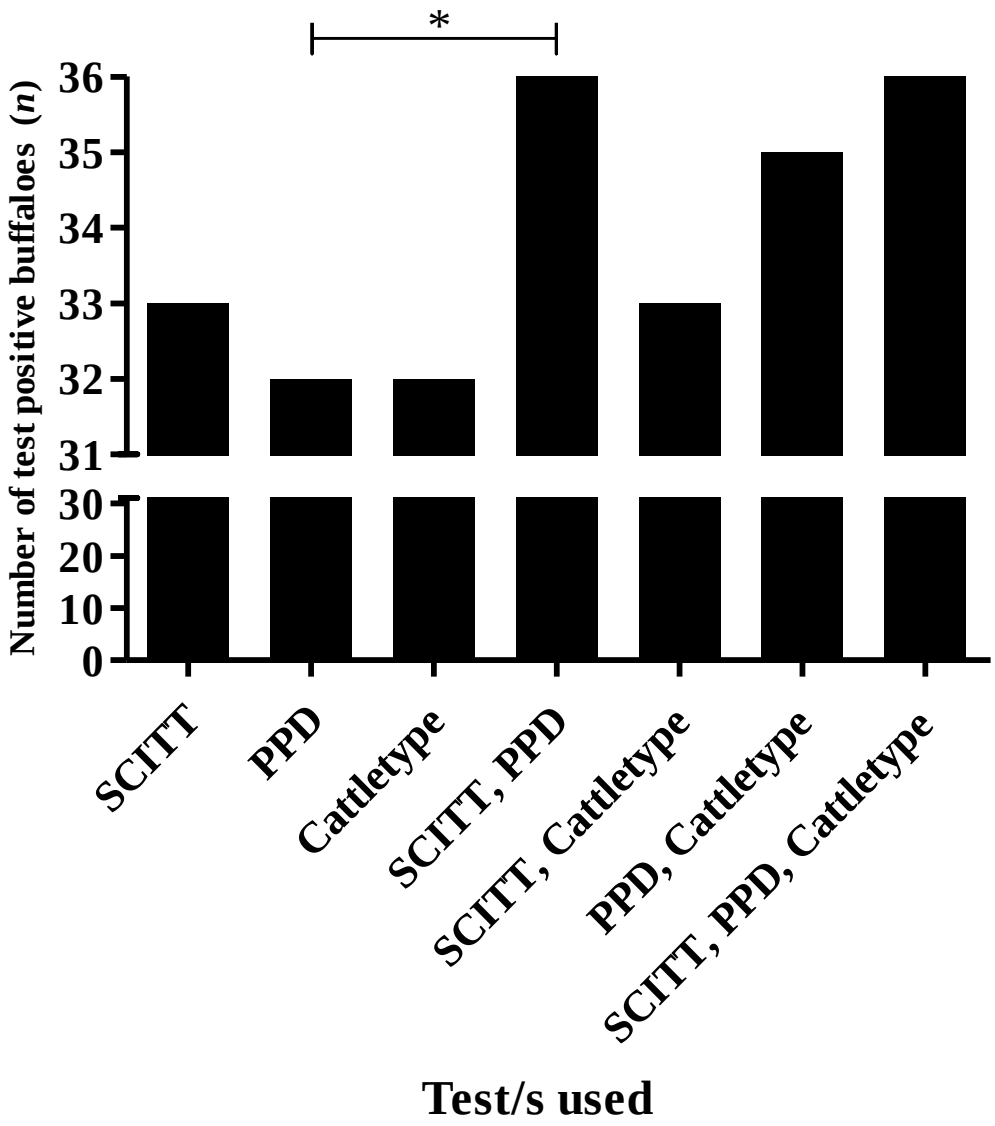

Figure 4.2 Thirty-six Mycobacterium bovis culture-confirmed buffaloes (Cohort B) were tested using three bovine tuberculosis tests (single comparative tuberculin skin test (SCITT), Bovigam® IGRA (PPD) and QFT IGRA) singly and in combination. The number of test-positive buffaloes for each test and various combinations are shown. Statistical analyses were performed and represented in Supplementary Table 4.2.

Results of the SCITT and Bovigam® IGRA performed on all 71 infected buffaloes were compared. As individual tests, the SCITT identified 63/71 buffaloes and the

Bovigam® IGRA identified 64/71 buffaloes. In parallel, the SCITT and Bovigam® IGRA identified all but one buffalo (70/71), a significantly greater number than identified by these tests individually ($p < 0.05$) (Figure 4.3). Other studies have shown that the SCITT and IGRAs identify different subsets of *M. bovis*-infected cattle (Vordermeier et al., 2006; Schiller et al., 2010) and consequently, when these tests are used in combination, a greater number of infected cattle are identified (Gonzàlez Llamazeres et al., 1990; Gormley et al., 2006). This is comparable to our findings that in parallel, the SCITT and an IGRA identified more infected buffaloes than these tests individually detected. Parallel testing using any two tests does not intrinsically increase Se since two tests with different performance characteristics are required to be performed in parallel to increase Se (Sheringham et al., 2015). For example, results from this study showed that the SCITT and QFT IGRA used in parallel detected the same number of buffaloes as the SCITT alone (cohort B). These two tests had high test agreement in a previous study (Bernitz et al., 2018), suggesting that they were detecting the same individual buffaloes.

Bovine PPD has been shown to be a more sensitive stimulating antigen than specific peptides such as Bovigam® PC-EC and PC-HP antigens in buffalo cytokine release assays (Goosen et al., 2014; van der Heijden et al., 2017). This has been demonstrated for the SCITT and Bovigam® IGRA in cattle (Schiller et al., 2010) and buffaloes (Michel et al., 2011). Therefore, in order to maximise detection of all infected animals from a known *M. bovis*-infected buffalo herd, such as in HiP and MGR, tests using PPD should be utilised. In the present study, the Bovigam® IGRA, which uses PPDs, consistently demonstrated that in parallel with the SCITT, the greatest number of *M. bovis*-infected buffaloes were identified. Parallel testing, using these two PPD-based

assays, has previously been advocated in cattle herds (Gormley et al., 2006; Vordermeier et al., 2006) and should be strategically applied when the goal of testing is to remove as many infected animals as possible. In herds where maximizing detection of all infected animals is the goal, then the expected decrease in Sp would be considered acceptable, especially in high prevalence herds. However, when testing herds with no-known history of *M. bovis* or extremely valuable animals, a different strategy focusing on increased Sp should be considered to minimise the risk of false-positive results.

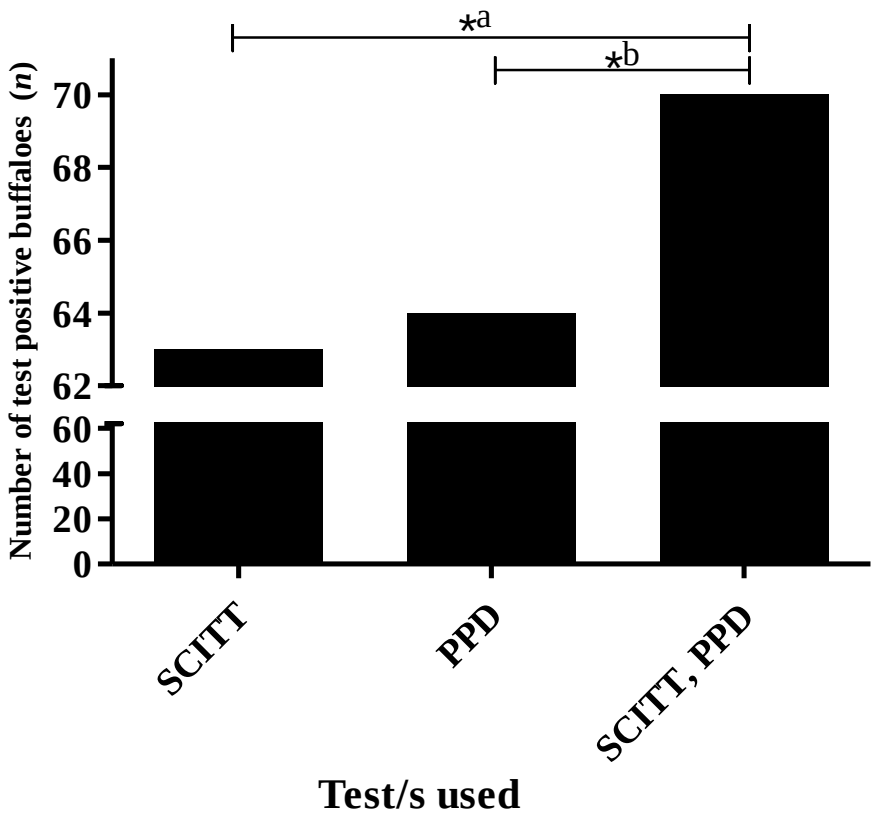

*Figure 4.3 Seventy-one Mycobacterium bovis culture-confirmed buffaloes were tested using two bovine tuberculosis tests [single comparative tuberculin skin test (SCITT) and Bovigam® IGRA (PPD)] singly and in parallel. The number of test-positive buffaloes for each test and in combination is shown. Proportions were compared using a two-tailed z-test. *p < 0.05, a z = 2.4 and b z = 2.1*

Due to the logistics and costs involved in performing the SCITT on wild animals such as buffaloes, an ideal parallel testing strategy would be based on blood-based assays with diagnostic performances comparable to or better than the SCITT in combination with an IGRA. However, results from this study demonstrated that parallel testing using two or three IGRAs did not identify as many *M. bovis*-infected buffaloes as the SCITT and an IGRA performed in parallel, although the Bovigam® and QFT IGRAs used in parallel identified all but one infected buffalo in cohort B. Therefore, these two IGRAs used in combination should be further investigated.

In conclusion, parallel testing, using the SCITT and an IGRA, increases detection of *M. bovis*-infected buffaloes when compared to detection using single diagnostic tests. Additionally, since the SCITT identified additional *M. bovis*-infected buffaloes which were not detected using a single or combination of IGRAs, it is not currently possible to replace the SCITT in a testing algorithm that includes an IGRA without losing Se. However, since buffaloes were culled based on a positive test result, it is not known if there were any additional truly *M. bovis*-infected buffaloes that were negative on all tests in these populations. Furthermore, because mycobacterial culture is an imperfect gold standard (de la Rua-Domenech et al., 2006), buffaloes that were culture-negative, but had evidence of *M. bovis* infection (based on immunological tests), were not included in our analysis. Additional strategies to develop a diagnostic algorithm using blood-based assays, including incorporation of other biomarkers, should be investigated.

*Supplemental Table 4.1 A summary of the statistical scores from 35 Mycobacterium bovis culture-confirmed buffaloes tested in Hluhluwe iMfolozi Park in 2015, that were test-positive using the single comparative tuberculin skin test (SCITT) and Bovigam® IGRA (PPD), Bovigam® PC-EC IGRA (EC) and Bovigam® PC-HP IGRA (HP) singly and in combination (Table 4.1). Proportions were compared using a two-tailed z-test. *p < 0.05 and **p < 0.01 and z scores are indicated in brackets.*

PPD[a]	NS[d]	-	-	-	-	-	-	-	-	-	-	-	-	-
EC[b]	NS	NS	-	-	-	-	-	-	-	-	-	-	-	-
HP[c]	NS	NS	NS	-	-	-	-	-	-	-	-	-	-	-
SCITT, PPD	NS	NS	*(2.2)	NS	-	-	-	-	-	-	-	-	-	-
SCITT, EC	* (2.3)	NS	**(2.7)	*(2.3)	NS	-	-	-	-	-	-	-	-	-
SCITT, HP	*(2.3)	NS	**(2.7)	*(2.3)	NS	NS	-	-	-	-	-	-	-	-
PPD, EC	NS	NS	NS	NS	NS	NS	NS	-	-	-	-	-	-	-
PPD, HP	NS	NS	NS	NS	NS	NS	NS	NS	-	-	-	-	-	-
EC, HP	NS	NS	NS	NS	NS	*(2.3)	*(2.3)	NS	NS	-	-	-	-	-
SCITT, PPD, EC	*(2.3)	NS	**(2.7)	*(2.3)	NS	NS	NS	NS	NS	*(2.3)	-	-	-	-
SCITT, PPD, HP	*(2.3)	NS	**(2.7)	*(2.3)	NS	NS	NS	NS	NS	*(2.3)	NS	-	-	-
SCITT, EC, HP	*(2.3)	NS	**(2.7)	*(2.3)	NS	NS	NS	NS	NS	*(2.3)	NS	NS	-	-
PPD, EC, HP	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-
SCITT, PPD, EC, HP	*(2.3)	NS	**(2.7)	*(2.3)	NS	NS	NS	NS	NS	*(2.3)	NS	NS	NS	NS
	SCITT[e]	**PPD**	**EC**	**HP**	**SCITT, PPD**	**SCITT, EC**	**SCITT, HP**	**PPD, EC**	**PPD, HP**	**EC, HP**	**SCITT, PPD, EC**	**SCITT, PPD, HP**	**SCITT, EC, HP**	**PPD, EC, HP**

[a] Bovigam® IGRA

[b] Bovigam® PC-EC IGRA

[c] Bovigam® PC-HP IGRA

[d] not statistically significant

[e] single comparative tuberculin skin test

*Supplemental Table 4.2 A summary of the statistical scores from 36 Mycobacterium bovis culture-confirmed buffaloes tested in Hluhluwe iMfolozi Park in 2016 and 2017 and in Madikwe Game Reserve in 2016, that were test-positive using the single comparative tuberculin skin test (SCITT), Bovigam® IGRA (PPD) and QFT IGRA singly and in combination (Table 4.2). Proportions were compared using a two-tailed z-test. * $p < 0.05$ and z scores are indicated in brackets.*

PPD[a]	NS[c]	-	-	-	-	-
QFT IGRA[b]	NS	NS	-	-	-	-
SCITT, PPD	NS	*(2.0)	*(2.0)	-	-	-
SCITT, QFT IGRA	NS	NS	NS	NS	-	-
PPD, QFT IGRA	NS	NS	NS	NS	NS	-
SCITT, PPD, QFT IGRA	NS	*(2.0)	*(2.0)	NS	NS	NS
	SCITT[d]	PPD	QFT IGRA	SCITT, PPD	SCITT, QFT IGRA	PPD, QFT IGRA

[a] Bovigam® IGRA

[b] QuantiFERON®-TB Gold IGRA

[c] not statistically significant

[d] single comparative tuberculin skin test

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Chapter 5 : Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*)

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Abstract

The QuantiFERON®-TB Gold (QFT) stimulation platform for cytokine release is a novel approach for diagnosis of bovine tuberculosis in wildlife species. Plasma interferon gamma (IFN-γ) is routinely measured to detect immune sensitization to *Mycobacterium bovis* (*M. bovis*). However, the cytokine interferon gamma-inducible protein-10 (IP-10) has been proposed as an alternative, more sensitive, diagnostic biomarker. In this study, we investigated the use of the QFT system with measurement of IFN-γ and IP-10 in parallel to identify *M. bovis*-infected African buffaloes (*Syncerus caffer*). The test results of either biomarker in a cohort of *M. bovis*-unexposed buffaloes (n = 70) led to calculation of 100% test specificity. Furthermore, in cohorts of *M. bovis* culture-positive (n = 51) and *M. bovis*-suspect (n = 22) buffaloes, the IP-10 test results were positive in a greater number of animals than the number based on the IFN-γ test results. Most notably, when the biomarkers were measured in parallel, the tests identified all *M. bovis* culture-positive buffaloes, a result neither the single comparative intradermal tuberculin test (SCITT) nor Bovigam® IFN-γ release assay (IGRA) achieved, individually or in parallel. These findings demonstrate the diagnostic potential of this blood-based assay to identify *M. bovis*-infected African buffaloes and a strategy to maximise the detection of infected animals while maintaining diagnostic specificity and simplifying test procedures.

