Identification of immunological biomarkers for detection of *Mycobacterium bovis* infection in African rhinoceros

by

Josephine Chileshe

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Supervisor: Prof Michele A. Miller

Co-supervisor: Prof Sven D.C.

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Declaration

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This dissertation includes 2 original papers published in peer-reviewed journals (Chapters 2 and 3), 1 chapter that is under review (Chapter 4), and 1 chapter prepared as a short communication publication (Chapter 5). The remaining 3 chapters include the general introduction (Chapter 1), general discussion (Chapter 6) and conclusion (Chapter 7).

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Summary

African rhinoceros have economic and ecological value, yet they are threatened by poaching and habitat loss, which causes reduction in population numbers. In addition, infection with *Mycobacterium bovis* (*M. bovis*), a causative agent of bovine tuberculosis (bTB), may be a threat to their conservation. This legislatively notifiable infectious disease has serious consequences on management of African rhinoceros by restricting their movements; therefore, it is imperative to have accurate and easily available diagnostic tools for the detection of *M. bovis* infection, to ensure that infected rhinoceros are not translocated. In order to address this need, this study aimed to (i) optimize enzyme-linked immunosorbent assay (ELISA) conditions for detecting rhinoceros interferon-gamma (IFN-γ), (ii) develop a diagnostic antigen-specific IFN-γ release assay (IGRA) for rhinoceros, (iii) identify cytokine gene signatures that distinguish between *M. bovis*-infected and uninfected rhinoceros, and (iv) identify cytokines other than IFN-γ with diagnostic potential to distinguish between *M. bovis*-infected and uninfected animals.

Whole blood samples were collected from *M. bovis*-infected rhinoceros and uninfected animals from the Kruger National Park and incubated overnight at 37°C in tubes of the QuantiFERON (QFT) system. Thereafter, harvested plasma samples and cell pellets preserved in RNAlater, were stored at -80°C. Archived plasma was used to validate the equine Mabtech IFN-γ ELISA^{PRO} and then develop a diagnostic IGRA for rhinoceros. Using archived blood pellets, RNA was extracted, reverse transcribed, and screened using the equine RT^2 profiler PCR array to identify candidate cytokine biomarkers. In addition, the Raybio® equine interferon gamma induced protein (IP-10) ELISA was used with rhinoceros plasma from QFT stimulated blood to investigate detection of rhinoceros IP-10.

The commercial Mabtech Equine IFN-γ ELISA^{PRO} kit was validated as a robust and highly reproducible assay for the detection of rhinoceros IFN-γ. Thereafter, the QFT IGRA was shown to distinguish between *M. bovis*-infected and uninfected animals. The optimal cut-off value was calculated as 21 pg/ml, although test results ranging between 22–61 pg/ml were interpreted as indeterminate, and a test result of $\geq$ 62 pg/ml as definitively positive. Using RNA extracted from QFT-stimulated rhinoceros whole blood, the equine RT^2 profiler PCR array identified six potential cytokine gene targets. Further development of a gene expression assay for *CXCL10* (IP-10) confirmed this cytokine as a promising biomarker based on significant upregulation in antigen-stimulated blood from *M. bovis*-infected rhinoceros. Based on this result, an equine IP-10 release assay (IPRA) was developed to demonstrate that antigen-specific IP-10 could discriminate between *M. bovis*-infected and uninfected white rhinoceros. Additionally, the IPRA was able to detect one additional truly infected animal when used in parallel with the IGRA, suggesting that this may have potential as an additional diagnostic biomarker in rhinoceros.

In summary, the novel tools developed in this project can be used to detect *M. bovis* infection in rhinoceros. Similar to other species, QFT IGRA and potentially IPRA may support testing of individual rhinoceros prior to translocation, as well as allow for disease surveillance. Future research should focus on additional biomarker discovery and understanding the rhinoceros' immune response to *M. bovis* infection.

Opsomming

Ten spyte van die groot ekonomiese en ekologiese waarde van Afrika-renosters, word hulle bedreig deur stroping en habitatverlies wat lei tot vermindering in die populasie se getalle. Daarby is infeksie met *Mycobacterium bovis* (*M. bovis*), die oorsaak van beestuberkulose (bTB), ‘n bedreiging vir hul bewaring. Hierdie wetlike aanmeldbare aansteeklike siekte het ernstige gevolge vir die bestuur van Afrika-renosters deur die beperking van hul beweging; gevolglik is akkurate and maklik verkrygbare diagnostiese toetse vir die identifisering van *M. bovis* infeksie noodsaaklik om te verseker dat geinfekteerde renosters nie geskuif word nie. Om hierdie behoefte aan te spreek het hierdie studie gepoog om i) ELISA kondisies te optimiseer vir identifisering van renoster interferon-gamma (IFN- γ), ii) ‘n diagnostiese antigeen-spesifieke IFN- γ vrystellingstoets (IGRA) vir renosters te ontwikkel, iii) sitokine geen paneel te identifiseer wat kan onderskei tussen *M. bovis*-geinfekteerde en ongeinfekteerde renosters, en iv) identifisering van ander sitokienes as IFN- γ wat diagnostiese potensiaal het om te kan onderskei tussen *M. bovis*-geinfekteerde en ongeinfekteerde diere. Heelbloed monsters van *M. bovis*-geinfekteerde en ongeinfekteerde renosters van die Kruger Nasionale Park is geneem en oornag geinkubeer by 37°C in buise van die QuantiFERON (QFT) sisteem. Daarna is plasma monsters geoes en sel klonte is gepreserveer en gestoor in RNAlater by -80°C.

Gestoorde plasma monsters is gebruik om die Equine Mabtech IFN- γ ELISA^{PRO} te toets en te ontwikkel vir 'n diagnostiese IGRA vir renosters. RNA is ge-ekstraëer vanaf gestoorde bloed, omgekeerd getranskribeer en gesif deur die perde RT^2 profiel PKR opstelling om kandidaat sitokine as biomerkers te identifiseer. Daarby is die Raybio® perde interferon gamma geinduseerde protein (IP-10) ELISA gebruik in kombinasie met die plasma van QFT

gestimuleerde bloed van renosters om IP-10 van hierdie diere te identifiseer. Die kommersiele Mabtech perde IFN-γ ELISA^{PRO} toets is beproef as ‘n geharde en herhaalbare toets vir die identifisering van renoster IFN-γ. Daarna is die QFT IGRA gebruik om te onderskei tussen *M.bovis*-infekteerde en ongeinfekteerde diere. Die optimale afsny waarde is bereken as 21 pg/ml, toets resultate tussen 22 – 61 pg/ml is geinterpreteer as intermediêr en ‘n toets resultaat van $\geq$ 62 pg/ml is beskou as positief. Die perde RT^2 profiel PKR opstelling het ses potensiële sitokien gene in die RNA wat geekstraëer is van QFT-gestimuleerde renoster heelbloed monsters geidentifiseer. Die ontwikkeling van ‘n geen uitdrukking toets vir *CXCL10* bevestig dat hierdie sitokien ‘n belowende biomerker is op grond van beduidende opregulering in antigeen-gestimuleerde bloed van *M. bovis*-geinfekteerde renosters. Gebasseer op hierdie resultaat is ‘n perde IP-10 vrystellings toets (IPRA) ontwikkel wat gedemonstreer het dat antigeen-spesifieke IP-10 kon diskrimineer tussen *M. bovis*-geinfekteerde en ongeinfekteerde wit renosters. Daarby kon die IPRA een waarlik geinfekteerde dier identifiseer wanneer dit in parallel gebruik is met die IGRA. Dit stel voor dat hierdie toets die potensiaal het om as ‘n addisionele biomerker vir renosters te dien.

Ter opsomming, in hierdie studie is nuwe tegnologie ontwikkel wat gebruik kan word om *M. bovis* infeksie in renosters te identifiseer. Soortgelyk aan ander spesies, kan die QFT IGRA en potensiëel ook IPRA die toetsing van individuele renosters ondersteun voor oorplasing/vervoer, asook vir siekte voorkoming. Toekomstige navorsing moet fokus op addisionele biomerker ontdekking en die verstaan van die renoster se immuun reaksie tot *M. bovis* infeksie.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

[IFN-γ^{Nil}]	IFN-γ concentration in QFT Nil tube
[IFN-γ^{TB}]	IFN-γ concentration in QFT TB antigen tube
[IFN-γ^{Mit}]	IFN-γ concentration in QFT Mitogen tube
[IP-10^{Nil}]	IP-10 concentration in QFT Nil tube
[IP-10^{TB}]	IP-10 concentration in QFT TB antigen tube
Ab	Antibody
AUC	Area under the curve
BB	Blocking buffer
BD	Becton Dickinson
BLAST	Basic Local Alignment Search Tool
bTB	Bovine tuberculosis
CA	California
cAb	Capture antibody
CCL8	Chemokine (C-C motif) ligand 8 (Protein: MCP2)
cDNA	Complementary deoxyribonucleic acid
CFP-10	10 kDa culture filtrate antigen
CI	Confidence interval
CMI	Cell-mediated immunity
Cq	Quantification cycle
CSF3	Colony stimulating factor 3
CXCL10	Chemokine (C-X-C motif) ligand 10

CV	Coefficient of variation
dAb	Detection antibody
DAFF	Department of Agriculture, Forestry and Fisheries
DALRRD	Department of Agriculture, Land Reform, and Rural Development
DC	Dendritic cells
DE	Delaware
DM	Derivative melt curve
DNA	Deoxyribonucleic acid
DPP® TB	Dual-path platform technology for tuberculosis
ESAT-6	6 kDa early secretory antigenic target
ELISA	Enzyme-linked immunoassay
ELISpot	Enzyme-linked immunospot
FPA	Fluorescence polarization assay
GA	Georgia
gDNA	Genomic deoxyribonucleic acid
GEA	Gene expression assay
h	Hours
HiP	Hluhluwe-iMfolozi Park
HRP	Streptavidin-horseradish peroxidase
IA	Iowa
IgG	Immunoglobulin G
IGRA	Interferon gamma release assay
IL	Interleukin

IL-23A	Interleukin 23 subunit alpha
IL-1β	Interleukin-1 beta
IFN-γ	Interferon gamma (Gene: *IFNG*)
IP-10	Interferon gamma induced protein 10
IPRA	IP-10 release assay
IUCN	International Union for Conservation of Nature Red List
IQR	Inter quartile range
kDa	Kilodalton
KNP	Kruger National Park
LOD	Limit of detection
LOQ	Limit of quantification
MA	Massachusetts
M. avium	*Mycobacterium avium*
MAPIA	Multi-antigen print immunoassay
M. bovis	*Mycobacterium bovis*
MGIT	Mycobacterial growth indicator tubes
MI	Michigan
MIG	Monokine induced by gamma interferon
	Chemokine (C-X-C motif) ligand 9 (Gene: *CXCL9*)
MIP-1β	Macrophage inflammatory protein
	Chemokine (C-C motif) ligand 4 (Gene: *CCL4*)
MN	Minnesota
MO	Missouri

MPB83	Major secreted immunogenic protein 83 (Gene: *Rv2873*)
mRNA	Messenger ribonucleic acid
MTBC	*Mycobacterium tuberculosis* complex
µg	Microgram
µl	Microliter
ml	Millilitre
N	Negative result
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
NJ	New Jersey
NK	Natural killer
NO	Nitric oxide
OD	Optical density
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
P	Positive result
PCR	Polymerase chain reaction
Pg	Picogram
PPD	Purified protein derivative
PWM	Pokeweed mitogen
QFT	QuantiFERON-TB Gold In-Tube
R^2	Correlation coefficient
RD	Regions of difference

rDNA	Recombinant deoxyribonucleic acid
RFLP	Restriction fragment length polymorphism
rIFN-γ	Recombinant interferon gamma
rIP-10	Recombinant interferon gamma induced protein 10
RNA	Ribonucleic acid
ROC	Receiver operator characteristics curve
RT	Room temperature
RT-qPCR	Real time quantitative polymerase chain reaction
SD	Standard deviation
Se	Sensitivity
Sp	Specificity
SU	Stellenbosch University
TB	Tuberculosis
TB Ag	Tuberculosis Antigen
T°C	Annealing temperature
Th1	T helper type-1
Th2	T helper type-2
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha (Gene: *TNFA*)
TST	Tuberculin skin test
TX	Texas
USA	United States of America
WB	Whole blood

WGS	Whole genome sequences
YWHAZ	Tyrosine-3-mono-oxygenase/tryptophan-5-monooxygenase activation protein zeta
ZN	Ziehl–Neelsen
VNTR	Variable-number tandem repeat typing

Chapter 1

General Introduction

This introductory chapter aims to provide a literature summary of the hosts, pathogen, and the diagnosis of *Mycobacterium bovis* (*M. bovis*) infection. Furthermore, it will highlight the justification for developing novel diagnostic tools to detect *M. bovis* infection in African rhinoceros, as well as setting the research questions and study aims, and describing the justification of the study.

Black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros

Rhinoceros species belong to a taxonomic order known as perissodactyla (odd-toed ungulate) (Murphy et al., 2001; Steiner and Ryder, 2011). The two extant African rhinoceros species, black and white rhinoceros, reside in sub-Saharan Africa (Standley and Emslie, 2013). According to the International Union for Conservation of Nature Red List (IUCN; http://www.iucnredlist.org/), the black rhinoceros is classified as 'critically endangered' while the white rhinoceros is 'near threatened'. In Africa, black rhinoceros populations are in decline, and white rhinoceros are following suit due to poaching for their horns and habitat loss (Standley and Emslie, 2013).

Rhinoceros are the second largest herbivores after the elephant and play an influential role in consuming large amounts of vegetation, which helps shape the African landscape. This benefits other animals and maintains ecosystem balance which may be disturbed if their numbers are affected (Waldram et al., 2008; Ripple et al., 2015). Additionally, they are also considered as ecosystem engineers by creating biological barriers when grass is grazed short, potentially altering the size, frequency, and distribution of fires in the landscape (Jones et al., 1994). Furthermore, rhinoceros species are iconic with tangible economic benefits through tourism at zoos and nature reserves, and wildlife ranching (Taylor et al., 2016). In order to promote conservation of natural resources and endangered wildlife, rhinoceros are translocated nationally and internationally as part of a sustainable utilization strategy (Taylor et al., 2016). While the illegal hunting of rhinoceros continues to impact their chances of survival, their susceptibility to infectious diseases such as bovine tuberculosis (bTB) may be an additional threat to their existence (Michel et al., 2017; Miller et al., 2017a).

Bovine tuberculosis

Tuberculosis (TB) may result from infection by bacteria belonging to the *Mycobacterium tuberculosis* complex (MTBC), a group of closely related multi-host pathogens of public health and veterinary importance, including *Mycobacterium tuberculosis* (*M. tb*) and *M. bovis* (Alexander et al., 2010). Members of the MTBC are slow growing, acid-fast bacilli (Ashford et al., 2001).

Bovine tuberculosis is a disease, caused by *M. bovis* infection, with a wide host range, which includes both captive and free-ranging wildlife (Morris et al., 1994; Alexander et al., 2002; Katale et al., 2017). Wildlife populations known to be endemically infected, including the African buffalo (*Syncerus caffer*) in South Africa, badger (*Meles meles*) in the United Kingdom, brushtail possum (*Trichosurus vulpecula*) in New Zealand, act as maintenance hosts, and allow transmission among individuals, as well as spill-over to other species (Alexander et al., 2002; Renwick et al., 2006).

Transmission of *M. bovis* occurs via different routes, depending on host species and infection burden in the population. Inhalation of *M. bovis* aerosols are the common route of transmission when there is prolonged contact between infected and healthy domestic, wild animals, or humans (Ashford et al., 2001; Michel et al., 2009). Close contact between animals usually occurs, for example, at points such as water sources and feeding sites (Renwick et al., 2007). In hot weather, animals tend to congregate under trees for parts of the day, or share wallows (Renwick et al., 2007). It has been demonstrated that infectious animals may shed *M. bovis* in several ways, through faeces, discharging lesions, urine, and saliva (Neill et al., 1991). Another route of pathogen spread may occur when *M. bovis* is ingested or inhaled from

contaminated material such as milk, pasture, or water (Ayele et al., 2004). Furthermore, consumption of infected tissues by a susceptible animal can result in transmission from prey to predators, especially carnivores (Renwick et al., 2007). Irrespective of the precise route of *M. bovis* spread, the period from infection to progression of chronic disease can last months to years, and leads to granulomatous inflammation in the lungs, lymph nodes and other organs (Keet et al., 2000; Ashford et al., 2001; Michel et al., 2010).

Impacts of TB in domestic livestock and wildlife include mortality, zoonotic transmission, loss in production, spread to other species, and potential for maintenance in the ecosystem (Michel, 2002). Furthermore, in South Africa, premises with infected animals are placed under veterinary quarantine with movement restrictions which affects livestock production system (Arnot and Michel, 2020) and wildlife conservation (pers. comm. Dr Peter Buss, 2016).

Bovine tuberculosis in South African wildlife

Bovine tuberculosis was introduced into South Africa during the colonial era, with immigration of European settlers and their livestock (Smith et al., 2011). This was confirmed by isolation of European 1 clonal complex of *M. bovis* in South Africa in cattle in 1880 (Renwick et al., 2007; Michel et al., 2008). Bovine TB was first recognized in Hluhluwe-iMfolozi Park (HiP) in 1986 and the Kruger National Park (KNP) in 1990 when *M. bovis* was isolated from buffaloes (Bengis et al., 2000). Since that time, African buffaloes, and greater kudu (*Tragelaphus strepsiceros*) have become maintenance hosts for bTB in South Africa (de Vos et al., 2001; Rodwell et al., 2001). Both KNP and HiP are considered endemic areas for *M. bovis* and there is an increasing number of African wildlife species reported with bTB (Keet, 2000; Michel, 2012). In South Africa, more than 20 wildlife species have been confirmed with TB to date (Keet et al.,

1996; Bengis et al., 2001; Michel et al., 2006; Buss, 2015; Olivier et al., 2015; Miller et al., 2017a; Miller et al., 2018; Roos et al., 2019; Higgitt et al., 2017; Higgitt et al., 2019; Hlokwe et al., 2019; Miller et al., 2019; Kerr et al., 2020). However, not all species are equally affected, but are often grouped as either spill-over (dead-end) hosts or maintenance hosts (Ayele et al., 2004). In South Africa, cattle and African buffaloes are considered the primary and most well-known reservoirs or maintenance hosts. Therefore, transmission of *M. bovis* infection from these maintenance hosts to other wildlife species or livestock is inevitable if not controlled, as well as potential spill-back (de Garine-Wichatitsky et al., 2013).

A bTB test-and-slaughter control strategy was implemented in South Africa in 1969 for cattle, which led to decreased prevalence (Hlokwe et al., 2014); however, lack of testing programs for communal livestock and paucity of diagnostic tests for wildlife species has allowed persistence of bTB in these populations (Michel et al., 2006). The interface between wildlife and livestock, especially in communities bordering bTB endemic parks, provides an opportunity for ongoing spread of *M. bovis* (Hlokwe et al., 2014). Spill-over and spill-back events between species continue to pose a significant risk unless bTB can be controlled within the wildlife maintenance hosts. Similar scenarios have been reported from Spain where a link was found between reinfection of livestock and infected badgers (Acevedo et al., 2019). In addition, the absence of bTB control in cattle results in potential ongoing spill-over to wildlife (Arnot et al., 2020). In order to manage and control bTB in South Africa, there is a need to understand the epidemiology of bTB in multi-host systems, which requires the development of tools to identify *M. bovis* infection and disease in a range of animal hosts.

Tuberculosis in rhinoceros

Tuberculosis has been reported in different rhinoceros species around the world. Infections with *M. tb* and *M. bovis* have resulted in morbidity and mortality in captive rhinoceros species (Mann, 1981; Barbiers, 1994; Stetter et al., 1995; Valandikar and Raju, 1996; Espie et al., 2009). Many of the historical *M. tb* cases have occurred in captive black rhinoceros, with only a few cases in white rhinoceros (Miller et al., 2016). This may be due to differences in housing or management of these species in zoological collections, or other risk factors such as their level of exposure to infected animals or people (Miller et al., 2016). Since the TB cases in zoo rhinoceros resulted in pulmonary disease, it is speculated that the route of transmission was through aerosolized mycobacteria (Oh et al., 2002).

In contrast, fewer cases of *M. bovis* infections are reported in rhinoceros. In South Africa, a case of mycobacteriosis was identified in a black rhinoceros in 1956 (Hofmeyr, 1956), and in another case in 1970, a black rhinoceros in HiP, which were presumptively diagnosed based on lesions consistent with TB (Keep and Basson, 1973). The first *M. bovis* culture-confirmed case occurred in a geriatric semi-captive black rhinoceros that had localised lesions (Espie et al., 2009). Yet it was only in 2016 that the first case of *M. bovis* was confirmed in a free-ranging black rhinoceros in KNP, followed by additional cases in white rhinoceros (Miller et al., 2017; Miller et al., 2018).

The route of *M. bovis* transmission in rhinoceros is unknown, however, ingestion of *M. bovis* through consumption of contaminated vegetation could be another route in grazing and browsing species (Ayele et al., 2004; Rewick et al., 2007). In South Africa, rhinoceros share range and resources with buffalo and greater kudu populations, which are important maintenance hosts for bTB (Michel et al., 2006; de Garine-Wichatitsky et al., 2010). Typical post-mortem

findings in infected rhinoceros are granulomatous lesions, primarily in lungs and lymph nodes (Song, 2001; Espie et al., 2009; Miller et al., 2016). This may be due to the overlap in grazing/browsing and water sources, frequented by rhinoceroses, buffaloes and greater kudus, could pose a threat for potential disease transmission (Michel et al., 2017), suggestive that infection may spread through aerosol transmission. However, irrespective of the exact route of infection, it may take years for clinical signs to develop (Keet et al., 2000).

Clinical signs of TB are generally related to the route of infection and target organs involved, although reports in rhinoceros are limited to those few cases that have been described in the literature. Clinical signs that have been associated with progressive TB include coughing, sneezing, nasal discharge, respiratory discharge, fever, lethargy, decreased appetite, weight loss, and anemia (Valandikar and Raju, 1996; Song, 2001; Espie et al., 2009; Miller et al., 2015; Miller et al., 2017a). Since KNP is endemic for *M. bovis* and home to large African rhinoceros populations, the finding of bTB in rhinoceros emphasizes the importance of developing sensitive, specific and accurate diagnostic tools to better understand the pathogenesis, transmission of *M. bovis* infection, and its impact on African free-ranging rhinoceros populations.

