The evaluation of novel biomarkers and antigens for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

Wynand Johan Goosen

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Supervisors: Prof Michele Miller and Dr S.D.C. Parsons

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This dissertation includes 4 original papers published in peer reviewed journals or books and 1 chapter unpublished work (Chapter 3). The development and writing of the papers (published and unpublished) were the principal responsibility of myself and for each of the cases where this is not the case a declaration is included in the dissertation indication the nature and extent of the contributions of co-authors.

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Summary

Mycobacterium bovis (*M. bovis*) forms part of the *Mycobacterium tuberculosis* complex (MTC), a group of genetically related bacteria that causes tuberculosis in humans and animals. African buffaloes (*Syncerus caffer*) are maintenance hosts of *M. bovis*, the causative organism of bovine tuberculosis (bTB). Since this species acts as a bTB reservoir for a wide range of domestic and wildlife species, the detection of *M. bovis*-infected animals is essential to control spread of the disease. However, diagnostic assays used for bTB management and control programmes, such as the single intradermal comparative tuberculin test (SICTT) and commercially available interferon-gamma release assays (IGRAs), are still believed to be sub-optimal for the diagnosis of bTB in bovids. A potential approach to improving detection of *M. bovis* infection could be the use of novel diagnostic antigens or the identification of alternative or ancillary biomarkers to interferon-gamma (IFN-γ). The studies presented in this dissertation aim to identify, develop and evaluate novel approaches for improving the detection of *M. bovis* infection in African buffaloes.

The first objective was to evaluate the performance of two new commercially available IGRAs, the Bovigam® PC-EC assay and the Bovigam® PC-HP assay, for the first time in buffaloes and compare their performance to that of two versions of an adapted human IGRA, the modified QuantiFERON® TB-Gold (mQFT) assay. In addition, the effect of increased blood incubation time on sensitivity of the mQFT assay was assessed along with whether centrifugation was a necessary step prior to harvesting the plasma fraction. Furthermore, the relative sensitivities of Bovigam® assays, a modified Bovigam assay that contains an additional stimulation criteria with *Mycobacterium fortuitum* tuberculin and the SICTT were compared in identified *M. bovis*-infected buffaloes. Combinations of these assays were evaluated to identify the optimal test algorithm (i.e., highest sensitivity) for detection of bTB in African buffaloes. Commercially available bovine ELISAs as well as a human IP-10 ELISA were used to identify selected

candidate biomarkers of *M. bovis*-specific immune activation and evaluate their diagnostic utility. Lastly, this study investigated what effect storing stimulated whole blood plasma under various conditions would have on the diagnostic performance of IP-10 assays in African buffaloes.

Agreement between the Bovigam® PC-EC and the Bovigam® PC-HP assays was high (κ = 0.86, 95% CI 0.75–0.97) and these detected the greatest number of test-positive animals suggesting that they were the most sensitive assays. Agreement between two versions of the mQFT assay was also high (κ=0.88, 95% CI 0.77-0.98); however, all buffaloes with discordant mQFT results (n=6), including 3 confirmed *M. bovis*-infected animals, were positive at 30 hours incubation and negative at 20 hours. These results suggest that the mQFT assay is more sensitive using the longer incubation period (i.e., 30 hours). There were no significant differences in IFN-γ concentrations in plasma samples harvested from QFT tubes prior to and after centrifugation, a step which may facilitate plasma sampling over multiple incubation times to improve sensitivity. When the test performance of IFN-γ assays was compared in buffaloes, the Bovigam PPD assay had a relative sensitivity between 91-93% while the sensitivity of the modified PPD assay was between 90-91%. Diagnostic sensitivity was improved by combining one or more IGRA together with the SICTT (95-100%). Investigation of alternative biomarkers to IFN-γ found that IP-10 levels were significantly increased in antigen-stimulated blood samples from *M. bovis*-infected buffaloes ($p < 0.0001$). In addition, IP-10 was produced in far greater abundance than IFN-γ, demonstrating its potential as a novel biomarker of bTB in buffaloes. Moreover, using IP-10, agreement between the mQFT assay and the Bovigam assays was increased while the excellent agreement between the Bovigam assays was retained. Since transport and storage of buffalo blood samples are important considerations for development of diagnostic tests for bTB, the effects of heat-inactivation and storage on protein saver cards (PSCs) on IP-10 performance were assessed. Incubation of

plasma at 65 °C for 20 min caused no statistically significant loss of IP-10 and this protein could be quantified in plasma stored on PSCs for 2 and 8 weeks. Moreover, for all storage conditions, IP-10 retained its excellent diagnostic characteristics.

Diagnostic tests for bTB in wildlife are limited by the lack of species-specific immunological reagents. Logistical constraints make validation of tests, using novel biomarkers such as IP-10, more difficult. These limitations may preclude definitive conclusions about the diagnostic utility of IP-10 in buffaloes. Some of the factors that may have influenced our conclusions include: 1) limited sample size which could affect calculation of appropriate IP-10 test cut-off values; 2) use of antigen-specific whole-blood incubation assay protocols which had been optimized for measurement of IFN-γ rather than IP-10, and 3) lack of post-mortem samples to confirm *M. bovis* infection status in Bovigam-negative IP-10-positive buffaloes.

In conclusion, both the Bovigam® PC-EC assay and the Bovigam® PC-HP assay were shown to be more sensitive than either the SICTT or mQFT assay in its current format. Plasma collected from the QFT tubes prior to centrifugation could be reliably utilized in this assay. Moreover, increasing the blood incubation time from 20h to 30h increased the mQFT assay's sensitivity. In an additional study, both the Bovigam PPD assay and modified PPD assay displayed greatest sensitivity for the detection of *M. bovis*-infected buffaloes. The SICTT detected additional IGRA-negative animals and maximum sensitivity was attained when these assays were used in combination. In addition to IFN-γ, IP-10 appears to be a useful marker of immune activation in buffaloes when using a commercially available IP-10 bovine ELISA. IP-10 shows promise as a diagnostic biomarker in *M. bovis*-infected buffaloes and measurement of IP-10 increased the sensitivity over conventional IGRAs. IP-10 could be measured in plasma stored on PSCs; however, the sensitivity of tests utilizing such samples was reduced with increased storage time. Plasma samples could be heated to 65 ºC for 20 min with no degradation of IP-10, demonstrating the thermal stability of this cytokine.

These findings are supported by previous cattle studies that advocate the parallel use of the SICTT and the Bovigam PPD assay. Moreover, these findings also highlight the potential application of IP-10 for the diagnosis of bTB in African buffaloes. Improved sensitivity of *M. bovis*-specific IGRAs is a significant advantage of using IP-10 as a preferred biomarker in this species. Other advantages of IP-10 are its thermal tolerance and stability on PSCs. These characteristics facilitate movement of diagnostic samples by permitting heat-inactivation of potential pathogens in plasma, and transport of samples by conventional delivery methods, respectively.

Opsomming

Mycobacterium bovis (*M. bovis*) vorm deel van die *Mycobacterium tuberculosis*-kompleks (MTK), ŉ groep genetiesverwante bakterieë verantwoordelik vir tuberkulose in mense en diere. Die Kaapse buffel (*Syncerus caffer*) is ŉ reservoir (instandhoudingsgasheer) vir *M. bovis*, die bakterie verantwoordelik vir beestuberkulose (bTB). Omdat hierdie spesie as reservoir van infeksie vir ŉ verskeidenheid van plaasmak- en wilde diere dien, is die suksesvolle diagnose van *M. bovis*-geïnfekteerde diere noodsaaklik om die verspreiding van die siekte te kan beheer. Daar word egter nog steeds geglo dat die diagnostiese toetse wat gebruik word gedurende bTB-beheerprogramme, soos die enkele intradermale vergelykende tuberkulien-toets ("single intradermal comparative tuberculin test"; SICTT) en kommersieel beskikbare interferon-gamma-vrystellingstoetse ("interferon gamma release assays"; IGRA's), nog steeds suboptimaal is vir die diagnose van bTB in beesverwante hoefdiere. ŉ Moontlike benadering tot die verbetering van sulke toetse mag dalk die gebruik wees van nuwe antigene of die identifisering van alternatiewe of aanvullende biomerkers anders as interferon-gamma (IFN-γ). Hierdie proefskrif bespreek studies wat ontwerp is om nuwe en oorspronklike benaderings te identifiseer, te ontwikkel en te evalueer met die doel om die diagnose van *M. bovis*-infeksie in Kaapse buffels te verbeter.

Die eerste doelwit was om die diagnostiese potensiaal van twee nuwe, beskikbare, kommersiële IGRA's, die Bovigam® PC-EC-toets en die Bovigam® PC-HP-toets, vir die eerste keer in buffels te evalueer, asook om hulle prestasie te vergelyk met diè van ŉ gemodifieerde mens-IGRA ("modified QuantiFERON® TB-Gold (mQFT) assay"). Verder is die uitwerking van ŉ verlengde inkubasietyd van bloed op die sensitiwiteit van die mQFT-toets bepaal, asook die noodsaaklikheid van sentrifugering voor isolering van die plasmafraksie van die bloed. ŉ Opvolgstudie het die relatiewe sensitiwiteit van die Bovigam-toetse, ŉ gemodifiseerde Bovigam-toets en die SICTT in geïdentifiseerde *M. bovis*-geïnfekteerde buffels vergelyk. Die

gebruik van hierdie toetse in kombinasie is ook geëvalueer in ń poging om die optimale toetsalgoritme vir die diagnose van *M. bovis*-geïnfekteerde buffels te identifiseer. Kommersieel beskikbare bees-ELISA's, asook ń mens-IP-10-ELISA is verder gebruik om geselekteerde kandidaatbiomerkers van *M. bovis*-spesifieke immuunaktivering te identifiseer, en om hulle diagnostiese potensiaal te evalueer. Laastens het hierdie studie ook bepaal wat die uitwerking van die stoor van bloedplasma onder verskillende omstandighede op die diagnostiese potensiaal van die gebruik van IP-10-toetse in buffels sal wees.

Ooreenstemming tussen die Bovigam® PC-EC- en die Bovigam® PC-HP-toetse was hoog (κ = 0.86, 95% CI 0.75–0.97) en hierdie toetse het die hoogste aantal toetspositiewe diere geïdentifiseer, wat aandui dat hulle die mees sensitiewe toetse was. Ooreenstemming tussen die mQFT-toetse was ook hoog (κ=0.88, 95% CI 0.77-0.98). Alle buffels met verskillende mQFT-resultate (n=6), asook 3 definitiewe *M. bovis*-geïnfekteerde diere, was egter positief na 30 uur inkubasie, maar negatief na 20 uur. Hierdie resultate impliseer dat die mQFT-toets die mees sensitiewe van die twee is wanneer langer inkubasieperiodes gebruik word. Verder is daar ook geen betekenisvolle verskil in IFN-γ-konsentrasies waargeneem tussen plasmafraksies geïsoleerd vanaf QFT-buise voor en na sentrifugering nie. Hierdie bevinding beteken dat verskillende inkubasieperiodes in ń poging om die toetssensitiwiteit te verbeter, aanvaarbaar is. Wanneer die betroubaarheid van die IFN-γ-toetse vergelyk is in buffels, het die Bovigam PPD-toets ń relatiewe sensitiwiteit van tussen 91-93% getoon, terwyl die sensitiwiteit van die gemodifieerde PPD-toets tussen 90-91% was. Diagnostiese sensitiwiteit is verder verbeter deur een of meer IGRA's met die SICTT te kombineer (95-100%). Verdere navorsing oor alternatiewe biomerkers anders as IFN-γ in ń poging om die sensitiwiteit van IGRA's te verhoog, het getoon dat IP-10-vlakke ń betekenisvolle verhoging in antigeen-gestimuleerde bloedmonsters van *M. bovis*-geïnfekteerde buffels toon ($p < 0.0001$). Daar is ook aangetoon dat IP-10 in groter hoeveelhede as IFN-γ geproduseer is, ń eienskap wat grootliks kan bydra

tot IP-10 se potensiaal as ń nuwe biomerker vir die diagnose van bTB in buffels. In aansluiting hierby is vasgestel dat deur IP-10 te gebruik, daar ń verbetering in ooreenstemming waargeneem is tussen die mQFT-toets en die Bovigam-toetse, terwyl die uitstekende ooreenstemming tussen die Bovigam-toetse onveranderd gebly het. Aangesien die vervoer en stoor van buffelbloed ń belangrike aspek is om by die ontwikkeling van diagnostiese toetse vir bTB in ag te neem, is die uitwerking van hitte-inaktivering en die stoor van plasma op proteïenstoorkaarte ("protein saver cards"; PSC's) op IP-10 se betroubaarheid as biomerker ondersoek. Daar is vasgestel dat die inkubasie van bloedplasma teen 65 °C vir 20 min geen betekenisvolle vernietiging van IP-10 getoon het nie en dat IP-10 nog steeds suksesvol gemeet kon word in plasma nà storing op PSC's vir 2 weke en 8 weke onderskeidelik. Verder het IP-10 uitstekende diagnostiese potensiaal onder alle stoortoestande behou.