1. Introduction

Bovine tuberculosis (bTB) is an infectious zoonotic disease caused by *Mycobacterium bovis* (*M. bovis*). In South Africa (SA), the existence of wildlife maintenance hosts of *M. bovis* poses the biggest challenge in bTB eradication. African buffaloes (*Syncerus caffer*) are key *M. bovis* wildlife maintenance hosts and detection of infected buffaloes is vital to lower the transmission risk between wildlife species and domestic cattle (Musoke et al., 2015; Olivier et al., 2015). The identification of infected buffaloes will also prevent the translocation of these animals and thus limit the expansion of the disease's geographic range (Grobler et al., 2002).

The diagnosis of bTB relies primarily on tests that quantify the cell-mediated immune response to *M. bovis* antigens (Vordermeier et al., 2000). In SA, the only ante mortem diagnostic test approved for the detection of *M. bovis* infection in African buffaloes is the single comparative intradermal tuberculin test (SCITT), using *M. bovis* purified protein derivative (PPD) (PPD_b) and *Mycobacterium avium* PPD (PPD_a). An *in vitro* alternative to the SCITT is the Bovigam® interferon gamma (IFN-γ) release assay (IGRA), a commercially available diagnostic test that was developed to detect *M. bovis* infection in cattle and may be less subject to operator bias and error than the SCITT (Wood and Jones, 2001). The Bovigam® IGRA is also used for bTB testing of buffaloes by measuring IFN-γ production in whole blood incubated in the presence of PPD (Grobler et al., 2002; van der Heijden et al., 2016). The use of PPD may result in false-positive test results due to cross-reactive immune responses with non-tuberculous mycobacteria.

The QuantiFERON®-TB Gold (QFT) IGRA is an assay using blood collection tubes containing the antigenic peptides simulating the proteins early secretory antigen target 6 kD

(ESAT-6), culture filtrate protein 10 kD (CFP-10) and TB7.7(p4). The use of specific antigenic peptides has shown to increase the specificity (Sp) of IGRAs (Vordermeier et al., 2001). Whole blood is incubated in the QFT tubes for 20-24 h, after which IFN-γ, released in response to the antigens, is measured in the plasma. Previous studies have demonstrated the utility of QFT tubes to stimulate antigen-specific IFN-γ responses in wildlife for detection of *Mycobacterium tuberculosis* (*Mtb*) infection in chacma baboons (*Papio ursinus*) (Parsons et al., 2009) and *M. bovis* infection in African buffaloes (Parsons et al., 2011; Bernitz et al., 2018a).

Assays using antigenic peptides are highly specific; however, this approach may compromise diagnostic sensitivity (Se) and thus additional biomarkers of immune activation have been identified. Interferon gamma-inducible protein-10 (IP-10), a cytokine produced at considerably higher levels than IFN-γ (Berry et al., 2010), has been proposed as a more sensitive diagnostic biomarker than IFN-γ for detecting host immune sensitization in humans, buffaloes and common warthogs (*Phacochoerus africanus*) (Ruhwald et al., 2009; Parsons et al., 2011; Roos et al., 2018). Preliminary work has shown that the QFT IP-10 release assay (QFT IPRA) has improved test Se compared to the QFT IGRA in buffaloes (Goosen et al., 2015), however, no study has investigated the use the QFT IGRA and QFT IPRA in parallel to detect *M. bovis*-infected wildlife.

The present study aimed to use the QFT stimulation platform in combination with both biomarkers IFN-γ and IP-10 to determine whether it can be used to maximise the detection of infected buffaloes, while maintaining test Sp and simplify bTB testing in field conditions. Furthermore, the study aimed to compare the detection of these assays with the SCITT and Bovigam® IGRA in *M. bovis*-exposed populations.

2. Materials and methods

2.1. Buffalo cohorts

Three buffalo cohorts were used for this study; 1) 70 *M. bovis*-unexposed; 2) 51 *M. bovis* culture-positive; and 3) 22 *M. bovis*-suspect animals.

Cohort 1 consisted of buffaloes from *M. bovis*-free farms in SA with no known history of *M. bovis*-infection or bTB disease in any species. These *M. bovis*-unexposed buffaloes were used as uninfected controls. Between 2016 and 2018, whole blood was opportunistically collected from buffaloes when animals were immobilised for reasons unrelated to this study. The QFT IGRA and QFT IPRA were performed on all samples.

Cohorts 2 and 3 consisted of buffaloes from *M. bovis*-endemic wildlife reserves in SA. In Madikwe Game Reserve in 2016 and Hluhluwe iMfolozi Park in 2016 and 2017, buffaloes were tested during bTB test-and-cull control programmes. Buffaloes were captured and immobilised to perform the SCITT, Bovigam® IGRA, QFT IGRA and QFT IPRA. No buffalo presented with any clinical signs of bTB disease, and only buffaloes that tested-positive on one or more of the tests evaluated in this study were culled. Mycobacterial culture is the gold standard for *M. bovis*-infection and samples collected during post mortem examinations from each of the culled buffaloes underwent culture to confirm infection. Cohort 2 consisted of all mycobacterial culture-confirmed buffaloes detected during the three test-and-cull programmes. Of these 51 buffaloes, data from 36 animals had previously been reported (Bernitz et al., 2018b), while data from the remaining 15 buffaloes had not. Cohort 3 consisted of buffaloes that were positive one or both of the PPD-based assays (SCITT and Bovigam® IGRA), but showed no bTB-like lesions upon post mortem examination and *M. bovis* could not be cultured from tissue samples (n = 22). These buffaloes were considered to

have a high probability of being infected with *M. bovis.* This is due to the Se of PPD and the fact that mycobacterial culture is an imperfect gold standard (de la Rua-Domenech et al., 2006). These buffaloes were classified as *M. bovis*-suspect.

2.2. SCITT and Bovigam® IGRA

The SCITT and Bovigam® IGRA were performed as previously described (Parsons et al., 2011; Bernitz et al., 2018b). A positive SCITT result was defined as a differential skin fold thickness measurement of ≥ 2 mm between the PPD_b injection site compared with the PPD_a injection site. A positive Bovigam® IGRA result was defined as a differential OD value (450 nm – 630 nm) of ≥ 0.1 between the PPD_b stimulation compared with the unstimulated control in addition to an OD value of ≥ 0.1 between the PPD_b stimulation compared with the PPD_a stimulation.

2.3. QFT IGRA

The QFT IGRA was performed on all buffaloes as previously described (Bernitz et al., 2018a). Briefly, aliquots of heparinised whole blood were aseptically transferred to blood collection tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands), i.e. an unstimulated control tube containing saline, an antigen tube containing ESAT-6, CFP-10 and TB-7.7(p4) peptides and a positive control tube (mitogen). Tubes were inverted ten times and incubated at 37 °C for 20 h. The plasma fraction was harvested following centrifugation and the Qiagen *cattletype*® IFN-gamma enzyme-linked immunosorbent assay (ELISA) was performed. Test results were interpreted per the manufacturer's protocol and a QFT IGRA-positive result was defined as S/P ≥ 35%.

2.4. QFT IPRA

Plasma harvested from QFT tubes was also assayed using an in-house bovine IP-10 ELISA (Kingfisher Biotech Inc., St Paul, MN, USA) as previously described (Parsons et al., 2016). The concentration of IP-10 in each sample was calculated using a standard curve generated from a dilution series of recombinant bovine IP-10 protein (Kingfisher Biotech Inc.). An assay result was defined as the concentration of IP-10 in the QFT Nil tube subtracted from the concentration of IP-10 in the QFT TB antigen tube, with a positive result being equal to or greater than 1486 pg/ml, based on a previously determined cutoff value (Goosen et al., 2015).

2.5. Data analyses

The Sp of the QFT IGRA and QFT IPRA, individually and in parallel, were calculated as the percentage of *M. bovis*-unexposed buffaloes that were test-negative for each test, respectively. For both the *M. bovis* culture-positive and *M. bovis*-suspect cohorts, the proportion of buffaloes that were test-positive on the: i) SCITT; ii) Bovigam® IGRA; iii) QFT IGRA; iv) QFT IPRA; v) parallel interpretation of the PPD-based assays; and vi) parallel interpretation of the QFT assays, were compared using the exact binomial McNemar's test with Bonferroni correction for multiple comparisons (http://scistatcalc.blogspot.com/2013/11/mcnemars-test-calculator.html). Results were considered statistically significant if $p < 0.05/15$ or $p < 0.003$. The QFT IGRA and QFT IPRA result values of *M. bovis* culture-positive buffaloes were compared to the QFT IGRA and QFT IPRA result values of *M. bovis*-suspect buffaloes, respectively, using the Kruskal-Wallis H test with a Dunn's Multiple Comparison Test. Results were considered statistically significant if $p < 0.05$. All statistical tests were performed on GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA).

3. Results

The QFT IGRA and QFT IPRA, individually and in parallel, classified all *M. bovis*-unexposed buffaloes as test-negative (Table 5.1) and thus demonstrated 100% (95% CI 95-100%) test Sp.

Table 5.1 The number of buffaloes that tested positive and negative on all individual tests evaluated in this study and selected parallel interpretations of these tests, performed on three buffalo cohorts; i) Mycobacterium bovis-unexposed and uninfected; ii) M. bovis culture-confirmed; and iii) M. bovis-suspect animals. Mycobacterial culture was used as the gold standard to confirm M. bovis infection in buffaloes.

Test/s	Uninfected (*n* = 70)		Culture-positive (*n* = 51)		Suspect (*n* = 22)	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
SCITT[a]	Not done		44	7	22	0
Bovigam[b]	Not done		41	10	11	11
QFT IGRA[c]	0	70	41	10	7	15
QFT IPRA[d]	0	70	45	6	18	4
SCITT & Bovigam	Not done		48	3	22	0
QFT assays[e]	0	70	51	0	18	4

[a] single comparative intradermal tuberculin test

[b] Bovigam® interferon gamma release assay

[c] QuantiFERON®-TB Gold interferon gamma release assay

[d] QuantiFERON®-TB Gold interferon gamma-inducible protein-10 release assay

[e] parallel interpretation of the QFT IGRA and QFT IPRA

No individual test identified all *M. bovis* culture-confirmed buffaloes (Cohort 2); the Bovigam® IGRA and QFT IGRA individually identified 80% (95% CI 67-90%) of animals, the SCITT identified 86% (95% CI 74-94%) of animals and the QFT IPRA identified 88%

(95% CI 76-96%) of animals (Table 5.1). Parallel interpretation of the SCITT and Bovigam® IGRA identified 94% (95% CI 84-99%) of *M. bovis* culture-positive buffaloes. Parallel interpretation of the QFT assays identified all [100% (95% CI 93-100%)] culture-positive buffaloes, and this was significantly more animals than either individual IGRA identified (p = 0.0019).

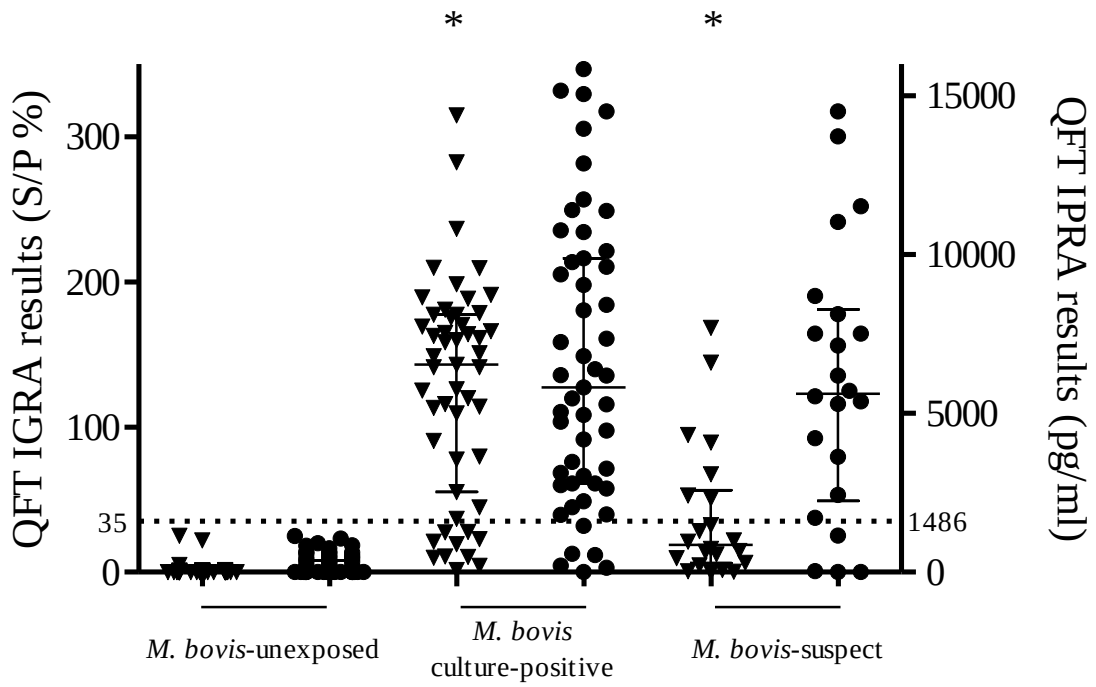

Figure 5.1 The magnitude of QuantiFERON®-TB Gold (QFT) interferon gamma release assay (IGRA) results (▼) and QFT interferon gamma-inducible protein-10 release assay (IPRA) results (●) for: i) Mycobacterium bovis-unexposed (n = 70); ii) M. bovis culture-positive (n = 51) and iii) M. bovis-suspect (n = 22) buffaloes. The cutoff values for each assay are indicated by the dotted line. Horizontal bars represent median and interquartile ranges. Median QFT IGRA results in M. bovis culture-positive animals were significantly greater (p < 0.001) than median QFT IGRA results in M. bovis-suspect buffaloes denoted by *.

Median QFT IGRA result values for *M. bovis* culture-positive buffaloes were significantly greater compared with the median QFT IGRA result values for *M. bovis*-suspect buffaloes ($p < 0.001$). In contrast to this result, the median QFT IPRA result values of the two cohorts were not significantly different (Figure 5.1).

All *M. bovis*-suspect buffaloes (Cohort 3) were SCITT-positive [100% (95% CI 85-100%)] and a subset of these were Bovigam® IGRA-positive [50% (95% CI 28-72%)] (Table 5.1). The QFT IPRA identified significantly more buffaloes [82% (95% CI 60-95%)] than the QFT IGRA [32% (95% CI 14-55%); $p = 0.0009$], but parallel interpretation of the QFT assays identified the same proportion of animals as the QFT IPRA alone (Table 5.1).

4. Discussion

In this study, measurement of both biomarkers IFN-γ and IP-10 in plasma harvested from the QFT stimulation system maximised the detection of *M. bovis* culture-confirmed buffaloes while maintaining test Sp.

Both the QFT IGRA and IPRA demonstrated 100% Sp. This in agreement with a pilot study that estimated the QFT IGRA to be 100% specific in buffaloes (Bernitz et al., 2018a) and previous reports of improved Sp when *M. bovis*-peptides are used as stimulating antigens for IGRAs in cattle and buffaloes (Vordermeier et al., 2001; Buddle et al., 2003; Bass et al., 2013; Goosen et al., 2014). Additionally, as for other species, the measurement of IP-10 in the QFT IPRA did not compromise the diagnostic Sp of the test (Ruhwald et al., 2011; Parsons et al., 2016). This characteristic is of particular importance in order to prevent negative financial and regulatory consequences of a false-positive test-result.

The QFT IPRA identified a greater number of *M. bovis* culture-positive buffaloes and a significantly greater number of *M. bovis*-suspect buffaloes than the QFT IGRA. In agreement, studies measuring antigen-specific IP-10 have reported increased Se over IGRAs for diagnosing *Mtb* and *M. bovis* infection in humans and buffaloes, respectively (Ruhwald et al., 2009; Goosen et al., 2015). Notably, median QFT IGRA result values were significantly lower in the *M. bovis*-suspect cohort than the culture-positive cohort, suggesting that the suspect animals were at an early stage of infection, as it has been reported that antigen-specific IFN-γ is a useful biomarker of disease severity (Vordermeier et al., 2002). In contrast, median QFT IPRA result values were similar in both groups, suggesting that this assay is not affected by disease status. We propose that not only is the measurement of IP-10 in the QFT system more sensitive than IFN-γ, but IP-10 may also be a better biomarker of early infection.