The immunology of tuberculosis

Generally, cell-mediated immunity (CMI) plays an important role in the control of TB (Orme and Cooper, 1999). As part of anti-mycobacterial immunity, there is development of a T helper type 1 (Th1) CMI response, along with the production of cytokines. Among the key cytokines, interleukin (IL)-12, interferon gamma (IFN-γ), and tumour necrosis factor alpha (TNF-α) are important in activating macrophage microbiocidal pathways (Orme and Cooper, 1999; Whiteside, 2002). Interferon-gamma (IFN-γ) is produced by $CD4^+$ T-cells, as well as some

$CD8^+$ T-cells, and natural killer (NK) cells. It acts on B cells, T cells, NK cells, and macrophages (Vivier et al., 2008) to promote protection against mycobacterial infection (Cooper et al., 1993). The T helper type 2 (Th2-type) cytokines (e.g. IL-3, IL-4, IL-5, IL-13) are involved in promoting antibody-mediated responses (Whiteside, 2002). Previous studies have suggested a shift in predominance from CMI to humoral responses occurs as infection progresses (Ritacco et al., 1991; Boussiotis et al., 2000; Dlugovitzky et al., 2000; Pollock et al., 2001; Welsh et al., 2005). Therefore, measurement of these different antigen-specific responses may provide indirect methods of diagnosing infection and disease.

Diagnosis of bovine tuberculosis

Bovine tuberculosis is diagnosed by either detecting the *M. bovis* organism or DNA (direct tests) or host immune responses to mycobacterial antigens (indirect tests). Direct diagnostic assays include identification of acid-fast bacilli in clinical samples, or mycobacterial culture and speciation by polymerase chain reaction (PCR) (Maas et al., 2013). Microscopic examination using acid-fast staining of respiratory secretions or tissues is a simple, inexpensive method and can provide a presumptive diagnosis (Uddin et al., 2013). However, this technique is non-specific and highly insensitive with less than 60% sensitivity under optimal conditions compared to culture (Apers et al., 2003). Mycobacterial culture isolation is still considered the "gold standard" for bTB diagnosis, however, the long processing time is a major limitation of this method (Almeida and Araujo, 2013). Due to the characteristic slow growth of mycobacteria in culture, the BACTEC™ MGIT™ (Becton Dickinson, Franklin Lakes, NJ, USA) was developed as an improved culture tool which provides faster and more accurate results than traditional solid culture methods (Robbe-Austerman et al., 2013). This automated mycobacterial culture system has been successfully used to culture MTBC organisms from post-mortem tissue

samples as well as ante-mortem bronchoalveolar lavage, trunk wash fluid and oropharyngeal swab samples from African wildlife (Miller et al., 2015; Bernitz et al., 2018; Miller et al., 2018; Roos et al., 2018; Higgitt et al., 2019). Molecular techniques, such as PCR, play an important role in identifying and differentiating *M. bovis* isolates from the other members of the MTBC that have been cultured or observed directly in samples (Ayele et al., 2004; Goosen et al., 2020). One of the most commonly used techniques is based on presence and absence of Regions of Difference (RD) in MTBC using PCR (Warren et al., 2006). In addition, a recent study demonstrated the use of the VetMAX MTBC PCR kit (ThermoFisher Scientific, MA, USA) as a new tool for the detection of *M. bovis* in various sample types from African wildlife species (Goosen et al., 2020). Furthermore, genotyping techniques to further identify strains, such as variable-number tandem repeat typing (VNTR), restriction fragment length polymorphism (RFLP) typing, spoligotyping, next generation sequencing (NGS) and whole genome sequences (WGS), provide additional data useful for disease epidemiologic investigations and identifying potential sources of *M. bovis* (Hlokwe et al., 2014; Musoke et al., 2015; Dippenaar et al., 2017).

Indirect detection of *M. bovis* depends on the development of bTB-specific immune responses. Cell-mediated immune responses develop soon after infection, after which, as the disease progresses, the humoral response is activated, and antibody titres increase (Pollock and Neill, 2002). Factors such as host species, immunological competence, initial infection dose, and stage of disease determine the presence and magnitude of CMI and humoral responses (Buddle et al., 2013). The tuberculin skin test (TST), which is an indirect in vivo detection method, has been a standard diagnostic test for bTB worldwide. The TST measures the delayed hypersensitivity response induced by purified protein derivatives (PPD) from *M. bovis* (and *M. avium*) injected intradermally (Pollock et al., 2005). The TST works well in some wildlife but is unreliable as a

diagnostic test in others (including rhinoceros), although it has been rarely validated and standardized for these species (Godfrey et al., 1990; Keet et al., 2010). Disadvantages of TST when applied in free-ranging wildlife include unknown standards for tuberculin doses and measurement interpretation, the requirement for recapture after three days to read the test (Chambers et al., 2009; Chambers, 2013), and influence of exposure to environmental mycobacteria on background responses (van Helden et al., 2008). Therefore, blood-based assays have been developed to avoid these limitations.

The IFN-γ release assay (IGRA) is an in vitro assay based on stimulation of whole blood or peripheral blood mononuclear cells (PBMC) with *M. bovis* antigens. Immune sensitization is detected by measuring release of antigen-specific IFN-γ by sandwich ELISA or enzyme-linked immunospot (ELISPOT) (Wood et al., 1990). These assays were initially developed with PPDs as antigens but had compromised specificity due to antigenic cross-reactivity (Lightbody et al., 1998). For cattle, the specificity was improved using more defined antigens (MPB83 and MPB70) (Buddle et al., 2001). The QuantiFERON-TB Gold In-Tube (QFT) (Qiagen, Venlo, Netherlands) system was developed for human IGRA and employs a peptide cocktail simulating the antigens early secreted antigenic target 6 kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) (Theel et al., 2018). The use of the QFT blood collection tubes, in combination with an appropriate IFN-γ ELISA, has been advocated for field testing of African wildlife because it is easy to use, provides high diagnostic specificity, and can be adapted for use in multiple wildlife species (Parsons et al., 2011). Additionally, IGRAs remain the most widely used and recommended immunological tests for *M. tb* diagnosis in humans (Pai et al., 2014) and *M. bovis* in cattle (Bezos et al., 2014; Parsons et al., 2016; Palmer et al., 2020), and in some wildlife species such as African buffaloes (Bernitz et al., 2019), pigs (Pesciaroli et al., 2012), and wild

dogs (*Lycaon pictus*) (Higgitt et al., 2019). However, studies have demonstrated that IGRAs may be suboptimal for diagnosis in bovids (Vordermeier et al., 2000; Bernitz et al., 2020) and results in false negatives in advanced TB cases in humans (Pai et al., 2005; 2014). Therefore, identification of alternative or ancillary biomarkers of bTB infection to IFN-γ is necessary to increase the sensitivity of *M. bovis*-specific assays (Waters et al., 2003; Blanco et al., 2011; Bernitz et al., 2019). Several host biomarkers of CMI responses have emerged as potential candidates for use in either gene expression or protein blood-based assays in humans (Walzl et al., 2011; Chegou et al., 2018) and animal species, including interleukin-1β (IL-1β) (Jones et al., 2010; Elnaggar et al., 2017), interleukin-2 (IL-2) (Rhodes et al., 2014), chemokine C-X-C motif ligand 9 (CXCL9) also known as monokine induced by IFN-γ (MIG) (Olivier et al., 2017; Kerr et al., 2020), chemokine C-X-C motif ligand 10 (CXCL10) also known as IFN-γ-induced protein-10 (IP-10) (Waters et al., 2012; Bernitz et al., 2019; Palmer et al., 2020), tumour necrosis factor-alpha (TNF-α), nitric oxide (NO) (Durbach et al., 2003), interleukin-17 (IL-17) (Blanco et al., 2011), and interleukin-22 (IL-22) (Steinbach et al., 2016).

Serological tests are also used as indirect diagnostic assays for detection of host antibody responses to mycobacterial antigens (Wood et al., 1992). There are a variety of antibody detection assays, including the indirect ELISA (Parsons et al., 2017; Roos et al., 2016), fluorescence polarization assay (FPA) (Jolley et al., 2007), lateral flow chromatography such as the STAT-PAK assay (Miller et al., 2015; Kerr et al., 2019; Miller et al., 2019), dual-path platform (DPP) technology (Roos et al., 2016; Kerr et al., 2019; Miller et al., 2019), and blotting methods like the multi-antigen print immunoassay (MAPIA) (Waters et al., 2011). Serodiagnosis has been useful as a screening tool for detecting *M. bovis* infection in some domestic and wildlife species, such as goats (Bezos et al., 2018), sheep (Muñoz-Mendoza et al., 2016), cattle (Casal et

al., 2017), warthogs (Roos et al., 2016), wild boar (Aurtenetxe et al., 2008), rhinoceros (Duncan et al., 2009), and elephants (Kerr et al., 2019; Miller et al., 2019). However, antibody production changes significantly during infection and is typically produced in advanced stages of infection (Pollock and Neill, 2002). Thus, tests based on humoral responses are generally not as sensitive as those based on the CMI response in many species, specifically during early stages of infection (Wood et al., 1992; Lightbody et al., 1998; Wiker et al., 1998; McNair et al., 2001). However, early antibody responses have been reported in some animal species such as experimentally infected cattle (Waters et al., 2006), naturally infected wild suids (Boadella et al., 2011; Roos et al., 2016), and elephants (Lyashchenko et al., 2006; Greenwald et al., 2009). Therefore, diagnostic tests need to be tailored for each species, based on knowledge of the patterns of specific host responses.

The development of improved and accurate diagnostic immunoassay tests for bTB also requires knowledge of the factors which impact host responses. Factors which influence test performance and selection of tests include the stage of infection, occurrence of disease, and species-specific inconsistency in immune responses (De La Rua-Domenech et al., 2006). It is therefore necessary to understand specific immune responses to bTB in each wildlife species.

Diagnostic tests for mycobacterial infection in African rhinoceros

Since clinical signs are vague and may only be noticed in advanced disease, a diagnostic tool to detect early infection is needed for rhinoceroses. Of the diagnostic methods available to diagnose TB in animals, mycobacterial culture has been the most used method to diagnose infection in rhinoceros (Espie et al., 2009; Miller et al., 2017a). Samples for culture are typically only obtained at post-mortem examination, although techniques for obtaining culture samples ante-mortem have been developed and include tracheal-bronchial or gastric lavage and nasal

swabs (Espie et al., 2009; Goosen et al., 2020; Hermes et al., 2018; Michel et al., 2017; Stetter et al., 1995). However, these sample types are only useful in animals that are shedding, resulting in a test that has low sensitivity (Michel et al. 2017; Miller et al., 2015). Furthermore, results from a recent study of three white rhinoceros experimentally infected with a virulent *M. bovis* showed no evidence of clinical disease, but the presence of minor and small tuberculosis lesions in the lung post mortem suggests a risk of inactive tuberculosis (Michel et al., 2017). Regardless of the low incidence of tuberculosis in free ranging rhinoceros, the possible intra and inter species transmission of *M. bovis* or in a captive setting demands for diagnostic tests (Lécu and Ball, 2011).

Diagnostic tests for TB based on host responses in rhinoceros are limited. The use of TST in species such as rhinoceroses, has not been standardized and has a high chance of false positive test results due to exposure to environmental mycobacteria (Godfrey et al., 1990; Espie et al., 2009). Therefore, the presence of serum antibodies to *M. bovis* and *M. tb*, using ELISA, multi-antigen print immunoassay (MAPIA) and ElephantTB STAT-PAK® have previously been used in black and white rhinoceros for diagnosis (Duncan et al., 2009; Miller et al., 2017a; Parsons et al., 2017). These assays allow retrospective analyses as well as show potential value for monitoring response to treatment of TB in rhinoceros. For example, serum antibodies have been shown to gradually decline when infected rhinoceros were treated with anti-tubercular therapeutics (Miller et al., 2017a).

Since serological assays may not detect early infection in rhinoceros, blood-based CMI assays have been investigated. To develop cytokine assays, the white rhinoceros IFN-γ gene has been cloned and sequenced (Morar et al., 2007). Antibodies were successfully produced to the recombinant rhinoceros IFN-γ protein, and an in-house ELISA was developed using these

antibodies to detect IFN-γ in plasma from mitogen-stimulated rhinoceros whole blood (Morar et al., 2007; Morar et al., 2015). Although, this rhinoceros-specific IFN-γ ELISA was able to detect IFN-γ in plasma, the assay was not validated for antigen-specific responses. Recently, the QFT blood stimulation platform, in combination with an in-house ELISA using commercial bovine IFN-γ antibodies, has been able to detect antigen-specific IFN-γ production in *M. bovis*-infected black and white rhinoceros (Miller et al., 2017a; Miller et al., 2018). In addition, results from an experimental *M. bovis* infection study of three white rhinoceroses support the use of the QFT bovine IFN-γ ELISA compared to rhinoceros-specific IFN-γ release assay for detection of immune responses in this species (Parsons et al., 2017). Additionally, the experimental *M. bovis* infection study of white rhinoceroses characterised antigen-specific immune response patterns to *M. bovis* infection for a duration of 20 months and all three rhinoceros reflected an effective control of *M. bovis* infection, suggesting the usefulness of IFN-γ to detect infection (Parsons et al., 2017). However, further studies investigating immune response patterns in naturally infected rhinoceros are required.

There is a lack of tests for diagnosing bTB in free-ranging wildlife species including African rhinoceros, which limits our understanding of the role and management of the disease in these species. Ante-mortem techniques are especially important for wildlife species which are indigenous, protected, individually valuable, and when post-mortem samples are unavailable (de Lisle et al., 2002; Morar et al., 2007). Therefore, further research is necessary to validate a QFT IFN-γ release assay (IGRA) and assess test performance in free-ranging African rhinoceros. Additionally, there is a need to identify and characterize other novel immunological cytokine biomarkers to improve diagnosis of *M. bovis* infection in rhinoceros.

Justification for study

The discovery of *M. bovis* infection in a free-ranging black rhinoceros in KNP along with additional sporadic cases of bTB (in black and white rhinoceros) in South Africa, highlights the threat that this infectious disease may have in disrupting conservation programs by restricting translocations (Espie et al., 2009; Miller et al., 2017b; Miller et al., 2018). The Department of Agriculture, Land Reform, and Rural Development (DALRRD) has already imposed a quarantine that prohibits movement of rhinoceros from KNP, since the discovery of bTB cases in 2016 (Dr. Peter Buss, pers. comm.). This has highlighted the large knowledge gaps regarding *M. bovis* transmission, susceptibility to infection, risk factors, progression to disease, prevalence in different rhinoceros species and populations, and impact on rhinoceros ecology and conservation. Understanding these aspects of bTB in rhinoceros requires reliable tools to diagnose individuals and screen populations. This study focuses on developing those tools.

Study research questions and aims:

1. What are the optimal ELISA conditions for detecting African rhinoceros IFN-γ?

Aims:

i) To select an optimal antibody pair to develop a rhinoceros IFN-γ ELISA.

ii) To characterize and validate a rhinoceros IFN-γ ELISA.

2. Can a diagnostic IGRA be developed to detect *M. bovis* infection in rhinoceros?

Aims:

i) To measure IFN-γ in QFT-stimulated whole blood from *M. bovis*-infected and uninfected rhinoceros using the validated IFN-γ ELISA.

ii) To determine if the QFT IGRA can distinguish between *M. bovis*-infected and uninfected rhinoceros.

iii) To determine a diagnostic cut-off value and test performance of an optimized QFT IGRA for use in rhinoceros.

3. Can cytokine gene expression assays be used to distinguish between *M. bovis*-infected and uninfected African rhinoceros?

Aims:

i) To screen cytokines and chemokines candidate genes for use as biomarkers of immune activation in rhinoceros QFT-stimulated whole blood.

ii) To optimize rhinoceros-specific qPCR assays for selected chemokine genes.

iii) To identify gene signatures to distinguish between *M. bovis*-infected and uninfected rhinoceros.

4. Can release assays for cytokines other than IFN-γ be used to differentiate *M. bovis*-infected and uninfected African rhinoceros?

Aims:

i) To detect selected cytokines (according to the outcomes of research question 3) in rhinoceros mitogen and antigen stimulated whole blood using selected ELISAs.

ii) To optimize selected cytokine ELISAs.

iii) To compare antigen-specific cytokine release in *M. bovis*-infected and uninfected rhinoceros to identify biomarkers with diagnostic potential.

Ethics and Other Permits

Permission for this research was acquired from the South African Department of Environment, Forestry and Fisheries according to Section 20 of the Animal Diseases Act, 1984

(Act No. 35 of 1984). In addition, ethical approval for this study was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2018-0966).

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Chapter 2

A commercial enzyme-linked immunosorbent assay for detection of interferon-gamma in white rhinoceros (*Ceratotherium simum*)

Josephine Chileshe[1], Wynand J. Goosen[1], Peter E. Buss[2], Paul D. van Helden[1], Robin Warren[1], Sven D. C. Parsons[1], Michele A. Miller[1]

[1]Department of Science and Technology/National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000, South Africa

[2]Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa

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Abstract

Kruger National Park (KNP), South Africa, home to the largest population of white rhinoceros (*Ceratotherium simum*) in the world, where bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*) is endemic. In 2016, the first cases of naturally occurring bTB were reported in white rhinoceros; however, there is a lack of understanding of infection and disease process in this species. Prevention and control of transmission depends on the availability of accurate tools to detect *M. bovis* infection. Interferon gamma (IFN-γ) assays are a reliable diagnostic method for TB in other animal species, and studies have indicated that these tests can be used in white rhinoceros. In this study, we sought to screen and optimize commercially available IFN-γ enzyme linked immunosorbent assay (ELISA) systems to detect endogenous white rhinoceros IFN-γ in mitogen-stimulated whole blood as a basis for developing a diagnostic test for *M. bovis* infection. Optimizations included identifying ELISA antibodies, determining the effect of sample matrix, ELISA linearity, effects of ELISA plate incubation temperature, assay reproducibility, and the assay's limit of quantification. The optimized assay employed an equine IFN-γ antibody pair that was used to create a commercially available ELISA kit. This ELISA showed a linear response to recombinant equine and endogenous rhinoceros IFN-γ (ranging from 7.8 to 125 pg/ml). When incubated at 37˚C, the ELISA was highly reproducible, with an optimal recovery and a low limit of quantification, indicating that the Mabtech Equine IFN-γ ELISA^{PRO} kit is a robust assay for measuring white rhinoceros IFN-γ.

Introduction

The white rhinoceros (*Ceratotherium simum*) is classified as "Near Threatened" by the International Union for Conservation of Nature and is under high poaching pressure in a number of African countries (Emslie, 2012; Miller et al., 2018). In South Africa, the majority of white rhinoceros are found in Kruger National Park (KNP), a bovine tuberculosis (bTB) endemic area rife with poaching. Even though *Mycobacterium bovis* infection has been reported in a wide range of wildlife, it was only in 2016 that the first cases were found in wild white rhinoceros (Miller et al., 2017). In order to understand *M. bovis* infection and disease processes, it is crucial to recognize the role of the host's immunological response. An effective immune response against *M. bovis* is dependent on T helper type-1 (Th1) cell-mediated immunity (CMI) (de la Rua-Domenech et al., 2006). Interferon gamma (IFN-γ) is a key cytokine in this response and has been shown to be an important diagnostic biomarker for the diagnosis of mycobacterial infections in domestic cattle, wildlife and humans (de la Rua-Domenech et al., 2006; Gormley et al., 2006). However, immune responses are not well characterized in most wildlife species such as rhinoceros. Therefore, understanding the comparative immunobiology of *M. bovis* infection requires the development of assays to detect and measure immune responses (Maas et al., 2013).

The white rhinoceros IFN-γ gene has been cloned and expressed, with the recombinant protein used for the production of rhinoceros IFN-γ specific antibodies (Morar et al., 2007). The inferred IFN-γ amino acid sequence was shown to have 90% homology to that of equines (Morar et al., 2007). Using rhinoceros-specific and commercial bovine IFN-γ antibodies in ELISAs, a previous study demonstrated that antigen-specific IFN-γ production is a promising immunological technique for the detection of *M. bovis* infection in white rhinoceros (Parsons et al., 2017). Notably, the bovine-specific IFN-γ antibody pair used in this study was cross-reactive

with equine IFN-γ and could detect endogenous white rhinoceros IFN-γ (Parsons et al., 2017). The findings in this study suggest that commercially available reagents may be utilized for developing immunoassays in wildlife (Parsons et al., 2017). Therefore, the aim of this study was to screen and optimize a commercially available IFN-γ ELISA system to detect and measure endogenous white rhinoceros IFN-γ in mitogen-stimulated whole blood. The optimized assay could then be further evaluated as a potential diagnostic test for *M. bovis* infection.

Materials and Methods

Animals

Blood samples were collected opportunistically from immobilized white rhinoceros in KNP, South Africa, during routine management procedures or for other approved activities according to the standard operating procedures for the capture, transportation and maintenance in holding facilities of wildlife (South African National Parks). Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (SU-0966), and a section 20 research permit was issued by the Department of Agriculture, Forestry and Fisheries (DAFF) (No. 12/11/1/7/2).

Whole blood stimulation

Rhinoceros whole blood was collected in sealed lithium heparin vacutainers (BD Biosciences, Franklin Lakes, NJ, USA) and for each animal, one ml aliquots were transferred to two empty serum vacutainer tubes with gas permeable caps. Pokeweed mitogen (PWM, Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline, pH 7.4 (PBS, Thermo Fisher Scientific, Waltham, MA, USA) was added to one tube at a final assay concentration of 10 µg/ml and 10 µl sterile PBS to the other tube. The tubes were designated as PWM and Nil, respectively, and incubated for 24 hours at 37°C in 5% CO_2. Thereafter, blood was transferred to 2 ml

microcentrifuge tubes and plasma was harvested following centrifugation at 2000 x g for five minutes. Plasma samples derived from mitogen-stimulated and unstimulated whole blood were screened using bovine antibodies as previously described (Parsons et al., 2017) and five samples with high IFN-γ concentrations (compared to nil concentrations for each animal) were selected and pooled to create a reference sample with sufficient volume for repeated ELISAs. Plasma samples were then stored at -80°C until analyzed.