Die onbeskikbaarheid van spesiesspesifieke immunologiese reagense beperk diagnostiese toetse vir bTB in wild grootliks. Verder is die validering van diagnostiese toetse, deur die gebruik van nuwe biomerkers soos IP-10, bemoeilik deur sekere logistieke beperkings. Sulke beperkings sluit in: 1) beperkte dieregetalle in ń studie wat die akkurate berekeninge van ń toepaslike afsnypunt vir IP-10 kan benadeel; 2) die gebruik van antigeenspesifieke toetsprotokolle vir bloedinkubasie wat alleenlik geoptimiseer is vir die meting van IFN-γ en nie noodwendig vir IP-10 nie, en 3) die gebrek aan nadoodse monsters vir die bevestiging van *M. bovis*-infeksie in Bovigam-negatiewe IP-10-positiewe buffels.

Ten slotte, die Bovigam® PC-EC-toets en die Bovigam® PC-HP-toets was meer sensitief as albei die SICTT- of die mQFT-toetse in hulle huidige formaat. Bloedplasma verkry vanaf die QFT-buise voor sentrifugering kon met vertroue gebruik word in die mQFT-toets. Verlenging van die inkubasietyd van bloed in die QFT-buise van 20 uur tot 30 uur, het gelei tot die aansienlike verbetering van die mQFT-toets se sensitiwiteit. Volgens die resultate van ń aanvullende studie het die Bovigam PPD-toets, asook ń gemodifiseerde PPD-toets die beste

sensitiwiteit vir die identifisering van *M. bovis*-geïnfekteerde buffels getoon. Die SICTT het addisionele IGRA-negatiewe diere geïdentifiseer en die hoogste sensitiwiteit is bereik wanneer hierdie toetse in kombinasie gebruik is. Addisioneel tot IFN-γ, wil dit ook voorkom of IP-10 as ń nuttige merker van immuunaktivering in buffels kan dien deur gebruik te maak van ń IP-10-bees-ELISA, wat kommersieel beskikbaar is. IP-10 toon verder goeie potensiaal as ń diagnostiese biomerker in *M. bovis*-geïnfekteerde buffels en die gebruik daarvan dui ook op ń verhoging in die sensitiwiteit van konvensionele IGRA's. IP-10 kon suksesvol gemeet word in plasma gestoor op PSC's, alhoewel die sensitiwiteit verminder het met ń verlengde stoortydperk in die toetse op hierdie monsters. Plasma verhit tot 65 ºC vir 20 min het geen degradering van IP-10 getoon nie en hierdie eienskap is ń verdere demonstrasie van die hittestabiliteit van hierdie sitokien.

Hierdie bevindinge word ondersteun deur vorige studies in beeste wat die gesamentlike gebruik van die SICTT- en die Bovigam PPD-toets sterk aanbeveel. Die huidige studie het ook grootliks die potensiaal van IP-10 vir die diagnose van bTB in die Kaapse buffel beklemtoon. Die verbetering in sensitiwiteit van *M. bovis*-spesifieke IGRA's, deur die gebruik van IP-10, is ń betekenisvolle voordeel van die gebruik van hierdie sitokien as voorkeurbiomerker in hierdie spesie. ń Verdere voordeel van diè sitokien is sy hittetoleransie en stabiliteit op PSC's. Hierdie nuttige eienskappe mag daarop dui dat diagnostiese monsters veilig vervoer kan word deur potensiële patogene in die plasma deur hitte te inaktiveer, of deur alternatiewelik gebruik te maak van konvensionele vervoer- of afleweringsmetodes.

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Audaces fortuna juvat – Fortune favours the bold

Abbreviations

ºC	Degrees Celsius
µg	Microgram
µl	Microliter
µM	Micro Molar
AA	Amino acid
Ab	Antibody
AUC	Area under the curve
BB	Blocking buffer
BCG	Bacillus Calmette-Guérin
BEC	Bovigam® EC assay
BHP	Bovigam® HP assay
bp	Base pair
BSA	Bovine serum albumin
bTB	Bovine tuberculosis
CD26	Cluster of differentiation 26
cDNA	Complimentary DNA
CFP-10	Culture filtrate protein
CMI	Cell-mediated immunity
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide Triphosphate
DTH	Delayed type hypersensitivity
EC	ESAT-6 and CFP-10 peptides
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target
GEA	Gene expression assay
h	Hours
H_2O_2	Hydrogen peroxide
HP	ESAT-6, CFP-10, Rv3615 and 3 additional peptides
IFN-γ	Interferon gamma
IGRA	Interferon gamma release assay

IL	Interleukin
IL1-RA	Interleukin 1 receptor antagonist
IP-10	Interferon gamma-induced protein 10
k	Cohen's kappa coefficient
kb	Kilo base
kDa	Kilo Daltons
M	Molar
mAb	Monoclonal antibody
MCP-1	Monocyte chemoattractant protein-1
MCP-2	Monocyte chemoattractant protein-2
MCP-3	Monocyte chemoattractant protein-3
MGIT™	Mycobacteria Growth Indicator Tube
MIG	Monokine induced by interferon gamma
min	Minutes
ml	Millilitre
mm	Millimetre
MMPs	Matrix metalloproteases
mQFT	modified QFT
mRNA	Messenger Ribonucleic acid
MTC	Mycobacterium tuberculosis complex
NCBI	National Centre for Biotechnology Information
ng	Nano gram
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pg	Pico gram
PHA	Phytohaemagglutinin
PPDav	Avian purified protein derivative
PPDbov	Bovine purified protein derivative
PSC	Protein saver card
QFT	QuantiFeron TB test

RD1	Region of difference 1
RNA	Ribonucleic acid
ROC	Receiver operating curve
RT	Room Temperature
s	Seconds
SCITT	Single intradermal comparative tuberculin test
SFT	Skin Fold Thickness
TB	Tuberculosis
Th0	Naïve T-cell
Th1	T-helper type-1
Th2	T-helper type-2
TST	Tuberculin skin test
USA	United States of America
UTR	Untranslated region
WB	Whole-blood
ΔOD	Delta-OD

Chapter 1 – General Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (bTB) in many free-ranging mammals, captive wildlife and livestock (1). This species forms part of the *Mycobacterium tuberculosis* complex (MTC) that consists of a genetically related group of bacteria that can cause tuberculosis in humans or many other species (2–4). *M. bovis* is known to have the widest host range of all the MTC bacteria (5) and infections have previously been reported in more than 60 mammal species worldwide (6). Garnier *et al.*, (2003) reported a worldwide annual loss to agriculture of $3 billion due to the increasing wildlife-livestock-human interfaces. In Africa, buffaloes (*Syncerus caffer*) are one of the most important maintenance hosts of bTB and spill-over of this pathogen to other wildlife or domestic cattle may not only result in reduced productivity or death of these animals (7), but also poses a serious zoonotic risk. In South Africa, the spill-over from infected buffaloes to other wildlife species may occur through predation, scavenging or contaminated environments (8). The occurrence of wildlife reservoirs of bTB such as African buffaloes, European badgers (*Meles meles*), brush-tail possum (*Trichosurus vulpecula*) and white-tailed deer (*Odocoileus virginianus*) complicates the eradication of this disease by posing a threat for reinfection of livestock (1).

Following infection with *M. bovis*, animals mount a cell mediated immune (CMI) response. This response is mediated by activated antigen-specific T-lymphocytes that might function as effector cells, orchestrate an inflammatory reaction, or recruit cells such as monocytes, neutrophils, macrophages, dendritic cells through secretion of certain cytokines. Key cytokines include interferon gamma (IFN-γ), interleukin (IL)-2, tumour necrosis factor (TNF)-α and TNF-β (9). The host response in the infected animal is a balance between effective inflammatory and immunological responses that contain and eliminate pathogens while minimizing tissue damage. Pro and anti-inflammatory cytokines play an essential role in

mediating the outcome of the host's response to pathogenic mycobacteria and pathogenesis of disease (10).

bTB is a chronic progressive disease and animals may be infected for months or years before developing lesions in affected tissues (11). Disease outcome in *M. bovis* infected animals is variable, and influenced by host species, behaviour, age, route of infection, infectious dose, concurrent disease states, and environmental conditions (12). In cattle, the most common routes of exposure are inhalation of aerosols containing *M. bovis* bacilli from infected animals or contact with contaminated pastures and/or water (13). Although typically asymptomatic in early infection, animals that develop advanced disease may show clinical signs such as coughing, debilitation, emaciation, and lagging behind the herd (14). For cattle and buffaloes, tuberculous lesions occur primarily in the thoracic cavity, specifically in the lungs, bronchial and mediastinal lymph nodes (15,16). Other commonly affected areas include lymphoid tissue in the head region such as the retropharyngeal lymph nodes, submandibular lymph nodes and tonsillar tissue (12,16). The slow development of lesions and location makes direct *ante-mortem* detection of *M. bovis* challenging. This complicates arriving at a definitive diagnosis of bTB, which is important for disease management and control.

Current control strategies in South Africa aim to effectively manage and prevent the spread of bTB in livestock by using a test and slaughter program (17). The program relies primarily on the single comparative intradermal tuberculin test (SCITT), a test that measures a delayed type hypersensitivity response following the intradermal injection of mycobacterial-specific antigens, i.e. *M. bovis* purified protein derivative (PPDbov) and *M. avium*-PPD (PPDav). Animals that react to PPDbov in this test are culled from the herd. Changes in skin thickness are measured three days after tuberculin injection of either the caudal fold (CFT) or opposite sides of the neck (SICTT). A positive skin test result is determined by an increase of 4 mm or greater in skin fold thickness (SFT) measurements at the PPDbov injection site compared to

the PPDav site at 72 hours. If the differential measurement is between 2 mm and 4 mm, the animal is considered to have a suspect test result (18). Even though the SICTT reportedly lacks both sensitivity and specificity (19,20), countries such as New Zealand, Australia and the U.S.A. have effectively controlled bTB in their livestock using tuberculin skin testing as the basis of their programs (21,22). This approach is complicated in countries such as South Africa and the U.K. where there are free-ranging maintenance hosts of this disease (23,24).

The SICTT has been used in African buffaloes for over 20 years, although not formally validated in this species (25). Unlike cattle, buffaloes require two chemical immobilizations to perform this test; once when administrating the injections and again when reading the dermal reactions. Buffaloes are typically confined to bomas for at least three days to minimize the cost and time associated with recapture. For wild animals such as buffaloes, the consequences of confinement are increased stress, risk of trauma, and possibly death. Furthermore, immunological sensitization to PPD antigens may occur following the SICTT, which prevents repeat testing for at least 90 days to avoid false positive reactions. Regulations require that any buffalo that will be moved to have a negative skin test. However, if there are reactors in a herd, retesting is necessary to confirm sequential negative results, leading to long delays before transport can occur. Extended confinement and prolonged testing has been highly problematic for the wildlife industry and conservation programs (19).

Due to the costs, risks to animals and staff, and logistical constraints of using the tuberculin skin test, novel diagnostic strategies are needed to effectively manage and prevent the spread of *M. bovis* in wildlife and livestock. One strategy would be early detection of infected animals, ideally prior to a stage in which they start shedding, to prevent the transmission to other animals and geographic spread of disease by natural or intentional movement of animals (26,27). One such way is use of the interferon-gamma (IFN-γ) release assay (IGRA) that measures the *in vitro* secretion of IFN-γ by lymphocytes in response to pathogen-specific antigen stimulation.

IGRAs quantify the production of IFN-γ in plasma supernatant using a sandwich enzyme-linked immunosorbent assay (ELISA) that utilizes either species-specific or cross-reactive monoclonal or polyclonal anti-IFN-γ antibodies (20). IGRAs have been shown to be more sensitive than the SICTT and have the added advantages of rapid results (usually within 24 hours) and single handling of the animal (20,28). A commercial IGRA, the Bovigam® assay (Prionics), is currently used as an ancillary test to the SICTT for the detection of bTB infection in cattle (29). Limitations of the Bovigam® assay, which uses PPDs as test antigens, are higher cost, logistical constraints such as sample handling for the incubation of blood in 96 well plates, and in return for high sensitivity, reduced specificity (30,31). More recently, the specificity of this IGRA has been improved by the inclusion of *M. bovis*-specific peptides, such as the 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10), both highly expressed by *M. bovis* and *M. tuberculosis,* but absent from *M. bovis* Bacillus Calmette-Guérin (BCG) strains and other non-tuberculous mycobacteria (29,32). Similarly, a human TB test, the QuantiFERON TB-Gold assay (Qiagen), which utilizes ESAT-6 and CFP-10, has been modified for bTB diagnosis in African buffaloes and has been shown to be a practical alternative to the Bovigam® assay (27). However, IGRAs are still believed to be suboptimal for bTB diagnosis in bovids (11). Therefore, identification of biomarkers other than IFN-γ, either as alternatives or ancillary markers of bTB infection, may increase the sensitivity of *M. bovis*-specific assays (33–36).

In humans, candidate immunological biomarkers of *M. tuberculosis* infection include the chemokine IFN-γ-induced protein 10 (IP-10) (37), monokine induced by IFN-γ (MIG) (38,39), monocyte chemoattractant protein (MCP)-1, MCP-2, MCP-3 and IL1-receptor antagonist (IL1-RA) (37,39). In patients with active tuberculosis, Ruhwald *et al*. (2009) reported significantly higher concentrations of IP-10, IL1-RA, MCP-1, MCP-2 and MCP-3 compared to IFN-γ in *M. tuberculosis*-antigen stimulated whole blood, showing the diagnostic potential of all five

biomarkers for TB infection. In this study, IP-10 and MCP-2 were the most promising cytokines. Furthermore, IP-10 has been shown to be a very stable protein in human blood plasma, stored at room temperature, as well as dried on filter membrane paper and transported via conventional postal services (40,41). MIG has also been identified as a novel biomarker of TB infection in a study of BCG-vaccinated controls and TB patients (38). There was a significant correlation between MIG and IFN-γ concentrations in these patients. In cattle, antigen-induced mRNA expression of *IP-10*, *MIG*, Granzyme A and *IL-22* suggests that these immunological markers may have value for detection of *M. bovis* infection. However, IP-10 was a poor indicator of immunological responses in *M. bovis* experimentally infected cattle using a human IP-10 ELISA (42,43).