Both the SCITT and Bovigam® IGRA in parallel, and the QFT IGRA and IPRA in parallel, identified a greater number of *M. bovis* culture-positive buffaloes than did any individual assay. Similarly, previous studies have advocated the parallel use of the SCITT and Bovigam® IGRA to maximise the detection of *M. bovis*-infected cattle and buffaloes (Gormley et al., 2006; Bernitz et al., 2018b). Furthermore, the parallel measurement of IFN-γ and IP-10 has been reported to improve the Se of detecting *Mtb* and *M. bovis* infection in humans and cattle, respectively (Ruhwald et al., 2009; Coad et al., 2019). In our study, the parallel use of the QFT assays detected all of the *M. bovis* culture-confirmed buffaloes, and significantly more of the suspect animals than the QFT IGRA alone. Due to the high Se of the PPD assays together with mycobacterial culture as an imperfect gold standard, it was hypothesised that a proportion of the *M. bovis*-suspect buffaloes were truly infected, especially since they were from an *M. bovis*-endemic population. Therefore, it is not

surprising that the QFT assays in parallel did not detect all animals in cohort 3, unlike the culture-confirmed group. However, in a control programme, detecting animals with early infection using the parallel QFT assays, although they may be culture negative, would potentially improve control and eradication. Thus, parallel testing may be strategically applied to maximise the detection of *M. bovis*-infected buffaloes in a disease control setting (Bernitz et al., 2018b).

In addition to the Sp and suggested Se of the QFT IGRA and IPRA in parallel, this test combination has a cost advantage associated with handling the animals. To perform the SCITT on buffaloes (and other wildlife species), animals need to be immobilised twice and are often held captive for the three days between immobilizations. For the QFT assays, animals need only be immobilised on a single occasion and blood can be collected directly into the test tubes. After incubation, tubes are centrifuged and the plasma is separated by a gel plug which allows for immediate testing of plasma or storage prior to shipping to a diagnostic laboratory. The QFT system also has the advantage of ease-of-use under field conditions compared to the Bovigam® IGRA, which requires trained laboratory technicians to perform antigen stimulations and plasma harvesting. Costs and effort are also reduced by measuring two biomarkers from the same plasma sample and, since there is no *in vivo* sensitization with these tests, they can be repeated without a waiting period, as is required for the SCITT.

A limitation of this study is that only 51 *M. bovis* culture-confirmed buffaloes were included in our analyses and therefore, true differences in assay performance may not have been detected due to the small sample size. No spectrum bias was introduced into this study due to the fact that: i) *M. bovis*-unexposed buffaloes were sourced from more than five geographically isolated bTB-free farms, and ii) *M. bovis* culture-positive buffaloes were

tested from two geographically isolated *M. bovis*-endemic reserves, and post mortem examinations of these buffaloes revealed a broad spectrum of bTB disease in these populations. Notably, the data from this study should be strategically applied to similar *M. bovis*-endemic populations. Finally, the diagnostic sensitivities of the assays could not be estimated due to the selection bias introduced by only using culture-positive buffaloes selected based on prior test results. Thus, only the differences between the proportion of animals identified by each test and parallel interpretations of selected tests were calculated. Future studies should evaluate the performance of tests to detect *M. bovis* infection in buffaloes in randomly selected, unbiased sample populations.

In conclusion, the parallel measurement of IFN-γ and IP-10 in the QFT system maximised detection of *M. bovis*-infected African buffaloes. This blood-based assay may be a preferred method to increase detection of infected animals while maintaining test Sp and simplifying bTB test procedures.

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Chapter 6 : Impact of *Mycobacterium bovis*-induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*)

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Abstract

The chemokine interferon gamma-inducible protein-10 (IP-10) is a sensitive biomarker of *Mycobacterium bovis* (*M. bovis*) infection in African buffaloes (*Syncerus caffer*). However, elevated levels of IP-10 in QuantiFERON®-TB Gold (QFT) unstimulated whole blood compromises the utility of this biomarker. In this study, IP-10 and interferon gamma (IFN-γ) concentrations in whole blood samples from *M. bovis* culture-confirmed buffaloes with varying degrees of pathological changes (n = 72) and uninfected controls (n = 70) were measured in IP-10 release assay (IPRA) and QFT IFN-γ release assay (IGRA), respectively. Findings suggest that concentrations of both IP-10 and IFN-γ in QFT Nil tubes were higher in infected buffaloes with macroscopic pathological changes consistent with bovine tuberculosis compared to uninfected controls, and IGRA values increased with more severe pathological changes in infected buffaloes ($p < 0.05$). Finally, in culture-confirmed buffaloes with IPRA-negative and IGRA-positive test results, most animals were also those with the most advanced pathology. We conclude that IP-10 and IFN-γ concentrations measured in QFT Nil tubes may provide insight into the presence of *M. bovis* pathology in infected buffaloes. Furthermore, this study highlights the value in evaluating cytokine production in both antigen-stimulated and unstimulated samples when interpreting cytokine release assay results.

1. Introduction

Bovine tuberculosis (bTB) is a chronic inflammatory disease caused by the pathogen *Mycobacterium bovis* (*M. bovis*). *Mycobacterium bovis* has a wide mammalian host range including domestic animals, wildlife, livestock and humans (Hlokwe et al., 2014; Dippenaar et al., 2017). The disease is characterised by granulomas, formed when host immune cells infiltrate the tissue at the site of infection. Aggregates of infected macrophages fuse to form multinucleated giant cells followed by localised fibrosis and encapsulation (Palmer et al., 2007). These lesions are initially microscopic but with chronic inflammation, they increase in size. Granulomas mature and multi-focal lesions may coalesce and become diffuse in the infected tissue, with caseous necrotic centers. Advanced bTB may result in morbidity and mortality (Domingo et al., 2014).

In South Africa (SA), African buffaloes (*Syncerus caffer*) are wildlife reservoirs of *M. bovis* and thus efforts to control bTB disease in buffaloes are aimed at reducing infection in this species and spread to others. Since bTB is a chronic disease, buffaloes may present with clinical signs only after months or years of infection. Subsequently, strategies to identify *M. bovis*-infected buffaloes utilise measurement of early antigen-specific cell-mediated immune responses against *M. bovis* antigens, rather than clinical disease. The early detection of infected buffaloes may enable management strategies to be implemented soon enough to limit the transmission of *M. bovis*.

In order to do this, *in vitro* cytokine release assays have been developed to quantify the production of cytokine biomarkers in plasma in response to antigen-specific whole blood stimulation. The QuantiFERON®-TB Gold (QFT) system is an easy-to-use stimulation platform comprised of a i) Nil tube containing saline (unstimulated control), ii) TB antigen

tube containing early secretory antigen target 6kD (ESAT-6), culture filtrate protein 10kD (CFP-10) and TB 7.7(p4) (stimulated) and iii) mitogen tube containing phytohemagglutinin (positive control). The interferon gamma-inducible protein-10 (IP-10) release assay (IPRA) and the interferon gamma (IFN-γ) release assay (IGRA) measure the cytokines IP-10 and IFN-γ in plasma, respectively, following QFT whole blood stimulation (Bernitz et al., 2019). The QFT IPRA and IGRA results are calculated as the plasma concentration of the cytokine in the QFT Nil tube subtracted from the plasma concentration of the cytokine in the QFT TB antigen tube. The QFT IPRA is highly sensitive and specific for detecting *M. bovis*-infection in buffaloes (Bernitz et al., 2019), however, the utility of the assay may be compromised by high concentrations of IP-10 in the QFT Nil tube ([IP-10^{Nil}]). This may cause false-negative test results in truly infected buffaloes and hinder the diagnostic performance of the QFT IPRA.

The phenomenon of elevated [IP-10^{Nil}] has been observed in humans and cattle and although not understood in the latter, has been associated with extent of disease in humans (Chen et al., 2011; Parsons et al., 2016). Therefore, the aim of this study was to evaluate the QFT IGRA and IPRA in terms of the spectrum of bTB pathology. The [IP-10^{Nil}] and the concentration of IFN-γ in the QFT Nil tube ([IFN-γ^{Nil}]) within groups of *M. bovis* culture-confirmed buffaloes with variable lesions were compared to uninfected controls. Furthermore, the magnitude of QFT IPRA and IGRA values and discordant QFT assay results between groups of culture-confirmed buffaloes with different pathological features were compared.

2. Materials and methods

2.1. Animals

A total of 786 buffaloes were tested using the QFT IPRA and IGRA during bTB test-and-cull programmes in Hluhluwe iMfolozi Park, South Africa during 2016, 2017 and 2018. All test positive animals were culled and underwent post mortem examination as described below (*n* = 145), of which all animals confirmed positive on mycobacterial culture were used in this study (*n* = 72). During the same period, buffaloes from eight farms in SA with no known history of bTB were opportunistically tested with the QFT IPRA and IGRA when immobilised for reasons unrelated to this study. These buffaloes were used as uninfected controls (*n* = 70).

2.2. QFT IPRA and IGRA

Both cytokine release assays were performed on all buffaloes as previously described (Bernitz et al., 2019). Briefly, within eight hours of blood collection, 1 ml aliquots of heparinised whole blood were aseptically transferred to tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands); i) Nil; ii) TB antigen, and iii) mitogen (respectively). Samples were inverted ten times and incubated for 20 h at 37 °C. Plasma was harvested following centrifugation. The cytokine IP-10 was measured using an in-house bovine-specific IP-10 enzyme-linked immunosorbent assay (ELISA) (Kingfisher Biotech Inc., St Paul, MN, USA) and IFN-γ was measured using the commercially available *cattletype*® IFN-gamma ELISA (Qiagen, Venlo, Limburg, Netherlands). The IPRA results were calculated as the [IP-10^{Nil}] subtracted from the concentration of IP-10 in the QFT TB antigen tube ([IP-10^{TB}]). An IPRA-positive result was defined as a differential concentration of IP-10 ≥ 1486 pg/ml (Goosen et al., 2015). In contrast, the IGRA results were calculated as sample to positive control (S/P) ratios per the manufacturer's protocol; (OD of QFT TB antigen – OD of QFT Nil)/ (OD

cattletype® IFN-gamma ELISA positive control – OD *cattletype*® IFN-gamma ELISA negative control) x 100. A positive IGRA result was defined as S/P ≥ 35%. All *M. bovis*-uninfected control buffaloes were test-negative on both the QFT IPRA and IGRA (data not shown).

2.3. Post mortem examinations and mycobacterial culture

Buffaloes that tested positive on one or both QFT assays were euthanased by gunshot and examined for gross bTB lesions during post mortem examination. Lymph nodes of the head and thoracic cavity, and lungs were carefully dissected. Macroscopic pathological changes consistent with bTB were scored as follows: L_1, one small focal lesion (diameter < 10 mm); L_2, several small foci or a single lesion (diameter ≥ 10 mm and < 30 mm); and L_3, a single lesion (diameter ≥ 30 mm) or multifocal/confluent lesions (Palmer et al., 2007). In addition to the lesion score, a subjective description was recorded. To limit variation in lesion scoring, a single experienced veterinarian scored the lesions from all buffaloes. The lesion scores were used as a measure of pathological changes due to *M. bovis* infection. If an animal had no visible lesions (NVL), this was recorded, and pooled tissue samples of head (parotid, retropharyngeal, submandibular, and tonsils) and thoracic (mediastinal and tracheobronchial) lymph nodes were collected. Mycobacterial culture and genetic speciation were performed on all lesions and pooled tissue samples to confirm *M. bovis* infection as previously described (Goosen et al., 2014).

2.4. Data analyses

A Kruskal-Wallis one-way ANOVA with a Dunn's post-test was used to compare i) [IP-10^{Nil}] and [IFN-γ^{Nil}] in *M. bovis*-unexposed buffaloes and groups of culture-confirmed buffaloes with different lesion scores, and ii) QFT IPRA and IGRA values in groups of

culture-confirmed buffaloes with different lesion scores [GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA)]. Differences were considered statistically significant if $p < 0.05$. A Cochran's Q test with Bonferroni correction for multiple comparisons was used to compare the proportions of culture-confirmed buffaloes with concordant and discordant QFT assay results in each of the lesion score groups (MedCalc Free Trial Version 19.03 https://www.medcalc.org/download.php). Differences were considered statistically significant if $p < 0.0125$.

3. Results

Table 6.1 Concordant and discordant QFT assay results in M. bovis culture-confirmed (n = 72) buffaloes with no visible lesions (NVL) and lesion scores of 1 to 3 (L1-3): L_1, one small focal lesion (diameter < 10 mm); L_2, several small foci or a single lesion (diameter ≥ 10 mm and < 30 mm); and L_3, a single lesion (diameter ≥ 30 mm) or multifocal/confluent lesions. Superscript numbers denote differences that are statistically significant (p = 0.002).

Lesion score	IPRA[a]+[b] IGRA[c]+	IPRA-[d] IGRA+	IPRA+ IGRA-
NVL	6	0^{1}	6
L_1	4	4	11
L_2	13	0^{2}	8
L_3	8	$8^{1,2}$	4
Total	**31**	**12**	**29**

[a] interferon gamma-inducible protein-10 release assay

[b] test positive

[c] interferon gamma release assay

[d] test negative

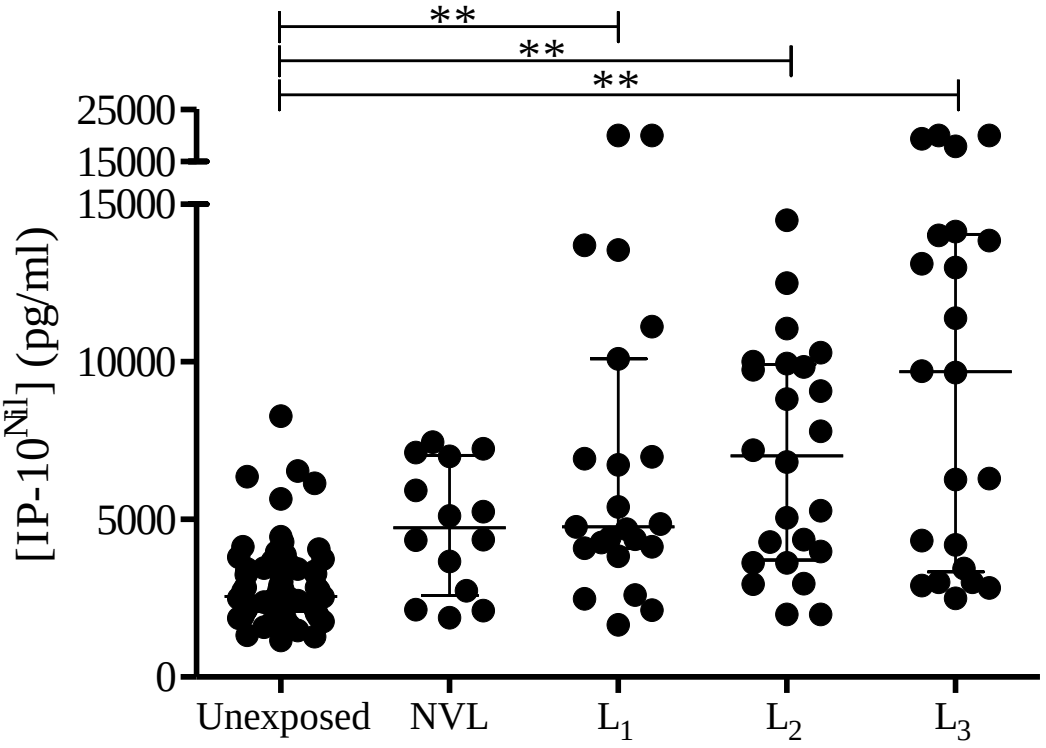

*Figure 6.1 The magnitude of IP-10 concentrations in unstimulated whole blood samples (QFT Nil tubes) from uninfected controls (n = 70) and M. bovis culture-confirmed buffaloes (n = 72) with no visible lesions (NVL) and lesion scores 1-3 (L1-3). Horizontal bars represent median and interquartile ranges. P-values were calculated, and differences were considered statistically significant if $p < 0.05$ (** $p < 0.001$).*

Median [IP-10^{Nil}] were significantly higher in *M. bovis* culture-confirmed buffaloes with lesion scores of L_1, L_2 and L_3 compared to uninfected controls ($p < 0.001$; Figure 6.1). Similarly, median [IFN-γ^{Nil}] were significantly higher in animals with lesions (L_2, L_3) than uninfected controls ($p < 0.001$). Moreover, culture-confirmed buffaloes with the highest lesion score (L_3) had median [IFN-γ^{Nil}] that were significantly higher than those with NVL ($p < 0.05$; Figure 6.2).