Screening of anti-IFN-γ antibodies

Commercially available ELISA antibody pairs were selected as potential candidates for the detection of rhinoceros IFN-γ (Table 2.1). Capture antibodies were diluted to 2 µg/ml in 1x PBS (Thermo Fisher Scientific). A 96-well microtiter plate (Greiner Bio-one, Heidelberg, Germany) was coated by adding 100 µl/well of diluted capture antibody and incubating the plate overnight at 4°C. The plate was washed four times (300 µl/well) with wash buffer solution (PBS with 0.05% Tween 20; Sigma Aldrich). Thereafter, 200 µl blocking buffer (BB, wash solution with 0.1% bovine serum albumin; Roche, Basel, Switzerland), was added to each well and the plate incubated at room temperature (RT, 19°C on the day of analysis) for one hour. After washing the plate four times, the pooled PWM plasma was diluted 1:2 in BB and 100 µl added to each well in duplicate. The plate was covered, incubated at RT for two hours then washed four times. Detection antibodies were diluted to 1 µg/ml in BB, 100 µl added to each well and incubated at RT for one hour. Following incubation, the plate was washed four times and 100 µl/well of streptavidin-horseradish peroxidase (HRP, R&D Systems, Minneapolis, MN, USA), diluted 1:200 in BB, was added and incubated at RT for one hour. The plate was washed as above and 100 µl of colorimetric 3,3',5,5'-tetramethylbenzidine (TMB) enzyme substrate (BD Biosciences) was added to each well and incubated at RT in the dark for 20 minutes. The

reaction was stopped by adding 100 µl of 2M H_2SO_4 solution to each well. The optical density (OD) of each test and control well was measured at 450 nm and 630 nm as reference wavelength, using a VersaMax™ ELISA Microplate reader with SoftMax® Pro Software (Molecular Devices, San Jose, CA, USA). The ELISA results were calculated as the OD value measured at 630 nm subtracted from that measured at 450 nm; negative assay controls were used to ensure that the test well signal was specific to the PWM plasma sample and normalize OD values. Those ELISAs with a detectable PWM signal (based on mean OD of test wells) were selected for further analysis.

Table 2.1 Commercial interferon gamma (IFN-γ) enzyme linked immunosorbent assay kits and development kits screened for the detection of endogenous rhinoceros IFN-γ.

Analyte	Antibody Type	Plasma dilution	Manufacturer	Product Information
Development ELISAs				
Bovine IFN-γ	MT17.1[a], MT30[b]	1:2	Mabtech Ab, Nacka Strand, Sweden	3119-1H-20
Bovine IFN- γ	bIFN-γ-1[a], PAN-biotin[b]	1:2	Mabtech	3115-1H-20
Canine IFN-γ	MT13[a], MT166-biotin[b]	1:2	Mabtech	3113-1H-6
Equine IFN-γ	MT166[a], MT13-biotin[b]	1:2	Mabtech	3117-1H-6 (Batch 11)
Ferret IFN-γ	MTF14[a], MTF19-biotin[b]	1:2	Mabtech	3112-1H-6
Porcine IFN-γ	pIFN-γ-1 [a], p2CII [b]	1:2	Mabtech	3130-1H-20
Precoated ELISA kits				
Equine IFN-γ	N/A	1:2	Sigma-Aldrich, St. Louis, MO, USA	RAB0583-1KT
Equine IFN-γ	N/A	1:2	RayBiotech, Peachtree Corners, GA, USA	MBS109347
Rhinoceros IFN-γ	N/A	Undiluted	MyBioSource, San Diego, CA, USA	ELE-IFNg

a = Capture antibody; b = Detection antibody; N/A = not applicable.

Selection of an IFN-γ ELISA

The pooled PWM rhinoceros plasma was diluted 1:8 in BB and assayed in duplicate using the Mabtech bovine IFN-γ Ab pair (kit 3115) and Mabtech equine IFN-γ Ab pair (kit 3117) as described above. Furthermore, these samples were assayed using three IFN-γ ELISA kits that included pre-coated plates and supplied reagents according to their manufacturer's instructions (Table 2.1). The ELISA displaying the greatest mean OD result for duplicate test wells was selected for further validation.

Validation of IFN-γ ELISA

A customized pre-coated equine IFN-γ ELISA^{PRO} kit (Mabtech, catalog number: 3117-1HP-10) utilizing the selected equine anti-IFN-γ antibody pair was used according to the manufacturer's instructions, except for the incubation temperature, as described below. A10 µl aliquot of recombinant equine IFN-γ (rIFN-γ) standard solution (500 000 pg/ml) was diluted in 5 ml sample diluent buffer (provided in the kit) to create a working solution of 1000 pg/ml. This was serially diluted 1:2 (2-fold dilutions) to produce a dilution series ranging from 1000 pg/ml to 7.8 pg/ml. The ELISA results were measured and calculated as above; the relationship between OD and IFN-γ concentration was described using linear regression analysis using a standard curve to determine rhinoceros IFN-γ concentrations, as previously described (Cox et al., 2012), using GraphPad Software, version 5 (GraphPad Software, March 2007, San Diego, CA, USA).

To characterize the assay performance at various incubation temperatures, the rIFN-γ dilution series described above was assayed in duplicate, with all incubations performed at either RT, 30°C or 37°C, except for the TMB substrate step, which was performed in the dark at RT. Results for each ELISA were analyzed by regression analysis as described above. Hereafter, all ELISA steps, except the TMB step, were performed at 37°C.

To determine the recovery of rIFN-γ in a rhinoceros plasma matrix, Nil plasma was utilized from three randomly selected rhinoceros. The concentrations of IFN-γ in the rhinoceros Nil samples were measured using the equine IFN-γ ELISA^{PRO} kit. For each animal, rIFN-γ was spiked into a reference sample consisting of 100% sample diluent, and two test samples, consisting of 50% plasma (1:2 dilution in sample diluent) and 25% plasma (1:4 dilution in sample diluent), respectively. Concentrations of rIFN-γ in each sample were calculated with reference to a standard curve as described above. The recovery (%) of spiked rIFN-γ in the test samples was calculated as: ([rIFN-γ] in test sample ÷ [rIFN-γ] in reference sample) x 100.

Assay linearity and parallelism were evaluated for IFN-γ ranging in concentration from 7.8 to 125 pg/ml. Pooled PWM plasma was diluted in sample diluent to obtain a plasma sample with approximately 125 pg/ml endogenous IFN-γ, as measured in the equine IFN- γ ELISA. The endogenous sample was serially diluted 1:2 in sample diluent to form a 6-point dilution series, and a replicate dilution series was performed starting with a1000 pg/ml solution of rIFN-γ. Duplicate samples were assayed, plasma IFN-γ concentrations were calculated as described above, and results for both dilution series were analyzed by regression analysis. The ELISA linearity was characterized as the correlation coefficient (R^2) value of the rIFN-γ regression. To determine the parallelism of the ELISA, regression slopes for the rIFN-γ and endogenous IFN-γ were compared using an F test (Graphpad Software, version 5).

Assay repeatability and reproducibility were determined using plasma from three rhinoceros. For each animal, PWM samples were diluted 1:5 in pooled Nil plasma and assayed in triplicate on the same ELISA plate, as above. This was repeated daily for three days. Intra-assay precision (within-run repeatability) was calculated as the coefficient of variation (CV) of the

results for the three replicates on day one. Inter-assay precision (between-run reproducibility) was calculated as the CV of the results of the three daily assays.

In order to determine the limit of detection (LOD) and limit of quantification (LOQ) of the ELISA, 24 replicates of sample diluent were analyzed on the same plate as a dilution series of rIFN-γ consisting of 7.8, 3.9, 2.0 and 1.0 pg/ml. For the 24 replicates, the mean OD value and standard deviation (SD) were calculated. The LOD (OD) was calculated as the mean value plus 3 x SD and the LOQ (OD) was calculated as the mean plus 10 x SD (Armbruster et al., 2008). The values of these parameters as a concentration of rIFN-γ were then extrapolated from the standard curve by regression analysis.

The final modified Mabtech Equine ELISA^{PRO} protocol was performed as follows. The pre-coated plate was washed five times with 1x wash solution provided in the kit. Hereafter, serially diluted (2-fold dilutions in sample diluent) equine IFN-γ recombinant standard and rhinoceros plasma samples were added to duplicate wells (100 µl/well), and the plate incubated at 37˚C for two hours. After incubation and five washes, equine detection antibody, prepared at a 1:500 dilution in sample diluent to a final concentration of 1 µg/ml, was added at 100 µl/well and the plate incubated at 37˚C for one hour. After five washes, 100 µl/well of streptavidin-HRP (1:1000 dilution) was added and incubated at 37˚C for one hour. After five washes, 100 µl of TMB enzyme substrate was added to each well and incubated in the dark at RT for 15 minutes. The reaction was stopped by addition of 100 µl/well stop solution (provided in kit). Results were determined as described above.

Results

Of the seven ELISA antibody pairs that were screened, the Mabtech bovine pair (kit 3115) and the Mabtech equine pair (kit 3117) resulted in a detectable signal when assaying

PWM-stimulated blood of white rhinoceros. Moreover, compared with the three precoated ELISA systems, the in-house equine IFN-γ antibody pair had the greatest signal for PWM plasma, and this was selected for further evaluation as a precoated ELISA (Fig. 2.1).

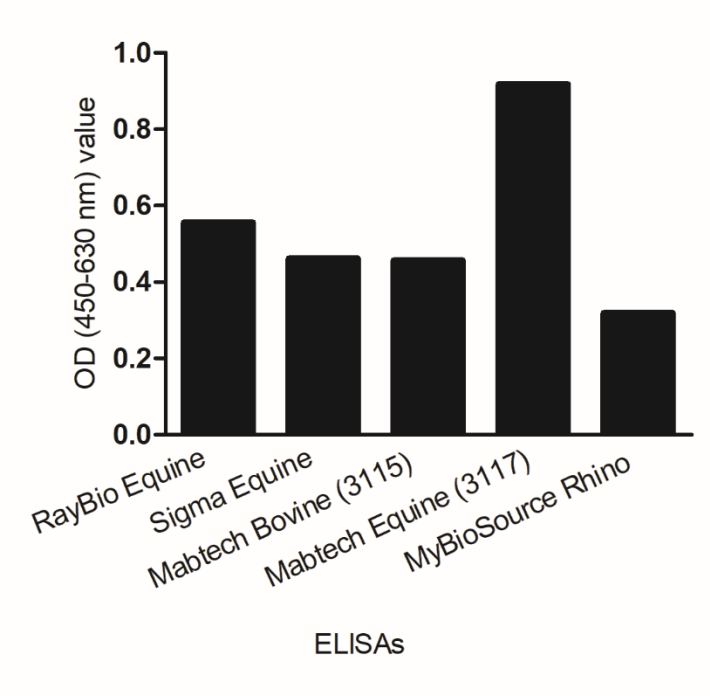

Figure 2.1 The comparative sensitivity of selected enzyme linked immunosorbent assays (ELISA) for rhinoceros IFN-γ. Whole blood from five rhinoceros was incubated overnight with pokeweed mitogen (10 ug/ml). Hereafter, pooled PWM rhinoceros' plasma was diluted 1:8 and assayed using selected IFN-γ ELISAs. The Mabtech equine ELISA displayed the greatest mean OD for this sample.

The Mabtech precoated equine ELISA^{PRO} kit displayed a linear response for rIFN-γ concentrations ranging from 7.8 pg/ml to 125 pg/ml ($R^2 > 0.99$), and this was consistent at incubation temperatures of RT (19°C), 30°C and 37°C (Fig. 2.2).

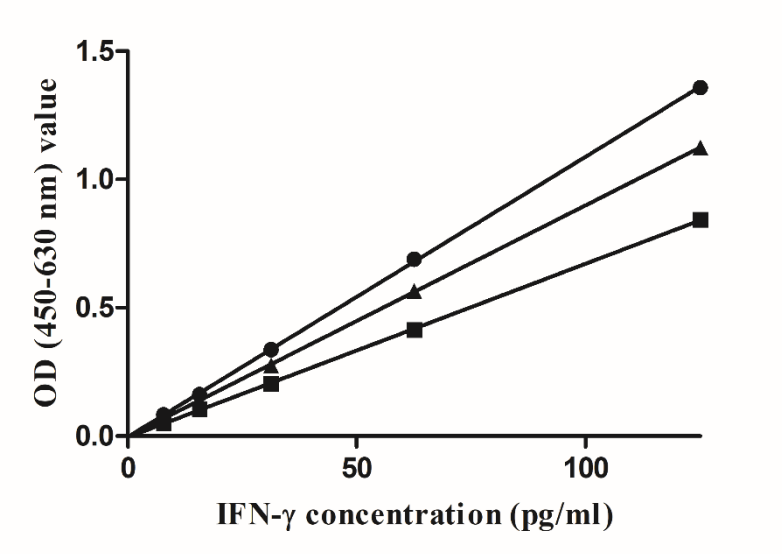

Figure 2.2 Regression analysis of a dilution series of recombinant equine IFN-γ, ranging from 7.8 to 125 pg/ml, and measured using the Mabtech Equine IFN-γ ELISA^{PRO} kit assay, at room temperature (■), 30˚C (●) and 37˚C (▲). At all temperatures, the assay displayed a linear response ($R^2 > 0.99$).

Hereafter, to facilitate reproducibility between laboratories, since most laboratories have incubators set at 37°C and because high ambient temperatures may be present in laboratories where rhinoceros occur, the ELISA was performed at this temperature. The individual IFN-γ concentrations of Nil plasma samples were less than 8 pg/ml (data not shown) and mean recovery of rIFN-γ in these rhinoceros' plasma was 93% in a 50% plasma sample and 88% in a 25% plasma sample (Table 2.2).

Table 2.2 Recovery of recombinant equine interferon-gamma (IFN-γ) in three rhinoceros plasma matrices using the Mabtech Equine IFN-γ ELISA^{PRO} kit assay.

Animal number	**Sample recovery**		
	50% plasma	**25% plasma**	**SD**
Animal 1	81	77	2.7
Animal 2	96	94	0.8
Animal 3	102	93	5.9
Mean recovery %	93	88	3.5

SD, standard deviation.

Subsequently, rhinoceros plasma was assayed at a 1:2 dilution in kit sample diluent. The linear response of the ELISA for rIFN-γ was not significantly different from that of endogenous rhinoceros IFN-γ (R^2 >0.99; F = 0.12; p > 0.5; Fig. 2.3).

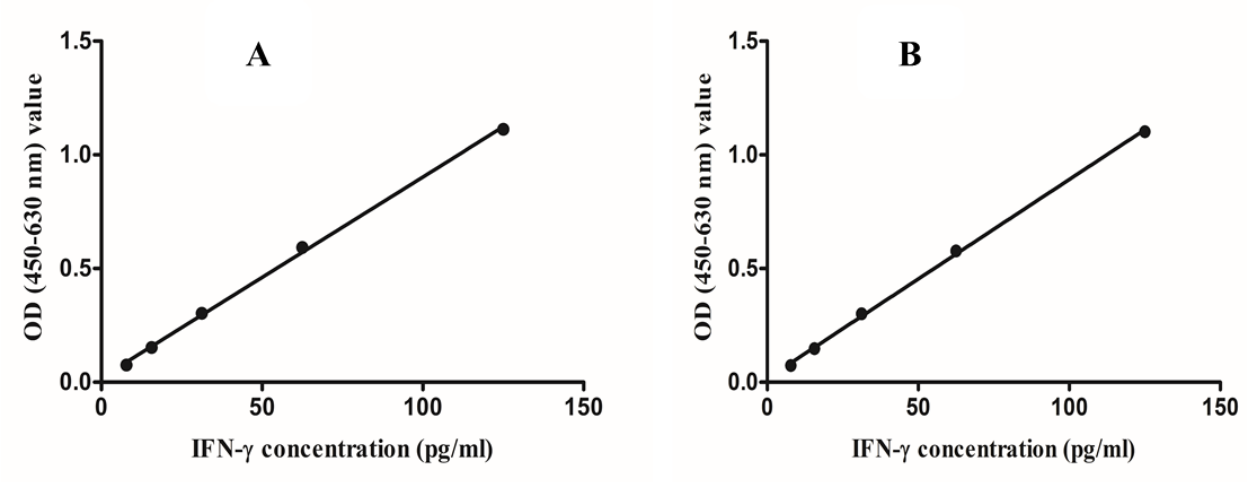

Figure 2.3 Regression analysis of dilution series of recombinant equine IFN-γ (A) and rhinoceros IFN-γ in plasma (B), ranging from 7.8 to 125 pg/ml, and measured using the Mabtech Equine IFN-γ ELISA^{PRO} kit assay. Both samples displayed linear responses ($R^2 > 0.99$) with no significant difference between lines ($F= 0.12$; $p > 0.5$).

Using three different rhinoceros samples, intra-assay precision ranged from 0.4% to 2.8% and inter-assay precision ranged from 3.4% to 6.4% (Table 2.3). The LOD and LOQ were calculated as 1.5 and 5.4 pg/ml, respectively. However, because these extrapolated values were regarded as imprecise, the nearest empirical values, i.e. 2 pg/ml and 7.8 pg/ml, were accepted as the LOD and LOQ, respectively.

Table 2.3 Intra-assay and inter-assay precisions of pokeweed mitogen-stimulated whole blood from three rhinoceros diluted in pooled Nil plasma assayed in triplicate for three days using the Mabtech Equine IFN-γ ELISA^{PRO} kit assay.

Animal number	**Intra-assay precision**			**Inter-assay precision**		
	Mean pg/ml	SD	CV	Mean pg/ml	SD	CV
Animal 1	111.0	1.7	1.6	104.0	6.2	5.9
Animal 2	105.0	2.6	2.8	102.0	3.5	3.4
Animal 3	112.0	0.6	0.4	104.0	6.7	6.4

CV, coefficient of variation; SD, standard deviation.

Discussion

In this study, the Mabtech Equine IFN-γ ELISA^{PRO} kit was selected as the optimal system for the measurement of white rhinoceros endogenous IFN-γ. When incubation steps were performed at 37°C, the ELISA displayed good recovery of IFN-γ in a rhinoceros plasma matrix, a linear response to both recombinant equine IFN-γ and endogenous rhinoceros IFN-γ, and high reproducibility.

Notably, the Equine IFN-γ ELISA^{PRO} kit results displayed greater sensitivity than the bovine in-house ELISA previously used (Parsons et al., 2017). The identification of an equine ELISA as the optimal assay for measuring white rhinoceros IFN-γ was anticipated given the phylogenetic relationship between rhinoceros and equine species (Steiner et al., 2011) and the

high homology of the interferon gamma sequences (Morar et al., 2007). Moreover, the antibodies used in this ELISA are known to cross-react with IFN-γ of other species (https://www.mabtech.com/knowledge-center/tutorials-and-guidelines/veterinary-reagents) and the use of ELISAs for IFN-γ detection in African wildlife has been previously reported (Goosen et al., 2014; Gormley et al., 2006).

The ELISA incubation steps were performed at 37˚C in contrast to the manufacturer's instructions. Rhinoceros typically are found in areas that are distant from environmentally controlled laboratories and temperature fluctuation could result in variation in ELISA results. Therefore, 37˚C was selected as the incubation temperature which can practically be achieved in most laboratories regardless of ambient temperatures. This temperature is utilized in a commercial cattletype® IGRA kit (Bernitz et al., 2018). A possible drawback of this protocol is that the LOQ of the ELISA^{PRO} kit was calculated as 7.8 pg/ml, which is greater than that reported by the manufacturer. Nonetheless, the ELISA displayed a linear response across a wide range of temperatures (19-37˚C). These characteristics of the ELISA^{PRO} kit highlight the utility of the assay for measuring rhinoceros IFN-γ under highly standardized conditions as applicable. The ELISA^{PRO} kit showed excellent performance in measuring endogenous rhinoceros IFN-γ in plasma samples. Rhinoceros plasma showed minimal interference with the ELISA, and recovery of IFN-γ in this matrix was within the acceptable range of 80-120% (ICH Harmonised Tripartite Guideline, 2005). Moreover, parallelism was excellent, and ELISA responses were indistinguishable for recombinant equine and endogenous rhinoceros IFN-γ. This is in contrast to other African wildlife species such as warthogs, in which detection of IFN-γ in stimulated blood samples has proven difficult (Roos et al., 2018). In addition, intra-and inter-assay precision showed CVs of less than 10% and 15%, respectively, which is considered an acceptable range

(Li et al., 2016), indicating the high reproducibility of the Mabtech Equine IFN-γ ELISA^{PRO} kit. Therefore, this kit is well suited for measuring rhinoceros IFN-γ in clinical samples. However, further research is underway to investigate the diagnostic use of this ELISA assay for the detection of *M. bovis* antigen-specific cytokine secretion from stimulated white rhinoceros whole blood samples.

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Chapter 3

An interferon-gamma release assay for the diagnosis of the *Mycobacterium bovis* infection in white rhinoceros (*Ceratotherium simum*)

Josephine Chileshe[1], Eduard O. Roos[1,3], Wynand J. Goosen[1], Peter Buss[2], Guy Hausler[2], Leana Rossouw[2], Tebogo Manemela[2], Paul van Helden[1], Robin Warren[1], Sven D.C. Parsons[1], Michele Miller[1]

[1]Department of Science and Technology/National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000, South Africa

[2] Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa

[3]Present address: The Pirbright Institute, Ash Rd, Pirbright, Woking GU24 0NF, United Kingdom

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Abstract

Mycobacterium bovis (*M. bovis*), the cause of bovine tuberculosis, is endemic in Kruger National Park (KNP), South Africa. The risk of spread of *M. bovis* infection currently prevents translocation of white rhinoceros (*Ceratotherium simum*) from this population. Therefore, accurate assays are necessary for screening this threatened species. Interferon gamma (IFN-γ) release assays (IGRA) are commonly used for tuberculosis diagnosis in humans and other wildlife species. Hence, the aim of this study was to develop an IGRA for *M. bovis* detection in white rhinoceros. Heparinized whole blood was collected from immobilized white rhinoceros in KNP (n=131) and incubated overnight in QuantiFERON®-TB Gold In-Tube (QFT) blood collection tubes, after which the plasma was harvested following centrifugation. Tissue samples for mycobacterial culture were available from a subset of 21 rhinoceros. The concentration of IFN-γ in plasma samples was measured using the Mabtech Equine IFN-γ ELISA^{PRO}. An IGRA result was calculated as the difference in IFN-γ concentrations in the QFT Nil and TB antigen tubes. Using test results for the white rhinoceros with known infection status, a diagnostic cut-off value was calculated as 21 pg/ml. Additionally, cut-off values for IFN-γ concentrations for plasma from QFT Nil and QFT Mitogen tubes were calculated to increase confidence in IGRA result interpretation. The combination of the QFT stimulation platform and Mabtech equine IFN-γ ELISA is a promising diagnostic test to distinguish between of *M. bovis*-infected and uninfected white rhinoceros.