In summary, bTB is a chronic and progressive disease that may be undetected for months or years before visible lesions develop in affected tissues. The disease is maintained by free ranging wildlife reservoirs such as African buffaloes that pose an infection risk to livestock and other animals. Therefore, the focus should be early diagnosis of bTB in buffaloes to minimize transmission and spread of disease. However, this goal is confounded by the lack of accurate practical tools for testing buffaloes.

Therefore, the present study aimed to investigate a number of approaches for improving the diagnosis of *M. bovis* infection in African buffaloes. These included the evaluation of new stimulation antigens for the Bovigam® assay, Peptide Cocktail Prionics® PC-EC and Peptide Cocktail Prionics® PC-HP; an estimation and comparison of diagnostic sensitivity of selected CMI assays; identification of novel biomarkers of *M. bovis* infection; assessment of the diagnostic performance of interferon-gamma induced protein-10 (IP-10) and investigation of IP-10 stability during storage and transport.

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Chapter 2 – Agreement between assays of cell-mediated immunity utilizing *Mycobacterium bovis*-specific antigens for the diagnosis of tuberculosis in African buffaloes (*Syncerus caffer*).

Wynand J. Goosen [a], Michele A. Miller [a], Novel N. Chegou [a], David Cooper [b], Robin M. Warren [a], Paul D. van Helden [a], Sven D.C. Parsons [a]

[a] DST/NRF Centre of Excellence for Biomedical TB Research/MRC Centre for Molecular and Cellular/Biology, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, P.O. Box 19063, Tygerberg 7505, South Africa

[b] Ezemvelo KZN Wildlife, P.O. Box 25, Mtubatuba 3935, South Africa

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My contribution to this research article:	Planning of project Blood collection Blood stimulation Running all assays *Post mortem* examinations Tissue sample collection Mycobacterial culturing Speciation by PCR Data interpretation All statistical analysis Writing of manuscript

Abstract

We assessed the use of *M. bovis*-specific peptides for the diagnosis of tuberculosis in African buffaloes (*Syncerus caffer*) by evaluating the agreement between the single intradermal comparative tuberculin test (SICTT), the Bovigam® EC (BEC) assay, the Bovigam® HP (BHP) assay and 2 assays utilizing the QuantiFERON® TB-Gold (in tube) system employing 20 h (mQFT20 assay) and 30 h (mQFT30 assay) whole blood incubation periods. Of 84 buffaloes, 45% were SICTT-positive, 48% were BEC-positive, 50% were BHP-positive, 37% were mQFT20-positive and 43% were mQFT30-positive. Agreement between the BEC and BHP Bovigam® assays was high (κ=0.86, 95% CI 0.75-0.97) and these detected the most test-positive animals suggesting that they were the most sensitive assays. Interferon-gamma release was significantly greater in buffaloes that were test-positive for all tests than in animals with discordant but positive Bovigam® results. Agreement between the mQFT assays was equally high (κ=0.88, 95% CI 0.77-0.98); however, all buffaloes with discordant mQFT results (n=6) were mQFT30-positive/mQFT20-negative, including 3 confirmed *M. bovis*-infected animals, suggesting that the mQFT30 assay is the more sensitive of the two. Agreements between the two Bovigam® and two mQFT assays were moderate, suggesting that in its current format the mQFT assay is less sensitive than either the BEC or BHP assays.

Keywords:

African buffalo, Bovigam®, bovine tuberculosis, CFP-10, ESAT-6, interferon-gamma.

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) and has a wide host range including cattle and various wildlife species (Michel *et al*., 2006). In cattle, BTB may cause reduced productivity and death but also poses an important zoonotic risk and the disease is therefore intensively controlled in many countries. In South Africa, African buffaloes (*Syncerus caffer*) are an important maintenance host of *M. bovis* and may act as a reservoir of this pathogen for cattle and other wildlife species such as lions (*Panthera leo*) (De Vos *et al*., 2001). Therefore, the detection of *M. bovis*-infected buffaloes is important in preventing the transmission of BTB to other species, including domesticated livestock, as well as the geographic spread of this pathogen through movement of infected animals (Grobler *et al*., 2002; Parsons *et al*., 2011).

Since BTB is a chronic and progressive disease, animals may be infected for months or years before developing lesions and early diagnosis relies primarily on the detection of cell mediated immunity (CMI) to *M. bovis* antigens (Vordermeier *et al*., 2000). Examples of such tests include the single intradermal comparative tuberculin test (SICTT) and the interferon gamma (IFN-γ) release assays (IGRA). These tests have historically measured CMI to *M. bovis* purified protein derivative (PPD) which comprises a broad range of *M. bovis* antigens. More recently, the specificity of IGRAs have been improved by the development of assays which detect immunological sensitization to *M. bovis*-specific antigens such as the 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) (Vordermeier *et al*., 1999; Vordermeier *et al*., 2001; Bass *et al*., 2013). Genes encoding these proteins are located in the genetic region of difference 1 (RD1) which is absent from most nontuberculous mycobacteria and the attenuated strain of *M. bovis*, Bacillus Calmette-Guérin (Vordermeier *et al*., 2001).

A modification of a human TB test, the modified QuantiFERON® TB-Gold (mQFT) assay, which utilises peptides simulating the proteins ESAT-6, CFP-10 and TB 7.7, has been described for the diagnosis of BTB in buffaloes (Parsons *et al*., 2011). However, the use of two newly available commercial Bovigam® assays which employ ESAT-6 and CFP-10 peptides (EC), and these together with peptides simulating Rv3615 and 3 additional mycobacterial antigens (HP), has not been reported in this species.

We therefore wished to evaluate the use of the Bovigam® EC (BEC) and Bovigam® HP (BHP) assays to detect *M. bovis*-infected buffaloes. Furthermore, we evaluated the effect of increased blood incubation time on the sensitivity of the mQFT assay, and as part of this study, assessed whether centrifugation of the QFT tubes, as previously reported (Parsons *et al*., 2011), was a necessary step prior to harvesting plasma supernatants. Finally, because mycobacterial culture is regarded as an imperfect gold standard of infection (de la Rua-Domenech *et al*., 2006), and because this data was not available for all tested animals, we characterized the agreement between the Bovigam® assays, the mQFT assays and the SICTT.

Material and Methods

Animals and SICTT:

Two hundred and thirty-one buffaloes from two herds (herd A and B) with known BTB exposure were captured during a test-and-slaughter control program in the Hluhluwe-iMfolozi Game Reserve (South Africa) and tested as previously described (Parsons *et al*., 2011). Following measurement of the skin fold thickness (SFT) at injection sites, 0.1 ml *M. bovis* PPD and 0.1 ml *M. avium* PPD (WDT, Hoyerhagen, Germany) were injected intradermally on the left and right side of the neck, respectively. After 3 days, the SFT was measured with callipers in all animals with palpable inflammation at the injection sites. SICTT-positive animals were defined as having an SFT increase at the *M. bovis* PPD injection site of 2 mm or greater than that at the *M. avium* PPD injection site. Eighty-four animals from Herd A (n=144) were

randomly selected to assess the agreement between the various diagnostic tests while all animals from Herd B (n=87) were used to compare the concentration of IFN-γ in QFT plasma samples before and after centrifugation. Ethical approval for this study was granted by the Stellenbosch University Animal Care and Use committee.

mQFT, BEC and BHP assays:

Ten ml of blood was drawn from each animal into heparinised blood collection tubes by venepuncture of the jugular vein. For all animals, 1 ml of blood was incubated for 20 h at 37ºC in the blood collection tubes of the QFT system (Qiagen, Venlo, Limburg, Netherlands), i.e. the Nil tube containing saline and TB Antigen tube containing ESAT-6, CFP-10 and TB7.7 peptides. Thereafter, for animals from Herd B, 150 µl of plasma was harvested from each tube. Tubes were then centrifuged at 1000 x *g* for 6 min and the remaining plasma was collected. For animals from Herd A, 150 µl of plasma was collected without centrifugation from each QFT tube and the tubes were further incubated for 10 h at 37ºC. Thereafter, tubes were centrifuged (as above) and the remaining plasma was collected. Plasma IFN-γ concentrations were determined using a bovine IFN-γ enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden). For the mQFT assays, a cut-off of 66 pg/ml was used to identify positive animals, as previously described (Parsons *et al.*, 2011). Tests performed with plasma collected after 20 h incubation and after a further 10 h incubation (30 h in total) were defined as mQFT20 and mQFT30, respectively.

All buffaloes from Herd A were tested with the BEC and BHP assays (Prionics, Schlieren-Zurich, Switzerland). The assays were performed according to the manufacturer's instructions with the following exceptions: because incubation was performed in a non-sterile incubator, whole blood samples were incubated in sealed 2 ml microcentrifuge tubes rather than tissue culture plates, and samples were centrifuged at 1000 x *g* for 6min prior to plasma collection.

Post mortem examination and mycobacterial culture:

All SICTT-positive animals and a single SICTT-negative/Bovigam®-positive/mQFT30-positive animal were killed by gunshot and inspected at necropsy for evidence of gross BTB lesions. The tonsils, retropharyngeal, parotid and sub-mandibular lymph nodes, the left- and right bronchial lymph nodes and lungs were excised and multiple parallel incisions were made into these tissues. Samples collected for mycobacterial culture included lesions suggestive of BTB, or if no such lesion was present, pooled samples from the bronchial and retropharyngeal lymph nodes. Tissue was homogenized (Bullet blender, Next Advance, Averill Park, NY, USA) after which 5ml BD MycoPrep™ (Becton Dickinson, Franklin Lakes, NJ, USA) was added to each 1 cm^3 of sample and incubated for 15 min at 37ºC. Thereafter, samples were neutralized with 5ml phosphate buffered saline (PBS). All samples were centrifuged for 15 min at 1000 x *g* and the supernatant decanted. Each pellet was resuspended in 1 ml PBS and 500 µl of this suspension was transferred to a Mycobacteria Growth Indicator Tube (MGIT™) and incubated in a BACTEC™ MGIT™ 960 Mycobacterial Detection System (both Becton Dickinson). Cultures which were ZN-positive were genetically speciated by polymerase chain reaction as previously described (Warren *et al*., 2006).

Statistical analysis:

For Herd A, the agreement between each of the diagnostic assays was calculated as Cohen's Kappa coefficient (κ) using the agreement calculator on the GraphPad Software website (http://graphpad.com/quickcalcs/kappa1/). For Herd B, plasma IFN-γ concentrations in pre- and post-centrifuged QFT samples were compared in SICTT-negative and -positive animals using a nonparametric paired student's t-test using GraphPad Prism version 5 (GraphPad Software, March 2007).

Results & Discussion

There were no significant differences in IFN-γ concentrations in plasma samples harvested from QFT tubes prior to and after centrifugation from SICTT-negative (n=70) and SICTT-positive (n=17) buffaloes (Fig 1). Additionally, mQFT results for these animals were identical for both methods (data not shown). This indicates, as for human samples (http://www.cellestis.com/IRM/content/aust/qftproducts_tbgoldintube_faqs.html), that buffalo plasma collected from the QFT tubes without centrifugation can reliably be used in the mQFT assay.

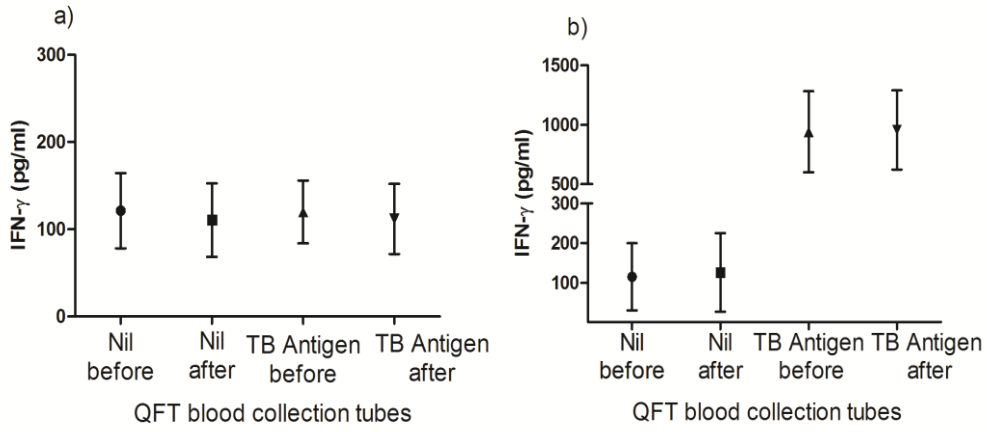

Fig 1. Mean IFN-γ concentrations (and 95% confidence intervals) in plasma from Nil and TB antigen QuantiFERON®-TB Gold tubes from a) SICTT-negative (n = 70) and b) SCITT-positive (n = 17) buffaloes showing no significant difference between samples collected before and after centrifugation ($p > 0.1$).