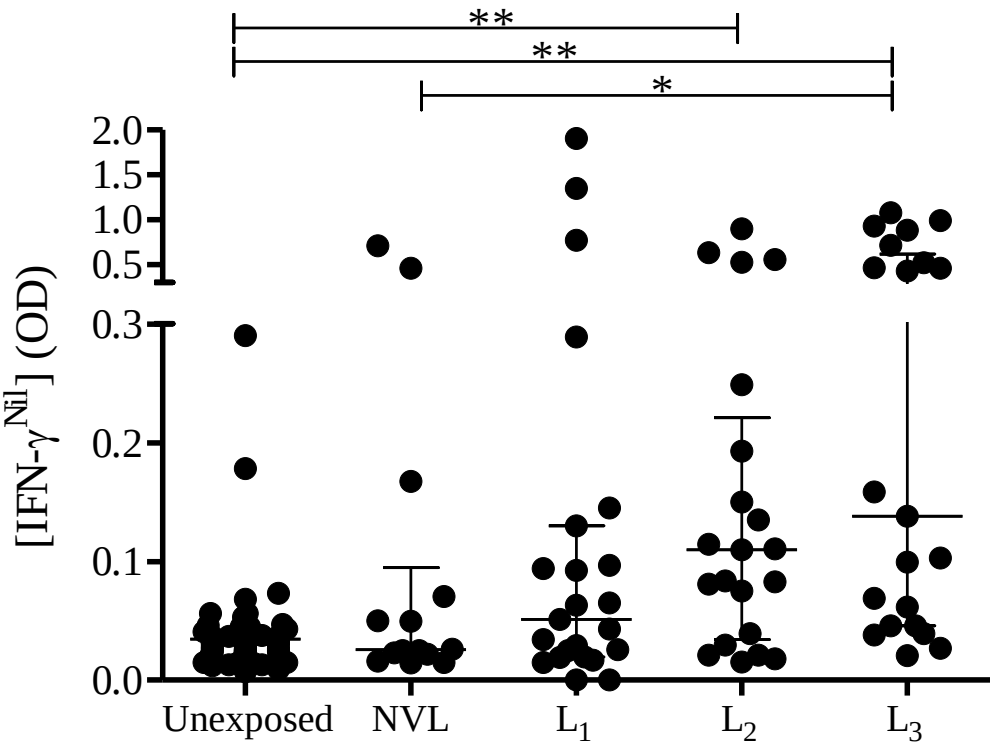

*Figure 6.2 The magnitude of IFN-γ concentrations in unstimulated whole blood samples (QFT Nil tubes) from uninfected controls (*n *= 70) and* M. bovis *culture-confirmed buffaloes (*n *= 72) with no visible lesions (NVL) and lesion scores 1-3 (L_{1-3}). Horizontal bars represent median and interquartile ranges.* P*-values were calculated, and differences were considered statistically significant if* $p < 0.05$ *(** $p < 0.05$ *and ** $p < 0.001$*).*

There were no statistical differences in IPRA values between culture-confirmed buffaloes with different lesion scores (Figure 6.3). However, median IGRA values for buffaloes with higher lesion scores (L_2 and L_3) were significantly higher than median values for buffaloes with NVL ($p < 0.001$) or a lesion score of L_1 ($p < 0.05$; Figure 6.4).

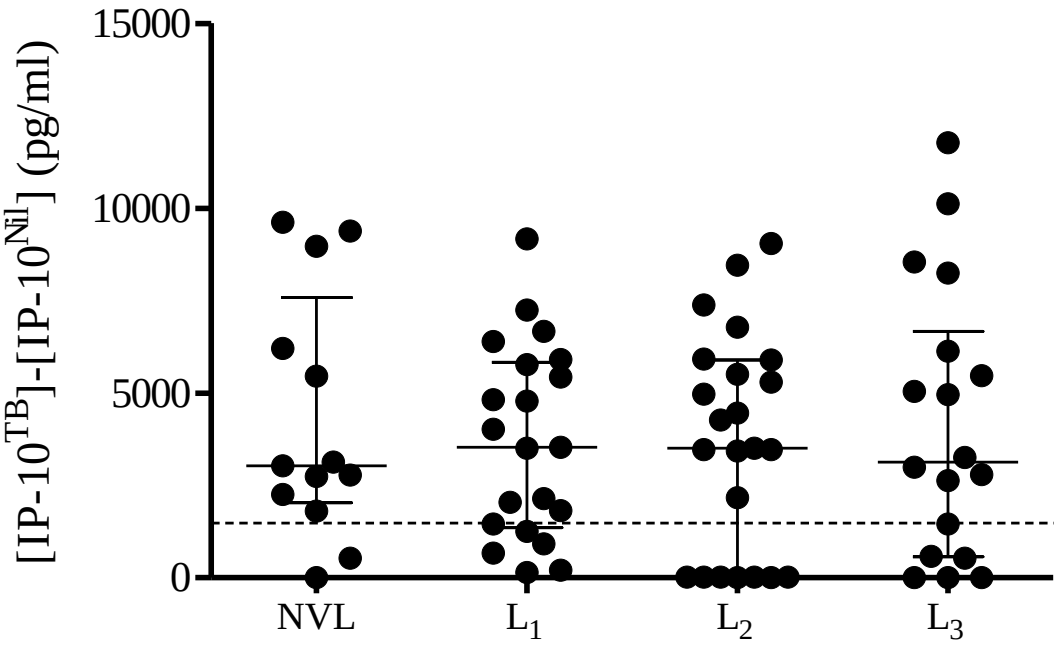

Figure 6.3 The magnitude of QFT IPRA values in M. bovis-culture confirmed buffaloes with no visible lesions (NVL) and lesion scores 1-3 (L1-3) (n = 72). The cutoff value for the assay is indicated by the dotted line (1486 pg/ml). Horizontal bars represent median and interquartile ranges. No statistical differences were observed between lesion score groups.

The proportion of IPRA-positive IGRA-positive buffaloes in different lesion score groups were not significantly different. In contrast, the greatest proportion of IPRA-negative IGRA-positive buffaloes had the highest lesion score (L_3) and this was a significantly greater than the proportion of animals with NVL or L_2 ($p = 0.002$). Furthermore, the greatest proportion of IPRA-positive IGRA-negative buffaloes had the lowest lesion score (L_1), although this was not significantly different from the proportions of buffaloes with other lesion scores (Table 6.1).

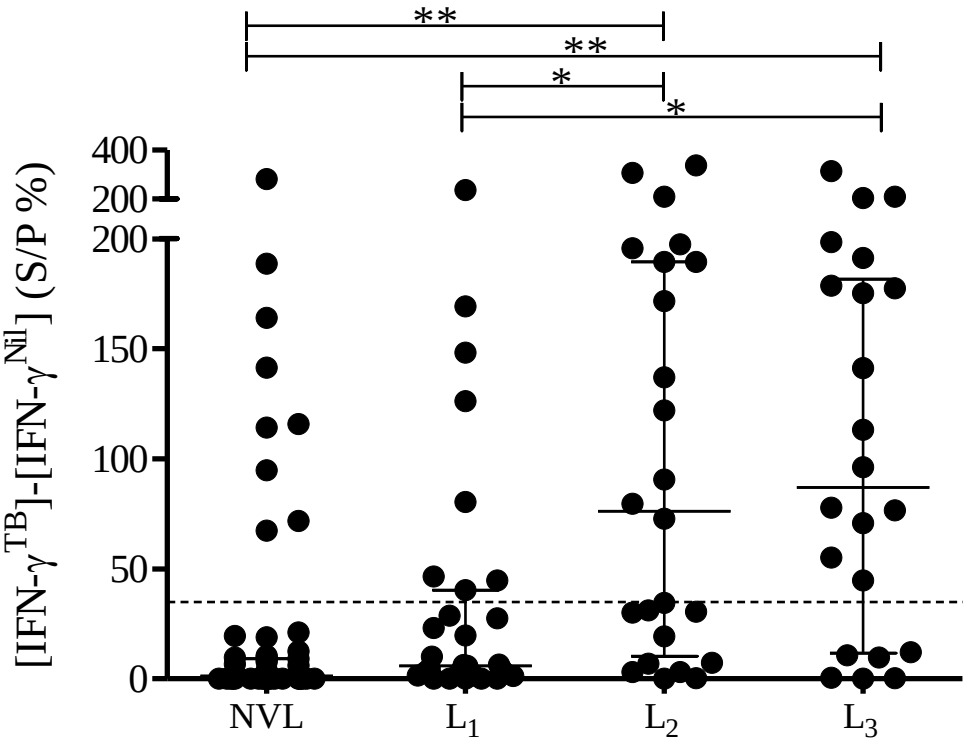

Figure 6.4 The magnitude of QFT IGRA values in M. bovis culture-confirmed buffaloes with no visible lesions (NVL) and lesion scores 1-3 (L1-3) (n = 72). The cutoff value for the assay is indicated by the dotted line (S/P = 35%). Horizontal bars represent median and interquartile ranges. P-values were calculated, and differences were considered statistically significant if p < 0.05 (p < 0.05 and ** p < 0.001).*

4. Discussion

In this study, pathological changes were assessed by the presence and size of macroscopic bTB lesions in the lymph nodes and lungs of *M. bovis* culture-confirmed buffaloes. We evaluated antigen-stimulated and unstimulated cytokine production to interpret the QFT IPRA and IGRA results in the context of bTB pathology in buffaloes.

Both the [IP-10^{Nil}] and [IFN-γ^{Nil}] were higher in buffaloes with culture-confirmed lesions than uninfected controls, and in culture-confirmed buffaloes, the [IFN-γ^{Nil}] increased with

lesion scores. This is consistent with studies showing the production of IP-10 and IFN-γ in unstimulated samples from human patients with active tuberculosis is higher than in uninfected controls, and unstimulated IFN-γ concentrations increase during the progression of active tuberculosis (Hasan et al., 2009). Additionally, both cytokines are included in an *ex vivo* biomarker signature for the diagnosis of active tuberculosis in humans (Hussain et al., 2010; Chen et al., 2011; Chegou et al., 2016). Therefore, the [IP-10^{Nil}] and [IFN-γ^{Nil}] should be interpreted along with antigen-stimulated results, when analysing QFT cytokine release assay results to improve the detection of bTB in buffaloes.

In culture-confirmed buffaloes, QFT IGRA values increased as pathological changes increased. The cytokine IFN-γ is the archetypal measure of the Th1 immune pathway activated in response to *Mycobacterium tuberculosis* (*Mtb*) or *M. bovis* infection. These findings are consistent with studies in cattle, humans and European badgers (*Meles meles*) that have demonstrated a correlation between antigen-stimulated IFN-γ concentrations and disease severity (Vordermeier et al., 2002; Sahiratmadja et al., 2007; Tomlinson et al., 2015). Therefore, antigen-stimulated IFN-γ may not only be a biomarker of *M. bovis* infection but may provide information regarding the presence of bTB pathology in buffaloes.

In contrast to the QFT IGRA, IPRA values did not differ among groups of culture-confirmed buffaloes with different lesion scores. We propose that high levels of antigen-stimulated IP-10 are produced in animals recently infected with *M. bovis* and so there is no marked difference between concentrations measured in culture-confirmed buffaloes with and without pathological changes. Supporting this, a study reported no significant differences between antigen-stimulated IP-10 in children with latent *Mtb*-infection and those with active disease (Whittaker et al., 2008).

In *M. bovis* culture-confirmed IPRA-positive IGRA-negative buffaloes, a greater proportion of animals presented with early signs of disease (L_1) compared to those with higher lesion scores. As discussed above, the cytokine IP-10 is a highly sensitive biomarker of infection and is suggested to detect buffaloes recently infected with *M. bovis*. The cytokine IP-10 is produced more than 2000-fold compared to IFN-γ (McInnis et al., 2005) and can therefore be measured at an earlier stage of infection when IFN-γ may not yet be detectable (Ruhwald et al., 2007). Therefore, we propose that IPRA-positive IGRA-negative buffaloes are animals that have recently been infected and this is supported by the findings of early lesions in these animals (Table 6.2). While IP-10 may not specifically be associated with development of pathological changes, this study confirms the use of IP-10 as a valuable biomarker of *M. bovis* infection in buffaloes.

Table 6.2 Interpretation of QFT assay result permutations to detect infection and macroscopic pathology in buffaloes from a Mycobacterium bovis-endemic population.

IPRA[a]	**IGRA[b]**	**Infection status**	**Further interpretation**
Negative	Negative	Uninfected	No macroscopic pathology
Positive	Negative*	Early-infection	No or early macroscopic pathology
Positive	Positive	Infected	Early macroscopic pathology
Negative**	Positive	Chronically infected	Advanced macroscopic pathology

[a] interferon gamma-inducible protein-10 release assay

[b] interferon gamma release assay

[*] IFN-γ may not yet be at detectable levels

[**] due to elevated concentrations of IP-10 in QFT Nil tube

In culture-confirmed IPRA-negative IGRA-positive buffaloes, a significantly greater proportion of animals had evidence of pathology compared to those with NVL. We propose that buffaloes with bTB lesions produce higher [IP-10^{Nil}] decreasing the IP-10 differential value between antigen-stimulated and unstimulated samples, resulting in an IPRA-negative result. This is supported by the observation that 5 of the 12 IPRA-negative IGRA-positive buffaloes in this study with bTB pathology, had higher [IP-10^{Nil}] compared to [IP-10^{TB}]. Thus, IPRA results with high [IP-10^{Nil}] should be regarded as inconclusive and not test-negative, whereas a buffalo that is IPRA-negative IGRA-positive should be suspected of having bTB pathology, if the [IP-10^{Nil}] is elevated (Table 6.2). An IPRA-negative (due to elevated [IP-10^{Nil}]) IGRA-positive result may have practical diagnostic relevance as a risk-based approach to culling the most infectious animals first, as bTB diseased animals are more likely to shed bacteria thereby transmitting infection.

A limitation of this study includes the low sample numbers that may mask statistically significant differences. Moreover, in this study, lesion scores were used as a measure of pathological changes due to *M. bovis* infection. However, to investigate the extent of disease, additional criteria including body condition, dissemination of bTB to other organs and histopathology would need to be examined. Future studies should be performed in larger buffalo cohorts to determine cutoff values for the [IP-10^{Nil}] and [IFN-γ^{Nil}] that may indicate *M. bovis* infection versus disease.

In conclusion, this study demonstrates that the magnitude of IP-10 and IFN-γ concentrations in QFT-processed whole blood can be used as an indicator of bTB pathology in buffaloes. Moreover, a negative QFT IPRA result, resulting from high [IP-10^{Nil}], in combination with a

positive QFT IGRA result, may be suggestive of the presence of disease due to *M. bovis* infection in buffaloes.

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Chapter 7 : Test performances of assays to detect *Mycobacterium bovis* infection in high prevalence African buffalo (*Syncerus caffer*) herds

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Abstract

A herd of buffaloes was tested for *Mycobacterium bovis* infection using three cytokine release assays. All animals were subsequently euthanased and mycobacterial culture was used to determine the infection prevalence (52%) and diagnostic performances. Sensitivities were lower than previously reported and results provide new insight into the practical utility of these assays.