Introduction

The white rhinoceros (*Ceratotherium simum*) is threatened (Emslie, 2012), and under severe poaching pressure. The largest populations of white rhinoceros are found in Kruger National Park (KNP), South Africa, where bovine tuberculosis (bTB) is endemic (Renwick et al., 2007). Bovine tuberculosis is a chronic disease caused by infection with *Mycobacterium bovis* (*M. bovis*), a member of the pathogenic *M. tuberculosis* complex (MTBC) (Brosch et al., 2002). Although white rhinoceros are susceptible to infection with *M. bovis*, there remains a knowledge gap concerning the extent of infection in exposed populations (Miller et al., 2018). In order to improve our understanding of bTB in white rhinoceros and prevent the spread of the disease through translocation of infected animals, new diagnostic tools are required to accurately identify infected and possibly diseased individuals.

Infection with *M. bovis* is usually diagnosed by detecting cell-mediated immune (CMI) responses towards pathogen-specific antigens (Welsh et al., 2005). This can be achieved by using an interferon-gamma (IFN-γ) release assay (IGRA) which includes antigenic stimulation of blood lymphocytes followed by measurement of the release of IFN-γ using an enzyme-linked immunosorbent assay (ELISA). Interferon-gamma is a commonly used cytokine biomarker for detection of MTBC infection and diagnostic IGRAs for detection of *M. bovis* infection have been developed for cattle (Bernitz et al., 2018), buffaloes (Goosen et al., 2014), and wild dogs (Higgitt et al., 2017).

The QuantiFERON-TB Gold In-Tube (QFT) system was developed for a human IGRA which provides sterile blood collection tubes containing a peptide cocktail simulating the antigens early secreted antigenic target 6kDa protein (ESAT-6), culture filtrate protein 10 (CFP-10) and TB7.7 (Ruhwald et al., 2007). Use of the QFT blood collection tubes has been advocated

for field testing of African wildlife because they are easy to use, provide high diagnostic specificity (Parsons et al., 2011), and can be adapted for use in multiple species (Bernitz et al., 2018; Higgitt et al., 2017; Roos et al., 2018). In addition, the QFT system has been previously used by Parsons et al. (2017) to measure immune sensitization of white rhinoceros to *M. bovis*. Therefore, the aim of this study was to describe a diagnostic QFT IGRA for detection of *M. bovis* infection in white rhinoceros by combining the commercially available QFT stimulation platform with a validated rhinoceros IFN-γ ELISA (Chileshe et al., 2019). This optimized IFN-γ ELISA was selected based on phylogenetic relationship between rhinoceros (Chileshe et al., 2019; Steiner and Ryder, 2011) and equine species and the high homology of the IFN-γ sequences (Morar et al., 2007).

Materials and Methods

Animals and samples

Whole blood was collected opportunistically in heparinized tubes (BD Biosciences, Franklin Lakes, NJ, USA) from 131 chemically immobilized white rhinoceros in KNP between 2015 and 2018, as previously described (Miller et al., 2018), during routine management or other approved activities. Twenty-one of these animals were subsequently euthanized due to severe poaching wounds. From these 21 rhinoceros, post-mortem tissue samples were collected (specifically, submandibular, retropharyngeal, cervical, prescapular, axillary, inguinal, mediastinal, tracheobronchial, mesenteric lymph nodes and lung) and frozen for transport to the lab, as previously described (Miller et al., 2018). All samples were processed for mycobacterial culture using the BACTEC™ MGIT™ 960 system (BD Biosciences) as described by Goosen et al. (2014). All Ziehl-Neelsen stain-positive bacterial cultures were speciated using genetic region

of difference analysis (Warren et al., 2006). Results were used to define animals as *M. bovis*-infected if any tissue was culture-positive, or uninfected if all tissues were culture-negative.

Also included in this study, 10 archived plasma sample sets obtained from 2 white rhinoceros that had been experimentally infected with *M. bovis* and tested monthly using the QFT system, as previously described (Parsons et al., 2017). These two white rhinoceros were included in the *M. bovis*-infected cohort.

Interferon gamma (IFN-γ) release assay (IGRA)

One ml aliquots of heparinized whole blood were incubated in tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands) comprising a Nil tube (containing saline) and a TB Antigen (TB Ag) tube containing mycobacterial peptides (ESAT6, CFP10, TB7.7). Additionally, one ml aliquots were incubated in either QFT Mitogen tubes (n = 90) or in tubes containing pokeweed mitogen (PWM, Sigma-Aldrich, St. Louis, MO, USA) at a final concentration of 10 µg/ml (n = 41). All tubes were incubated for 24 h at 37˚C. After incubation, plasma was harvested following centrifugation at 800 x g for 10 min, transferred into a 2 ml microcentrifuge tube, and stored at -80˚C until analysis.

All plasma samples, including samples from the experimentally infected animals, were analyzed using the equine IFN-γ ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden; custom precoated plates using kit reagent product 3117-1H-6) as previously described (Chileshe et al., 2019). The IFN-γ concentrations in plasma from the Nil, TB Ag and mitogen tubes were defined as [IFN-γ^{Nil}], [IFN-γ^{TB}], [IFN-γ^{Mit}], respectively, and the QFT interferon gamma release assay (IGRA) result for each rhinoceros was calculated as [IFN-γ^{TB}]-[IFN-γ^{Nil}], measured in pg/ml.

Data analysis

The QFT test values of the culture-confirmed naturally *M. bovis*-infected and uninfected rhinoceros (n = 21) were compared using the unpaired Student t-test. A p value of 0.05 was used to determine statistical significance.

A receiver operator characteristic (ROC) curve analysis was performed to determine the optimal diagnostic cut-off value for the QFT assay, calculated as the value that yielded the highest Youden's index (Youden, 1950). Hereafter, alternative cut-off values for the QFT assay were evaluated in the remaining 110 free-ranging rhinoceros (without culture results) by comparing the proportion of animals testing positive for each alternative value to the proportion testing positive using the ROC derived cut-off. This was done using a Z test (GraphPad Software, version 5, Inc., La Jolla, CA, USA). For this analysis, a stringent p value of ≤ 0.01 was considered statistically significant. In order to ensure the validity of future tests, exclusion criteria for the QFT Nil and QFT Mitogen samples were calculated as the 95th percentile of all [IFN-γ^{Nil}] values (n=131) and the 5th percentile of all [IFN-γ^{Mit}] values (n=90), respectively. The cut-off values were used to determine thresholds for negative (QFT Nil) and positive (QFT Mitogen) control samples.

Results

Of the 21 white rhinoceros that had mycobacterial culture results, 8 were *M. bovis* culture-positive and 13 were negative. The 21 test results for these animals are shown in Figure 3.1. The mean QFT IGRA value for *M. bovis*-infected animals (n=8) was significantly greater than the mean value for uninfected animals (Fig. 3.1; $p < 0.001$).

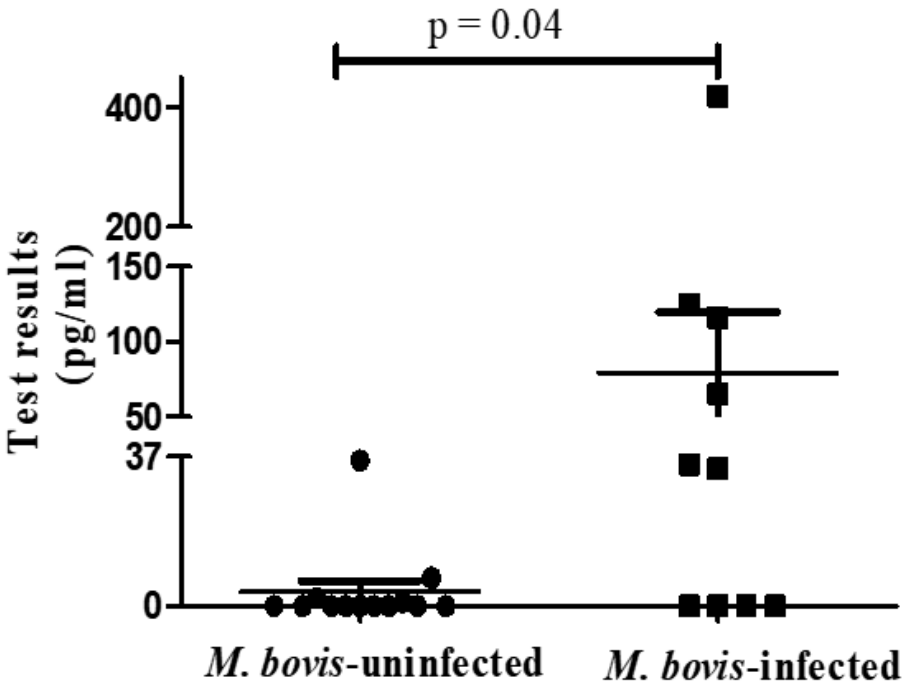

Figure 3.1 The QuantiFERON-TB Gold In-Tube interferon gamma release assay results for *M. bovis*-infected and uninfected white rhinoceros, showing a significant difference between the means of these groups (unpaired Student *t*-test p = 0.04). Means and 95% confidence intervals are shown by horizontal bars.

Based on ROC curve analysis, the optimal QFT IGRA cut-off value was 21 pg/ml (Se = 78%, 95% CI 52-93%; Sp = 92%, 95% CI 63-99%; AUC = 0.84) (Fig. 3.2, Table 3.1).

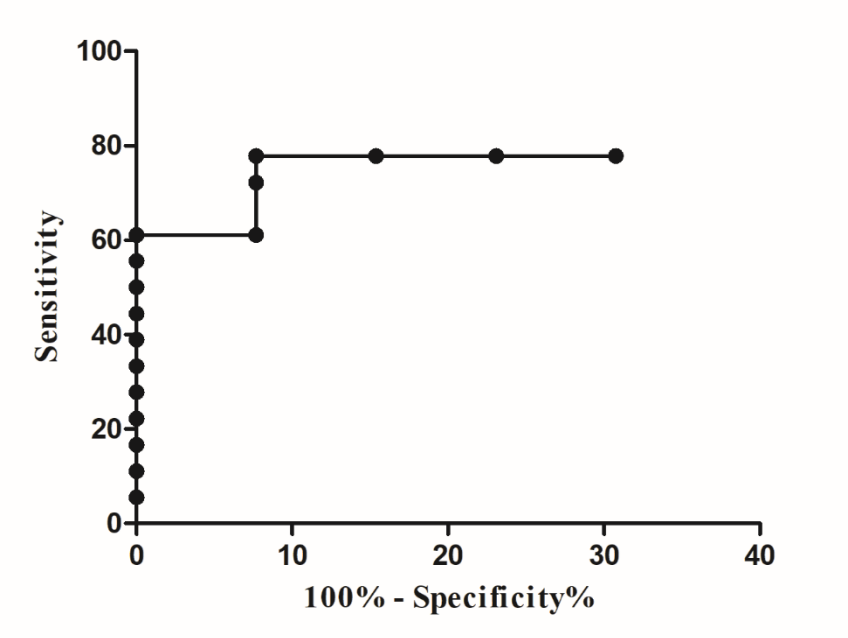

Figure 3.2 Receiver operating characteristics curve analysis of QuantiFERON-TB Gold In-Tube interferon gamma release assay results for *Mycobacterium bovis*-infected and uninfected white rhinoceros (Area Under the Curve = 0.84; 95% CI, 0.70-0.98; $p < 0.001$).

Using this cut-off value, 21 out of 110 (19 %) white rhinoceros (with no culture results) were test positive. The distribution of QFT IGRA responses for *M. bovis*-exposed rhinoceros is shown in Figure 3.3. The proportion of animals testing positive using a cut-off value of 21 pg/ml was compared with the proportion of animals testing positive using proposed positive cut-off values (as listed in column 1, Table 3.1). Cut-off values lower than 21 pg/ml and greater than 61 pg/ml resulted in significantly different proportions of test-positive rhinoceros.

Table 3.1 Test performance of the QuantiFERON-TB Gold In-Tube interferon gamma release assay, for selected cut-off values as determined by receiver operating characteristic curve analysis using samples (n = 31) from *M. bovis*-infected and uninfected white rhinoceros.

Cut-off value (pg/ml)	Sensitivity %	95% CI	Specificity %	95 % CI	Youden's Index %
1	78	52.3% to 93.5%	69	38.5% to 90.9%	47
2	78	52.3% to 93.5%	77	46.1% to 94.9%	55
5	78	52.3% to 93.5%	85	54.5% to 98.1%	62
21[a]	78	52.3% to 93.5%	92	63.9% to 99.8%	70
35	72	46.5% to 90.3%	92	63.9% to 99.8%	65
36	61	35.7% to 82.7%	92	63.9% to 99.8%	53
41	61	35.7% to 82.7%	100	75.3% to 100%	61
48	56	30.7% to 78.4%	100	75.3% to 100%	56
54	50	26.0% to 73.9%	100	75.3% to 100%	50
61	44	21.5% to 69.2%	100	75.3% to 100%	44
69	39	17.3% to 64.2%	100	75.3% to 100%	39
77	33	13.3% to 59.0%	100	75.3% to 100%	33
85	28	9.6% to 53.4%	100	75.3% to 100%	28
99	22	6.4% to 47.6%	100	75.3% to 100%	22
112	17	3.5% to 41.4%	100	75.3% to 100%	17
121	11	1.3% to 34.7%	100	75.3% to 100%	11
272	6	0.1% to 27.2%	100	75.3% to 100%	6

[a] Optimal diagnostic cut-off value; CI, confidence interval

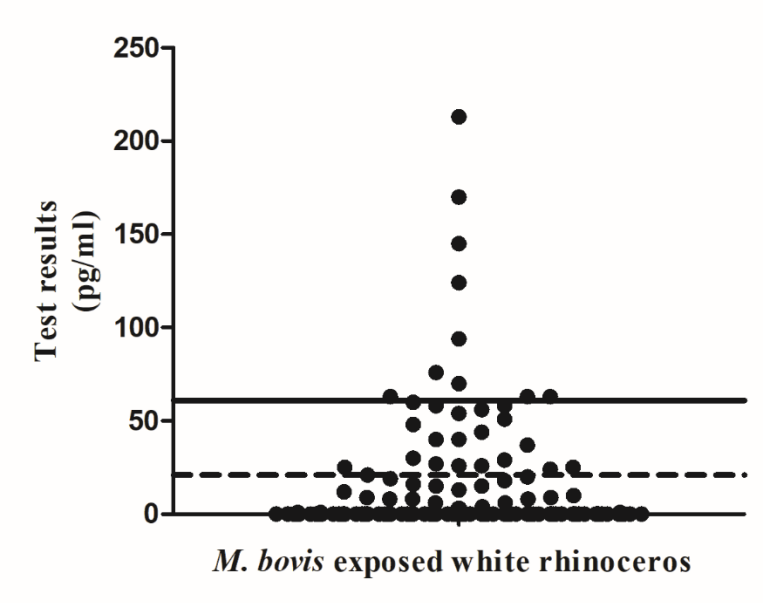

Figure 3.3 QuantiFERON-TB Gold In-Tube interferon gamma release assay values of 110 *M. bovis*-exposed white rhinoceros from Kruger National Park. The optimal cut-off value of 21 pg/ml is indicated by the dotted line (------) and alternative cut-off value of 61 pg/ml indicated by straight line (——).

In order to determine exclusion criteria for the QFT IGRA, the 95th percentile of all [IFN-γ^{Nil}] values were calculated as 28 pg/ml and the 5th percentile of [IFN-γ^{Mit}] values as 84 pg/ml (Table 3.2).

Table 3.2 Interpretation criteria for the QuantiFERON-TB Gold In-Tube interferon gamma release assay.

Test result	[IFN-γ^{Nil}]		[IFN-γ^{Mit}]		[IFN-γ^{TB}] minus [IFN-γ^{Nil}]
Negative	$\leq$ 28 pg/ml	AND	$\geq$ 84 pg/ml	AND	< 21 pg/ml
Positive	$\leq$ 28 pg/ml	AND	$\geq$ 84 pg/ml	AND	$\geq$ 62 pg/ml
Re-test	$\leq$ 28 pg/ml	AND	$\geq$ 84 pg/ml	AND	21-61 pg/ml
Inconclusive	> 28 pg/ml	OR	< 84 pg/ml		N/A

IFN-γ, interferon gamma; [IFN-γ^{Nil}] concentration in nil tube; [IFN-γ^{Mit}], concentration in Mitogen tube; [IFN-γ^{TB}], concentration in TB antigen tube; N/A, not applicable

Discussion

In this study, a new diagnostic IGRA, combining the QFT stimulation platform and Mabtech Equine IFN-γ ELISA^{PRO} kit, was evaluated for detection of *M. bovis* infection in white rhinoceros. The IGRA results for *M. bovis*-infected animals were significantly greater than those for uninfected animals, indicating that it could be used to distinguish between these groups. The optimal cut-off value was calculated as 21 pg/ml of antigen-specific IFN-γ. In addition, we calculated cut-off values for IFN-γ concentrations for plasma from QFT Nil and QFT Mitogen tubes to increase confidence in IGRA result interpretation.

White rhinoceros with confirmed *M. bovis* infection status were used to determine the diagnostic cut-off value. The value of 21 pg/ml is similar to cut-off values determined for QFT IGRAs in wild dogs (51 pg/ml) (Higgitt et al. 2019), African buffaloes (66 pg/ml) (Parsons et al., 2011), and humans (18 pg/ml) (Ruhwald et al., 2007). Therefore, this diagnostic cut-off value appears appropriate for white rhinoceros. However, the low number of animals used for this analysis, and the wide confidence intervals obtained for test parameters, suggests that this value should be considered as a preliminary finding. However, in a larger cohort, QFT test outcomes using a cut-off of 21 pg/ml were statistically similar to those using cut-off values ranging from

35 to 61 pg/ml. For these reasons, we propose that QFT IGRA results be interpreted as follows: a test result $\leq$ 21 pg/ml indicates that the rhinoceros is uninfected; a test result $\geq$ 62 pg/ml, that the rhinoceros has detectable immune sensitization to *M. bovis* antigens, consistent with infection; and results ranging from 21 – 61 pg/ml should be considered suspect for infection. Interpretation of the latter should be informed by epidemiological data and risk management considerations. Therefore, serial testing is recommended since a single result only provides an insight into the current status and not progression of initial infection.

In order to confirm the validity of test results, cut-off values for the QFT Nil and QFT Mitogen samples were calculated as $\leq$ 28 pg/ml and $\geq$ 84 pg/ml, respectively. The latter value is stringent when compared to the human QFT and Qiagen Cattletype® IGRAs that use cut-off values for mitogen samples that are similar to the diagnostic cut-off value (Ruhwald et al., 2007; Bernitz et al., 2018). The interpretation criterion for the mitogen sample is important for confirming viability and function of cells throughout the stimulation process. Individual rhinoceros samples with QFT Mitogen values that do not have an IFN-γ concentration $\geq$ 84 pg/ml should be excluded since a negative QFT IGRA value cannot be reliably interpreted as negative. With regards to background IFN-γ concentration in the QFT Nil sample, high nonspecific values may occur if there is a technical issue in blood processing or if the individual has other conditions causing in vivo immune stimulation (Pai et al., 2014). Therefore, these cut-off values provide increased confidence in test interpretation.

The QFT IGRA has shown utility for screening white rhinoceros to detect *M. bovis* infection. In addition, this IGRA is easy to use and incorporates commercially available reagents to improve reproducibility. Notably, the use of the prepared tubes of the QFT stimulation platform is especially suited for field use.

Limitations of the study included the low number of culture-confirmed white rhinoceros in the *M. bovis*-infected and uninfected groups. Also, the true infection status of the larger cohort of 110 white rhinoceros was unconfirmed. This highlights the difficulty of validating diagnostic tests in threatened species with few individuals with confirmed infection. In addition, a test result < 21 pg/ml indicates that the rhinoceros is uninfected, although it could be an early infection that has not yet resulted in a detectable immune response. With a test result ≥ 62 pg/ml, the rhinoceros has detectable immune sensitization to *M. bovis* antigens, consistent with infection, although it could also have recently cleared infection.

In conclusion, this study shows the combination of the QFT stimulation platform and Mabtech Equine IFN-γ ELISA^{PRO} is a promising diagnostic test for the detection of *M. bovis* infection in white rhinoceros. However, further research is necessary to recalculate the cut-off value of this assay using a ROC curve analysis with samples from larger infected and unexposed populations, respectively.

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Chapter 4

Cytokine biomarker discovery in the white rhinoceros (*Ceratotherium simum*)

Josephine Chileshe[1], Tanya J. Kerr[1], Craig Kinnear[1], Peter E. Buss[2], Paul D. van Helden[1], Robin M. Warren[1], Michele A. Miller[1], Sven D.C. Parsons[1]

[1]DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research; South African Medical Research Council Centre for Tuberculosis Research; Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000, South Africa

[2]Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa

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Abstract

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* (*M. bovis*) infection, disrupts conservation programs of threatened species such as the white rhinoceros (*Ceratotherium simum).* Interferon gamma release assays have been developed for the diagnosis of *M. bovis* infection in rhinoceros, however, the discovery of additional diagnostic biomarkers might improve the accuracy of case detection. The aim of this pilot study was therefore to evaluate a novel unbiased approach to candidate biomarker discovery and preliminary validation. Whole blood samples from 12 white rhinoceros were incubated in Nil and TB antigen tubes of the QuantiFERON® TB Gold In-Tube (QFT) system after which RNA was extracted and reverse transcribed. Using the equine RT^2 profiler PCR array, relative gene expression analysis of samples from two *M. bovis* immune sensitized rhinoceros identified *CCL4*, *CCL8*, *IL23A*, *LTA*, *NODAL*, *TNF*, *CSF3*, *CXCL10* and *GPI* as upregulated in response to antigen stimulation. Novel gene expression assays (GEAs) were designed for selected candidates, i.e. *CCL4*, *CXCL10* and *IFNG*, and analysis of QFT-processed samples showed the *CXCL10* GEA could distinguish between five *M. bovis*-infected and five uninfected rhinoceros. These findings confirm the value of the equine RT^2 profiler PCR array as a useful tool for screening biomarkers for the diagnosis of *M. bovis* infection in rhinoceros.