Of the 84 buffaloes tested with all diagnostic assays, 38 (45%) tested positive with the SICTT, 31 (37%) with the mQFT20 assay, 36 (43%) with the mQFT30 assay, 41 (48 %) with the BEC assay and 42 (50%) with the BHP assay. Of these, 29/84 (35%) tested positive for all tests, 36/84 (43%) tested negative for all tests and 19/84 (22%) had discordant test results (Table 1 and Supplementary Table 1 under Appendix I). *Mycobacterium bovis* infection was confirmed by mycobacterial culture and genetic speciation following post mortem examination in 27/34 SICTT-positive buffaloes and 1 BEC/BHP/mQFT30-positive animal which tested negative with the SICTT and the mQFT20 assays (animal A67, Table 1 and Supplementary Table 1

under Appendix I).

Table 1
Test results for African buffaloes with discordant bovine tuberculosis test outcomes.

	BEC [a]		BHP [b]		mQFT20 [c]		mQFT30 [d]			*M.bovis*
Animal	Result	ΔOD [g] (450nm)	Result	ΔOD (450nm)	Result	ΔIFN-γ [h] (pg/ml)	Result	ΔIFN-γ (pg/ml)	SICTT [e]	isolation [f]
A1	+[i]	0.158	+	0.131	-	16	-	57	-	n.d. [j]
A8	-[k]	0.05	+	0.17	-	0	-	17	-	n.d.
A26	+	2.262	+	2.192	+	1192	-	0	+	+
A30	-	0.042	-	0.091	-	46	-	35	+	-
A32	+	0.15	+	0.206	-	0	-	0	-	n.d.
A33	+	0.26	+	0.165	-	0	-	11	-	n.d.
A34	+	0.095	+	0.098	-	0	-	20	-	n.d.
A36	+	0.168	+	0.191	-	46	+	236	+	+
A41	+	0.643	-	0	-	14	-	0	-	n.d.
A42	+	0.253	+	0.184	-	36	+	74	+	-
A43	+	0.254	+	0.226	-	26	+	108	+	+
A48	-	0.065	-	0.057	-	44	-	3	+	+
A49	+	0.111	-	0.083	-	12	-	27	+	+
A50	+	0.393	+	0.41	-	1	-	2	-	n.d.
A52	-	0.085	-	0.073	-	45	-	41	+	-
A67	+	0.191	+	0.212	-	22	+	162	-	+
A68	-	0	+	0.288	+	191	+	549	+	+
A70	-	0.067	+	0.103	-	26	+	68	-	n.d.
A75	-	0.088	+	0.154	+	129	+	142	-	n.d.
A94	-	0.075	-	0.075	-	36	+	121	-	n.d.

[a] Bovigam® assay utilising peptides derived from ESAT-6 and CFP-10.
[b] Bovigam® assay utilising peptides derived from ESAT-6, CFP-10, Rv3615 and 3 mycobacterial antigens.
[c] modified QuantiFERON® TB-Gold assay (20 h blood incubation).
[d] modified QuantiFERON® TB-Gold assay (30 h blood incubation).
[e] Single intradermal comparative tuberculin test
[f] Warren *et al*. (2006).
[g] Optical density of the peptide stimulated sample minus that of the Nil sample.
[h] IFN-γ concentration in the TB Antigen-stimulated sample minus that in the Nil sample
[I] Positive.
[j] Not done.
[k] Negative

The agreement between the two Bovigam® assays was very good (κ=0.86, 95% CI 0.75-0.97, Table 2), however, the limitations of this study precluded confirmation of *M. bovis* infection in 9 BEC- and BHP-positive animals. Nonetheless, the excellent agreement between these assays

suggests that they may indicate true infection and that they were the most sensitive detection methods used in this study. Furthermore, the BHP assay yielded the greatest number of positive tests, and may be more sensitive than the BEC, in agreement with the manufacturer's guidelines for cattle (http://www.prionics.com/diseases-solutions/tuberculosis/bovigamR-2g/). Notably, for both assays, the amount of IFN-γ release was significantly greater in buffaloes that were test-positive for all tests than in animals with discordant test results (Fig. 2). Higher levels of

Table 2

The Kappa (κ) and 95% confidence interval estimates of agreement between five tests for bovine tuberculosis in African buffaloes (n=84).

	SICTT[a]	mQFT20[b]	mQFT30[c]	BHP[d]	BEC[e]
SICTT	1	0.78 (0.65-0.91)	0.81 (0.68-0.93)	0.72 (0.57-0.86)	0.76 (0.62-0.90)
mQFT20	0.78 (0.65-0.91)	1	0.88 (0.77-0.98)	0.74 (0.60-0.88)	0.69 (0.54-0.84)
mQFT30	0.81 (0.68-0.93)	0.88 (0.77-0.98)	1	0.81 (0.69-0.93)	0.71 (0.56-0.86)
BHP	0.72 (0.57-0.86)	0.74 (0.60-0.88)	0.81 (0.69-0.93)	1	0.85 (0.75-0.97)
BEC	0.76 (0.62-0.90)	0.69 (0.54-0.84)	0.71 (0.56-0.86)	0.85 (0.75-0.97)	1

[a] single intradermal comparative tuberculin test.
[b] modified QuantiFERON® TB-Gold assay (20 h blood incubation).
[c] modified QuantiFERON® TB-Gold assay (30 h blood incubation).
[d] Bovigam® assay utilising peptides derived from ESAT-6, CFP-10, Rv3615 and 3 mycobacterial antigens.
[e] Bovigam® assay utilising peptides derived from ESAT-6 and CFP-10.

IFN-γ release in response to ESAT-6 and CFP-10 stimulation have been shown to correlate with increased pathology in cattle (Vordermeier *et al*., 2002) and the buffaloes with discordant test results may therefore represent a group of animals with early infection or limited disease progression. These interpretations of our results should be investigated in future studies in which BEC- and BHP-positive animals are examined at post mortem for evidence of *M. bovis* infection and disease.

As expected, agreement between the mQFT20 and mQFT30 assays was equally high (κ=0.88, 95% CI 0.77-0.98, Table 2). However all animals with discordant mQFT results were mQFT30-positive and mQFT20-negative (n=6; Table 1). Five of these were BHP-positive and 3 were confirmed to be *M. bovis*-infected at post mortem (Table 1). However, a single mQFT20-negative/mQFT30-positive animal (A94) tested negative for all other tests (Table 1). Notably, Min *et al*. (2013) showed that in healthy human controls the number of QFT-positive individuals increased from 2/33 to 4/33 when the duration of blood incubation was increased from 24 to 48 h. However, in patients with suspected tuberculosis, the sensitivity of the QFT assay decreased when the duration of QFT blood incubation was increased from 24 to 48 and 72 h (Min *et al.*, 2013). Our findings on the increased incubation time are confounded by the concurrent reduction in plasma volume in the mQFT30 assay during the last 10 h of incubation. In itself, this lower plasma volume may have allowed for the accumulation of a higher IFN-γ concentration in antigen-stimulated samples. Nonetheless, although specificity of the assay may be compromised, our findings suggest that increasing the duration of blood incubation in IGRAs may increase the sensitivity of this assay in buffaloes and should be further investigated.

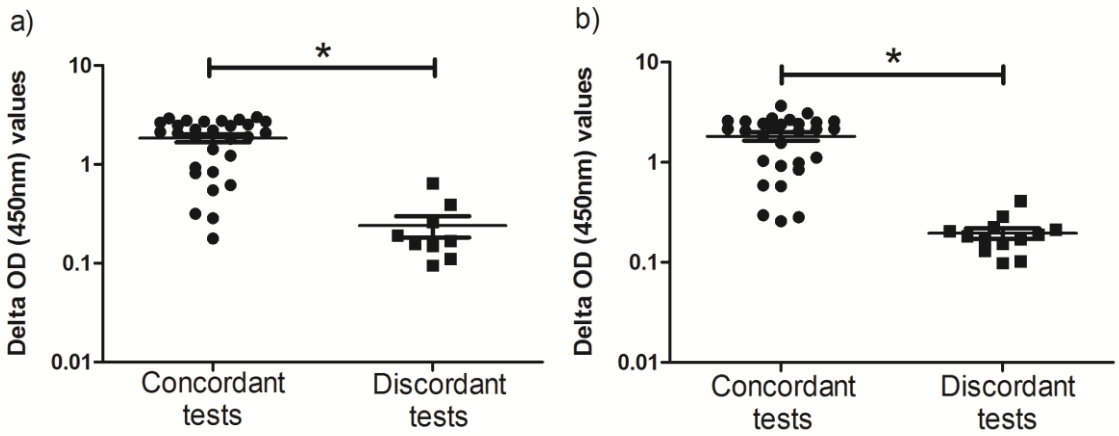

Fig 2. Test results for (a) the Bovigam® EC assay, and (b) the Bovigam® HP assay for buffaloes with concordant and discordant SICTT, mQFT20 and mQFT30 test results showing mean and 95% confidence intervals. *, $p < 0.001$.

The moderate agreement between the two Bovigam® and two mQFT assays was surprising (Table 2). Interpretation of this finding is speculative given the proprietary nature of their components, however it may in part be attributable to differences in the sensitivities of the ELISAs used in each assay and improvement of the mQFT ELISA might increase its utility. Furthermore, in humans, Gaur *et al*. (2013) showed that reducing the volume of blood in QFT tubes increases the sensitivity of this assay, possibly as a result of the increased antigen concentration in the blood sample. Indeed, higher TB antigen concentrations have been shown to result in greater IFN-γ release in IGRAs (Rose *et al*., 2013). Any difference in the concentrations of antigenic peptides in the Bovigam® and mQFT assays may have resulted in differences in their sensitivities. Reducing the volume of blood incubated in the QFT tubes might therefore increase the sensitivity of this assay. Additionally, the QFT assay has been specifically developed for TB diagnosis in humans and presumably utilizes ESAT-6 and CFP-10 peptides which are immunodominant in this host (Mustafa *et al*., 2008; Arlehamn *et al*., 2012). However, a different set of such peptides is immunodominant in cattle (Vordermeier *et al*., 1999, 2000, 2001) and differences in the composition of the ESAT-6/CFP-10 peptide cocktail of the mQFT and Bovigam® assays may have contributed towards the observed discrepancies in test results.

In conclusion, this study shows that plasma collected from the QFT tubes prior to centrifugation can reliably be used in the mQFT assay and that increasing the incubation time of this assay from 20 to 30 h may increase its sensitivity. This is also the first report of the use of the Bovigam® peptide assays for the diagnosis of BTB in buffaloes and our findings suggest that these assays are more sensitive than either the SICTT or mQFT in its current format. Nonetheless, the mQFT assay remains a highly practical test for BTB in buffaloes, especially under field conditions (Parsons *et al*., 2011), and improvements to this assay, as discussed above, may increase its utility.

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Chapter 3 - Evaluation of the comparative sensitivity of selected tests for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

Wynand J. Goosen, David Cooper, Charlene Clarke, Paul D. van Helden, Michele A. Miller, Sven D.C. Parsons

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My contribution to this research article:	Planning of project Blood collection Blood stimulation Running all assays *Post mortem* examinations Tissue sample collection Mycobacterial culturing Speciation by PCR Data interpretation All statistical analysis Writing of manuscript

1. Abstract

In South Africa, African buffalo (*Syncerus caffer*) are a major maintenance host of *Mycobacterium bovis*, the cause of bovine tuberculosis (bTB), and may act as a source of this infection for domestic cattle. The control of bTB in buffaloes relies on the use of the single intradermal comparative tuberculin test (SICTT) and interferon gamma release assays (IGRAs). In this study we aimed to evaluate relative sensitivities of the SICTT, the Bovigam PPD assay, a modified Bovigam PPD assay, the Bovigam PC-EC assay and the Bovigam PC-HP assay in *M. bovis*-infected animals and explore the possibility of using these tests in combinations to report the optimal algorithm for the detection of bTB in African buffaloes. Of these assays, the Bovigam PPD assay had a relative sensitivity between 91-93% and the modified PPD assay a relative sensitivity between 90-91%. Moreover, combining one or more IGRA together with the SICTT showed an overall diagnostic sensitivity between 95-100%. In conclusion, the Bovigam PPD and modified PPD assays displayed greatest sensitivity for the detection of *M. bovis*-infected buffaloes. Nonetheless, the SICTT detected additional IGRA-negative animals and maximum sensitivity was attained when these assays were used in combination.

Keywords: African buffalo, Bovigam®, bovine tuberculosis, diagnosis, *Mycobacterium bovis*, SICTT

2. Introduction

Mycobacterium bovis, the causative agent of bovine tuberculosis (bTB), has a wide host range that includes numerous wildlife and domestic species (Michel *et al*., 2006). The disease not only causes loss of productivity or death of infected animals but also poses an important zoonotic risk and is intensively controlled in many countries (Olmstead and Rhode, 2004). In South Africa, African buffaloes (*Syncerus caffer*) are a major maintenance host of *M. bovis* and may act as a source of infection for domestic cattle (Musoke *et al*., 2015) or other wildlife

species such as lions (*Panthera leo*) (Olivier *et al*., 2015). For this reason, the detection of *M. bovis*-infected buffaloes is important for preventing the transmission and geographic spread of this disease (Grobler *et al*., 2002; Parsons *et al*., 2011). However, animals may be infected for months or even years before developing detectable disease and early diagnosis relies primarily on tests that quantify the cell mediated immune response to *M. bovis* antigens (Vordermeier *et al*., 2000).