1. Introduction

The Bovigam® interferon-gamma (IFN-γ) release assay (IGRA), QuantiFERON®-TB Gold (QFT) IGRA and QFT IFN-γ-inducible protein-10 (IP-10) release assay (IPRA) have been used to detect *Mycobacterium bovis (M. bovis)* infection in African buffaloes (*Syncerus caffer*) (Goosen et al., 2015; Bernitz et al., 2018). The sensitivity (Se) and specificity (Sp) of these assays have previously been calculated to evaluate their diagnostic performances (Michel et al., 2011; Bernitz et al., 2019). Sensitivity was calculated as the proportion of test-positive culture-confirmed buffaloes (i.e., animals selected based on a positive *M. bovis* test result which had confirmed infection based on culture) correctly diagnosed. Specificity was calculated as the proportion of test-negative buffaloes correctly diagnosed in *M. bovis*-unexposed controls.

Sensitivity and Sp describe the performance of an assay at a given cutoff, and are independent of the population's infection prevalence. From a clinical perspective, the predictive values of a test are more relevant, i.e. the probability of an individual with a positive or negative test outcome being truly infected or uninfected, respectively (Trevethan et al., 2017). Positive and negative predictive values (PPV and NPV) are dependent on both the technical characteristics of the test (Se and Sp) as well as the infection prevalence (Grunau and Linn 2018). Therefore, in order to evaluate the diagnostic performance of an assay in a clinical context, gold standard criteria need to be applied to an unbiased sample, independent of prior selection. In the case of *M. bovis* infection, mycobacterial culture is the gold standard, and therefore, an entire herd would need to be euthanased to determine the infection status of each animal and subsequently, the infection prevalence of the herd.

2. Materials and methods

During 2018 in Hluhluwe iMfolozi Park (HiP), South Africa (SA), a herd of 50 buffaloes was captured, immobilised and whole blood collected as described by Parsons et al. (2011).

The Bovigam® IGRA (Prionics AG, Schlieren-Zurich, Switzerland) was performed as previously described (Bernitz et al., 2018). The QFT IGRA (Qiagen, Venlo, Limburg, Netherlands) and QFT IPRA were performed as described by Bernitz et al. (2019) with the exception that the QFT Plus system was used for whole blood stimulation. The QFT-Plus system has two antigen tubes, namely i) TB1 which contains the same ESAT-6 and CFP-10 antigen peptides as the original QFT system, but not TB7.7(p4), and ii) TB2 which contains the same peptides as TB1 plus additional peptides for human application (Theel et al., 2018). No differences were observed when either antigen tubes (TB1 or TB2) were used to interpret the assays and on instruction from the manufacturer, TB2 was used to calculate assay results.

All animals were euthanased due to a suspected high infection prevalence based on test results (> 50% of buffaloes were test-positive on one or more assay). Buffaloes underwent necropsy as previously described in detail (Bernitz et al. 2019b) and mycobacterial culture, using the BACTEC™ MGIT™ 960 Mycobacterial Detection System (Becton Dickinson, Franklin Lakes, NJ, USA).

The infection prevalence, Se, Sp and predictive values for each assay and parallel interpretation of the QFT IGRA and QFT IPRA assays ($QFT^{parallel}$), a $QFT^{parallel}$-positive result defined as one or both assays being test-positive (Bernitz et al., 2019a), were calculated using an online tool (https://www.medcalc.org/calc/diagnostic_test.php).

3. Results and discussion

The infection prevalence of the herd was 52%, based on culture. The Se of assays determined in this study (Table 7.1) were lower than those previously calculated. Studies have reported Se of 80% for QFT IGRA (Bernitz et al., 2019), 80-100% for Bovigam® IGRA (Michel et al., 2011; Bernitz et al., 2018) and 100% for both the QFT IPRA and QFT^{parallel} (Bernitz et al., 2019). However, only test-positive culture-confirmed buffaloes were used in these studies and therefore, truly infected test-negative buffaloes would not have been included, resulting in an overestimation of Se. Results of this study suggest biased sampling (when the reference test is interpreted with knowledge of the test result) may lead to overestimations of Se, and lower detection rates of truly infected animals when applied in control programmes.

The assay Sp calculated in this study (Table 7.1) were also lower than those previously reported based on *M. bovis*-unexposed controls, which were 95% for Bovigam® IGRA and 100% for individual QFT assays and QFT^{parallel} (Michel et al., 2011; Bernitz et al., 2019). Although it is common practise to use endemic controls to calculate the Sp of assays in human studies, it was expected that use of culture-negative endemic controls in this study would result in an underestimation of Sp due to the suboptimal performance of culture as a gold standard (de la Rua-Domenech et al., 2006). Future studies should use *M. bovis*-unexposed buffaloes to calculate assay Sp.

Table 7.1 Test performances [sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV)] with 95% confidence intervals of in vitro cytokine release assays in detecting Mycobacterium bovis infection in African buffaloes (Syncerus caffer), based on mycobacterial culture results in a herd with an infection prevalence of 52% (n = 50).

Test/s	**Culture-positive (*n* = 26)**		**Culture-negative (*n* = 24)**		**Se (95% CI)**	**NPV (95% CI)**	**Sp (95% CI)**	**PPV (95% CI)**
	True positive	**False negative**	**False positive**	**True negative**				
Bovigam® IGRA [a]	19	7	2	22	73 (52-88)	76 (62-86)	92 (73-99)	90 (71-97)
QFT IGRA [b]	10	16	1	23	38 (20-59)	59 (51-66)	96 (79-100)	91 (58-99)
QFT IPRA [c]	18	8	8	16	69 (48-86)	67 (51-79)	67 (45-84)	69 (55-81)
QFT[parallel d]	21	5	9	15	81 (61-93)	75 (56-87)	63 (41-81)	70 (57-80)

[a]Bovigam® interferon gamma release assay

[b]QuantiFERON®-TB Gold Plus interferon gamma release assay

[c]QuantiFERON®-TB Gold Plus interferon gamma-inducible protein-10 release assay

[d]parallel interpretation of the QuantiFERON®-TB Gold Plus IGRA and IPRA

The assay NPVs were 59-76% and the PPVs 69-91% (Table 7.1) in this high prevalence herd. These metrics have not been previously reported for these assays. Assays with high NPVs would be useful for herds in eradication schemes to increase confidence in a negative test result, as leaving a test-negative infected animal may maintain infection (Clegg et al., 2019). Similarly, assays with high PPVs would be useful in testing valuable infected or previously uninfected herds, where confidence in a positive test result is required, as a false-positive result may result in the unnecessary euthanasia of a healthy animal or a farm being placed under quarantine.

A limitation of this study is that culture is an imperfect gold standard, especially during early infection, and the QFT IPRA has high Se in buffaloes with early infection (Bernitz et al., under review). Thus, the infection status of some IPRA-positive buffaloes may be misinterpreted as negative (Bernitz et al., 2019). Furthermore, the small sample size limited statistical analyses and interpretation of data in this study. Despite this, the predictive values calculated provide estimated levels of confidence in test results in high prevalence herds.
In this pilot study, a whole buffalo herd tested using three cytokine release assays was euthanased, enabling the infection prevalence of the herd and the test performances of the assays to be calculated using an unbiased sample. Our results suggest assay Se have previously been overestimated, while the assay Sp were underestimated in this study. Future studies using larger unbiased sample populations are required so that statistical analyses can be performed to compare test performances and avoid any selection bias that may prejudice test results. The consequence of this study is that eradication of *M. bovis* infection in South Africa may be prolonged by the diagnostic performances of established assays that are lower than previously estimated.

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Chapter 8 : Flow cytometric analysis of interferon gamma-inducible protein-10 by cattle leukocytes: a pilot study

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Unpublished chapter

Abstract

The QuantiFERON® TB Gold (QFT) interferon gamma-inducible protein-10 (IP-10) release assay (IPRA) is a sensitive and specific assay to detect *Mycobacterium bovis* (*M. bovis*) infection in African buffaloes (*Syncerus caffer*). However, elevated levels of IP-10 in unstimulated QFT whole blood of some buffaloes may compromise the utility of this IPRA. In buffaloes and cattle, the cellular source of IP-10 in peripheral blood is unknown and the aim of the present study was to develop a multiplex flow cytometry protocol to determine the major phenotypes of immune cells that produce IP-10 in cattle peripheral blood. Leukocytes from unstimulated and mitogen-stimulated whole blood samples of ten cattle were labelled with a panel of bovine cross-reactive antibodies in order to detect IP-10 production in selected cell phenotypes. An IP-10 signal was detected in all cell subsets and no differences were observed between unstimulated and mitogen-stimulated samples. As a result, the IP-10 antibody signal was not specific for the cytokine and therefore, further optimisation of the protocol is required.

1. Introduction

Strategies to identify *Mycobacterium bovis* (*M. bovis*) infection in buffaloes rely on measurements of early cell-mediated immune responses towards mycobacterial antigens (Goosen et al., 2014). *In vitro* cytokine release assays quantify the production of cytokine biomarkers in plasma in response to *M. bovis* antigen-specific whole blood stimulation. The interferon gamma-inducible protein-10 (IP-10) release assay (IPRA) measures the cytokine IP-10 in QuantiFERON® TB-Gold (QFT) stimulated whole blood to detect *M. bovis* infection in buffaloes (Bernitz et al., 2019). The QFT system is an easy-to-use stimulation platform comprised of i) Nil tube containing saline (unstimulated control), ii) TB antigen tube containing specific mycobacterial peptides (stimulated) and iii) mitogen tube containing phytohemagglutinin (positive control).

The cytokine IP-10 is a sensitive biomarker of *M. bovis* infection in buffaloes (Goosen et al., 2015), however the utility of the biomarker in the QFT IPRA is compromised by high concentrations of IP-10 in the QFT Nil tube. This may cause false-negative test results in truly infected buffaloes and hinders the diagnostic performance of the QFT IPRA. Elevated levels of IP-10 in the QFT Nil tube have also been reported in cattle, but this phenomenon is not understood in this species (Parsons et al., 2016). In humans, studies have suggested that elevated levels of IP-10 in unstimulated whole blood samples may be due to active tuberculosis (Chen et al., 2011). In buffaloes, a study is underway to determine if this is also true for buffaloes and bovine tuberculosis (Bernitz et al., under review). In order to understand the mechanisms behind IP-10 production in unstimulated buffalo whole blood, the cellular source of IP-10 needs to be identified. In humans, IP-10 has numerous cellular sources including antigen-presenting cells (such as monocytes), neutrophils and B-

lymphocytes (Cassatella et al., 1997; Gasperini et al., 1999; Antonelli et al., 2014; Hoff et al., 2015), while in cattle and buffaloes, the cellular source of IP-10 is unknown.

Collecting blood samples from buffaloes is logistically challenging and for this reason, this study was performed in cattle with the intention of using the optimised methods and results to investigate IP-10 production in buffaloes. This study aimed to identify the cellular sources of IP-10 in cattle peripheral blood using flow cytometric analysis by identifying the phenotypes of leukocytes that are able to produce IP-10 under stimulation conditions.

1. Materials and methods

1.1. Sample collection and blood processing

On day one, 6 ml of blood was collected into heparinised tubes from the tail vein of ten Holstein cattle at the Stellenbosch University's Welgevallen Experimental Farm, South Africa. All animals were in the milking line-up and therefore considered healthy. Within four hours, blood from each cow was processed in 5 ml tubes under two conditions, i.e., i) stimulated, for which a 2 ml aliquot of blood was incubated with 25 µl pokeweed mitogen (PWM), 5 µg/ml final concentration (Sigma-Aldrich, St. Louis, Missouri, USA), and ii) unstimulated, for which a 2 ml aliquot of blood was incubated with phosphate buffered saline. The samples were thoroughly mixed and incubated at 37 °C for 6 h after which 10 µl brefeldin-A, 10 µg/ml final concentration (Sigma-Aldrich) was added to all samples. The samples were once more thoroughly mixed and incubated for a further 18 h.

1.2. Titrations

Titrations were performed prior to the final experiment to determine the optimal concentration of antibodies to use. The datasheet of each antibody was consulted to determine

the concentration of antibody recommended by the manufacturer. A dilution series was prepared including a dilution to achieve this recommended antibody concentration, as well as half this dilution (double the recommended concentration) and double this dilution four times (four concentrations lower than the recommended concentration). For the titrations, 200 µl aliquots of whole blood in 96-well plates were centrifuged and the supernatant removed. The cells were thereafter stained in a total volume of 20 µl. The staining index, the ratio of the separation between the positive and negative populations divided by two times the standard deviation of the negative population, was used to determine each optimal antibody concentration (Appendix 8.1).

1.3. Surface marker staining

Following 24 h incubation, 200 µl aliquots of stimulated and unstimulated whole blood was added to wells of a 96-well plate and centrifuged at 400 x *g* for 5 min. The supernatant was removed and a master mix (MM) of surface marker antibodies and a viability cell marker at optimised concentrations, in BD Perm/Wash™ buffer (BD Biosciences, San Jose, CA, USA), diluted in water and freshly prepared, was added to the cell pellet (Table 8.1). Plates were incubated in the dark for 1 h at room temperature (RT).

1.4. Red cell lysis of whole blood

Incubated whole blood samples were centrifuged and the supernatant was removed. BD FACS™ lysing solution 10X concentrate (BD Biosciences), diluted in water, was added to the cell pellet and red cell lysis was performed in the dark for 15 min at RT. Samples were centrifuged and washed twice in BD Perm/Wash™ buffer.

1.5. Intracellular maker staining

Cells were incubated in BD Perm/Wash™ buffer in the dark for 15 min at RT. Samples were centrifuged and washed twice in BD Perm/Wash™ buffer. Cells were resuspended in a MM of the biotinylated anti-IP-10 and anti-CD3 (Alexa Flour® 700) antibody, at optimised concentrations (Table 8.1), and incubated in the dark for 40 min at RT. After incubation, cells were washed twice in BD Perm/Wash™ buffer and then stained with PE-Cy™7-conjugated streptavidin at an optimised concentration (Table 8.1). Cells were washed twice and resuspended in BD Perm/Wash™ buffer. All samples were processed and stained on the same day. The BD LSR II flow cytometer (BD Biosciences) was used for cell acquisition (≥ 10 000 events). The cytometer was calibrated per the manufacturer's instructions and compensation settings were adjusted using BD™ CompBeads (BD Biosciences).

1.6. Data analyses and gating strategies

Data were analysed using FlowJo Version 10 software (Tree Star, Ashland, OR, USA). Initially, a gating strategy was applied in order to obtain data that included a stable flow stream and excluded cell doublets, debris and non-viable cells: i) time versus side scatter area (SSC-A) was used to ensure an even flow of acquired cells; ii) forward scatter area (FSC-A) versus FSC height (FSC-H) was used to identify and include singlets and exclude doublets; iii) FSC-A versus SSC-A was used to exclude cell debris based on size and granularity of cells; and iv) a viability stain was used to include viable cells and exclude non-viable cells (Table 8.2, Figure 8.1A-D). A diagonal belt of signal believed to be autofluorescence and appearing to express the surface marker CD3, was excluded from all analyses (Figure 8.1E).

Table 8.1 Reagents optimised to identify subsets of leukocytes that produce IP-10 in cattle whole blood.

Target	Clone	Fluorophore	Reactivity	Company	Catalog Number	Dilution	
						Recommended	Used
CD3	CD3-12	AF700	human, bovine	Bio-Rad	MCA1477A700	neat	1:4
CD4	CC8	AF647	bovine	Bio-Rad	MCA1653A647	neat-1:10	1:20
CD21	LT21	PE-Cy5®	human, bovine	Abcam	ab201303	1:5	1:12.5
CD335	AKS1	PE	bovine	Bio-Rad	MCA2365PE	Neat	1:5
CD16	KD1	FITC	human, bovine	Bio-Rad	MCA5665F	neat-1:10	1:8
CD14	M5E2	APC/Cy7	human, bovine	BioLegend	301819	none given	1:80
biotinylated IP-10	pAb	none	bovine	Kingfisher	PBB0393B-050	none given	1:10 000
streptavidin	N/A	PE-Cy™7	N/A	BD Biosciences	557598	none given	1:6400
Viability stain	N/A	BV510	N/A	BD Biosciences	564406	none given	1:24

The proportion of each cell subset that produced IP-10 was calculated as the proportion of $CD3^+/CD4^+$, $CD3^+/CD4^-$, $CD3^+/CD335^+$, $CD3^-/CD335^+$, $CD3^-/CD21^+$, $CD3^-/CD14^+$ and $CD3^-/CD16^+$ cells displaying an $IP\text{-}10^+$ signal (Figure 8.1F-I). A one-way ANOVA was used to compare median IP-10 signals in unstimulated and stimulated samples using GraphPad Prism version 5 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered statistically significant if $p < 0.05$.