Introduction

Bovine tuberculosis (bTB) caused by *Mycobacterium bovis* (*M. bovis*) is endemic in the Kruger National Park (KNP), South Africa (Miller et al., 2017). The presence of disease in this park has disrupted conservation programs by restricting translocations of wildlife and may also have an impact on the health of infected animals (Emslie, 2011; Miller et al., 2018). The first case of *M. bovis* infection in rhinoceros in KNP was recorded in 2016 (Miller et al., 2017) with additional cases reported subsequently (Miller et al., 2018). The current management plan for bTB screening in rhinoceros in KNP relies on ante-mortem testing to identify infected animals before translocation, thereby reducing the risk of introducing *M. bovis* into new ecosystems (P. Buss pers. comm).

Ante-mortem tests for the diagnosis of bTB are based on detecting cell-mediated immune (CMI) responses. In rhinoceros, the equine interferon gamma (IFN-γ) ELISA^{PRO} kit (Mabtech Ab, Nacka Strand, Sweden) has been validated to measure antigen-specific cytokine release using the QuantiFERON® TB Gold In-Tube (QFT) system for stimulation of whole blood (Chileshe et al., 2019). The QFT system uses mycobacterial peptide antigens early secretory antigenic target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) to provide specificity to the IFN-γ release assay (IGRA). However, studies in other species have shown that cytokine biosignatures provide greater diagnostic accuracy than a single analyte (Bernitz et al., 2019; Palmer et al., 2020). Therefore, there is a need to investigate additional biomarkers for detection of *M. bovis* infection in rhinoceros.

Antigen-induced cytokine gene expression, measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR), has been used to identify potential biomarkers for TB diagnosis in humans (Kim et al., 2013) and cattle (Palmer et al., 2020). This

approach may be useful for wildlife where there is a lack of species-specific reagents (de Lisle et al., 2002). For example, gene expression assays (GEAs) using candidate biomarkers in the *CXCL* family have shown promise as diagnostic tools in different wildlife species (Olivier et al., 2015; Higgitt et al., 2017; Roos et al., 2019). Therefore, the aim of this study was to investigate GEAs as tools to identify potential biomarkers of *M. bovis* infection in white rhinoceros.

Materials and Methods

Animals, sample collection and blood stimulation

Samples were collected from 12 white rhinoceros originating from the KNP as previously described (Chileshe et al., 2019). Two animals that had previously been confirmed to be immunologically sensitized to *M. bovis* antigens (R1 and R2), based on the validated interferon-gamma release assay, provided convenient blood samples for initial biomarker discovery. Additionally, blood and tissue samples were opportunistically collected from severely injured animals that were euthanized for welfare reasons (R3 – R12). Mycobacterial cultures, performed on tissue samples as previously described (Miller et al., 2018), were used to classify animals as either *M. bovis*-infected (n = 5) or uninfected (n = 5).

Whole blood was collected in heparinized vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA) as previously described (Chileshe et al., 2019). Within 6 h of collection, one ml of heparinized blood was added to each tube of the QuantiFERON® TB Gold Plus (In-Tube) system (QFT) (Qiagen, Venlo, The Netherlands), comprising a Nil tube (QFT Nil, containing saline) and the TB2 antigen tube (QFT TB, containing ESAT-6 and CFP-10 peptides). Samples from R1 and R3-R12 were incubated for 24 hours at 37°C, while samples from R2 were incubated for 6 hours. After incubation, the blood was transferred to a 2 ml microcentrifuge tube

and centrifuged at 800 x g for 10 min. Plasma was harvested and the cell pellets resuspended in 1.3 ml RNAlater® (Ambion, Austin, TX, USA) and stored at -80˚C until analyzed.

RNA extraction

The RNAlater®-stabilised samples were centrifuged at 15 000 × g for 2 min and the supernatant discarded before RNA was extracted from the remaining pellet using the RiboPure™ Blood Kit (Ambion), then eluted in a volume of 60 µl, according to the manufacturer's guidelines. Total RNA concentration (ng/µl) and quality (A260/A280 and A260/A230 ratio) were measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Biomarker screening using the equine RT^2 profiler™ PCR array

The QFT Nil and QFT TB antigen samples for R1 (24 hours) and R2 (6 hours) were analysed using the equine RT^2 profiler PCR array (Qiagen). This quantitative real-time (RT-qPCR) array consists of expression assays for 84 cytokine genes, 5 reference genes, a genomic DNA control, a reverse-transcription control, and a PCR reagents control. The assay was performed according to the manufacturer's instructions. Briefly, 200 ng of RNA from each sample of 60 µl was treated for genomic DNA (gDNA) with DNA elimination mix and reverse-transcribed using the RT^2 First Strand Kit (Qiagen), in a final volume of 20 µl. The PCR reaction mix for each cDNA sample was prepared using the RT^2 SYBR Green qPCR Mastermix (Qiagen) in a final volume of 1300 µl. Ten µl of the reaction mix was dispensed into duplicate wells of a 384-well plate format using an automated electronic pipette (epMotion®, Eppendorf, Hamburg, Germany).

The RT-qPCR was initiated at 95˚C for 10 min followed by 50 cycles at 95˚C for 15 s and 60˚C for 1 min using a 7900HT™ Thermo Cycler (Applied Biosystems, Foster City, CA, USA)

and quantification cycle (Cq) values for each assay were automatically determined. For each animal, the difference in gene expression in the QFT TB Antigen sample, relative to the QFT Nil sample was calculated according to the manufacturer's instructions using the ΔΔCq method (Livak and Schmittgen, 2001). Potential candidate targets were selected for further analysis based on (i) intra-assay variability of $\leq$ 5% and (ii) to limit the number of potential biomarkers identified, we chose a fold change cut-off value for upregulated cytokines following antigen stimulation of > 2 in either R1 or R2. The RT-qPCR products for R2 were visualized in a 1% agarose gel (Lonza Group, Basel, Switzerland) by electrophoresis.

Sequencing of selected white rhinoceros gene transcripts

Based on findings from the RT^2 profiler, as described below, candidate biomarkers were selected for further analysis by RT-qPCR, i.e. *CCL4* and *CXCL10*. In addition, *IFNG* was included since interferon-gamma protein (IFN-γ) has been shown to be a biomarker for *M. bovis* infection in rhinoceros (Chileshe et al., 2019). The gene *YWHAZ* was selected as a reference transcript as its expression is stable in whole blood of numerous other species (Olivier et al., 2015; Higgitt et al., 2017; Roos et al., 2019). In order to confirm that published messenger RNA (mRNA) sequences of the white rhinoceros were valid, *CXCL10*, *CCL4*, *IFNG*, and *YWHAZ* sequences of the domestic horse and white rhinoceros were obtained from the National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) and aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Using the Primer3Plus online tool (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi), forward and reverse PCR primers were designed to anneal to regions with high homology (Table 4.1).

Partial or complete coding sequences of these transcripts were amplified from samples of two randomly selected rhinoceros in a total volume of 25 µl consisting of 1 µl of cDNA (reverse

transcribed from 200 ng total RNA using QuantiTect Reverse Transcription kit; Qiagen), 12.5 µl of One*Taq*® Hot Start 2x Mastermix with Standard Buffer (New England BioLabs® Inc., Ipswich, MA, USA), 0.5 µl of each gene-specific forward and reverse primer (final concentration of 0.2 µM; Integrated DNA Technologies, Coralville, IA, USA) and 10.5 µl nuclease-free water. The reaction was initiated at 94°C for 2 min, followed by 45 cycles of 94°C for 30 s, a PCR-specific annealing temperature (Table 4.1) for 30 s, and an extension step of 68°C for 1 min, and concluded with a final extension step at 68°C for 10 min using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems).

The qPCR products, visualized in a 1% agarose gel (Lonza Group) by electrophoresis, were submitted to the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa) for sequencing using a 3130xl Genetic Analyzer (Applied Biosystems), according to the manufacturer's guidelines, and analysed using Sequencher software version 5.4.6 (Gene Codes, Ann Arbor, MI, USA). The resulting white rhinoceros mRNA sequences were submitted to the GenBank® genetic sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 4.1).

qPCR design and evaluation of biomarkers using qPCR assays

For the white rhinoceros mRNA sequences, putative exon-exon boundaries and intron sizes were inferred from domestic horse sequences obtained from the Ensembl Genome Browser (http://www.ensembl.org/index.html). Primer3 online software was used to design qPCR primers, at least one of which spanned an exon–exon boundary (Table 4.2).

Table 4.1 Reference sequence accession numbers**,** primer sequences, and annealing temperatures of PCRs used to amplify selected white rhinoceros mRNA targets.

Gene	NCBI accession No.		Primer sequence (5'-3')		T (˚C)
	Horse	Rhinoceros	Forward	Reverse	
CCL4	XM_001503888	XM_004434846	GCACCAATGGGCTCAGAC	TCACAAAGTTGCGAGGAAGC	60
CXCL10	NM_001114940	XM_004430848	CACGTTTTCTGAGACACTGCTC	CAAGGGATCTTTCCACCTCT	60
IFNG	NM_001081949	XM_004429381	GTCCTTGGGACCTGATCAGC	TTGGGTGCAGTCACAGTTGT	65
YWHAZ	XM_001492988	XM_004431216	GAAAGGTCCCGGATGTTGCT	GGGGCTCAGCAGGCTCTG	65

T, annealing temperature

Table 4.2 Reference sequence accession numbers, primer sequences, and selected parameters of RT-qPCRs for transcripts of the white rhinoceros.

Gene	**NCBI accession No.**	**Primer sequence (5'-3')**		**DM Peak (◦C)**	**E%**	**CV (%)**
		Forward	Reverse			
CCL4	MT465464;MT465465	CTCTCAGCACCAATGGGCTCAG	GCTTCCTCGCAACTTTGTGA	81	104	1.32
CXCL10	MT465462;MT465463	CCACGTGTTGAGATCATTGCC	AATTCTGGATGGTCTGGGACTC	76	112	2.47
IFNG	MT465460;MT465461	GAAGAACTGGAAAGAGGAGAGTG	TCCATGCTCTTTTGAATGACCTG	78.5	108	1.97
YWHAZ	MT465466	TGGTGACAAGAAAGGGATTG	ACAGAGAAGTTAAGGGCCAGAC	76.5	105	1.79

E%, amplification efficiency; DM, derivative melt curve peak temperature; CV, intra-assay variability

All qPCRs were performed in triplicate using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA) and consisted of 10 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 1 µl of each gene-specific forward and reverse qPCR primer (at a final primer concentration of 0.5 µM; Integrated DNA Technologies), 2 µl of cDNA, and 6 µl of nuclease-free water. The reaction was initiated at 95˚C for 30 s, followed by 40 cycles of 95˚C for 5 s and 60˚C for 30 s, and concluded with a standard melt-curve analysis which was used to confirm the specificity of subsequent qPCRs. Initially, qPCR products were visualized in a 1% agarose gel and sequenced as described above. In order to determine the efficiencies of each qPCR, a dilution series (1:5, 1:25, 1:125, 1:625, 1:3125) of a pooled cDNA sample was assayed and analysed as previously described (Pfaffl et al., 2001). To validate the use of the relative quantification method, amplification efficiencies of *YWHAZ* and target genes were compared as previously described (Livak and Schmittgen, 2001). For each of the five *M. bovis*-infected and five uninfected rhinoceros, RT-qPCRs were performed in triplicate for the reference and each of the target genes using cDNA derived from blood incubated in QFT Nil and QFT TB antigen tubes.

Data analysis

To determine the intra-assay variability of replicate reactions of each qPCR, the coefficient of variation (CV) was calculated for each sample and reported as the mean of all samples (n = 20). For each sample, the relative expression of each target gene was normalised by subtracting the Cq value of *YWHAZ* from the Cq value of the target gene in order to calculate the relative abundance of the target gene mRNA for each sample (i.e. ΔCq). Thereafter, the ΔCq value derived from the QFT Nil sample was subtracted from the ΔCq value derived from the QFT TB sample for all animals (i.e. ΔΔCq). The relative fold change ($2^{-\Delta\Delta Cq}$) was used to derive

a QFT GEA result as a measure of difference in expression of the target transcript in response to antigen stimulation (Livak and Schmittgen, 2001).

The GEA results for all target genes were then analysed in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA). A Mann-Whitney U-test was used to determine if there was a difference in results for each gene between five *M. bovis*-infected and five uninfected rhinoceros. Results with a p value < 0.05 were considered statistically significant.

Results

The RNA yields from blood incubated in the QFT TB Antigen and QFT Nil tubes ranged from 21 – 193 ng (mean of 52 ng) with a mean A260/A280 ratio of 1.84 (range: 1.53 – 2.09) and a mean A260/A230 ratio of 1.43 (range: 0.78 – 2.38). For the equine RT^2 profiler assay, electrophoresis of RT-qPCR products revealed clear and reproducible bands of 80 to 200 base pairs for four of five reference genes, i.e. *ACTB*, *B2M*, *HPRT1*, and *LOC100056766*. Duplicate assays of these genes showed a CV of < 5% and these four reference genes were therefore used to normalize relative expression calculations (Supplementary Table 1 and 2). In addition, C_q values of the RT^2 profiler controls were all within the manufacturer's recommended ranges (data not shown). A total of 32 genes were successfully amplified in R1 (Supplementary Table 1) and 51 genes in R2 (Supplementary Table 2). However, nine target genes met the screening criteria (Table 4.3).

Table 4.3 Selected equine RT^2 profiler assay results, for two white rhinoceros, using mRNA isolated from whole blood processed with the QuantiFERON® TB Gold In-Tube system (QFT).

		QFT TB			QFT Nil			Assay result	
Animal ID	Gene	Mean target Cq	Mean Ref Cq	ΔCq TB	Mean target Cq	Mean Ref Cq	ΔCq	ΔΔCq	Fold Change ($2^{-\Delta\Delta Cq}$)
R1	*CCL4*	18.2	22.3	-4.0	19.1	21.4	-1.9	-2.1	4.3
	CCL8	30.7	22.3	8.5	32.2	21.4	11.2	-2.6	6.3
	IL23A	29.2	22.3	7.0	29.7	21.4	8.7	-1.7	3.2
	LTA	28.5	22.3	6.2	29.9	21.4	8.5	-2.2	4.7
	NODAL	29.0	22.3	6.7	30.2	21.4	8.7	-2.1	4.1
	TNF	25.7	22.3	3.4	26.2	21.4	5.2	-1.7	3.3
R2	*CCL4*	25.7	23.9	1.9	28.2	24.2	4.1	-2.2	4.7
	CSF3	34.3	23.9	10.5	37.6	24.2	13.5	-3.0	7.9
	CXCL10	33.8	23.9	10.0	36.3	24.2	12.2	-2.2	4.6
	GPI	28.1	23.9	4.2	30.1	24.2	5.9	-1.7	3.3

CV, Coefficient of variation; Ref, Reference genes; QFT TB, QFT TB antigen tube containing ESAT-6 and CFP-10 peptides; QFT Nil (containing saline); Cq, quantitation cycle

Two of these potential candidates were selected for further analysis using RT-qPCR assays, i.e. *CXCL10* since this has shown promise in numerous species and *CCL4* because it was identified in both R1 and R2. In addition, *IFNG* was included because there is a validated IGRA for TB diagnosis in white rhinoceros. Sequenced gene transcripts for *CCL4*, *CXCL10*, *IFNG* and *YWHAZ* showed high homology with published white rhinoceros sequences (Table 4.2).

All RT-qPCRs displayed low intra-assay variability with characteristic melt curves (data not shown) and melt peaks (Table 4.2). In addition, amplification efficiencies ranged from 104 to 112% (Table 4.2) and the relative efficiencies of all target gene qPCRs were confirmed to be similar to the reference gene (data not shown). No products were obtained in the no-RT and no-template control reactions. Visualization following gel electrophoresis and sequencing of qPCR products confirmed the amplification of appropriate targets (data not shown).

In samples from *M. bovis*-infected rhinoceros, *CXCL10* showed a significant upregulation in response to antigen stimulation compared to samples from uninfected rhinoceros (Fig. 4.1). The median fold increases in antigen-specific *CXCL10* expression for the *M. bovis*-infected and uninfected animals were 20.5 and 1.6, respectively (Fig. 4.1). In contrast, no significant differences in *CCL4* and *IFNG* expression were seen between these groups (Fig. 4.1).

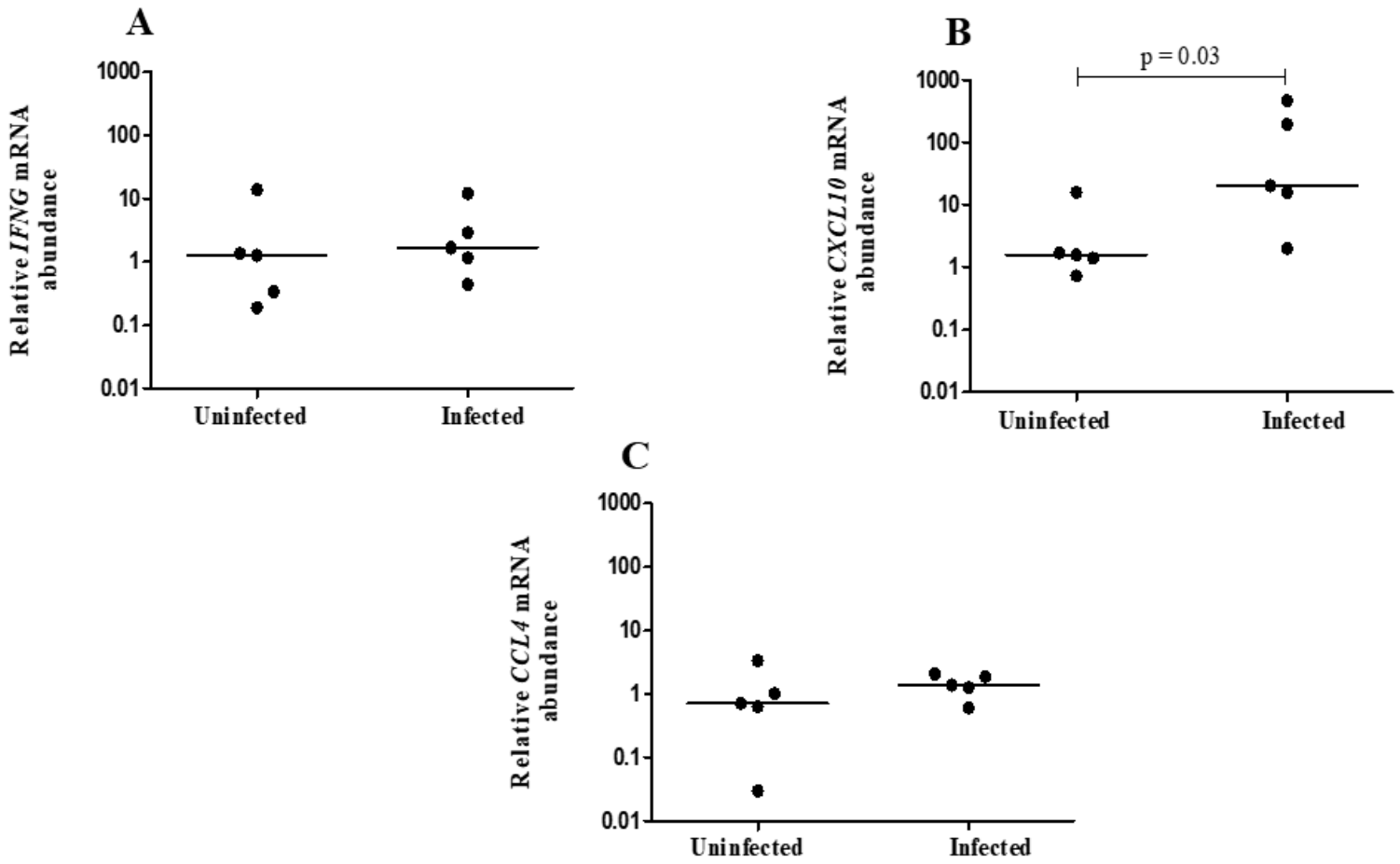

Figure 4.1 Test results for QuantiFeron TB Gold In-Tube gene expression assays targeting *IFNG* (A), *CXCL10* (B) and *CCL4* (C), for *M. bovis*-infected and uninfected rhinoceros. The *CXCL10* assay results were significantly greater for *M. bovis*-infected rhinoceros than for uninfected animals (Mann-Whitney U-test). Median assay results are shown by horizontal bars.

Discussion

This study describes the successful amplification of white rhinoceros cytokine gene transcripts from whole blood using the equine RT^2 profiler array. Using this assay, nine genes were identified as potential biomarkers of antigen recognition in blood incubated in the QFT stimulation system. In addition, novel RT-qPCR assays were developed to further evaluate these cytokine targets. Using this approach, measurement of *CXCL10* expression was confirmed to be able to distinguish between *M. bovis*-infected and uninfected white rhinoceros.