The most commonly used tests are the tuberculin skin tests which measure the delayed-type hypersensitivity response to intradermal injection of purified protein derivative (PPD) derived from *M. bovis* (PPDbov). Of these, the most specific test is the single intradermal comparative tuberculin test (SICTT) which compares this response to that against *Mycobacterium avium* PPD (PPDav) (Schiller *et al*., 2010). Similarly, the *in vitro* Bovigam® PPD assay measures the comparative release of interferon gamma (IFN-γ) to these antigens in whole blood samples (Grobler *et al*., 2002). However, in buffaloes, this assay displays reduced specificity and has been modified to include *Mycobacterium fortuitum* PPD (modified PPD assay) as an additional comparative antigen (A.L. Michel *et al*., 2011). The specificity of IGRAs have also been increased through the use of the antigens 6-kDa early secreted antigenic target (ESAT-6) and 10-kDa culture filtrate protein (CFP-10) (Parsons *et al*., 2011). Peptides simulating these are utilized in the Bovigam PC-EC assay, while the Bovigam PC-HP assay utilizes these together with peptides simulating Rv3615 and 3 additional mycobacterial antigens (Goosen *et al*., 2014). However, recent studies have reported conflicting results on the comparative sensitivities of these assays and the SICTT, in part, due to limitations in their design (Goosen *et al*., 2014; van der Heijden *et al*., 2016).

In order to advance insight into the diagnosis of *M. bovis* infection in African buffaloes, we therefore aimed to compare the sensitivities of the Bovigam PPD assay, the modified PPD

assay, the Bovigam PC-EC assay, the Bovigam PC-HP assay and the SICTT, as well as combinations of these, in *M. bovis* infected animals.

3. Material and methods

3.1. Animals

In 2015, 283 buffaloes from three herds (Herd A, B and C) with known exposure to *M. bovis* were captured and chemically immobilized, as previously described (Parsons *et al*., 2011), during an annual test-and-slaughter program in Hluhluwe-iMfolozi Game Reserve (HiP, South Africa). Following immobilization, 10 ml of heparinized whole blood was collected by jugular venepuncture. Ethical approval for the capture and testing of these animals was granted by the Stellenbosch University Animal Care and Use Committee.

3.2. IGRAs

All buffaloes were tested using three standard commercially available Bovigam® assays (Prionics, Schlieren-Zurich, Switzerland) according to the manufacturer's instructions as well as a modified testing protocol of the Bovigam® PPD assay that includes the additional stimulation of whole blood with *M. fortuitum* PPD as previously described (A.L. Michel *et al*., 2011). For each animal, 250 µl of whole blood was added to 2 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing, respectively, 25 µl solutions of: 1) phosphate buffer saline (PBS), 2) *M. bovis* PPD (Thermo Fisher Scientific Prionics AG, Schlieren, Switzerland) at a final concentration of 24 µg/ml, 3) *Mycobacterium avium* PPD (Thermo Fisher Scientific Prionics AG, Schlieren, Switzerland) at a final concentration of 24 µg/ml, 4) *M. fortuitum* PPD (Tuberculosis Laboratory, Onderstepoort Veterinary Institute, South Africa) at a final concentration of 3.3 µg/µl, 5) peptides simulating ESAT-6 and CFP-10 (Bovigam® PC-EC stimulation antigen, Prionics, Schlieren-Zurich, Switzerland), 6) the above peptides together with peptides simulating Rv3615 and 3 additional *M. bovis* antigens (Bovigam® PC-HP stimulation antigen, Prionics, Schlieren-Zurich, Switzerland) and 7) pokeweed mitogen

(PWM) (Sigma-Aldrich, St. Louise, MO, USA) at a final concentration of 5 µg/ml, respectively. Following incubation for 20 h at 37 ºC, each tube was centrifuged at 1500 x g for 10 min and plasma fractions were harvested and stored at -80 ºC. For the standard commercial assays, test analyses were done according to the manufacturer's instructions. For the modified Bovigam PPD protocol, the standard test criteria were used unless an animal was classified as *M. fortuitum* PPD responsive ($OD_{M.\ fortuitum\ PPD} - OD_{PBS} \geq 0.15$). In such cases, an animal was defined as test-positive if $OD_{M.\ bovis} - OD_{PBS} \geq 0.2$ as well as $OD_{M.\ bovis} - OD_{M.\ avium} \geq 0.2$.

3.3. SICTT

Following blood collection, 0.1 ml *M. bovis* PPD and 0.1 ml *M. avium* PPD (WDT, Hoyerhagen, Germany) were injected intradermally on the left and right side of each animal's neck, respectively. Seventy-two hours post PPD administration, the skin fold thickness (SFT) at injection sites of all animals that tested positive on any of the Bovigam assays, as well as animals with distinctly visible skin responses, was measured with Hauptner callipers (Kyron Laboratories, Benrose, South Africa). An animal with an increase of 2 mm or greater in SFT at the *M. bovis* PPD injection site compared to the *M. avium* PPD injection site was defined as SICTT-positive.

3.4. *Post mortem* (PM) examination and mycobacterial culture

All IGRA and SICTT-positive buffaloes were killed by gunshot and examined for gross bTB lesions at necropsy. The tonsils and retropharyngeal, parotid, sub-mandibular, trachea-bronchial, mediastinal and prescapular lymph nodes as well as the lungs were excised and multiple incisions were made into these tissues. Gross pathological changes consistent with bTB were scored as follows: 0 – no visible lesions; 1 – one small (< 10 mm in diameter) focal lesion; 2 – several small focal (more than 1 lesion < 10 mm in diameter) or single large (> 10 mm in diameter) lesion and 3 – multifocal or confluent lesions (Palmer *et al*., 2007). Tissue samples with bTB-like lesions were collected and stored separately. For animals with no visible

lesions, samples of head and thoracic lymph nodes were pooled by anatomical site. All tissue samples were processed for mycobacterial culture using Mycobacterial Growth Indicator Tubes (MGIT™) and the BACTEC™ MGIT™ 960 Mycobacterial Detection System (Becton Dickinson, Franklin Lakes, NJ, USA), previously described (Goosen *et al.*, 2014). All Ziehl-Neelsen positively stained bacterial cultures were genetically speciated by polymerase chain reaction as previously described (Warren *et al.*, 2006).

3.5. Statistical analysis

The agreement between each diagnostic assay was calculated as Cohen's kappa coefficient (*k*) using the freely available agreement calculator on the GraphPad Software website (http://graphpad.com/quickcalcs/kappa1/). *M. bovis*-infected animals were defined as 1) those confirmed to be *M. bovis*-infected by culture of the pathogen (stringent criterion); and 2) those confirmed to be either *M. bovis* culture-positive or having bTB-like lesions with a score of 2 or greater (lenient criterion). Using both criteria, the relative sensitivity of each assay was calculated as the proportion of *M. bovis*-infected animals testing positive for a particular IGRA. Furthermore, the relative sensitivity of all possible test combinations was evaluated using both reference criteria. All analyses were done using GraphPad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

4. Results

4.1. SICTT, IGRAs and Mycobacterial culture

Of the 283 buffaloes, 50/283 (17.7%) tested positive with the Bovigam PPD assay, 49/283 (17.3%) tested positive with the modified PPD assay, 44/283 (15.5%) tested positive for the SICTT and 43/283 (15.2%) tested positive for the PC-EC assay and PC-HP assay, respectively (Appendix III, Supplementary Table S1). Of these, 31/283 (10.9%) tested positive for all tests, 220/283 (77.7%) tested negative for all tests and 32/283 (11.3%) had discordant test results. The PC-EC and PC-HP assays showed excellent agreement (k = 0.89, 95% CI 0.82-0.97).

Moreover, the Bovigam PPD and modified PPD assays showed near-perfect agreement (k = 0.99, 95% CI 0.96-1.00) with a single Bovigam PPD-positive animal testing modified PPD-negative. The lowest agreement in test results was between the PC-EC assay and the SICTT (k = 0.69, 95% CI 0.63-0.85, Table 1).

Of 63 buffaloes which tested positive on any test, 35/63 (55.6 %) were confirmed to be *M. bovis* infected and 41/63 (65%) were either confirmed to be *M. bovis*-infected or to have bTB-like lesions with a score of 2 or more.

4.2. Test performance

The relative sensitivities of each assay and of selected assay combinations, for both the stringent and lenient criteria of infection, are summarized in Table 2. The Bovigam PPD and modified PPD assay showed the greatest individual test sensitivity, respectively. However, combinations of one or more IGRAs together with the SICTT showed the greatest overall diagnostic sensitivity (Table 2). Notably, 5/35 (14.3%) buffaloes confirmed to be *M. bovis*-infected, tested negative for the SICTT but positive for one or more IGRAs.

Table 1

The kappa (*k*) and 95% confidence interval values of agreement between five tests for bovine tuberculosis in African buffaloes (n = 283).

	SICTT[a]	Bovigam PPD[b]	modified PPD[c]	PC-EC[d]	PC-HP[e]
SICTT	1	0.77 (0.67 to 0.87)	0.78 (0.68 to 0.88)	0.69 (0.57 to 0.81)	0.74 (0.63 to 0.85)
Bovigam PPD	0.77 (0.67 to 0.87)	1	0.99 (0.96 to 1.00)	0.73 (0.62 to 0.84)	0.81 (0.71 to 0.90)
modified PPD	0.78 (0.68 to 0.88)	0.99 (0.96 to 1.00)	1	0.74 (0.63 to 0.85)	0.82 (0.73 to 0.91)
PC-EC	0.69 (0.57 to 0.81)	0.73 (0.62 to 0.84)	0.74 (0.63 to 0.85)	1	0.89 (0.82 to 0.97)
PC-HP	0.74 (0.63 to 0.85)	0.81 (0.71 to 0.90)	0.82 (0.73 to 0.91)	0.89 (0.82 to 0.97)	1

[a] Single intradermal comparative tuberculin test.
[b] Bovigam® assay utilizing *M. bovis* purified protein derivatives (PPD) and *M. avium* PPD.
[c] Modified Bovigam® assay utilizing *M. bovis*, *M. avium* and *M. fortuitum* PPDs.
[d] Bovigam® assay utilizing peptides derived from ESAT-6 and CFP-10.
[e] Bovigam® assay utilizing peptides derived from ESAT-6, CFP-10, Rv3615 and 3 additional mycobacterial antigens.

Table 2

The total number of positive animals, relative sensitivity (%) and 95% confidence interval for each test and possible test combinations calculated as the proportion of positive animals in a cohort where 1) either *M. bovis* was isolated or there were several small lesion foci to multifocal or confluent lesions (n = 41) and 2) only *M. bovis* was isolated (n = 35).

	M. bovis and/or visible lesions		*M. bovis* isolated	
Test and test combinations	n = 41	Sensitivity (%) (95%CI)	n = 35	Sensitivity (%) (95%CI)
SICTT[a]	34/41	83 (0.71 to 0.95)	30/35	86 (0.74 to 0.97)
Bovigam PPD[b]	38/41	93 (0.85 to 1.00)*[c]	32/35	91 (0.82 to 1.00)
modified PPD[d]	37/41	90 (0.82 to 0.99)	32/35	91 (0.82 to 1.00)
PC-EC[e]	31/41	76 (0.63 to 0.89)	28/35	80 (0.67 to 0.93)
PC-HP[f]	34/41	83 (0.71 to 0.95)	30/35	86 (0.74 to 0.97)
modified PPD & Bovigam PPD	38/41	93 (0.85 to 1.00)*	32/35	91 (0.82 to 1.00)
modified PPD & PC-EC	38/41	93 (0.85 to 1.00)*	33/35	94 (0.87 to 1.00)
modified PPD & PC-HP	38/41	93 (0.85 to 1.00)*	33/35	94 (0.87 to 1.00)
modified PPD & SICTT	39/41	95 (0.89 to 1.00)*	34/35	97 (0.92 to 1.00)
Bovigam PPD & PC-EC	39/41	95 (0.89 to 1.00)*	33/35	94 (0.87 to 1.00)
Bovigam PPD & PC-HP	39/41	95 (0.89 to 1.00)*	33/35	94 (0.87 to 1.00)
Bovigam PPD & SICTT	40/41	98 (0.93 to 1.00)*#[g]	34/35	97 (0.92 to 1.00)
PC-EC & PC-HP	34/41	83 (0.71 to 0.95)	30/35	86 (0.74 to 0.97)
PC-EC & SICTT	39/41	95 (0.89 to 1.00)*	35/35	100 (1.00 to 1.00)*
PC-HP & SICTT	39/41	95 (0.89 to 1.00)*	35/35	100 (1.00 to 1.00)*

[a] Single intradermal comparative tuberculin test.

[b] Bovigam® assay utilizing *M. bovis* purified protein derivatives (PPD) and *M. avium* PPD.

[c] Statistically different ($p<0.05$) compared to the PC-EC assay within that respective *M. bovis* isolated group.

[d] Modified Bovigam® assay utilizing *M. bovis*, *M. avium* and *M. fortuitum* PPDs.

[e] Bovigam® assay utilizing peptides derived from ESAT-6 and CFP-10.

[f] Bovigam® assay utilizing peptides derived from ESAT-6, CFP-10, Rv3615 and 3 additional mycobacterial antigens.