2. Results and discussion

2.1. Phenotyping

Scatter plots of Alexa Flour® 700 (CD3) versus SSC-A identified two distinct populations: $CD3^+$ cells and $CD3^-$ cells. The mean percentages of the $CD3^+$ and $CD3^-$ cells were 36.9% (± 16.2%) and 59.8% (± 16.13%) of all leukocytes, respectively (Figure 8.1E, Table 8.3).

All $CD3^+$ cells were gated on to analyse CD4 (Figure 8.1F) and CD335 expression (Figure 8.1G). Three distinct populations were identified, namely $CD3^+/CD4^+$ cells designated as CD4 T lymphocytes, $CD3^+/CD4^-$ cells designated as CD8 T lymphocytes, and $CD3^+/CD335^+$ cells designated as Natural Killer (NK) T (NK-T) lymphocytes (Kim et al., 2016). The mean percentages of $CD3^+/CD4^+$ and $CD3^+/CD4^-$ cells were 12.5% (± 7.7%) and 24.2% (± 8.5%) of all leukocytes, respectively (Table 8.3). This finding is in agreement with studies reporting peripheral blood from cattle is comprised of more $CD8^+$ cells than $CD4^+$ cells (Soltys and Quinn, 1999; Guzman et al., 2014). The stage of lactation has shown to affect the frequencies of leukocytes in peripheral blood of healthy cattle, however it appears that CD8 remains the predominant marker at all stages of lactation in cattle (Kulberg et al., 2002; Soltys and Quinn, 1999).

Table 8.2 The percentage of cells selected by specific gating strategies as a percentage of parent population from 10 ex vivo cattle whole blood samples after red blood cell lysis.

Cow ID	Time (%)	Single cells (%)	Size and granularity (%)	Viable cells (%)
1	93.2	88.3	92.2	78.6
2	93.3	82.8	79.5	74.4
3	96.3	86.0	84.5	70.6
4	86.7	77.8	69.1	69.5
5	91.3	75.1	65.0	87.4
6	88.5	71.9	61.2	69.9
7	79.4	76.8	69.6	79.4
8	93.7	86.9	88.7	87.7
9	98.4	82.1	82.5	78.4
10	93.3	74.0	64.8	76.3
Mean	91.4	80.2	75.7	77.2
SD	5.4	5.8	11.1	6.6

The small proportion of $CD3^{+}/CD335^{+}$ cells (1.1 ± 0.9% of total leukocytes) identified in this study (Table 8.3) is comparable to the 0.1-1.7% of NK-T cells previously reported for cattle in isolated peripheral blood mononuclear cells (Connelley et al., 2014). This non-conventional T lymphocyte subset that shares functional properties with both conventional NK and T-cells, was only identified in cattle in 2014 (Connelley et al., 2014). Limited literature on the frequencies of NK-T cells in whole blood is available with which to directly compare the frequencies observed in this study.

All $CD3^-$ cells were gated on to analyse CD335 (Figure 8.1H), CD21 (Figure 8.1I), CD14 and CD16 expression (Figure 8.1J). Four distinct populations were identified, namely $CD3^-/CD335^+$ cells designated as NK cells (Hussen et al., 2013), $CD3^-/CD21^+$ cells designated as B lymphocytes (Hussen et al., 2013), $CD14^+/CD16^-$ cells designated as classical monocytes, and $CD14^-/CD16^+$ cells designated as nonclassical monocytes (Corripio-Miyar et al., 2015; Hussen et al., 2013). The mean percentage of $CD3^-/CD335^+$, $CD3^-/CD21^+$, $CD3^-/CD14^+$ and $CD3^-/CD16^+$ cells was 0.5% (± 0.4%), 7.1% (± 3.3%), 1.7% (± 0.4%), and 0.3% (± 0.1%) of all leukocytes, respectively (Table 8.3).

A study in periparturient dairy cattle, in which the CD21 surface marker was used to identify B lymphocytes, calculated the percentage of B lymphocytes in peripheral blood to be 5-10% of all lymphocytes (Bromfield et al., 2018). Thus, the percentage of B lymphocytes of all leukocytes will be lower than this, as observed in this study. However, as previously mentioned, the lactation stage of cattle influences leukocyte counts in peripheral blood making direct comparisons difficult (Kulberg et al., 2002). Moreover, leukocytes, especially NK cells and monocytes, constitute only a small percentage of the total population of cells in cattle peripheral blood and percentages fluctuate regularly (Roland et al., 2014; Hamilton et al., 2017). Furthermore, only a small proportion of NK cells circulate in peripheral blood while most occur in afferent lymph and lymph nodes (Hamilton et al., 2017).

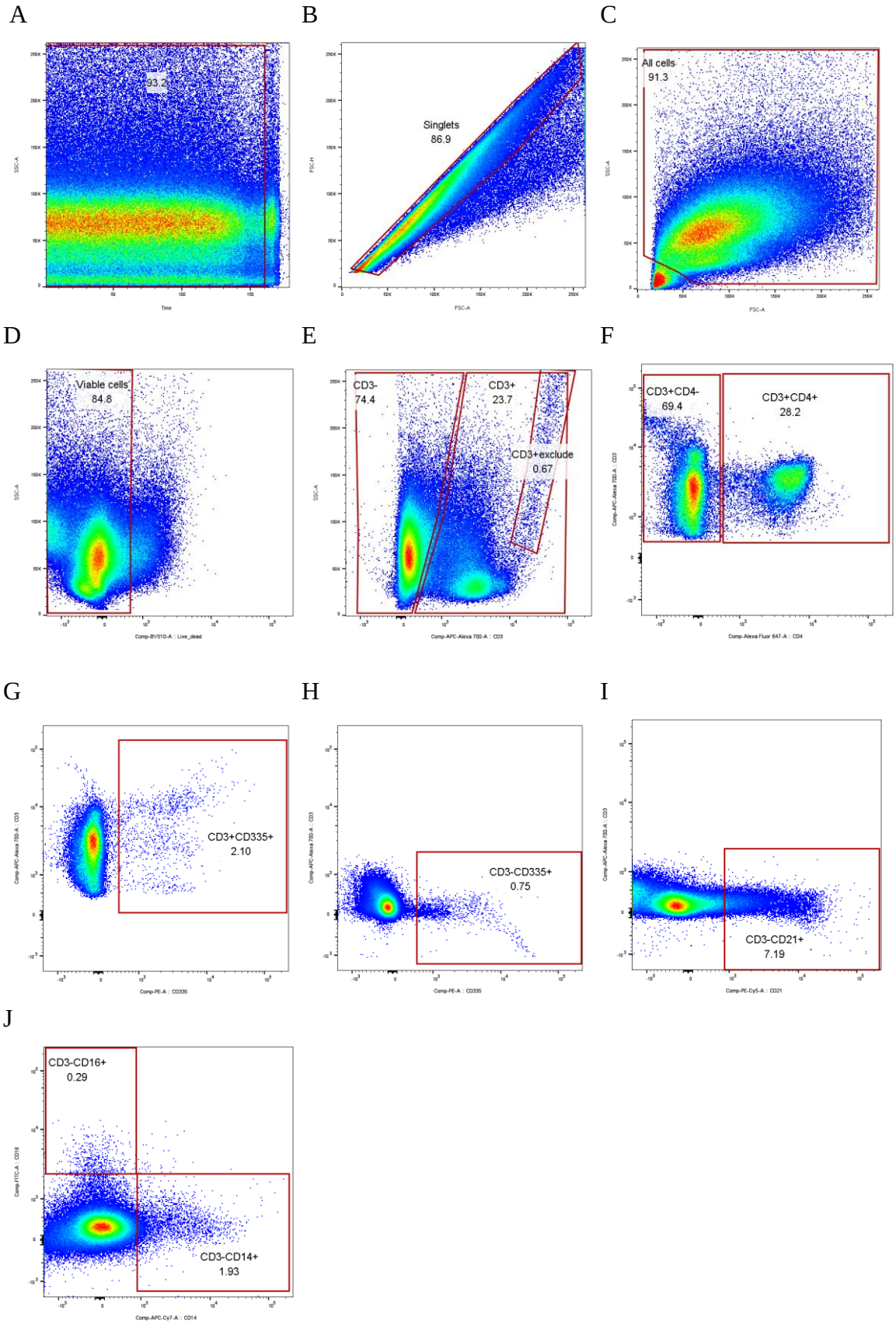

Figure 8.1 Representative flow cytometric scatter plots of ex vivo cattle leukocytes from whole blood samples. A) Time versus side scatter area (SSC-A) scatter plot with a gate on a

stable flow stream of cells, B) Forward scatter area (FSC-A) versus forward scatter height (FSC-H) scatter plot with a gate on single cells, C) FSC-A versus side scatter area (SCC-A) scatter plot with a gate on all cells excluding cell debris, D) Viability stain versus SSC-A scatter plot with a gate on viable cells, E) Alexa Fluor® 700 (CD3) versus SSC-A scatter plot with a gate on $CD3^-$ cells, a gate on $CD3^+$ cells, and a gate to exclude selected cells appearing to be $CD3^+$, F) Alexa Fluor® 647 (CD4) versus Alexa Fluor® 700 (CD3) scatter plot with a gate on $CD3^+/CD4^+$ cells and a gate on $CD3^+/CD4^-$ cells, G) PE (CD335) versus Alexa Fluor® 700 (CD3) scatter plot with a gate on $CD3^+/CD335^+$ cells, H) PE (CD335) versus Alexa Fluor® 700 (CD3) scatter plot with a gate on $CD3^-/CD335^+$ cells, I) APC/Cy7 (CD14) versus FITC (CD16) scatter plot with a gate on $CD3^-/CD21^+$ cells and J) APC/Cy7 (CD14) versus FITC (CD16) scatter plot with a gate on $CD3^-/CD14^+$ cells and a gate on $CD3^-/CD16^+$ cells.

A neutrophil surface marker was not included in the panel of antibodies optimised in this study. In humans, neutrophils are one of the key cellular sources of IP-10 (Gasperini et al., 1999) and therefore, the exclusion of cell surface markers was used to try to identify bovine neutrophils in this study. Specifically, $CD3^-/CD335^-/CD21^-/CD14^-/CD16^-$ cells were identified and designated as potential neutrophils (data not shown). The mean percentage of these cells was 55.7% (± 12.6%) of all leukocytes (Table 8.3). However, in peripheral blood of adult cattle, the neutrophil-to-lymphocyte ratio is approximately 1:2 with neutrophils being the second largest leukocyte population after lymphocytes (Kim et al., 2016; Roland et al., 2014). Moreover, FSC-A versus SSC-A scatter plot of these designated neutrophils indicated no uniform dispersion of cells based on size and granularity (data not shown). Based on these findings, the $CD3^-/CD335^-/CD21^-/CD14^-/CD16^-$ cells that were identified as potential neutrophils, may have included neutrophils but also included other cell types.

Table 8.3 The percentage of cell populations characterized by phenotype as a percentage of total leukocytes in 10 ex vivo cattle whole blood samples.

Cow ID	$CD3^+$ exclude	$CD3^+$	$CD3^+$			$CD3^-$	$CD3^-$				
			$CD4^+$	$CD4^-$	$CD335^+$		$CD335^+$	$CD21^+$	$CD14^+$	$CD16^+$	$CD335^-/CD21^-/CD14^-/CD16^-$
1	0.6	23.6	6.7	16.8	0.5	73.3	0.5	5.1	1.4	0.2	68.6
2	0.3	26.3	7.2	18.8	0.9	68.6	0.2	5.2	1.3	0.3	72.1
3	0.1	33.4	13.1	20.2	0.3	64.7	1.4	13.9	1.6	0.3	55.1
4	0.8	62.0	23.3	38.3	0.7	35.3	0.3	4.3	1.9	0.1	48.2
5	1.0	23.9	7.0	16.8	0.7	72.9	0.2	5.9	1.3	0.1	67.8
6	1.6	63.6	25.5	37.8	1.9	32.6	0.8	2.9	1.5	0.2	40.2
7	0.1	36.9	12.0	24.2	3.2	60.4	0.6	11.3	2.1	0.2	48.9
8	0.9	30.4	9.4	20.8	0.6	66.7	0.4	6.6	2.3	0.5	62.2
9	0.6	32.1	8.2	23.7	0.8	63.6	0.3	8.2	2.0	0.3	59.2
10	1.0	59.7	24.3	35.0	0.8	36.6	0.2	6.4	1.8	0.3	34.3
Mean	0.7	36.9	12.5	24.2	1.1	59.8	0.5	7.1	1.7	0.3	55.7
SD	0.5	16.2	7.7	8.5	0.9	16.1	0.4	3.3	0.4	0.1	12.6

* cells excluded from the analyses due to autofluorescence

2.2. IP-10 production

All cell subsets displayed an IP-10 signal, however in each cell subset, no significant difference was observed when the median proportions of unstimulated and stimulated cells displaying an IP-10 signal were compared (Figure 8.2). This suggests the IP-10 signal observed may not have been related to the cytokine IP-10, and was due to non-specific binding of either the biotinylated anti-IP-10 antibody or PE-Cy™7-conjugated streptavidin. Notably, the proportion of cells in each cell subset displaying an IP-10 signal in both unstimulated and stimulated sample were similar for all animals (Figure 8.2). This suggests the reagent that caused the non-specific binding, bound to the same cellular component in the unstimulated and stimulated samples. The molecules biotin and streptavidin have a high affinity for one another (Weber et al., 1989), and so it is unlikely that PE-Cy™7-conjugated streptavidin would have bound to anything other than the biotinylated anti-IP-10 antibody. Therefore, it seems probable that the biotinylated anti-IP-10 antibody bound to an unknown cellular component, but this cannot be confirmed. The mean fluorescent intensity of all unstimulated and stimulated samples in the various cell subsets did not differ, indicating no upregulation of the cellular component causing an IP-10 signal (data not shown).

A limitation of this study included detecting the cytokine IP-10. A bovine anti-IP-10 antibody directly conjugated to a fluorophore was not available at the time this study was designed, and therefore a biotinylated anti-IP-10 antibody was used in combination with PE-Cy™7-conjugated streptavidin. This indirect method of labelling IP-10 may have resulted in an amplification of signal as many streptavidin molecules could have bound to one biotinylated antibody, but as observed, non-

specific binding is more likely to occur. Moreover, dairy cattle may not be the most suitable animals to use to investigate IP-10 production in leukocyte subsets, as the periparturient period and stage of lactation may influence frequencies of leukocytes. Additionally, neutrophils are a major cellular source of IP-10 in humans (Cassatella et al., 1997; Gasperini et al., 1999) and therefore, future studies investigating IP-10 production in cattle and buffaloes should include a neutrophil surface maker such as CD11b or CD19 (Hoff et al., 2015; Alhussien et al., 2016).