Seven of the genes identified by the equine RT^2 profiler array as displaying antigen-specific expression, i.e. *CCL4*, *CCL8*, *CSF3*, *CXCL10*, *IL23A*, *LTA* and *TNF*, have all previously been associated with both mRNA and protein biomarkers of mycobacterial infection and disease in humans and animals. In particular, *CXCL10* expression (encoding IP-10) is a highly sensitive diagnostic biomarker of TB in humans (Ruhwald et al., 2008) and other species (Roos et al., 2018; Bernitz et al., 2019) and is discussed, as is *CCL4*, in greater detail below. Similarly, antigen-induced *TNF* expression (detected as either mRNA or protein) is a TB biomarker common to humans and acttle but is diagnostic of disease severity rather than infection (Thacker et al., 2007; Sutherland et al., 2010; Wang et al., 2013). In contrast, while protein products of *CSF3* and *IL23A* expression protein have been identified as biomarkers of TB disease in humans (Sutherland et al., 2010; Wei et al., 2015), this is the first report of expression of these genes as candidate biomarkers in an animal species. The expression of *CSF3* and *IL23A* are both closely associated with *IL17* (Stark et al., 2005), which has been described as a diagnostic biomarker in cattle (Blanco et al., 2011; Waters et al., 2016). The need for species-specific studies is highlighted by *CCL8* (which encodes the protein MCP-2), the expression of which has shown promise as a biomarker of *M. tuberculosis* infection in humans (Ruhwald et al., 2008) but not of

M. bovis infection in African buffaloes (Goosen et al., 2014). Multiplex gene expression assays have the advantages of screening large numbers of potential target genes using limited samples and have been widely used for biomarker identification as well as diagnostic applications (Shukla et al., 2018; Palmer et al., 2020). Given the scarcity of samples from *M. bovis*-sensitized white rhinoceros and the lack of species-specific reagents, these findings support the use of the equine RT^2 profiler as a useful tool for screening analytes for the diagnosis of *M. bovis* infection in rhinoceros.

Of the candidate biomarkers selected for further evaluation by RT-qPCR, only antigen-specific *CXCL10* expression distinguished between *M. bovis*-infected and uninfected rhinoceros. This finding contrasts with reports that measurement of antigen-induced IFN-γ protein release is diagnostic of *M. bovis* infection in this species (Parsons et al., 2017; Chileshe et al., 2019). Similarly, measurement of *CXCL10* gene transcription, rather than that of *IFNG*, has shown promise as a diagnostic biomarker in other animal species such as lions (Olivier et al., 2015) and spotted hyena (*Crocuta crouta*) (Higgett et al., 2017). This may be related to differences in the kinetics of gene expression of these markers or the relative abundance of effector cells expressing these transcripts (Ruhwald et al., 2007). Importantly, the expression of *CXCL10* protein (IP-10) during antigen-induced immune activation is a sensitive and valuable biomarker of TB diagnosis in humans (Ruhwald et al., 2008), African buffaloes (Goosen et al., 2015), warthogs (Roos et al., 2018) and cattle (Parsons et al., 2016; Coad et al., 2019; Palmer et al., 2020). These studies strongly support the present findings that *CXCL10*/IP-10 should be further investigated as a diagnostic biomarker of *M. bovis* infection in rhinoceros.

Despite *CCL4* initially showing promise as a candidate biomarker of antigen recognition, no difference in *CCL4* responses was detected in QFT-processed samples from *M. bovis*-infected

and uninfected rhinoceros. This gene encodes for MIP-1β, which, in combination with other cytokines, has been identified as a biomarker of TB disease in humans when measured in both unstimulated and antigen-stimulated blood (Chegou et al., 2009). However, MIP-1β levels are also elevated in unstimulated plasma from human patients with other respiratory diseases (Chegou et al., 2018) and antigen-stimulated samples of healthy controls (Wei et al., 2015). This might, in part, explain the discrepancy in the results obtained using the RT^2 profiler and RT-qPCR assays. While *CCL4* may display antigen-induced upregulation, the present findings do not support further investigation of this cytokine as a diagnostic marker of *M. bovis* infection in rhinoceros.

A major limitation for the optimal measurement of antigen-induced gene transcription of activated lymphocytes and other immune cells is the dynamic nature of this process. Gene expression may be induced directly by lymphocyte antigen recognition (e.g. *IFNG*), or indirectly in response to cytokine release (e.g. *CXCL10*), resulting in gene-specific expression kinetics (Abdalla et al., 2003). For this reason, sample incubation times must be optimized for assays designed to measure specific targets (Bibova et al., 2012). In cases where numerous gene transcripts are analysed, no single incubation time will be optimal, and multiple sampling time points post-infection can be of value (Palmer et al., 2020). This is supported by findings from the present study in which different targets were identified after 6 and 24 h of sample incubation.

In conclusion, despite limitations in this pilot study, the target gene *CXCL10,* identified by the RT^2 profiler, was confirmed as a promising biomarker by demonstrating significant upregulation in antigen-stimulated blood from *M. bovis*-infected rhinoceros in a novel cytokine GEA. This study demonstrates the potential that GEA may have for developing diagnostic tools

for *M. bovis* infection in rhinoceros; future studies should investigate optimizing these assays in a larger cohort of rhinoceros.

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Chapter 5

Evaluation of interferon gamma-induced protein 10 (IP-10) responses for the detection of *Mycobacterium bovis* infection in white rhinoceros (*Ceratotherium simum*)

Josephine Chileshe[1], Peter E. Buss[2], Paul D. van Helden[1], Robin M. Warren[1], Sven D.C. Parsons[1], Michele A. Miller[1]

[1]DSI-NRF Centre of Excellence for Biomedical Tuberculosis Research; South African Medical Research Council Centre for Tuberculosis Research; Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 241, Cape Town 8000, South Africa

[2]Veterinary Wildlife Services, South African National Parks, Kruger National Park, Private Bag X402, Skukuza, 1350, South Africa

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Abstract

Interferon gamma (IFN-γ)-induced protein 10 (IP-10) has shown promise as a diagnostic biomarker of mycobacterial infection in humans, cattle, and African wildlife. The aim of this pilot study was to evaluate IP-10 as an additional biomarker to interferon gamma in order to increase accuracy in interpreting results to diagnose *M. bovis* infection in white rhinoceros. Archived plasma sample sets stored at -80°C, harvested from whole blood incubated in QuantiFERON TB Gold Plus (QFT) tubes from experimentally *M. bovis*-infected and free-ranging *M. bovis* uninfected white rhinoceros, were used for this study. Sample selection was based on a range of antigen-specific IFN-γ release assay (IGRA) responses. The Raybio® equine IP-10 ELISA kit was used for the detection of white rhinoceros IP-10. The IP-10 assay was able to distinguish between *M. bovis*-infected and uninfected white rhinoceros. Furthermore, using a preliminary calculated cut-off value, IPRA detected an additional truly infected animal with low IGRA response. These results indicate that white rhinoceros develop a measurable antigen-specific IP-10 response, which can distinguish between *M. bovis*-infected and uninfected animals. Thus, the IP-10 assay shows moderate promise as a diagnostic biomarker and may increase detection of truly infected animals.

Introduction

The Kruger National Park (KNP), South Africa, is an endemic area for bovine tuberculosis (bTB), caused by infection with *Mycobacterium bovis* (*M. bovis*) (Renwick et al., 2007). *Mycobacterium bovis* infection has been reported in a number of different wildlife species, including African rhinoceros (de Lisle et al., 2002; Hlokwe et al., 2014; Miller et al., 2018). The white rhinoceros (*Ceratotherium simum*) is classified as "Near Threatened" by the International Union for Conservation of Nature and is under high poaching pressure in Africa (Emslie, 2012). Although white rhinoceros are susceptible to *M. bovis* infection, the extent of infection in exposed individuals or populations and its impact on conservation of this species are still unknown (Chileshe et al., 2019; Miller et al., 2018). Therefore, to understand *M. bovis* infection in white rhinoceros and prevent the spread of disease through translocation of infected animals, accurate diagnostic tests are necessary to screen individuals.

Previous studies focused on bTB diagnosis in wildlife have shown the accuracy of blood-based tests utilizing the QuantiFERON TB Gold Plus (QFT) system, which includes peptides simulating the *M. bovis* proteins 6 KDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) (Bernitz et al., 2018; Roos et al., 2018). The basis of current blood-based assays is detection of cell-mediated immune (CMI) responses through the release of cytokines, such as interferon gamma (IFN-γ) (Parsons et al., 2011; Higgitt et al., 2019). Notably, antigen-specific IFN-γ responses in experimentally *M. bovis*-infected white rhinoceros were observed to peak 5 to 6 months post infection and gradually declined to low or undetectable levels within 12 to 20 months after infection (Parsons et al., 2017). However, these immune response patterns, together with limited bTB pathology and failure to isolate viable *M. bovis* organisms at post-mortem examination may reflect the possibility that *M. bovis* infection is

controlled in white rhinoceros (Parsons et al., 2017). An IFN-γ release assay (IGRA) has been validated for use in white rhinoceros, findings suggested that rhinoceros with IGRA results should be interpreted as test negative (< 21 pg/ml), indeterminate (21–61 pg/ml), and test positive (> 61 pg/ml) (Chileshe et al., 2019). Due to uncertainty associated with indeterminate results, repeat testing of these individuals is recommended, which is costly in terms of time, risk to animal, and expenses to immobilize and perform additional assays. Therefore, additional biomarkers of *M. bovis* infection in white rhinoceros may be useful to increase accuracy in interpreting results to diagnose bTB in this species, especially if the same sample could be used.

In addition to IFN-γ, *CXCL10*/interferon gamma induced protein 10 (IP-10) has emerged as a potential biomarker for TB in humans (Ruhwald et al., 2007), cattle (Palmer et al., 2020), and wildlife (Goosen et al., 2015; Roos et al., 2018). Interestingly, findings from a recent study showed that an antigen-specific *CXCL10* gene expression assay was able to distinguish between *M. bovis*-infected and uninfected white rhinoceros (Chileshe et al., manuscript submitted). Furthermore, in African buffalo (*Syncerus caffer*), the antigen-specific IP-10 release assay (IPRA) increased sensitivity (Se) when used in parallel with the IGRA for detection of *M. bovis* infection (Bernitz et al., 2018; Bernitz et al., 2019). Therefore, in the present study, archived QFT plasma samples from animals with a range of IGRA results were used to answer three questions: (i) can white rhinoceros IP-10 be detected in plasma from QFT-stimulated whole blood; (ii) can antigen-specific IPRA results differentiate between *M. bovis*-infected and uninfected white rhinoceros; and (iii) can antigen-specific IPRA results be used to increase detection of truly infected white rhinoceros?

Materials and Methods

Sample selection

Archived sample sets (n = 23) from 16 white rhinoceros were selected for this study. Each sample set had been stored at -80°C and included plasma harvested from whole blood incubated in the Nil, TB Antigen, and Mitogen tubes of the QFT system (Qiagen, Venlo, The Netherlands), as previously described (Chileshe et al., 2019). Initially, QFT Nil and QFT Mitogen plasma samples from three *M. bovis* uninfected animals were used to determine if there was detection of rhinoceros IP-10 using the selected ELISA. The remaining 20 sample sets were grouped into 4 categories based on the *M. bovis* infection status and IGRA responses of the individual animals, as determined in previous studies (Michel et al., 2017; Parsons et al., 2017; Chileshe et al., 2019). These included samples from uninfected animals with antigen-specific IGRA responses that were < 21 pg/ml (Group A, n = 5) or 21-61 pg/ml (Group B, n = 5), and *M. bovis*-infected animals with IGRA responses that were ≤ 61 pg/ml (Group C, n = 5) or > 61 pg/ml (Group D, n = 5) (Table 5.1). Ethical approval for this project was granted by Stellenbosch University Animal Care and Use Committee (SU-ACU-2018-0966).

Interferon gamma induced protein 10 (IP-10) release assay (IPRA)

Due to the high homology between equine and rhinoceros *CXCL10* mRNA sequences (Chileshe et al., manuscript submitted), the RayBio® equine IP-10 ELISA kit (RayBiotech Inc., Norcross, GA, USA) was selected as a candidate assay for detection of rhinoceros IP-10. As a pilot study, QFT Nil and QFT Mitogen plasma from three uninfected white rhinoceros were assayed in single ELISA wells. Hereafter, a serial dilution of equine recombinant IP-10 (rIP-10) (RayBiotech Inc.), a negative control consisting of kit dilution buffer, and all other samples were assayed in duplicate. In all cases, plasma samples were diluted 1:4 in kit dilution buffer and the

ELISA was performed according to the manufacturer's instructions. The optical density (OD) of each well was measured at wavelengths of 450 nm (OD_{450}) and 630 nm (OD_{630}) using a VersaMax ELISA microplate reader with SoftMax Pro® software (Molecular Devices, San Jose, CA, USA). The sample OD was calculated as OD_{450}–OD_{630} to account for light absorbance of the polystyrene plate. The OD result was calculated as the mean sample OD value for the duplicate samples minus the mean sample OD value of the background control.

Data analysis

White rhinoceros IP-10 OD results for the QFT Nil and QFT Mitogen-stimulated plasma from three *M. bovis* uninfected animals were compared using a one-tailed Mann-Whitney U-test (GraphPad Software, Inc., San Diego, CA, USA). Where applicable, the absolute concentration (pg/ml) of IP-10 for each sample was calculated from the mean OD results with reference to the standard curve (linear regression using GraphPad Software). The antigen-specific IP-10 release assay (IPRA) result for each rhinoceros was calculated as the concentration of IP-10 in the QFT TB Antigen tube minus the concentration of IP-10 in the QFT Nil tube measured in pg/ml. Negative values were assigned a value of 0 pg/ml. The median IPRA results were calculated along with interquartile range (IQR) and compared between *M. bovis*-infected and uninfected white rhinoceros cohorts using a one-tailed Mann-Whitney U-test (GraphPad Software).

A preliminary diagnostic cut-off value for QFT IPRA was calculated using the formula mean + (2 x SD) using *M. bovis* uninfected animal samples and IPRA results were designated as either positive or negative based on this value. To determine if the IPRA sensitivity was similar for samples with low and high IGRA responses, the proportions of IPRA test-positive animals in groups C and D were compared using a Z test (GraphPad Software). A p value of 0.05 was used to determine statistical significance.

Results

The plasma from QFT Nil and QFT Mitogen-stimulated whole blood of three *M. bovis* uninfected white rhinoceros showed a detectable IP-10 signal using the RayBio® equine IP-10 ELISA kit (Fig. 5.1). Although IP-10 was detected in QFT Nil (unstimulated plasma) from all three animals, it was particularly high in one animal. Notably, OD values for plasma from QFT Mitogen-stimulated samples of all three animals were higher than those of the matched QFT Nil samples. However, there was no significant difference ($p > 0.05$) between median OD values of 0.36 (range: 0.27–0.58) and 0.19 (range: 0.06–0.53) in the QFT Mitogen and QFT Nil samples, respectively.

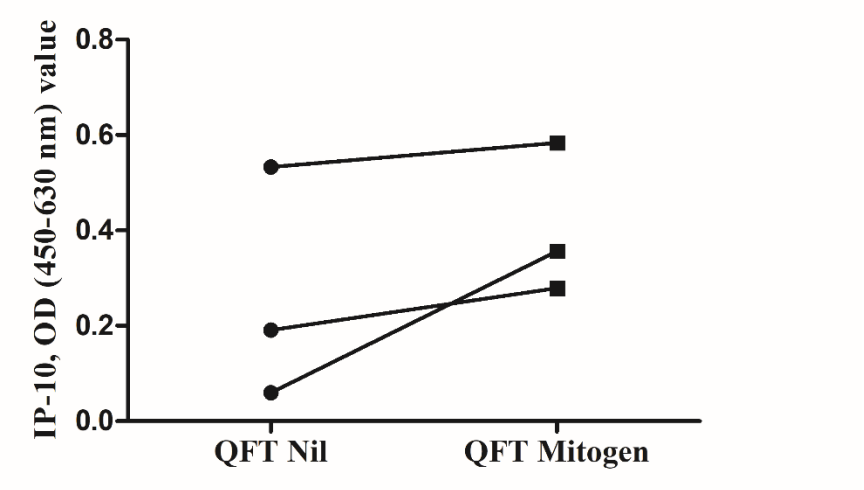

Figure 5.1 Optical density (OD) values of interferon gamma induced protein 10 (IP-10) in plasma harvested from QFT Nil and QFT Mitogen tubes (24 hours incubation at 37°C) from *M. bovis* uninfected white rhinoceros (n = 3), measured using the RayBio® equine IP-10 ELISA kit. Values of QFT Nil and QFT Mitogen samples were not statistically different ($p = 0.20$; one-tailed Mann Whitney U-test).

In order to determine if antigen-specific IP-10 could be detected, concentrations of IP-10 in plasma from QFT Nil and QFT TB Antigen tubes stimulated whole blood from 10 sets of

samples from *M. bovis*-infected (n = 3 animals) and 10 uninfected rhinoceros were measured using the equine IP-10 ELISA. Concentrations of IP-10 in each sample and the IPRA results, along with previously determined IGRA results, are shown in Table 5.1.). The cut-off value of 203 pg/ml was calculated and the distribution of QFT IPRA responses for *M. bovis*-infected and uninfected white rhinoceros is shown in Figure 5.2. Using this cut-off value IPRA results were categorized as test positive or negative (Table 5.1). Five out of 10 samples from the *M. bovis*-infected rhinoceros were considered IPRA positive.

Table 5.1 Test results of interferon gamma release assay and interferon gamma induced protein 10 release assay from *Mycobacterium bovis* experimentally infected (n=10 sample sets from three animal) and uninfected (n=10) free-ranging white rhinoceros.

Group	Group Description	Animal ID	Post-infection (month)	IGRA (pg/ml)	IP-10 (pg/ml)		IPRA	
					QFT Nil	QFT TB	Value (pg/ml)	Outcome
A	*M. bovis* uninfected;	18/515	N/A	0	722	506	0	Neg[a]
	< 21 pg/ml IGRA response	18/630	N/A	0	68	81	13	Neg
		19/70	N/A	0	1251	205	0	Neg[a]
		17/632	N/A	2	79	77	0	Neg[a]
		17/648	N/A	7	344	357	13	Neg
B	*M. bovis* uninfected;	19/306	N/A	22	699	724	25	Neg
	21-61 pg/ml IGRA response	19/281	N/A	34	1374	1561	186	Neg
		17/683	N/A	36	1640	1453	0	Neg[a]
		19/88	N/A	49	585	577	0	Neg[a]
		19/76	N/A	52	1407	1609	202	Neg
C	*M. bovis* infected;	PB4	1	14	169	880	711	Pos
	≤ 61 pg/ml IGRA response	PB2	18	37	683	798	115	Neg
		PB1	19	41	1207	962	0	Neg[a]
		PB4	18	48	449	436	0	Neg[a]
		PB2	16	52	7372	6355	0	Neg[a]
D	*M. bovis* infected;	PB4	19	75	6148	6727	579	Pos
	> 61 pg/ml IGRA response	PB2	6	557	686	2066	1380	Pos
		PB4	5	657	982	1123	141	Neg
		PB2	3	731	5696	6872	1176	Pos
		PB1	8	983	462	2373	1912	Pos

M. bovis, *Mycobacterium bovis*; IGRA, Interferon gamma release assay; QFT, QuantiFERON TB Gold Plus tubes; IP-10, interferon gamma induced protein 10; IPRA, Interferon gamma induced protein 10 release assay; IPRA value, was calculated as the concentration of IP-10 in the QFT TB Antigen tube minus the concentration of IP-10 in the QFT Nil tube; Neg, negative; Pos, positive; N/A, not applicable,

[a] Due to elevated concentrations of IP-10 in QFT Nil tube, the IPRA value was assigned a value of 0 pg/ml

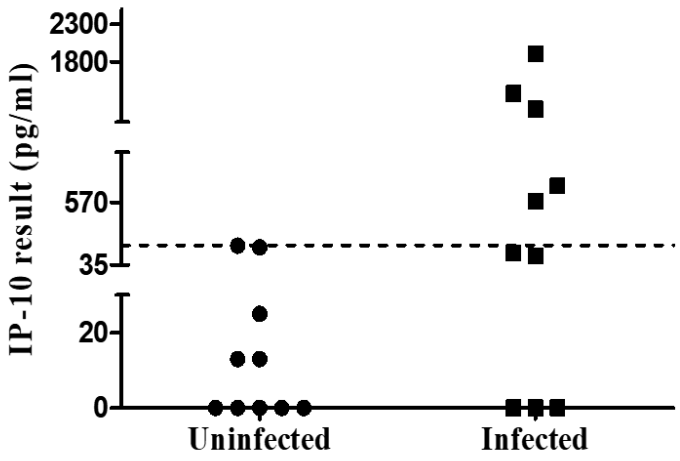

Figure 5.2 Antigen-specific interferon gamma-induced protein 10 (IP-10) production in samples from rhinoceros with known *M. bovis* infection status. Whole blood of *M. bovis*-infected (10 sample sets from 3 experimentally infected individuals) and uninfected (n=10) white rhinoceros was stimulated for 24 hours using the QuantiFERON TB Gold Plus system. Cytokine production was measured in plasma supernatant using the RayBio® equine IP-10 ELISA kit (RayBiotech Inc.). The diagnostic cut-off value of 203 pg/ml is indicated by the dotted line.

Rhinoceros QFT plasma samples were selected based on previously determined antigen-specific IGRA results to determine if antigen-specific IPRA results could increase detection of *M. bovis*-infected individuals using the calculated cut-off value. In group A, all 5 uninfected animals with negative IGRA results (< 21 pg/ml) had negative IPRA results. Additionally, all 5 uninfected rhinoceros with indeterminate IGRA results (21–61 pg/ml) in group B were also IPRA negative. Among the *M. bovis*-infected cohorts, one IGRA negative sample in group C (IGRA ≤ 61 pg/ml) was IPRA positive. Among group D, which had positive IGRA responses (> 61 pg/ml), 4 out of 5 samples were IPRA positive. The proportions of IPRA positive results among the *M. bovis*-infected cohorts showed that group D was significantly higher than group C ($p = 0.001$). If the IGRA and IPRA results were used in parallel to determine *M. bovis* infection status, all the uninfected rhinoceros would be considered negative and one additional *M. bovis* infected animal would be test-positive.

Discussion

In this pilot study, white rhinoceros IP-10 release in whole blood was detected using the QFT stimulation platform and Raybio® equine IP-10 ELISA. In addition, this IPRA was able to detect an additional test-positive sample from a *M. bovis*-infected rhinoceros with a negative IGRA result. Therefore, IP-10 appears to be a promising biomarker to increase detection of truly infected white rhinoceros when used in parallel with IFN-γ.