[g] Statistically different ($p<0.05$) compared to the SICTT within that respective *M. bovis* isolated group.

5. Discussion

As individual tests, the Bovigam PPD assay and modified PPD assay detected the greatest number of culture-confirmed *M. bovis*-infected buffaloes as well as buffaloes classified as *M. bovis*-infected based on culture or pathological criteria. However, the estimated diagnostic sensitivities of the PPD assays were not shown to be significantly greater than those of the SICTT or PC-HP assay. In contrast, the sensitivity of the PC-EC assay was significantly lower than that of the Bovigam PPD assay for the detection of animals with bTB-like lesions or confirmed *M. bovis* infection.

When tests were used in combination, the SICTT together with either the PC-EC or PC-HP assays detected the greatest number of culture-confirmed *M. bovis*-infected animals. Respectively, these combinations were significantly more sensitive than either the PC-EC or PC-HP assays alone. Similarly, the SICTT and Bovigam PPD assay together detected the greatest number of animals that were classified as *M. bovis*-infected based on both culture or the presence of VL. Moreover, this test combination was significantly more sensitive than the use of the SICTT alone.

Our finding that the Bovigam PPD assay shows greater sensitivity than the SICTT for the diagnosis of *M. bovis* infection in buffaloes is generally held to be true for cattle too (de la Rua-Domenech *et al*., 2006; Schiller *et al*., 2010; Vordermeier *et al*., 2006). However, comparing the diagnostic sensitivities of these assays for cattle is problematic given the large variation in reported test conditions (de la Rua-Domenech *et al*., 2006) and because direct comparisons of the two tests in the same study have rarely been made (Vordermeier *et al*., 2006). A retrospective study in Ireland compared these tests in 767 *M. bovis*-infected cattle and estimated the IGRA to have a sensitivity of 88% while that of the SICTT was estimated to be 60% and 74%, for standard and severe test interpretations, respectively (Gormley *et al*., 2006). Similarly, in two smaller studies, the sensitivities of the IGRA and intradermal test were

estimated to be 85% and 80% (González Llamazares *et al*., 1999), and 95% and 90% (Ameni *et al*., 2000), respectively. The wide range in reported sensitivities is indicative of the bias introduced by local study conditions (de la Rua-Domenech *et al*., 2006; Schmidt and Factor, 2013) and highlights the benefit of evaluating comparative values in a single study, as done here. Nonetheless, a limitation of the present study is the likelihood that the sensitivities of the various assays were overestimated, as truly infected animals which tested negative on all tests were not included in the study.

Importantly, while most infected buffaloes were both SICTT- and IGRA-positive, the agreement between these tests was only moderate and using them in combination detected a greater number of infected animals. In cattle, numerous studies have shown the occurrence of distinct subsets of infected individuals that only test positive with one or the other of these tests (Neill *et al*., 1994; Schiller *et al*., 2010; Vordermeier *et al*., 2006). For this reason, a greater number of *M. bovis*-infected cattle are detected when they are used in combination (Ameni *et al*., 2000; González Llamazares *et al*., 1999; Gormley *et al*., 2006). The parallel use of the SICTT and Bovigam PPD assay has therefore been advocated for testing of infected cattle herds (Gormley *et al*., 2006; Vordermeier *et al*., 2006) and the present study supports this approach for buffaloes.

While the Bovigam PPD assay detected the greatest number of *M. bovis*-infected animals, limitations in the specificity of this assay have been reported for both buffaloes (A. L. Michel *et al*., 2011) and cattle (Schiller *et al*., 2010). When greater test specificity is desired, additional or alternative IGRA test antigens have been advocated for both species (Goosen *et al*., 2014; A. L. Michel *et al*., 2011; P J Cockle, 2006). Such approaches have commonly been associated with decreased test sensitivity (Schiller *et al*., 2010). However, in the present study, the modified PPD assay, which utilizes *M. fortuitum* PPD as an additional antigen, showed no significant difference in sensitivity compared to the Bovigam PPD assay. Moreover, these

assays showed extremely high agreement and detected an equal number of animals confirmed to be *M. bovis* infected, supporting the use of this method for improving diagnostic specificity. In contrast, the PC-HP assay detected fewer such animals, in agreement with the manufacturer's guidelines for cattle (Anonymous, n.d.). Moreover, the PC-EC assay detected still fewer and was significantly less sensitive than the Bovigam PPD assay. Again, these findings are in agreement with the manufacturer's guidelines, findings from published studies in cattle (Schiller *et al*., 2010) and previous studies in buffaloes (Goosen *et al*., 2014; van der Heijden *et al*., 2016).

In conclusion, the Bovigam PPD and modified PPD assays displayed greatest sensitivity for the detection of *M. bovis*-infected buffaloes. Nonetheless, the SICTT detected additional IGRA-negative animals and maximum sensitivity was attained when these assays were used in combination. The SICTT, however, is logistically challenging in buffaloes, given that animals must be chemically immobilized on two occasions. For this reason, further strategies for improving the diagnostic performance of IGRAs should be investigated in future work.

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Chapter 4 – The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

Wynand J. Goosen [a], David Cooper [b], Robin M. Warren [a], Michele A. Miller [a], Paul D. van Helden [a], Sven D.C. Parsons [a]

[a] DST/NRF Centre of Excellence for Biomedical TB Research/MRC Centre for Tuberculosis Research/Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, P.O. Box 19063, Tygerberg 7505, South Africa

[b] Ezemvelo KZN Wildlife, P.O. Box 25, Mtubatuba 3935, South Africa

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My contribution to this research article:	Planning of project All optimizations necessary Sample preparations Running of all cytokine ELISAs Sequencing of all selected cytokines Submission of all sequences to NCBI All statistical analysis Data interpretation Writing of manuscript

Abstract

We evaluated commercially available bovine enzyme linked immunosorbent assays (ELISA) and a human IP-10 ELISA to measure IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA in buffalo plasma in order to identify sensitive markers of the immune response to *M. bovis*-specific peptides. Additionally, we found that all coding mRNA sequences of these cytokines showed very high homology with their homologues in domestic cattle (97% - 99%) as did the derived amino acid sequences (97% - 99%). This high sequence homology between cattle and buffaloes supports the use of bovine ELISAs for the detection these cytokines in buffaloes. MCP-1 concentration showed a positive correlation with that of IFN-γ ($p = 0.0077$) and appears to occur in far greater abundance in buffaloes when compared to humans. Using a bovine IP-10 ELISA, levels of this cytokine were found to be significantly increased in antigen-stimulated blood samples from *M. bovis* test positive buffaloes ($p < 0.0001$) and IP-10 was detected in far greater abundance than IFN-γ. Measurement of IP-10 with this ELISA may prove to be a sensitive marker of *M. bovis* infection in African buffaloes.

Keywords:

African buffalo, Bovine tuberculosis, Enzyme linked immunosorbent assay, Interferon gamma-induced protein 10, Interferon gamma

Introduction

In Africa, buffaloes (*Syncerus caffer*) are one of the most important maintenance hosts of a variety of pathogens which cause disease in domestic cattle (Michel and Bengis, 2012). These include indigenous diseases such as foot-and-mouth disease, Corridor disease and alien diseases such as bovine brucellosis and bovine tuberculosis (BTB) (Michel and Bengis, 2012). In order to better control such diseases, the development of improved tests for their diagnosis and an understanding of their pathogenesis and epidemiology is essential.

In the case of BTB, the immunological diagnosis of infection relies primarily on the detection of a cell mediated immune response to *Mycobacterium bovis* antigens (Vordermeier *et al*., 2000). Examples of such assays include the single intradermal tuberculin skin test (SICTT) and the *in vitro* interferon gamma release assays (IGRA), the latter of which quantify antigen-stimulated release of this cytokine. Recently, the specificity of IGRAs have been improved with the availability of the Bovigam PC-EC assay (BEC) utilizing peptides simulating the *M. bovis* proteins 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) and the Bovigam PC-HP assay (BHP) utilizing ESAT-6, CFP-10, peptides simulating Rv3615 and 3 additional mycobacterial antigens (Bass *et al*., 2013; Goosen *et al*., 2014). However, the sensitivity of BTB diagnosis in bovids remains suboptimal (Vordermeier *et al*., 2000) and it is possible that the quantification of biomarkers other than IFN-γ may improve the sensitivity of tests such as the BEC and BHP assays (Waters *et al*., 2003; Vordermeier *et al*., 2009; Jones *et al*., 2010; Blanco *et al*., 2011).

In humans, candidate biomarkers for the diagnosis of *Mycobacterium tuberculosis* infection include monocyte-derived chemokine IFN-γ-induced protein 10 (IP-10) (Ruhwald *et al*., 2009), monokine induced by interferon gamma (MIG) (Abramo *et al*., 2006; Chakera *et al*., 2011), monocyte chemoattractant protein (MCP)-1, MCP-2, MCP–3 and interleukin 1 receptor antagonist (IL1-RA) (Ruhwald *et al*., 2009; Chakera *et al*., 2011). Ruhwald *et al*., (2009) reported significantly higher concentrations of IP-10, IL1–RA, MCP-1, MCP-2 and MCP-3 compared to IFN-γ in antigen-stimulated blood samples from patients with active tuberculosis suggesting that all 5 biomarkers hold promise as immunological markers of tuberculosis in humans, with IP-10 and MCP-2 showing the greatest potential. Similarly, Abramo *et al*., (2005) showed a correlation between MIG concentration and IFN-γ production in 29 BCG-vaccinated controls and 24 TB patients, identifying MIG as a novel biomarker for *M. tuberculosis* infection. In cattle, Aranday-Cortes *et al*., (2012) reported IP-10, MIG, Granzyme A and IL-

22 mRNA expression to show their promise as biomarkers of antigen-induced immune responses which might be utilized for the diagnosis of *M. bovis* infection. However, using a human IP-10 ELISA, a study in cattle which were experimentally infected with *M. bovis* concluded that IP-10 is a poor diagnostic biomarker for the detection of this infection in this species (Waters *et al*., 2012).

The aim of this study was to evaluate the use of commercially available bovine ELISAs as well as a human IP-10 ELISA (Waters *et al*., 2012) for the measurement of selected candidate biomarkers of immune activation in response to *M. bovis* antigens in whole blood from African buffaloes.

Material and methods

Animals and sample preparation

In 2012, randomly selected buffaloes in the Hluhluwe-iMfolozi Park were captured and tested for *M. bovis* infection using the SICTT as previously described (Parsons *et al*., 2011). After SICTT assessment, whole blood (WB) was collected from all SICTT-positive buffaloes into sodium heparin tubes by jugular venipuncture. Immediately after collection, one ml of WB from each animal was transferred to a QFT TB Antigen tube (containing peptides simulating ESAT-6, CFP-10 and TB7.7) and a QFT Nil tube (containing saline), respectively. The QFT tubes were shaken vigorously, and within 4 h of WB transfer to the QFT tubes, these were incubated at 37°C for 20 h, centrifuged at 3000 × *g* for 6 min and the plasma fraction stored at -80°C. To identify positive QFT reactors, plasma samples harvested from the QFT tubes were used in a modified QFT (mQFT) assay using a commercially available bovine IFN-γ ELISA (Table 1) and a cut-off of 66 pg/ml, as previously described (Parsons *et al*., 2011). As previously reported, the mQFT assay is a highly specific test of *M. bovis* infection (Parsons *et al*., 2011, 2012; Goosen *et al*., 2014) and buffaloes that tested positive on both the SICTT and mQFT assays were considered to be infected for the purposes of this study.

Sequencing of selected buffalo cytokine mRNAs

Cytokine mRNA sequences of domestic cattle and sheep were obtained for IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA from the Ensemble Genome Browser (http://www.ensembl.org/index.html). These mRNA sequences were aligned using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalo/) and highly homologous regions were used to generate primers with Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Two forward and two reverse primers were designed, one of each within the untranslated regions flanking the translated region and one of each within the latter sequence (Table 2). Eight hundred ng of mRNA which had previously been extracted from *M. bovis* antigen-stimulated whole blood of two *M. bovis*-infected buffaloes (Parsons *et al*., 2012) was reverse transcribed using the QuantiTect Reverse Transcription (RT) Kit (Qiagen, Venlo, Netherlands) in a final volume of 80 µl. For each cytokine, each of the 4 primer combinations (0.4 µM of each primer) were used in a 25 µl PCR reaction containing 1 µl of cDNA , 2.5 µl of 10 x buffer and 0.1 µl of HotStarTaq DNA polymerase (reagents from Qiagen) and 0.4 mM dNTPs (Promega Corporation, Fitchburg, WI, USA). The PCR reactions were initiated by incubation at 95 °C for 15 min and consisted of 45 cycles of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min after which PCR products were incubated for a further 10 min at 72 °C. These amplification products were sequenced with the 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. Cytokine protein sequences were then inferred from cDNA sequences using the EMBOSS Transeq software (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The degree of homology between all buffalo sequences and homologous sequences of cattle were calculated using ClustalW2 software.

Cytokine ELISAs

The concentration of IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA in the QFT-processed plasma samples was measured using commercially available ELISAs (Table 1) according to the manufacturer's instructions. Furthermore, the absolute concentration of IP-10 in these samples was calculated with reference to a dilution series of recombinant human and bovine IP-10 for the human and bovine ELISAs, respectively (Table 1).