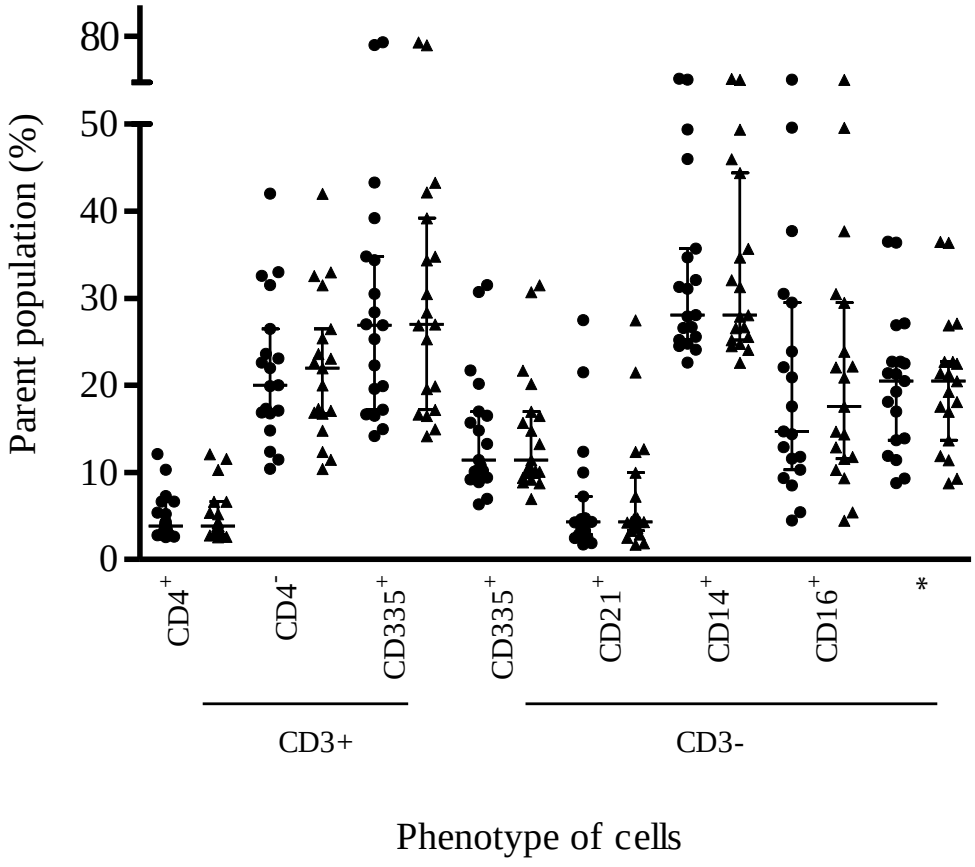

*Figure 8.2 The proportion of leukocyte populations characterized by phenotype as a percentage of the parent cell population eliciting an IP-10 signal. The IP-10 antibody signal in unstimulated (●) and pokeweed mitogen stimulated (▲) cattle whole blood of 10 cattle. The * indicates $CD3^-/CD335^-/CD21^-/CD14^-/CD16^-$ cells. Horizontal bars represent medians and interquartile ranges.*

In this study, immunophenotyping was used to identify cattle leukocytes subsets. Although the cytokine IP-10 could not be detected, this pilot study provides a foundation for future research to investigate which leukocytes produce IP-10 in cattle and buffalo peripheral blood.

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Appendix 8.1

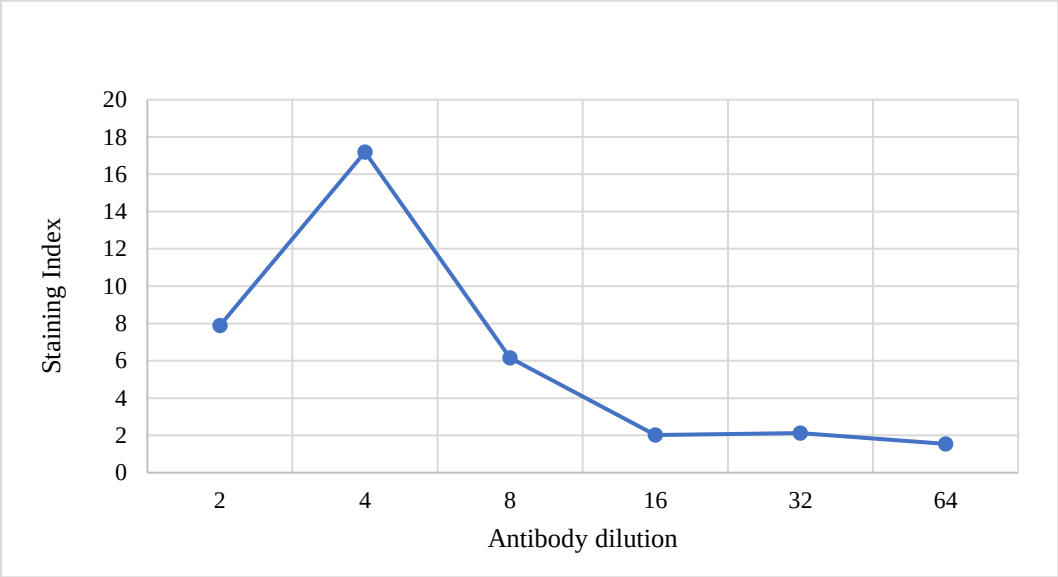

Figure 8.1 Titration of anti-CD3 [CD3-12] (Alexa Flour® 700) on cattle whole blood after red cell lysis.

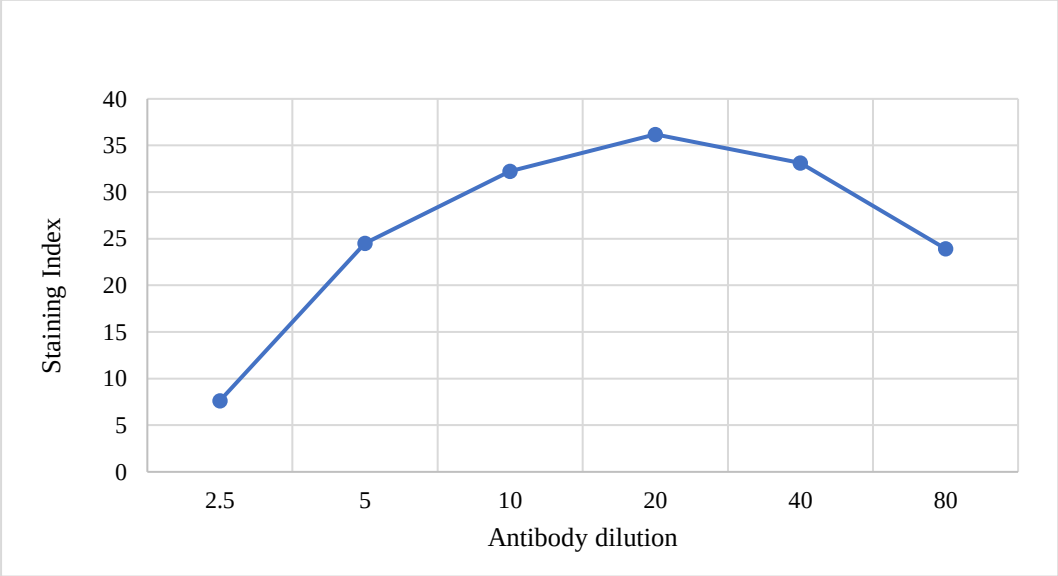

Figure 8.2 Titration of anti-CD4 [CC8] (Alexa Flour® 647) on cattle whole blood after red cell lysis.

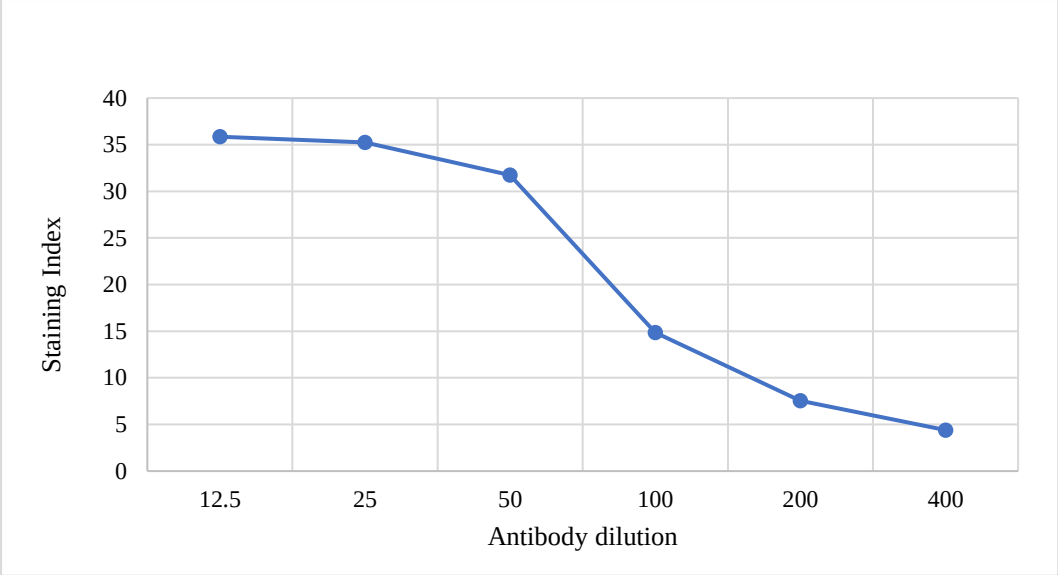

Figure 8.3 Titration of anti-CD21 [LT21] (PE/Cy5®) on cattle whole blood after red cell lysis.

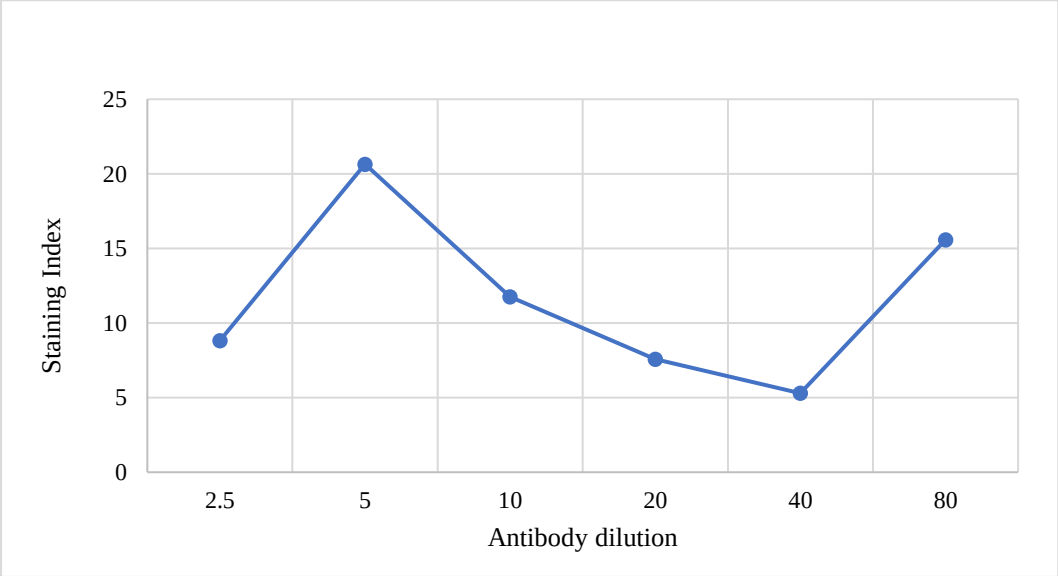

Figure 8.4 Titration of anti-CD335 [AKS1] (PE) on cattle whole blood after red cell lysis.

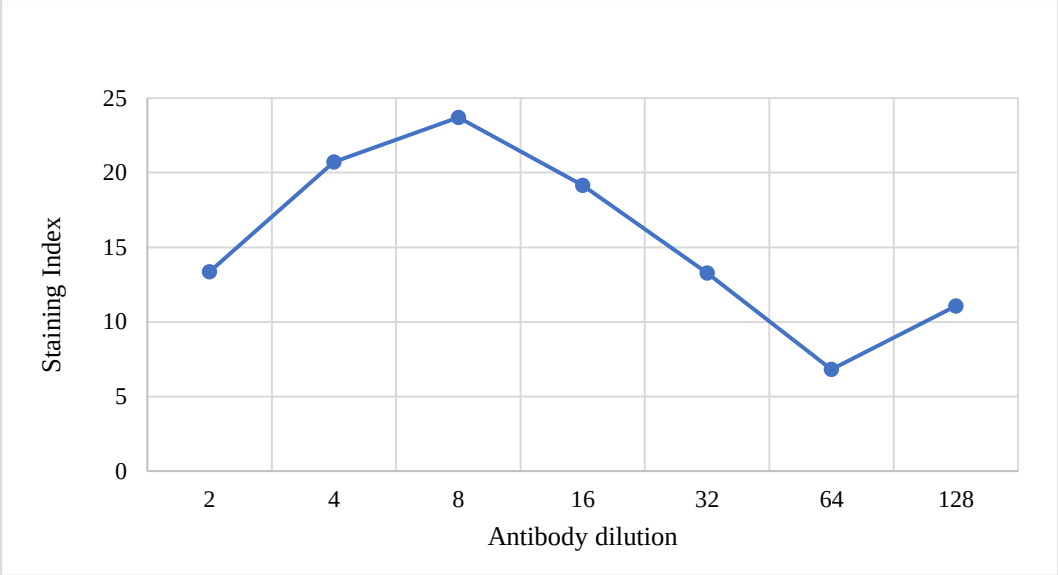

Figure 8.5 Titration of anti-CD16 [KD1] (FITC) on cattle whole blood after red cell lysis.

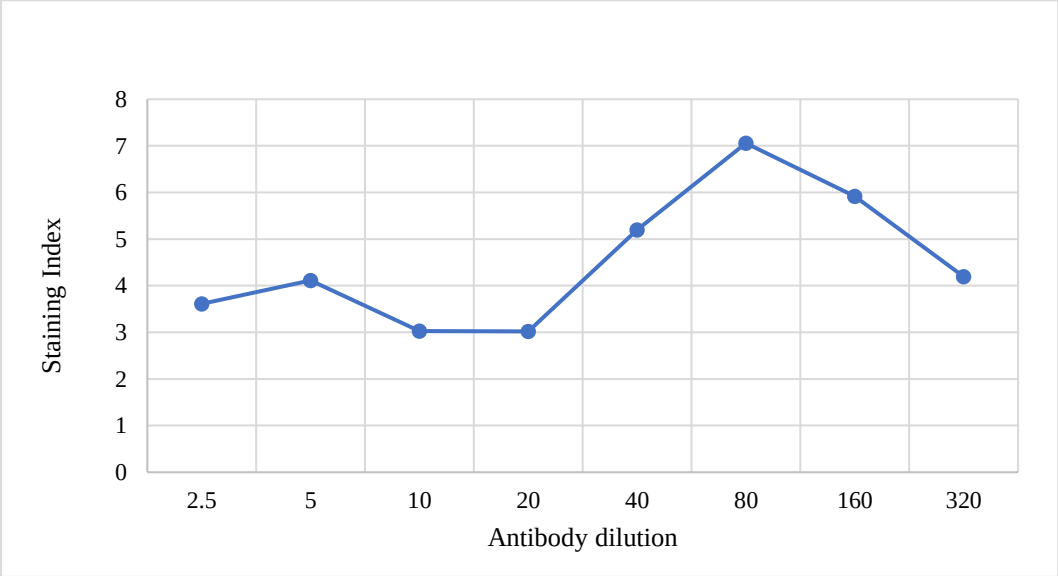

Figure 8.6 Titration of anti-CD14 [M5E2] (APC/Cy7) on cattle whole blood after red cell lysis.

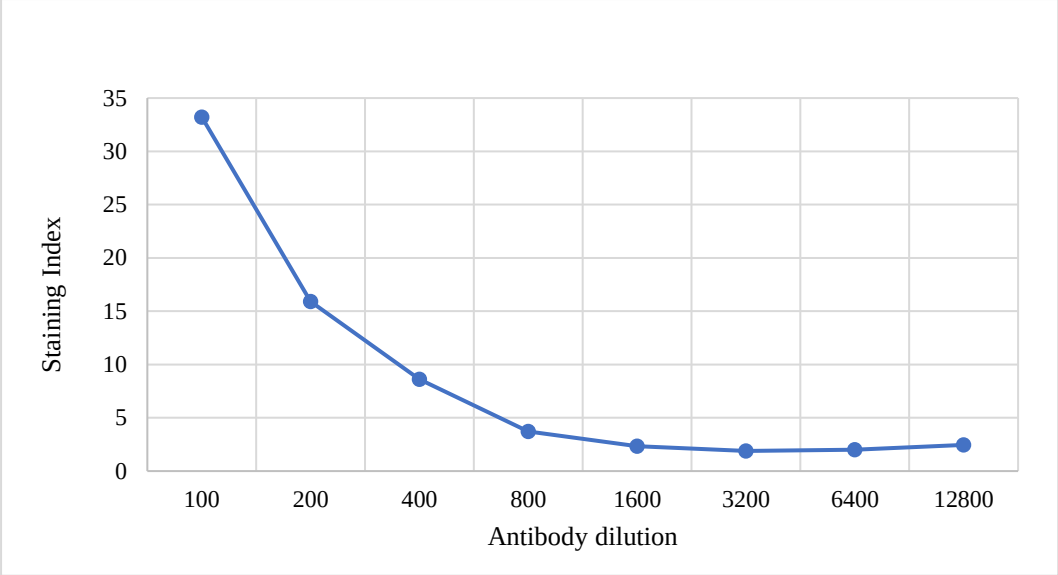

Figure 8.7 Titration of PE-Cy™7 streptavidin on cattle whole blood after red cell lysis.