The Raybio® equine IP-10 ELISA could detect white rhinoceros IP-10 in plasma harvested from QFT stimulated whole blood. The high homology between white rhinoceros and equine cytokine sequences (Chileshe et al., manuscript submitted) predicted the anti-equine IP-10 antibody cross-reactivity with the rhinoceros cytokine. Similarly, the use of commercially available reagents such as anti-bovine IP-10 antibodies are known to cross react with IP-10 in

African buffaloes and common warthogs (*Phacochoerus africanus*), based on phylogenetic relationships (Goosen et al., 2014; Roos et al., 2018). Findings from this study indicate that the equine IP-10 ELISA kit may provide a readily available standardized method for cytokine detection in white rhinoceros.

Notably, a wide range of IP-10 concentrations was detected in QFT Nil (unstimulated) plasma samples from both infected and uninfected white rhinoceros. This finding of spontaneous production of IP-10, in incubated whole blood from white rhinoceros, is similar to studies in cattle (Waters et al., 2012). Furthermore, similar to cattle, measurements of antigen-specific IP-10 responses in rhinoceros were compromised by high levels of IP-10 in some unstimulated plasma samples (Parsons et al., 2016; Palmer et al., 2020). In addition, studies conducted in cattle (Palmer et al., 2020) and humans (Hong et al., 2014) have reported the measurements of IP-10 in serum samples, suggesting that in vivo induction of this cytokine contributed to the increased production. Furthermore, the secretion of IP-10 is enhanced by T cell secreted IFN-γ and numerous pro-inflammatory cytokines, such as IL-2, IFN-α, IFN-β, IL-27, IL-17, IL-23, TNF-α, and IL -1β which are secreted by antigen-presenting cells (Hong et al., 2014).

Therefore, results from the present study highlight the potential confounding effect of high levels of IP-10 in QFT Nil (unstimulated) plasma samples from white rhinoceros. However, further investigation is necessary to determine if spontaneous IP-10 release will compromise the diagnostic performance of this assay in white rhinoceros.

Antigen-specific IP-10 could differentiate between *M. bovis*-infected and uninfected white rhinoceros. Similarly, prior studies have demonstrated the use of IP-10 as a biomarker in the diagnosis of bTB in cattle (Parsons et al., 2016; Coad et al., 2019; Palmer et al., 2020), African buffaloes (Bernitz et al., 2019), and common warthogs (*Phacochoerus africanus*) (Roos

et al., 2018). Moreover, in the present study, a single QFT IGRA-negative sample from an *M. bovis*-infected rhinoceros tested IPRA positive. This sample was collected one month after experimental infection and may indicate the potential of the IPRA to detect early infection. This finding agrees with others showing that the parallel measurement of IFN-γ and IP-10 increased the detection of *M. bovis*-infected cattle and buffaloes (Parsons et al., 2016; Bernitz et al., 2019). However, the sensitivity of the IPRA was significantly greater for rhinoceros with positive IGRA results, reflecting the association between IFN-γ and IP-10 production. Nonetheless, our findings show the diagnostic potential of QFT IPRA for *M. bovis* infection in white rhinoceros with increased detection of truly infected animals when used in parallel with QFT IGRA.

Limitations of this pilot study prevent definitive conclusions on the diagnostic utility of IP-10 in white rhinoceros. The small sample size of *M. bovis*-infected and uninfected white rhinoceros may have influenced the calculation of appropriate IP-10 test cut-off values. Also, the inclusion of plasma samples from *M. bovis* experimentally infected, rather than naturally infected, white rhinoceros may not be a true reflection of host immune responses. In natural infections, rhinoceros are exposed to a number of environmental risk factors, such as drought, which could alter immune responses (Miller et al., 2017), and affect calculation of cut-off values.

In conclusion, while recent advancements have been made in the development of a QFT IGRA for the diagnosis of *M. bovis* infection in white rhinoceros, there remains a need for increased diagnostic sensitivity (Chileshe et al., 2019). This pilot study indicates that IP-10 has moderate promise as a diagnostic biomarker in this species and that its measurement in antigen-stimulated whole blood may increase detection of truly infected animals. Future investigations should include recalculation of the diagnostic cut-off value with samples from larger cohorts of naturally *M. bovis* culture-positive and negative populations. In addition, further investigation of

the observed spontaneous release of IP-10 in QFT-Nil samples is necessary to interpret IPRA results in this species.

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Chapter 6

General discussion

This chapter aims to synthesise all the results from the previous chapters and to contextualize the findings regarding the development of immunological tools for diagnosis of *M. bovis* infection in African rhinoceros.

In 2016-2017, the first cases of *M. bovis* infection in a free-ranging black and white rhinoceros were discovered in KNP (Miller et al., 2017; Miller et al., 2018). Prior to finding naturally infected animals, a study to investigate susceptibility to *M. bovis* infection, progression to disease, and immune responses in white rhinoceros was performed by experimentally infecting three individuals (Michel et al., 2017; Parsons et al., 2017). This study demonstrated the potential utility of antigen-specific rhinoceros IGRAs for the detection of *M. bovis* in rhinoceros. However, even with findings from the experimentally and naturally infected rhinoceros, there were significant knowledge gaps regarding *M. bovis* transmission, diagnosis, and pathogenesis in these species.

Understanding bTB in rhinoceros requires reliable and accurate tests to diagnose infected individuals and screen populations. Therefore, the studies presented in this PhD thesis aimed to identify and develop novel and optimized approaches for improving the detection of *M. bovis* infection in African rhinoceros. The thesis describes a novel IGRA for white rhinoceros that can distinguish between *M. bovis*-infected and uninfected individuals as well as pilot studies on the cytokine IP-10 release assay and *CXCL10* (IP-10) gene expression assay. The results of these studies showed promise that IP-10 may be an additional cytokine biomarker for the detection of *M. bovis* in rhinoceros.

Use of equine reagents for rhinoceros assays

The use of commercially available equine reagents for rhinoceros diagnostic assays proved to be useful in this study. Although there is a recognized need to develop tests for infectious diseases and research to understand wildlife health, one of the major challenges is the lack of available reagents that can be used in wildlife diagnostic tests (Stallknecht, 2007). In addition, selecting the appropriate reagents, especially sufficient antibody validation, has

important consequences for the quality and applications of an assay (Ryser-Degiorgis, 2013; Andersson et al., 2017). To develop indirect tests such as ELISAs, which are based on detecting the host immune response to a pathogen, one option is to develop rhinoceros-specific antibodies to cytokines, such as IFN-γ and IP-10, for the optimization of antigen-specific release assays. This has been done in a previous study where white rhinoceros IFN-γ was cloned, sequenced, and purified to produce a monoclonal antibody for the development of an IFN-γ ELISA (Morar et al., 2007). However, in the current study, commercially available antibodies and reagents were screened for cross-reactivity because the aim was to develop novel tests which would be easily accessible for use by other veterinary laboratories. Notably, cross-reactive binding was observed between anti-equine cytokine antibodies and rhinoceros cytokines, likely due to structurally similar epitopes of the equine and rhinoceros proteins because of the phylogenetic relationship between the species (Benjamin et al., 1984). Furthermore, results from our study demonstrated cross-reactivity of equine primers for rhinoceros transcripts, suggesting that these equine reagents could be used to develop qPCR assays to study immune responses in rhinoceros. Therefore, the approach of using commercially available reagents of closely related species provides a toolbox for assay development for rhinoceros and could be employed in other wildlife species to create new diagnostic tests.

Validation of an IFN-γ ELISA

In this study, the equine IFN-γ ELISA was validated for the diagnosis of *M. bovis* infection in white rhinoceros. Evaluation of an assay through various validation steps is important to ensure robustness and reproducibility (Andreasson et al., 2015). Notably, the IFN-γ ELISA tests previously used in rhinoceros for detecting cytokine responses have not been validated (Parsons et al., 2017). Therefore, the validation of the equine IFN-γ ELISA provided a

method that could be used to detect antigen-specific responses, not just to mycobacterial antigens, but could be modified to investigate IFN-γ responses to other antigens and provide a useful technique to study immunological responses in rhinoceros. For example, various haemoparasites have been identified in African rhinoceros, particularly protozoan parasites including trypanosomes, *Theileria bicornis,* and *Babesia bicornis,* which have been shown to cause mortalities (Penzhorn et al., 1994; Nijof et al., 2003; Otiende et al., 2015; Yam et al., 2018). Notably, previous studies have shown the role of IFN-γ in protective immunity to *Babesia* and *Theileria* infections in cattle (Ahmed, 2002). In addition, increased IFN-γ production was associated with resistance to African trypanosomes in experimentally infected mice (Hertz et al., 1998). In another study, high levels of IFN-γ were involved in protection against acute babesiosis in infected mice (Aguilar-Delfin et al., 2002). Therefore, this validated ELISA could be used to investigate IFN-γ response in infection or vaccination of African rhinoceros to provide a basis of understanding the comparative immunology in these species.

Diagnostic IGRA for rhinoceros

In order to use a diagnostic test, it is essential to calculate a diagnostic cut-off value that results in acceptable performance and provides a level of statistical confidence for the purpose of the assay (Šimundić, 2009). In this study, the calculated diagnostic cut-off value for the QFT IGRA provided a method to distinguish between *M. bovis*-infected and uninfected white rhinoceros. In contrast, a previous study only used samples from uninfected rhinoceros to determine a diagnostic cut-off value (Morar et al., 2013). The use of ROC curve analysis in our study provided both an optimal cut-off value and as well as diagnostic accuracy of the test (Zou et al., 2007). Sensitivity and specificity are qualities of a diagnostic test which describe the assay's ability to accurately classify animals as positive and negative; however, it is important to

understand the inverse relationship between sensitivity and specificity when setting the threshold for continuous data and that this may impact interpretation of results (Zou et al., 2007; Šimundić, 2009). Hence, a range of cut-off values was calculated in order to inform the optimal discriminative potential of the IGRA for rhinoceros. Additionally, to increase confidence and diagnostic accuracy, we sought to investigate other biomarkers for inclusion into a panel to diagnose *M. bovis* in African rhinoceros, similar to the approach used for human TB (Ruhwald et al., 2007; Chegou et al., 2018).

Alternative biomarker discovery

This study is the first to describe a potential alternative biomarker to IFN-γ for the diagnosis of *M. bovis* infection in white rhinoceros. An equine mRNA multiplex assay was used for the discovery of alternative cytokine/chemokine biomarkers that could potentially detect immunological changes in *M. bovis*-infected rhinoceros. This approach was selected since it allowed screening of multiple candidate target genes with minimal sample volume and a presumed likelihood of conserved cytokine gene sequences between equine and rhinoceros targets, compared to using a protein-based multiplex approach. Our results, along with previous studies in humans and animals, suggest that *CXCL10*/IP-10 can be used as an early diagnostic biomarker of TB (Whittaker et al., 2008; Roos et al., 2018; Bernitz et al., 2019; Palmer et al., 2020). The current study also highlighted the potential improvement in detection of *M. bovis*-infected rhinoceros using parallel measurements of IFN-γ and IP-10, which increases confidence in test result interpretation. High accuracy, sensitivity, and specificity of the tests are important because of the implications a positive test result has on the farm or park, which could include being put under quarantine. For this reason, identification and validation of a biomarker panel that can reliably distinguish between *M. bovis*-infected and uninfected rhinoceros is of utmost

importance, particularly because early detection of infection is crucial to permit movement of rhinoceros locally, regionally and internationally, with confidence in their infection status. For example, if a rhinoceros from South Africa is imported into Australia, where bTB is eradicated (More et al., 2015), the risk of a false-negative test result could have widespread impacts on their agricultural trade status. In addition to determining current infection status, it will be important to follow infected rhinoceros over time to assess whether they can shed mycobacteria, eliminate, or contain infection, or progress to disease. Therefore, the development and validation of cytokine assays provide the first tools for studying the epidemiology and pathogenesis of bTB in African rhinoceros.

Translational impact of study

Translocation is a frequently applied approach in wildlife management, although this may be associated with risks of spreading disease (Daszak et al., 2000). Since the discovery of *M. bovis*-infected rhinoceros in KNP, the ability to translocate individuals, as a strategy to prevent loss due to poaching, has been curtailed due to the unknown risk of disease spread until an acceptable method of screening candidates could be developed. With the development of the QFT IGRA, the Department of Agriculture, Land Reform, and Rural Development, South Africa, has approved a rhinoceros TB management program using this assay to reduce this risk from animals translocated from KNP.

Disease surveillance is important for understanding the impact and risks associated with a pathogen in a population. Wildlife disease surveillance is based on detection of infection using microbiological and immunological techniques (Artois et al., 2009; Ryser-Degiorgis et al., 2013). In this study, the application of the QFT IGRA was used to assess *M. bovis* infection in the exposed rhinoceros population in KNP, resulting in an estimated 19% prevalence (95% CI

13-25%), based on a positive test result (chapter 3). These initial results provide a foundation for evaluating the presence and impact of *M. bovis* infection in KNP and other rhinoceros populations. Furthermore, tools for surveillance and monitoring of *M. bovis* infections are not only essential for wildlife conservation but also to inform public health and agricultural disease management (Artois et al., 2009). Therefore, the development and validation of a blood-based IGRA, and potential for a diagnostic biomarker panel, for detection of *M. bovis* infection is an important advance for screening individual rhinoceros before movement between zoos or wildlife ranges, as well as contributing to understanding this disease at the population level.

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Chapter 7

Conclusion

This PhD thesis describes the development of novel diagnostic tools (IGRA, GEA and IPRA), which could identify and distinguish between *M. bovis*-infected and uninfected white rhinoceros. For the first time, we validated an IGRA, although its test performance had limitations in accurately interpreting results and the additional biomarker IP-10 was shown to have promise for increasing detection of *M. bovis* infection, suggesting that the use of both IGRA and IPRA in parallel improves diagnostic accuracy in rhinoceros. Therefore, the application of these tests to detect *M. bovis* infection by screening rhinoceros prior to translocation could prevent the spread of bTB and may contribute towards the control of the disease through surveillance and monitoring of their health. In addition, our study highlights a practical approach for the evaluation of CMI responses in white rhinoceros and identifies multiple candidate biomarkers worthy of further investigation for diagnosis of *M. bovis* infection in African rhinoceros. Furthermore, the findings create a platform for researchers to study the immunology of rhinoceros in the context of bTB and other conditions that may impact African rhinoceros species.

Future studies

To successfully advance this research, the diagnostic cut-off values of IGRA and IPRA should be further investigated in a larger population of African rhinoceros with known culture-confirmed infection status. Additionally, samples from *M. bovis* uninfected rhinoceros populations should be included to determine if there are any factors influencing the performance of these diagnostic assays in animals from different locations. Additional studies should be conducted to assess the performance of parallel testing using the IPRA and IGRA in a large cohort with known infection status, which will confirm whether IPRA increases the detection of *M. bovis*-infected animals. Furthermore, a study should explore whether IP-10 is produced

during early infection before IFN-γ becomes detectable and if high levels of IP-10 are indicative of disease in African rhinoceros. Since IP-10 appears to be produced spontaneously in unstimulated whole blood from both infected and uninfected rhinoceros, it will be important to investigate the mechanism behind its production and how this may cofound interpretation of assay results. Lastly, further studies should be performed to evaluate cytokines identified in this study as biomarker candidates for use in whole blood assays in addition to IFN-γ and IP-10, which would facilitate the development of a diagnostic panel for *M. bovis* detection in African rhinoceros. These studies would facilitate the understanding of immunology in the context of *M. bovis* infection as well as applications to other disease diagnosis in these species.

Appendix 1

Supplementary Table 1

House Keeping genes (a) Qiagen RT^2 Profiler PCR Array results for Housekeeping genes for QFT TB and QFT Nil samples obtained from rhinoceros R1.

HK Gene	TB					Nil				
	Rep 1 (CT)	**Rep 2 (CT)**	**Mean (CT)**	**SD**	**CV (%)**	**Rep 1 (CT)**	**Rep 2 (CT)**	**Mean (CT)**	**SD**	**CV (%)**
GAPDH	38.64	ud	n.d.	n.d.	n.d.	43.73	ud	n.d.	n.d.	n.d.
ACTB	22.33	21.48	21.90	0.60	2.73	21.68	21.30	21.49	0.13	0.62
B2M	18.56	17.99	18.28	0.40	2.22	17.60	16.28	16.94	0.47	2.76
HPRT1	25.31	26.55	25.93	0.88	3.38	26.36	26.20	26.28	0.06	0.22
LOC100056766	23.91	21.96	22.94	1.38	6.01	21.65	20.41	21.03	0.44	2.09
HKV			**22.26**					**21.43**		

HK, Housekeeping
TB, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON TB Antigen Tube

Nil, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON Nil Tube

Rep 1, Replicate 1

Rep 2, Replicate 2

CT, Reverse Transcription PCR Threshold Cycle

SD, Standard Deviation

CV, Coefficient of Variation

ud, Undetermined

n.d., Not Done

HKV, Housekeeping value

Valid QFT assay (b) Results for the Qiagen RT2 Profiler PCR Array and QFT Gene Expression Assay for rhinoceros R1. Results regarded as valid as replicate RT2 Profiler measurements have a CV < 10%.

Target Gene	TB					Nil					HKV		ΔCT (TB)	ΔCT (Nil)	ΔΔCT	EFT
	Rep 1 (CT)	Rep 2 (CT)	Mean TB (CT)	SD	CV (%)	Rep 1 (CT)	Rep 2 (CT)	Mean Nil (CT)	SD	CV (%)	TB (CT)	Nil (CT)				
BMP2	39.80	34.28	37.04	3.90	10.53	34.81	35.72	35.27	0.64	1.83	22.26	21.43	14.78	13.83	0.94	0.52
BMP6	32.21	27.81	30.01	3.11	10.38	32.39	26.73	29.56	4.00	13.54	22.26	21.43	7.75	8.13	-0.38	1.30
CCL5	24.46	24.18	24.32	0.19	0.79	24.26	26.83	25.54	1.82	7.11	22.26	21.43	2.06	4.11	-2.05	4.15
CCL8	31.54	29.98	30.76	1.10	3.58	31.78	32.62	32.20	0.59	1.83	22.26	21.43	8.50	10.77	-2.27	4.83
CXCL10	41.44	35.63	38.53	4.11	10.67	42.31	38.91	40.61	2.41	5.93	22.26	21.43	16.27	19.18	-2.91	7.50
GPI	31.68	27.06	29.37	3.27	11.12	27.42	26.86	27.14	0.40	1.47	22.26	21.43	7.11	5.71	1.40	0.38
IL10	46.68	43.33	45.01	2.37	5.27	38.53	40.33	39.43	1.27	3.22	22.26	21.43	22.74	18.00	4.74	0.04
IL1B	42.66	36.69	39.68	4.22	10.64	40.38	33.20	36.79	5.07	13.79	22.26	21.43	17.42	15.36	2.06	0.24
IL23A	29.37	29.15	29.26	0.16	0.53	30.58	28.92	29.75	1.17	3.95	22.26	21.43	7.00	8.31	-1.31	2.49
IL5	40.26	33.37	36.81	4.87	13.24	42.59	38.53	40.56	2.87	7.08	22.26	21.43	14.55	19.13	-4.58	23.89
CXCL8	34.31	34.79	34.55	0.34	0.99	30.91	30.36	30.64	0.39	1.26	22.26	21.43	12.29	9.20	3.08	0.12
CD40LG	27.70	26.72	27.21	0.69	2.55	23.10	27.45	25.27	3.07	12.17	22.26	21.43	4.95	3.84	1.11	0.46
IL20	38.73	34.62	36.67	2.90	7.91	37.73	33.53	35.63	2.97	8.34	22.26	21.43	14.41	14.20	0.22	0.86
IL24	32.77	31.44	32.10	0.94	2.94	29.86	30.12	29.99	0.18	0.60	22.26	21.43	9.84	8.56	1.28	0.41
CCL4	18.25	18.32	18.29	0.05	0.26	19.57	18.80	19.18	0.55	2.85	22.26	21.43	-3.98	-2.25	-1.73	3.31
LTA	29.47	27.54	28.51	1.37	4.79	29.94	29.88	29.91	0.04	0.14	22.26	21.43	6.24	8.48	-2.23	4.69
LTB	27.99	27.74	27.87	0.18	0.65	28.41	27.48	27.94	0.65	2.34	22.26	21.43	5.60	6.51	-0.91	1.88
NAMPT	23.38	21.99	22.68	0.99	4.34	21.01	20.68	20.84	0.24	1.15	22.26	21.43	0.42	-0.59	1.01	0.50
TNFFSF11	33.51	29.51	31.51	2.83	8.98	29.13	28.39	28.76	0.52	1.80	22.26	21.43	9.25	7.33	1.92	0.26
CXCL16	36.65	35.26	35.95	0.98	2.73	33.53	32.20	32.86	0.95	2.88	22.26	21.43	13.69	11.43	2.26	0.21
IL32	37.94	36.22	37.08	1.22	3.28	35.07	37.69	36.38	1.85	5.09	22.26	21.43	14.81	14.95	-0.14	1.10
CCL14	26.56	26.20	26.38	0.25	0.95	26.25	25.10	25.67	0.81	3.17	22.26	21.43	4.12	4.24	-0.12	1.09
NODAL	30.00	27.90	28.95	1.48	5.12	30.18	30.16	30.17	0.01	0.05	22.26	21.43	6.69	8.74	-2.05	4.14
CSF1	24.36	23.53	23.94	0.59	2.46	23.72	23.54	23.63	0.13	0.53	22.26	21.43	1.68	2.20	-0.52	1.43
LIF	32.62	30.36	31.49	1.60	5.07	31.52	29.88	30.70	1.16	3.79	22.26	21.43	9.23	9.27	-0.04	1.03

CCL20	41.25	34.36	37.81	4.87	12.89	32.62	35.98	34.30	2.37	6.92	22.26	21.43	15.55	12.87	2.68	0.16
CXCL13	34.74	33.96	34.35	0.55	1.59	33.62	40.30	36.96	4.72	12.77	22.26	21.43	12.09	15.53	-3.44	10.85
IL3	37.02	35.49	36.25	1.08	2.98	34.56	35.50	35.03	0.66	1.88	22.26	21.43	13.99	13.60	0.39	0.76
TNF	26.54	24.86	25.70	1.18	4.61	26.78	25.62	26.20	0.82	3.12	22.26	21.43	3.43	4.77	-1.33	2.52
TNFRSF11B	38.44	36.29	37.37	1.52	4.07	25.08	23.57	24.33	1.06	4.38	22.26	21.43	15.10	2.89	12.21	0.00
TNFSF13B	31.72	27.79	29.76	2.78	9.34	23.77	23.27	23.52	0.36	1.51	22.26	21.43	7.50	2.08	5.41	0.02
VEGFA	28.50	26.14	27.32	1.67	6.11	23.57	25.12	24.34	1.10	4.50	22.26	21.43	5.06	2.91	2.15	0.23

TB, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON TB Antigen Tube
Nil, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON Nil Tube
HKV, Housekeeping Value
CT, Reverse Transcription PCR Threshold Cycle
ΔCT (TB), Mean TB - HKV (TB)
ΔCT (Nil), Mean Nil - HKV (Nil)
ΔΔCT, ΔCT (TB) - ΔCT (Nil)
EFC, Expression Fold Change
Rep 1, Replicate 1
Rep 2, Replicate 2
SD, Standard Deviation
CV, Coefficient of Variation
ud, Undetermined
n.d., Not Done

Invalid QFT assays (c) Results for the Qiagen RT^2 Profiler PCR Array and QFT Gene Expression Assay for rhinoceros R1. Results regarded as invalid as RT2 Profiler replicates not available or CV < 10%.