Table 1
Enzyme linked immunosorbent assay kits and components, and buffalo plasma dilutions, used to measure selected cytokines in QFT-processed whole blood from African buffaloes.

Analyte	**Plasma dilution [a]**	**Manufacturer**	**Product information**
Bovine IFN-γ	1:2	Mabtech AB, Nacka Strand, Sweden	Kit no. 3115-1H-20
Bovine IP-10 (BIP-10)	1:10	Kingfisher Biotech Inc., St Paul, USA	Primary antibody: PB0385B-100 Secondary antibody: PBB0393B-050 Recombinant protein: RP0079B-005
Bovine MIG	1:2	Bethyl Laboratories Inc., Texas, USA	Kit no. E11-803
Bovine MCP-1	1:1000	Bethyl Laboratories Inc.	Kit no. E11-800
Bovine MCP-3	1:10	MyBioSource, California, USA	Kit no. MBS739771
Bovine MCP-2	1:10	MyBioSource	Kit no. MBS913274
Bovine IL1-RA	1:2	MyBioSource	Kit no. MBS740784
Human IP-10 (HIP-10)	1:2	PeproTech®, Rocky Hill, NJ	Kit no. 900-K39

[a] Final plasma assay dilution in each well.

Statistical analysis

For each animal, the concentration of each analyte was characterised as the ELISA optical density (OD) and the antigen-dependent secretion of each analyte (OD^{TB-Nil}) was defined as the OD value of the QFT TB Antigen plasma sample (OD^{TB}) minus that of the QFT Nil plasma sample (OD^{Nil}). Using these OD values, a Wilcoxon signed-rank test was used to determine if the median concentration of each analyte was significantly different in plasma from the QFT Nil and TB Antigen tubes. Furthermore, absolute concentrations of antigen-specific IFN-γ and IP-10 were also compared using a Wilcoxon signed-rank test. The correlation between the

OD^{TB} value for each analyte and that of IFN-γ was calculated as Spearman's correlation coefficient.

Results and Discussion

The coding mRNA sequences of IP-10, MIG, MCP-1, MCP-2, MCP-3 and IL1-RA were obtained for two African buffaloes and submitted to the NCBI sequence database (http://www.ncbi.nlm.nih.gov/genbank/) (Table 2). The availability of these sequences should prove useful for the investigation of buffalo immunology by means of gene expression assays as previously described (Parsons *et al*., 2012). The sequences showed very high homology with those of cattle (97% - 99%) as did the inferred amino acid sequences (97%-99%) (Table 2). This degree of homology between buffalo and cattle suggests that commercial bovine ELISA kits could be used to quantify these proteins in plasma samples from buffaloes.

Table 2
Sequencing data of selected cytokine mRNAs of the African buffalo: primers used for sequencing, NCBI Genbank accession numbers and homology with the equivalent sequences of domestic cattle.

	Primer sequence (5' – 3')				NCBI accession number:	Homology with cattle (%)	
	External[a]		Internal[b]				
Analyte	Forward	Reverse	Forward	Reverse		mRNA	Inferred amino-acid sequence
IP-10	GCCTTTGCAAATATAT ACTGCATCT	ACTACGGTTTTC ACCTACATTTCC	TCCTGCCCACGT GTCGAGAT	AGTCCACGGACA ATTAGGGCTTGA	KM111562 KM111563	98%	97%
MIG	GTGACTCAGTAGAAC AAACACAGG	TAACACAAGATA GTGGTTGGTGA	AGGAATGGACGC TGTTCCTG	CTGGGTTTAGGC AGGCTTCA	KM111564 KM111565	98%	98%
MCP-1	CCACCCTCTCGGTTTT CAAT	ATTCTTGCGAGG ACACTTCC	AGGCCAAACCAG AGACCAAC	TATAGCAGCAGG CGACTTGG	KM111566 KM111567	99%	99%
MCP-2	CCCATGCACTCTGCTC CCTATAA	TCCACTTACAGG AGCACTGA	CAGTTTCTACCC CAATCACCTG	AGACATCCCTGT CCGCTTTG	KM111558 KM111559	99%	98%
MCP-3	AAGACTGCGAGCCCT GAGAA	ACTGGGACATCC CTTGCCAC	GCCCAGAT GACCCTCATGTC	GTCCACGTAGCC CAGCAC	KM111568 KM111569	98%	98%
IL1-RA	CTCGAGGTCACAGGA TGGAC	GCCACAGGGTGT GGACTTTA	GTTCAGAAACAG CCTGCCAC	TCTGGAGTCATG GACGTGCT	KM111560 KM111561	98%	99%

[a] External primers annealing in the untranslated regions, flanking the translated region of interest.
[b] Internal primers annealing within the translated region of interest.

These cytokines have been shown to be useful diagnostic biomarkers of cell activation following antigen recognition in humans (Abramo *et al*., 2006; Ruhwald *et al*., 2007, 2009). However, using commercial bovine ELISAs, only IFN-γ and IP-10 levels were found to be significantly increased in antigen-stimulated blood samples from SICTT-/mQFT-positive buffaloes (Figs. 1 and 2). Furthermore, MCP-1 was the only cytokine to show a significant

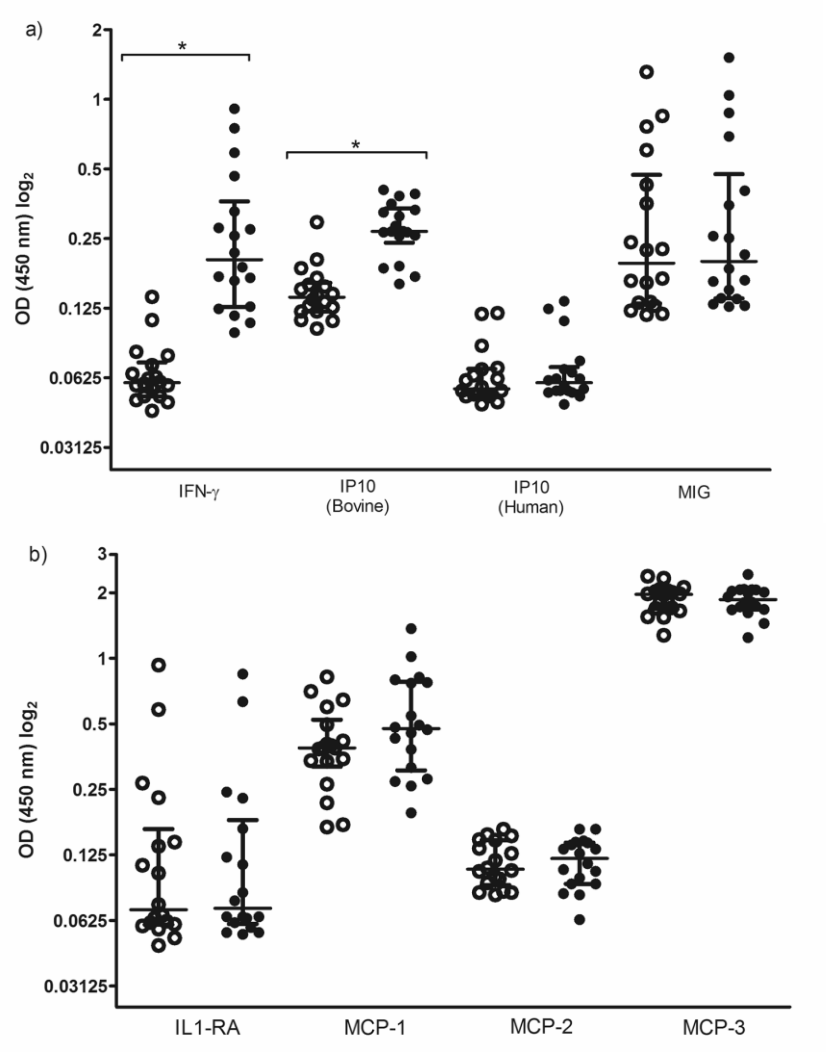

Fig. 1 Optical densities (including median and interquartile range) obtained in ELISAs of plasma samples harvested from QFT Nil tubes (○) and QFT TB Antigen tubes (●) following overnight whole blood incubation at 37 °C for (a) IFN-γ, IP-10, and MIG, and (b) IL1-RA, MCP-1, MCP-2 and MCP-3 (n=18 SICTT-positive/mQFT-positive African buffaloes; * $p < 0.0001$).

correlation with IFN-γ ($p = 0.0077$). The results for the remaining analytes were unexpected and we conclude that these are either poor indicators of antigen stimulation in buffaloes under the conditions described or that the bovine ELISAs did not efficiently detect these buffalo proteins. The latter is unexpected given the high degree of homology observed between cattle and buffalo transcripts of these analytes (Table 2). However, we cannot rule out that transcriptional, translational and post-translational differences between cattle and buffaloes may alter protein epitopes. Particularly surprising were the results for the human IP-10 ELISA which detected negligible levels of IP-10 in all buffalo plasma samples (0 – 137 pg/ml; Fig. 1). This is in contrast to findings from a study which used the same ELISA reagents to detect high levels of IP-10 in plasma from cattle following experimental infection with *M. bovis* (Waters *et al*., 2012). Our findings may indicate that the anti-human IP-10 antibodies used in this study have poor cross-reactivity to this protein of buffaloes despite their high predicted homology with cattle IP-10 (Table 2).

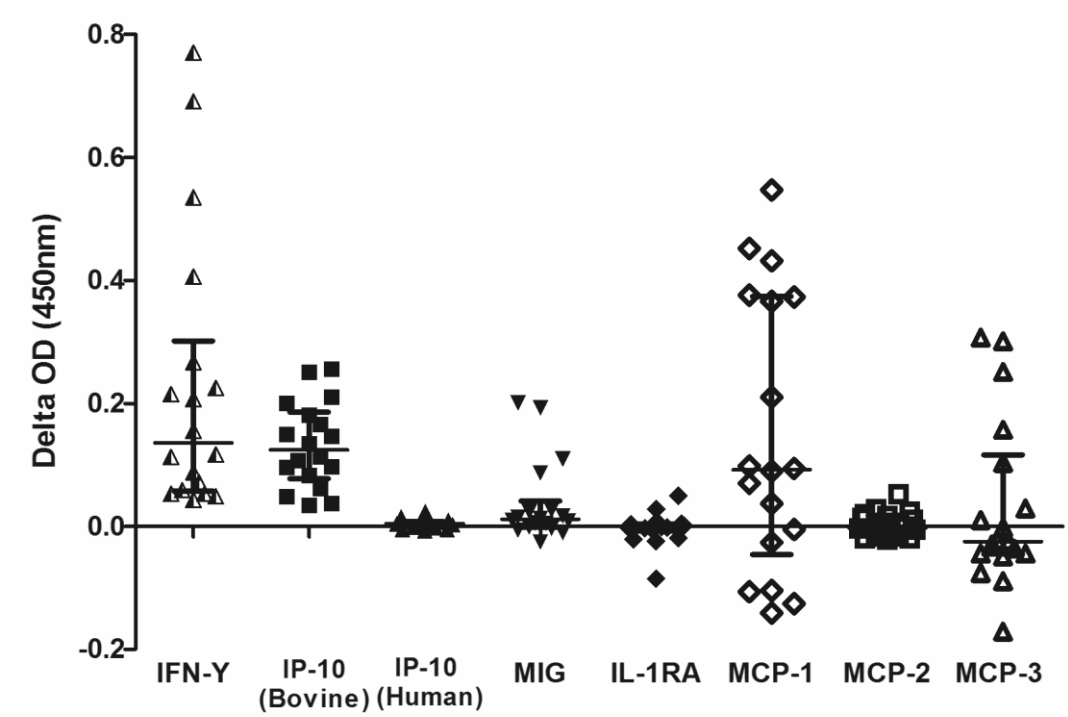

Fig. 2 The difference in plasma levels of IFN-γ, IP-10, MIG, IL1-RA, MCP-1, MCP-2 and MCP-3 harvested from QFT TB antigen tubes and QFT Nil tubes following overnight whole blood incubation at 37 ºC (n=18 SICTT-positive/mQFT-positive African buffaloes).

For the MCP-1 ELISA, plasma samples were diluted 1:1000. Nonetheless, MCP-1 ELISA signals were highly correlated with IFN-γ ($p = 0.0077$) suggesting that the results are a true reflection of MCP-1 abundance. Absolute concentrations of MCP-1 were not calculated; however, in humans, a typical plasma dilution factor for such an assay is 1:8 (Ruhwald *et al*., 2009) and our findings suggest that MCP-1 is particularly abundant in antigen-stimulated buffalo plasma. This cytokine is associated with a Th2 bias and enhanced production of IL-4 (Deshmane *et al*., 2009), both of which are associated with greater BTB pathology in cattle (Thacker *et al*., 2007). Moreover, an increase in MCP-1 plasma concentration due to a polymorphism in the MCP-1 gene promoter region has been shown to be associated with an increased susceptibility to pulmonary tuberculosis in humans (Flores-Villanueva *et al.*, 2005). Our findings, therefore, warrant further investigation of the role of MCP-1 in buffalo BTB.