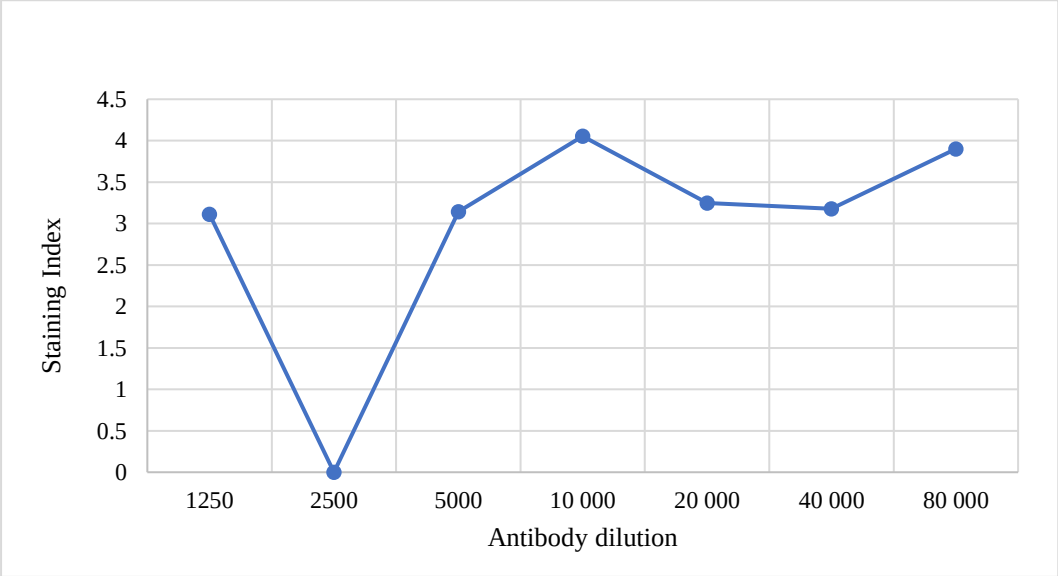

Figure 8.8 Titration of biotinylated anti-IP-10 on cattle whole blood after red cell lysis.

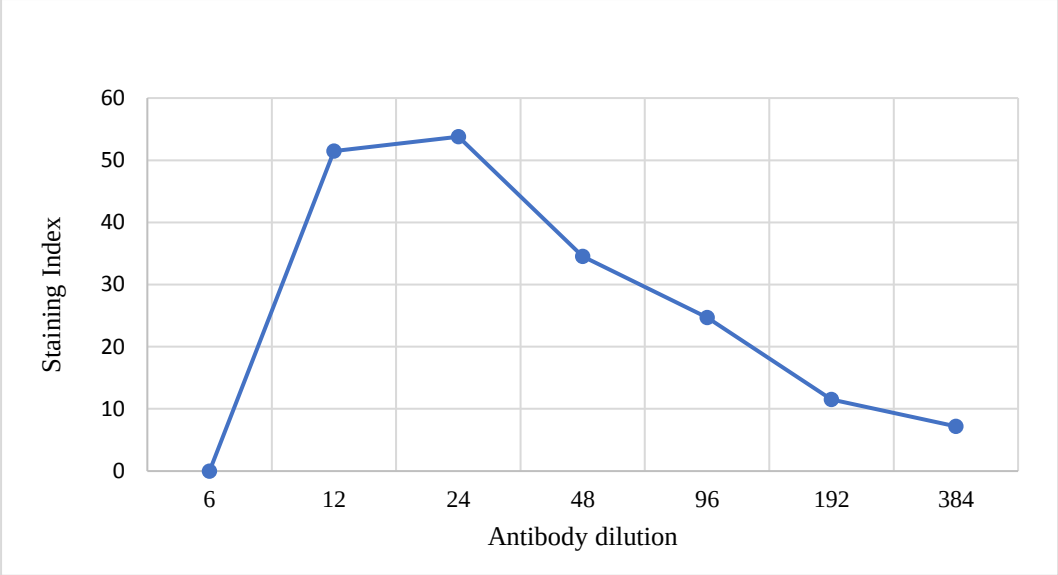

Figure 8.9 Titration of Fixable Viability Stain (BV510) on cattle whole blood after red cell lysis.

Chapter 9 : General discussion

This chapter aims to synthesise all the results from the previous chapters and to contextualize the findings with regard to the detection of *M. bovis* infection in African buffaloes.

The diagnostic performance of CMI-based assays, namely the SCITT and *in vitro* cytokine release assays, to detect *M. bovis* infection in buffaloes are suboptimal. The development of novel tests and the optimisation of established tests with improved diagnostic performances are urgently required. The feasibility of performing a test is as important as the performance and therefore, both features need to be considered when evaluating a new test. Moreover, it is unlikely that a single test will have the desired level of performance, and so tests can be combined to increase Se or Sp (Gormley et al., 2006). In addition, the required performance parameters of a test will depend on the population being tested and the testing goals, i.e. screening versus confirmation of infection (Cousins and Florisson, 2005), and for different circumstances (high or low infection prevalence), interpretation of a test requires modification or a different test needs to be utilised. A holistic approach that incorporates bTB history, testing goals, infection prevalence and exposure risk of the population being tested will determine what test(s) would be most suitable and effective. This thesis describes novel CMI-based approaches to diagnose *M. bovis* infection in African buffaloes.

QFT IGRA

The QFT IGRA, using the QFT stimulation system in combination with the newly available commercial ruminant-specific *cattletype*® IFN-gamma ELISA, is a highly practical blood-based assay, with high Sp but relatively poor Se to detect *M. bovis* infection in buffaloes. It has been previously reported that the Sp of IGRAs are improved by using specific mycobacterial peptides (Parsons et al., 2011), with a possible decrease in Se (Goosen et al., 2014). The most accurate way to calculate the Se of an assay is to perform unbiased sampling of a population of known infection

prevalence (Trevethan, 2017). Therefore, using samples from a small unbiased buffalo herd with high prevalence, the Se of the QFT IGRA was calculated to be poor, while the Se was overestimated in test-positive culture-confirmed buffaloes due to selection bias. Throughout this thesis, the Sp of assays were calculated using historical *M. bovis*-unexposed buffalo populations and not culture-negative *M. bovis*-endemic controls, as mycobacterial culture is an imperfect gold standard (de la Rua-Domenech et al., 2006). Thus, the QFT IGRA would be best utilised as a test in buffalo herds with no history of bTB, where the goal of testing is to limit false-positive test results by using highly specific tests. Moreover, the standardised and commercially available components of the QFT IGRA will simplify future validation and enable assay reproducibility between laboratories.

QFT IPRA

Measuring the cytokine IP-10 in the QFT system, namely in the QFT IPRA, detected a greater number of culture-confirmed buffaloes than the QFT IGRA, however the Se of the IPRA remained suboptimal to detect *M. bovis* infection in buffaloes. It has been previously reported that measuring IP-10 in the QFT system improves the Se of detection in buffaloes compared to IFN-γ (Goosen et al., 2015), as IP-10 is a more sensitive biomarker since the cytokine is produced more than 2000-fold compared to IFN-γ (McInnis et al., 2005). Thus, the decrease in Se of the QFT IGRA as a result of using specific mycobacterial peptides in the QFT system (Goosen et al., 2014) is compensated in the IPRA by increased Se of this assay. Moreover, the QFT IPRA detects recently infected buffaloes as IP-10 can be measured at an earlier stage of infection when IFN-γ may not yet be detectable (Ruhwald et al., 2007). This is supported by the finding that most buffaloes with IPRA-positive and IGRA-negative

test results presented with no or early macroscopic pathology. In addition, as for other species, the measurement of IP-10 in the IPRA did not compromise the diagnostic Sp of the test (Ruhwald et al., 2009; Parsons et al., 2016). Thus, the QFT IPRA would be best utilised as a test in previously uninfected buffalo herds to identify recently *M. bovis*-infected buffaloes.

Parallel testing

Parallel interpretation of the SCITT and Bovigam® IGRA identified all test-positive culture-confirmed buffaloes, significantly more buffaloes than each individual test identified, indicating an improvement in the detection of infected buffaloes. In agreement, in cattle, the Bovigam® IGRA is used as an ancillary test to the SCITT to improve the detection of *M. bovis* infection (de la Rua-Domenech et al., 2006) and a study advocated the parallel use of the SCITT and Bovigam® IGRA to maximise the detection of *M. bovis*-infected animals (Gormley et al., 2006). However, the use of this parallel testing strategy in buffaloes is limited by: i) the use of PPDs that result in cross-reactive immune responses to non-tuberculous mycobacteria (Michel, 2008); ii) the SCITT requirement for animals to be chemically immobilised twice and held captive during this time (Parsons et al., 2011); iii) the SCITT being subject to operator bias and error (A. McCall, personal communication); iv) the SCITT causing *in vivo* sensitization (Clarke et al., 2018) or desensitization which affects future test results; and v) the need for Bovigam® IGRA stimulations to be performed by skilled technicians, which cannot be easily performed in the field (Michel et al., 2011). Therefore, development of a blood-based assay, which only requires one immobilisation for blood collection and utilises a whole blood stimulation system that

can be performed in the field and contains specific mycobacterial antigens, would be beneficial for bTB testing of buffaloes.

Parallel interpretation of the QFT assays, the IGRA and IPRA, identified all *M. bovis*-infected buffaloes which was more than the individual QFT assays identified. It has been previously reported in humans and cattle that the parallel measurement of antigen-specific IFN-γ and IP-10 improves the detection of *Mtb* and *M. bovis* infection, respectively (Ruhwald et al., 2008; Coad et al., 2019). However, the lower Se of the QFT assays in parallel calculated in a small unbiased buffalo herd with high prevalence, indicates the Se is lower than previously reported. Importantly, the Sp of the individual QFT assays were maintained when the assays were interpreted in parallel. These findings indicate this *in vitro* parallel testing strategy, where only one set of QFT whole blood stimulation tubes are required in which two biomarkers are measured from the same plasma sample, should be used to increase detection of *M. bovis*-infected buffaloes while maintaining Sp and simplifying the testing procedure. Moreover, since there is no *in vivo* sensitization, these tests can be repeated without a waiting period, unlike the SCITT (Clarke et al., 2018). This advances our diagnostic approach to bTB diagnosis in that the parallel interpretation of tests may increase Se for optimal detection of infected buffaloes. However, further research into different interpretations of test combinations are required to see if Se can be improved.

bTB Pathology

The [IP-10^{Nil}] and [IFN-γ^{Nil}] were higher in buffaloes with culture-confirmed pathological lesions than uninfected controls, indicating the cytokines IP-10 and IFN-γ are not only biomarkers of infection, but may be biomarkers of pathological changes

in infected buffaloes. Therefore, the cytokines IP-10 and IFN-γ may be biomarkers of disease in buffaloes, as previously shown in humans (Whittaker et al., 2008; Hasan et al., 2009; Chegou et al., 2016), although additional investigation is required.

In culture-confirmed buffaloes, the [IFN-γ^{Nil}] and [IFN-γ^{TB}] - [IFN-γ^{Nil}] values increased with lesion score, suggesting that the magnitude of unstimulated and antigen-stimulated IFN-γ indicate the degree of pathology in *M. bovis*-infected buffaloes. In humans, unstimulated IFN-γ concentrations increase during the progression of active TB disease (Hasan et al., 2009) and in humans, cattle and badgers, antigen-stimulated IFN-γ concentrations and disease severity are correlated (Vordermeier et al., 2002; Sahiratmadja et al., 2007; Tomlinson et al., 2015). Further investigation into quantification of [IFN-γ^{Nil}] and [IFN-γ^{TB}] - [IFN-γ^{Nil}] to determine cutoff values that are indicative bTB pathology in buffaloes will be valuable for detecting bTB disease.

In *M. bovis*-infected culture-confirmed IPRA-negative IGRA-positive buffaloes, a significantly greater proportion of animals had evidence of pathology compared to those with NVL. Buffaloes with bTB lesions may produce higher [IP-10^{Nil}], thus decreasing the IP-10 differential value between antigen-stimulated and unstimulated samples, resulting in an IPRA-negative result. This was supported by the observation that 5 of the 12 IPRA-negative IGRA-positive buffaloes with bTB pathology had higher [IP-10^{Nil}] compared to [IP-10^{TB}]. Therefore, IPRA results with high [IP-10^{Nil}] should be regarded as inconclusive and not test-negative, whereas a buffalo that is IPRA-negative IGRA-positive should be suspected of having bTB pathology, if the [IP-10^{Nil}] is elevated. Therefore, it is beneficial to analyse cytokine concentrations in

the QFT Nil tubes when interpreting the final QFT assay results as this may provide extra information on the infection status and pathological state of the buffalo.

Immunobiology of IP-10 production

In cattle and buffaloes, the cellular source of IP-10 is unknown. Therefore, in order to understand the mechanism behind elevated IP-10 in some unstimulated buffalo whole blood samples, the cellular source of IP-10 was investigated in cattle, with the intention of using the optimised method to investigate IP-10 production in buffaloes. In humans, IP-10 has numerous cellular sources including antigen-presenting cells and lymphocytes (Gasperini et al., 1999; Hoff et al., 2015). Multiplex flow cytometry was used to determine the phenotype of immune cells that produce IP-10 in cattle. An IP-10 signal was detected in all cell phenotypes and no differences were observed between stimulated and unstimulated samples. Therefore, we could not conclude that the signal was specific to IP-10 and we were unable to identify the immune cells that produce the cytokine IP-10 in cattle.

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Chapter 10 : Conclusion

The QFT system is an innovative platform to detect *M. bovis* infection in buffaloes utilizing field-friendly whole blood stimulation tubes and specific mycobacterial antigens that elicit highly specific cell-mediated immune responses. The QFT IGRA, combining the QFT system and novel *cattletype*® IFN-gamma ELISA, is a practical useful assay with high Sp that would especially have value for testing *M. bovis*-unexposed herds to reduce the risk of false-positive test results. The measurement of the chemokine IP-10 in the QFT system increases the detection of *M. bovis*-infected buffaloes compared to IFN-γ, and is able to detect recently infected buffaloes that IGRAs may not. Notably, when the cytokines IP-10 and IFN-γ are measured in parallel in the QFT system, the detection of *M. bovis*-infected buffaloes is maximised while Sp is maintained and the test procedure is simplified using solely blood-based assays and a single stimulation platform. Finally, the magnitude of IP-10 and IFN-γ concentrations in QFT-processed whole blood may offer additional information as indicators of bTB pathology in buffaloes, providing a new insight into the disease status of infected buffaloes.

Future directions

Future investigations of the diagnostic performances of assays to detect *M. bovis* infection in African buffaloes should use unbiased sample populations with known infection prevalences to avoid overestimating performance metrics and should include buffalo herds with varying infection prevalences to determine the performances of assays in different populations. Moreover, investigations into different interpretations of the same test in buffalo populations with varying infection prevalences will allow the adaption of a single test for use in multiple populations, i.e. calculating different cutoff values or interpreting tests in parallel or series. Additionally, studies

investigating the performance of tests in different stages of *M. bovis* infection and disease will confirm whether IP-10 is produced during early infection before IFN-γ becomes detectable and whether elevated levels of IP-10 are indicative of disease in buffaloes. Future characterization of the immunobiology of IP-10 production in cattle and buffaloes will enable a deeper understanding of the mechanism behind unstimulated production of this cytokine in *M. bovis* infection in bovids.