Target Gene	QFT TB					QFT Nil				
	Rep 1 (CT)	Rep 2 (CT)	Mean	SD	CV (%)	Rep 1 (CT)	Rep 2 (CT)	mean	SD	CV (%)
BMP4	ud	42.64	n.d.	n.d.	n.d.	46.00	43.76	44.88	1.12	2.49
BMP7	7.97	ud	n.d.	n.d.	n.d.	44.43	45.09	44.76	0.33	0.74
CCL11	ud	ud	n.d.	n.d.	n.d.	33.46	ud	n.d	n.d.	n.d.
CCL13	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
CCL2	38.47	43.73	41.10	2.63	6.40	39.97	ud	n.d	n.d.	n.d.
CCL3	ud	ud	n.d.	n.d.	n.d.	13.71	ud	n.d	n.d.	n.d.
CSF3	ud	37.83	n.d.	n.d.	n.d.	39.20	38.24	38.72	0.48	1.24
CXCL1	ud	7.76	n.d.	n.d.	n.d.	ud	47.55	n.d	n.d.	n.d.
CXCL2	17.85	36.89	27.37	9.52	34.79	36.25	36.24	36.24	0.00	0.01
CXCL6	34.86	14.93	24.89	9.97	40.05	42.93	ud	n.d	n.d.	n.d.
CXCL9	26.90	40.31	33.61	6.71	19.95	48.14	38.34	43.24	4.90	11.33
FASLG	33.28	26.83	30.06	3.23	10.74	31.30	35.97	33.64	2.33	6.94
IFNG	ud	43.28	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
IL12A	34.16	33.77	33.96	0.19	0.57	40.57	ud	n.d	n.d.	n.d.
IL12B	38.15	ud	n.d.	n.d.	n.d.	36.35	21.84	29.10	7.25	24.93
IL13	47.75	ud	n.d.	n.d.	n.d.	47.64	47.02	47.33	0.31	0.66
IL15	ud	43.65	n.d.	n.d.	n.d.	ud	36.30	n.d	n.d.	n.d.
IL16	12.77	46.33	29.55	16.78	56.79	45.26	43.15	44.21	1.05	2.38
IL17A	38.54	32.96	35.75	2.79	7.81	43.57	12.88	28.23	15.34	54.36
IL18	ud	ud	n.d.	n.d.	n.d.	31.64	ud	n.d	n.d.	n.d.
IL1A	7.17	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
IL1RN	34.48	ud	n.d.	n.d.	n.d.	26.08	27.41	26.74	0.66	2.47
IL2	40.35	41.33	40.84	0.49	1.20	37.13	ud	n.d	n.d.	n.d.
IL4	15.32	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.

IL6	ud	35.63	n.d.	n.d.	n.d.	36.16	35.46	35.81	0.35	0.98
TGFB2	42.51	17.88	30.19	12.31	40.78	ud	ud	n.d	n.d.	n.d.
MIF	39.30	26.76	33.03	6.27	18.99	43.59	46.38	44.98	1.40	3.11
IL22	ud	33.54	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
IL7	33.77	36.63	35.20	1.43	4.07	35.42	46.60	41.01	5.59	13.62
THPO	43.96	43.70	43.83	0.13	0.29	39.33	ud	n.d	n.d.	n.d.
IL33	13.21	11.89	12.55	0.66	5.26	9.12	38.99	24.05	14.94	62.10
LOCI00060619	33.71	25.96	29.84	3.88	13.00	33.55	29.57	31.56	1.99	6.30
CCL24	ud	ud	n.d.	n.d.	n.d.	ud	47.59	n.d	n.d.	n.d.
CCL22	19.89	45.02	32.45	12.56	38.72	ud	36.58	n.d	n.d.	n.d.
IL9	46.93	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
IL21	40.12	23.57	31.85	8.28	25.98	28.98	30.86	29.92	0.94	3.15
OSM	ud	ud	n.d.	n.d.	n.d.	ud	48.03	n.d	n.d.	n.d.
TNFSF10	ud	ud	n.d.	n.d.	n.d.	ud	40.33	n.d	n.d.	n.d.
LOC100064468	45.38	ud	n.d.	n.d.	n.d.	5.95	ud	n.d	n.d.	n.d.
CD70	24.52	15.35	19.94	4.58	22.99	ud	40.58	n.d	n.d.	n.d.
IL32	37.94	36.22	37.08	0.86	2.32	35.07	37.69	36.38	1.31	3.60
CCL25	ud	45.79	n.d.	n.d.	n.d.	42.27	ud	n.d	n.d.	n.d.
C5	11.11	44.18	27.64	16.54	59.82	45.60	7.63	26.61	18.98	71.32
IL17F	ud	46.74	n.d.	n.d.	n.d.	36.38	40.24	38.31	1.93	5.04
CCL15	45.59	47.42	46.51	0.92	1.98	47.60	ud	n.d	n.d.	n.d.
CCL16	44.73	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
CCL28	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d	n.d.	n.d.
CNTF	ud	ud	n.d.	n.d.	n.d.	ud	37.68	n.d	n.d.	n.d.
CXCL11	28.01	26.77	27.39	0.62	2.26	35.89	29.06	32.48	3.42	10.52
CCL26	45.64	ud	n.d.	n.d.	n.d.	ud	42.11	n.d	n.d.	n.d.
CCL19	37.26	29.86	33.56	3.70	11.03	29.46	ud	n.d	n.d.	n.d.
CCL17	46.57	ud	n.d.	n.d.	n.d.	34.53	35.80	35.16	0.63	1.80
MSTN	6.91	42.06	24.49	17.58	71.79	33.37	48.68	41.03	7.66	18.67

TB, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON TB Antigen Tube

Nil, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON Nil Tube
HKV, Housekeeping Value
CT, Reverse Transcription PCR Threshold Cycle
Rep 1, Replicate 1
Rep 2, Replicate 2
SD, Standard Deviation
CV, Coefficient of Variation
ud, Undetermined
n.d., Not Done

Appendix II

Supplementary Table 2

House keeping genes (a) Qiagen RT^2 Profiler PCR Array results for Housekeeping genes for samples obtained from rhinoceros R2.

	TB					Nil				
HK Gene	**Rep 1 (CT)**	**Rep 2 (CT)**	**Mean (CT)**	**SD**	**CV (%)**	**Rep 1 (CT)**	**Rep 2 (CT)**	**Mean (CT)**	**SD**	**CV (%)**
GAPDH	43.24	45.72	n.d.	n.d.	n.d	47.90	40.49	n.d	n.d	n.d
ACTB	24.70	24.88	24.79	0.13	0.52	25.77	25.52	25.65	0.17	0.68
B2M	19.86	19.63	19.75	0.16	0.79	19.57	19.75	19.66	0.13	0.65
HPRT1	26.67	26.83	26.75	0.11	0.43	26.44	26.53	26.48	0.07	0.25
LOC100056766	23.80	24.47	24.13	0.48	1.97	24.48	25.32	24.90	0.59	2.37
HKV			**23.85**					**24.17**		

HK, Housekeeping
TB, Sample obtained after 24 hour incubation of whole blood in the QuantiFERON TB Antigen Tube
Nil, Sample obtained after 24 hour incubation of whole blood in the QuantiFERON Nil Tube
Rep 1, Replicate 1
Rep 2, Replicate 2
CT, Reverse Transcription PCR Threshold Cycle
SD, Standard Deviation
CV, Coefficient of Variation
ud, Undetermined
n.d., Not Done
HKV, Housekeeping value

Valid QFT assays (b) Results for the Qiagen RT^2 Profiler PCR Array and QFT Gene Expression Assay for rhinoceros R2. Results regarded as valid as replicate RT2 Profiler measurements have a CV < 10%.

Target Gene	TB					Nil					HKV		ΔCT (TB)	ΔCT (Nil)	ΔΔCT	EFT
	Rep 1 (CT)	Rep 2 (CT)	Mean TB (CT)	SD	CV (%)	Rep 1 (CT)	Rep 2 (CT)	Mean Nil (CT)	SD	CV (%)	TB (CT)	Nil (CT)				
BMP2	34.40	33.56	33.98	0.59	1.74	33.72	32.79	33.26	0.66	1.98	23.85	24.17	10.13	9.09	1.04	0.49
BMP4	34.95	42.31	38.63	5.20	13.46	36.69	36.76	36.72	0.05	0.13	23.85	24.17	14.78	12.55	2.22	0.21
BMP6	27.45	27.21	27.33	0.17	0.63	26.57	26.91	26.74	0.24	0.89	23.85	24.17	3.47	2.57	0.91	0.53
CCL13	41.16	39.99	40.58	0.82	2.03	49.22	40.46	44.84	6.20	13.82	23.85	24.17	16.72	20.67	-3.95	15.44
CCL5	27.01	26.92	26.96	0.06	0.24	27.35	27.72	27.54	0.26	0.96	23.85	24.17	3.11	3.37	-0.26	1.20
CCL8	35.12	34.37	34.75	0.53	1.53	32.79	33.74	33.27	0.67	2.01	23.85	24.17	10.89	9.10	1.80	0.29
CSF3	33.90	34.75	34.32	0.60	1.74	36.60	38.65	37.62	1.45	3.86	23.85	24.17	10.47	13.45	-2.98	7.91
CXCL10	33.81	33.85	33.83	0.03	0.08	35.60	37.07	36.34	1.04	2.85	23.85	24.17	9.98	12.16	-2.19	4.56
CXCL9	36.00	37.24	36.62	0.87	2.39	36.52	36.38	36.45	0.10	0.29	23.85	24.17	12.77	12.28	0.49	0.71
GPI	27.68	28.45	28.06	0.55	1.96	29.59	30.60	30.09	0.72	2.38	23.85	24.17	4.21	5.92	-1.71	3.27
IL10	41.79	36.32	39.05	3.87	9.90	42.77	38.26	40.52	3.19	7.87	23.85	24.17	15.20	16.35	-1.15	2.21
IL12A	44.42	42.89	43.65	1.08	2.48	43.37	44.15	43.76	0.56	1.27	23.85	24.17	19.80	19.59	0.21	0.86
IL17A	32.82	31.83	32.32	0.69	2.15	30.40	29.95	30.17	0.32	1.06	23.85	24.17	8.47	6.00	2.47	0.18
IL1A	41.87	43.65	42.76	1.26	2.94	42.63	41.40	42.01	0.87	2.06	23.85	24.17	18.91	17.84	1.07	0.48
IL1B	32.14	31.74	31.94	0.28	0.89	32.60	33.20	32.90	0.42	1.29	23.85	24.17	8.08	8.73	-0.65	1.57
IL23A	28.12	28.38	28.25	0.18	0.65	27.19	27.76	27.47	0.40	1.46	23.85	24.17	4.40	3.30	1.10	0.47
IL5	36.90	42.46	39.68	3.93	9.92	47.61	44.45	46.03	2.24	4.86	23.85	24.17	15.82	21.86	-6.04	65.58
IL6	32.94	34.48	33.71	1.09	3.23	34.58	34.58	34.58	0.00	0.01	23.85	24.17	9.86	10.41	-0.55	1.47
CXCL8	36.02	36.60	36.31	0.41	1.12	37.30	36.55	36.92	0.53	1.45	23.85	24.17	12.45	12.75	-0.30	1.23
CD40LG	27.27	27.59	27.43	0.22	0.81	26.74	27.12	26.93	0.27	0.99	23.85	24.17	3.58	2.76	0.82	0.57
IL20	33.29	33.01	33.15	0.20	0.59	32.24	32.32	32.28	0.06	0.18	23.85	24.17	9.30	8.11	1.19	0.44
IL24	29.32	29.78	29.55	0.32	1.09	29.70	30.13	29.91	0.31	1.03	23.85	24.17	5.70	5.74	-0.05	1.03
CCL4	25.64	25.77	25.71	0.09	0.35	28.52	28.00	28.26	0.37	1.31	23.85	24.17	1.85	4.09	-2.24	4.72
LTB	27.49	27.77	27.63	0.20	0.71	26.90	27.83	27.37	0.66	2.41	23.85	24.17	3.78	3.20	0.58	0.67

IL22	28.77	28.94	28.85	0.12	0.42	28.32	28.77	28.55	0.32	1.12	23.85	24.17	5.00	4.37	0.62	0.65
THPO	33.37	33.96	33.66	0.42	1.24	33.92	38.60	36.26	3.31	9.12	23.85	24.17	9.81	12.09	-2.28	4.86
TNFFSF11	28.10	28.01	28.06	0.07	0.24	27.21	27.37	27.29	0.11	0.41	23.85	24.17	4.20	3.12	1.08	0.47
CCL24	42.60	40.72	41.66	1.32	3.18	47.81	47.40	47.60	0.29	0.60	23.85	24.17	17.81	23.43	-5.63	49.41
IL9	43.33	39.12	41.22	2.98	7.22	47.12	39.54	43.33	5.36	12.36	23.85	24.17	17.37	19.16	-1.79	3.45
TNFSF10	46.96	49.89	48.42	2.08	4.29	48.49	47.14	47.82	0.95	1.99	23.85	24.17	24.57	23.64	0.93	0.53
CD70	25.06	25.20	25.13	0.10	0.41	24.71	24.90	24.81	0.13	0.52	23.85	24.17	1.28	0.63	0.64	0.64
IL32	27.77	27.48	27.62	0.21	0.75	26.77	26.83	26.80	0.04	0.15	23.85	24.17	3.77	2.63	1.14	0.45
C5	25.98	25.99	25.98	0.01	0.02	26.62	27.24	26.93	0.44	1.62	23.85	24.17	2.13	2.76	-0.63	1.54
IL17F	36.41	37.29	36.85	0.62	1.68	40.88	37.57	39.23	2.34	5.97	23.85	24.17	13.00	15.05	-2.06	4.16
NODAL	31.71	32.25	31.98	0.38	1.19	30.76	31.96	31.36	0.85	2.71	23.85	24.17	8.13	7.19	0.94	0.52
CCL16	30.90	31.79	31.34	0.63	2.00	31.55	33.26	32.40	1.21	3.73	23.85	24.17	7.49	8.23	-0.74	1.67
CSF1	35.91	38.03	36.97	1.50	4.05	36.13	38.28	37.20	1.52	4.08	23.85	24.17	13.11	13.03	0.08	0.95
LIF	41.23	40.48	40.85	0.53	1.30	40.92	37.67	39.30	2.30	5.84	23.85	24.17	17.00	15.13	1.87	0.27
CNTF	26.47	26.82	26.64	0.25	0.93	25.78	25.88	25.83	0.07	0.26	23.85	24.17	2.79	1.66	1.13	0.46
CXCL11	40.67	41.18	40.93	0.36	0.88	38.49	38.67	38.58	0.13	0.34	23.85	24.17	17.07	14.41	2.66	0.16
CCL20	39.69	43.84	41.77	2.93	7.02	48.02	44.42	46.22	2.55	5.51	23.85	24.17	17.91	22.05	-4.13	17.54
CXCL13	27.67	27.75	27.71	0.05	0.19	26.90	27.48	27.19	0.41	1.50	23.85	24.17	3.86	3.02	0.84	0.56
IL3	35.91	38.67	37.29	1.95	5.22	37.87	36.01	36.94	1.31	3.55	23.85	24.17	13.43	12.77	0.66	0.63
CCL26	29.29	29.77	29.53	0.34	1.16	29.61	31.01	30.31	0.99	3.27	23.85	24.17	5.67	6.14	-0.47	1.38
CCL19	28.51	29.44	28.97	0.65	2.26	29.67	30.75	30.21	0.77	2.54	23.85	24.17	5.12	6.04	-0.92	1.89
CCL17	24.70	24.88	24.79	0.13	0.52	25.77	25.52	25.65	0.17	0.68	23.85	24.17	0.93	1.48	-0.54	1.46
MSTN	19.86	19.63	19.75	0.16	0.79	19.57	19.75	19.66	0.13	0.65	23.85	24.17	-4.11	-4.51	0.41	0.76
TNF	43.24	45.72	44.48	1.75	3.94	47.90	40.49	44.19	5.24	11.85	23.85	24.17	20.63	20.02	0.60	0.66
TNFRSF11B	26.67	26.83	26.75	0.11	0.43	26.44	26.53	26.48	0.07	0.25	23.85	24.17	2.90	2.31	0.59	0.67
TNFSF13B	23.80	24.47	24.13	0.48	1.97	24.48	25.32	24.90	0.59	2.37	23.85	24.17	0.28	0.73	-0.45	1.37
VEGFA	34.76	36.70	35.73	1.37	3.85	34.75	35.29	35.02	0.38	1.08	23.85	24.17	11.88	10.85	1.03	0.49

TB, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON TB Antigen Tube
Nil, Sample obtained after 6 hour incubation of whole blood in the QuantiFERON Nil Tube

HKV, Housekeeping Value
CT, Reverse Transcription PCR Threshold Cycle
ΔCT (TB), Mean TB - HKV (TB)
ΔCT (Nil), Mean Nil - HKV (Nil)
ΔΔCT, ΔCT (TB) - ΔCT (Nil)
EFC, Expression Fold Change
Rep 1, Replicate 1
Rep 2, Replicate 2
SD, Standard Deviation
CV, Coefficient of Variation
ud, Undetermined
n.d., Not Done

Invalid QFT assays (c) Results for the Qiagen RT^2 Profiler PCR Array and QFT Gene Expression Assay for rhinoceros R2. Results regarded as invalid as RT2 Profiler replicates not available or CV < 10%.

Target Gene	QFT TB					QFT Nil				
	Rep 1 (CT)	Rep 2 (CT)	Mean	SD	CV (%)	Rep 1 (CT)	Rep 2 (CT)	mean	SD	CV (%)
BMP7	ud	ud	n.d.	n.d.	n.d.	44.57	ud	n.d.	n.d.	n.d.
CCL11	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
CCL2	43.63	ud	n.d.	n.d.	n.d.	38.03	39.21	38.62	0.59	1.52
CCL3	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
CXCL1	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
CXCL2	36.16	44.57	40.37	4.20	10.41	44.32	42.82	43.57	0.75	1.73
CXCL6	25.17	ud	n.d.	n.d.	n.d.	46.13	ud	n.d.	n.d.	n.d.
FASLG	42.40	37.27	39.84	2.57	6.44	35.64	45.85	40.74	5.11	12.53
IFNG	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
IL12B	44.61	40.44	42.52	2.09	4.91	48.90	39.56	44.23	4.67	10.56
IL13	44.21	45.96	45.08	0.87	1.94	ud	45.90	n.d.	n.d.	n.d.
IL15	ud	49.99	n.d.	n.d.	n.d.	44.69	42.55	43.62	1.07	2.46
IL16	46.54	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
IL18	42.97	38.74	40.86	2.12	5.18	48.79	ud	n.d.	n.d.	n.d.
IL1RN	37.85	41.89	39.87	2.02	5.06	46.87	37.40	42.13	4.74	11.24
IL2	ud	43.35	n.d.	n.d.	n.d.	ud	40.51	n.d.	n.d.	n.d.
IL4	42.07	44.84	43.46	1.38	3.18	36.75	ud	n.d.	n.d.	n.d.
TGFB2	46.78	ud	n.d.	n.d.	n.d.	ud	48.00	n.d.	n.d.	n.d.
MIF	29.91	42.33	36.12	6.21	17.19	45.96	41.94	43.95	2.01	4.57
LTA	ud	49.74	n.d.	n.d.	n.d.	ud	41.44	n.d.	n.d.	n.d.
IL7	41.65	40.96	41.30	0.35	0.84	ud	ud	n.d.	n.d.	n.d.
NAMPT	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
IL33	45.90	48.20	47.05	1.15	2.45	ud	ud	n.d.	n.d.	n.d.
LOCI00060619	ud	ud	n.d.	n.d.	n.d.	ud	48.00	n.d.	n.d.	n.d.
CXCL16	48.97	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.

CCL22	44.19	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
IL21	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
OSM	39.31	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
LOC100064468	45.19	ud	n.d.	n.d.	n.d.	ud	40.91	n.d.	n.d.	n.d.
CCL25	49.26	ud	n.d.	n.d.	n.d.	43.53	ud	n.d.	n.d.	n.d.
CCL15	ud	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
CCL14	49.10	ud	n.d.	n.d.	n.d.	ud	ud	n.d.	n.d.	n.d.
CCL28	ud	45.55	n.d.	n.d.	n.d.	49.27	ud	n.d.	n.d.	n.d.

TB, Sample obtained after 24 hour incubation of whole blood in the QuantiFERON TB Antigen Tube
Nil, Sample obtained after 24 hour incubation of whole blood in the QuantiFERON Nil Tube
CT, Reverse Transcription PCR Threshold Cycle
Rep 1, Replicate 1
Rep 2, Replicate 2
SD, Standard Deviation
CV, Coefficient of Variation
ud, Undetermined
n.d., Not Done