Using the bovine IP-10 ELISA, levels of this cytokine were shown to be elevated in antigen-stimulated samples in all SICTT-positive/mQFT-positive buffaloes (Figs. 1 and 2). In addition, antigen-specific IP-10 was released in significantly greater abundance than IFN-γ ($p<0.0001$). This suggests that IP-10 may be at least as sensitive as IFN-γ as a diagnostic biomarker in buffaloes. Furthermore, because of its abundance, it may prove to be a more sensitive marker of *M. bovis* infection as has been shown for *M. tuberculosis* infection in humans (Ruhwald *et al*., 2007, 2009, 2011). The IP-10 molecule is also highly stable at room temperature (Aabye *et al*., 2011) and human plasma samples dried on filter membrane paper and transported via conventional postal service showed no loss of IP-10 signal (Aabye *et al*., 2013). This could prove particularly useful for the transport of buffalo samples from remote locations. Moreover, human studies have also shown *M. tuberculosis*-specific IP-10 responses to be less affected by co-infection with other bacteria and viruses, including HIV (Kabeer *et al*., 2010; Ruhwald *et al*., 2011). Again, this attribute might be particularly useful in buffaloes, a species which is known to be infected with a number of livestock pathogens (Michel and Bengis, 2012).

In conclusion, we have shown that IP-10 is a useful marker of immune activation by *M. bovis* antigens when using the bovine IP-10 ELISA. Our results highlight the limitations imposed on veterinary research by the lack of optimal immunological reagents and the need to interpret experimental results in this light. Our findings also suggest that the diagnostic potential of IP-10 for BTB in cattle be re-evaluated using species-specific reagents. Studies are currently under way to compare the sensitivity of IP-10 and IFN-γ as diagnostic biomarkers of *M. bovis* infection in African buffaloes.

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Chapter 5 – IP-10 is a sensitive biomarker of antigen recognition in whole blood stimulation assays used for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

Wynand J. Goosen[a], David Cooper[b], Michele A. Miller[a], Paul D. van Helden[a], Sven D.C. Parsons[a]

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/SAMRC Centre for Tuberculosis Research/Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa[a]; Ezemvelo KZN Wildlife, South Africa[b]

Running head: IP-10 for the diagnosis of *M. bovis* in buffaloes

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My contribution to this research article:	Planning of project Blood collection Blood stimulation Running all assays *Post mortem* examinations Tissue sample collection Mycobacterial culturing Speciation by PCR Data interpretation All statistical analysis Writing of manuscript

Abstract

African buffaloes (*Syncerus caffer*) are maintenance hosts of *Mycobacterium bovis*, the causative agent of bovine tuberculosis. They act as reservoirs of this infection for a wide range of wildlife and domestic species and the detection of infected animals is important to control the geographic spread and transmission of the disease. Interferon-gamma (IFN-γ) release assays utilizing pathogen-derived peptide antigens are highly specific tests of *M. bovis* infection; however, the diagnostic sensitivities of these assays are suboptimal. We evaluated the diagnostic utility of measuring antigen-dependent interferon gamma-induced protein 10 (IP-10) release as an alternative to measuring IFN-γ. *M. bovis*-exposed buffaloes were tested using the Bovigam PC-EC and Bovigam PC-HP assays and a modified QuantiFERON TB-Gold (mQFT) assay. IP-10 was measured in the harvested plasma and was produced in significantly greater abundance in response to *M. bovis* antigens in Bovigam-positive than in Bovigam-negative animals. For each assay, using the Bovigam results as a reference, receiver operating characteristic curve analysis was done to determine diagnostically relevant cut off values for IP-10. Hereafter, mQFT test results derived from measurement of IP-10 and IFN-γ were compared and a larger number of Bovigam-positive animals were detected using IP-10 as a diagnostic marker. Moreover, using IP-10, agreement between the mQFT assay and the Bovigam assays was increased while the excellent agreement between the Bovigam assays was retained. We conclude that IP-10 is a sensitive marker of antigen recognition and that measurement of this cytokine in antigen-stimulated whole blood might increase the sensitivity of conventional IGRAs in African buffaloes.

Keywords: African buffalo, bovine tuberculosis, enzyme linked immunosorbent assay, interferon gamma-induced protein 10, gamma interferon

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB) in a wide range of domestic animals and wildlife (1). BTB in cattle populations is intensively controlled in many countries as it can result in reduced productivity or death of infected animals and poses a serious zoonotic risk. In South Africa, the African buffalo (*Syncerus caffer*) is a maintenance host of *M. bovis* and the early detection of infected animals is important to control the transmission of the pathogen to other wildlife and domestic species and to prevent the geographic spread of this disease by translocation (2, 3).

The most sensitive method for diagnosing *M. bovis* infection is by detection of the host's cell mediated immune response to pathogen-specific antigens (4). Examples of such tests are the *in vivo* tuberculin skin test (TST) and *in vitro* interferon gamma release assays (IGRAs). The latter detect the release of interferon gamma (IFN-γ) in whole blood or from isolated peripheral blood mononuclear cells (PBMCs) in response to *M. bovis* purified protein derivative (PPD) (5) or more specific antigens such as the 6 kDa early secreted antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10) (6, 7). Recently, IGRAs utilizing the latter antigens have been described and evaluated for the diagnosis of BTB in buffaloes, i.e. the modified QuantiFERON TB-Gold (mQFT) assay and the commercially available Bovigam PC-EC assay (PC-EC) and Bovigam PC-HP assay (PC-HP)(3, 8). Notably, the mQFT assay, which is highly practical for use under field conditions, and the highly specific PC-EC assay are both less sensitive than the less specific PC-HP IGRA (8). As such, the utility of the mQFT and PC-EC assays would be increased by improving the detection of immune sensitization to ESAT-6/CFP-10.

One possibility for this might be the detection of additional or alternative biomarkers other than IFN-γ. In humans, a number of immunological proteins have been investigated as potential markers of immune activation in response to antigenic peptides and IFN-γ-induced protein 10

(IP-10) has proven to be a very strong candidate for the diagnosis of infection with *M. tuberculosis* (9–11). Similarly, and in contrast to reports from cattle studies (5), in *M. bovis*-infected buffaloes, IP-10 is significantly elevated in whole blood stimulated with ESAT-6/CFP-10 and is produced in much greater abundance than IFN-γ, suggesting its potential as an alternative or ancillary biomarker to IFN-γ in this species (10).

The aim of this pilot-study was, therefore, to determine if the measurement of IP-10 in antigen-stimulated whole blood is a useful diagnostic marker in buffaloes and whether it could be used to increase the sensitivity of established IGRAs. Because the PC-EC and PC-HP assays have been shown to have a greater sensitivity than the TST in this species (8) and because human studies have solely investigated IP-10 responses to peptide antigens (11), we used the PC-EC and PC-HP assays as reference tests. Moreover, since mycobacterial culture is considered to be an imperfect gold standard of *M. bovis* infection (12) and because this data was not available for Bovigam-negative animals in this study, an optimal diagnostic cut off value for IP-10 could not be calculated. We therefore evaluated the diagnostic utility of IP-10 by calculating a diagnostically relevant cut off value for this cytokine and thereafter, using both IFN-γ and IP-10 as diagnostic markers, the agreement between the mQFT assay and the highly sensitive and specific Bovigam peptide assays.

Materials and methods

Animals

In 2013, two hundred and thirty-one buffaloes were captured during an annual BTB test-and-slaughter program in the Hluhluwe-iMfolozi Game Reserve (South Africa) as previously described (3). Of these, eighty-four buffaloes were randomly selected and following chemical immobilization as previously described (8), ten ml of heparinized whole blood was collected from each animal by jugular venipuncture. Ethical approval for the capture and testing of these animals was granted by the Stellenbosch University Animal Care and Use Committee.

mQFT, PC-EC and PC-HP assays

One ml of blood was transferred to a QFT Nil tube, containing saline, and a QFT TB Antigen tube, containing peptides simulating ESAT-6, CFP-10, and TB 7.7 (Qiagen, Venlo, Limburg, Netherlands), respectively. Tubes were mixed thoroughly and incubated for 20h at 37ºC. After incubation, 150 µl of plasma was collected without centrifugation from each QFT tube. The concentration of IFN-γ in all plasma samples was measured using a bovine enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden). For each animal, the IFN-γ concentration in the Nil tube was subtracted from that in the TB Antigen tube and a differential value greater than 66 pg/ml was defined as a positive mQFT test result, as previously determined (3).

Performance of the PC-EC and PC-HP assays (Prionics, Schlieren-Zurich, Switzerland) included the incubation of 250 µl of whole blood with 25 µl saline, a 25 µl solution of peptides simulating ESAT-6 and CFP-10 (PC-EC assay) and a 25 µl solution of these peptides together with peptides simulating Rv3615 and 3 additional *M. bovis* antigens (PC-HP assay), respectively, for 20h at 37 ºC. All further analyses were done according to the manufacturer's instructions.

Bovine IP-10 ELISA and IP-10 assays

The concentration of IP-10 in all plasma samples was measured as follows: 100 µl of a 5 µg/ml solution of anti-bovine IP-10 capture antibody (Kingfisher Biotech Inc., St Paul, USA, Catalog number PB0385B-100) in phosphate buffered saline (PBS) was added to each well of a 96-well flat bottom polystyrene plate (NUNC, Roskilde, Denmark) and incubated overnight at 4 ºC. Thereafter, and after each subsequent step, plates were washed with a wash buffer consisting of 0.05% Tween-20 (Sigma-Aldrich, St. Louis, USA) in PBS. Blocking buffer (200 µl), consisting of 0.1% bovine serum albumin (BSA) (Roche, Basel, Switzerland) and 0.05% Tween-20 in PBS, was added to each well and the plates were incubated for 1h at room

temperature (RT). Then, aliquots of 25 µl of plasma in 75 µl blocking buffer as well as a dilution series (0 – 5000 pg/ml) of recombinant bovine IP-10 protein (Kingfisher, Catalog Number RP0079B-005) were incubated in duplicate wells for 2h at RT. Hereafter, the plates were incubated at RT for 1h with 100 µl/well of 0.01 µg/ml biotinylated anti-bovine IP-10 antibody (Kingfisher, Catalog Number PBB0393B-050) diluted in blocking buffer and subsequently at RT for 1h with 100 µl/well of a streptavidin-horse radish peroxidase solution (R&D Systems, Minneapolis, USA) diluted in blocking buffer. After a final wash, plates were incubated for 30 min at RT with 100 µl of 0.4 mg/ml o-phenylenediamine dihydrochloride/well (Sigma-Aldrich, St. Louis, USA). Optical densities of each of the wells was measured at 450 nm with a LT-4000 Microplate Reader (Labtech, Vienna, Austria) and the concentration of IP-10 in each well was calculated using the standard curve generated from the dilution series of recombinant bovine IP-10 protein.

For each diagnostic assay, the IP-10 test result was calculated as the IP-10 concentration in the antigen-stimulated sample minus the IP-10 concentration in the sample incubated with sterile PBS. IP-10 tests using plasma obtained from the mQFT assay, PC-EC assay and PC-HP assay were defined as the IP-10(QFT), IP-10(EC) and IP-10(HP) tests, respectively.

Statistical analysis

Animals which tested positive by either the PC-EC or PC-HP assay were defined as Bovigam-positive while animals which tested negative for both assays were defined as Bovigam-negative. IP-10(QFT), IP-10(EC) and IP-10(HP) test results for animals which were Bovigam-positive and Bovigam-negative were compared using the Wilcoxon signed-rank test. For each IP-10 test, an optimal diagnostic cut off value for discrimination between Bovigam-positive and Bovigam-negative animals was calculated using receiver operating characteristic (ROC) curve analysis. The relevant cut off value for each assay was determined by selecting the maximum value of Youden's index (YI), i.e. sensitivity + specificity – 1, which corresponds

to the point nearest the upper left corner on the ROC curve (13). These cut off values were used to define animals as IP-10 test-positive or -negative. The diagnostic performance of each IP-10 assay was calculated as the area under the curve (AUC) of the respective ROC curve. All analyses were done using GraphPad Prism version 5 (GraphPad Software, March 2007). Lastly, using GraphPad software (http://graphpad.com/quickcalcs/kappa1/), agreement between selected assays was calculated as Cohen's kappa coefficient (*k*).

Results

IGRA test results

Of 84 buffaloes tested, the mQFT, PC-EC and PC-HP assays identified 31 (37%), 40 (48%) and 42 (50%) test-positive animals based on IFN-γ values, respectively. Forty-four animals were defined as Bovigam-positive, and of these, the mQFT, PC-EC and PC-HP assays detected 71%, 91% and 95%, respectively (Table 1).

Table 1
The number and percentage of Bovigam-positive animals (n = 44) detected by measuring *M. bovis*-specific IFN-γ and IP-10 following the processing of whole blood with the mQFT, PC-EC and PC-HP assays.

	No (%) of positive cases	
Assay	IFN-γ	IP-10
mQFT	31 (71)	38 (86)
PC-EC	40 (91)	36 (82)
PC-HP	42 (95)	41 (93)

Antigen-induced IP-10 release in whole blood

In order to confirm IP-10 as a useful biomarker of antigen-dependent immune activation, the IP-10 test results for Bovigam-positive and -negative buffaloes were compared using the Wilcoxon signed-rank test. For Bovigam-positive and –negative animals, the median IP-10(QFT), IP-10(EC) and IP-10(HP) test results were 5458 pg/ml and 5 pg/ml, 10269 pg/ml and 671 pg/ml, and 6773 pg/ml and 734 pg/ml, respectively (Fig. 1). For all assays, the IP-10 test results were significantly greater for Bovigam-positive than -negative animals ($p < 0.001$; Fig. 1).

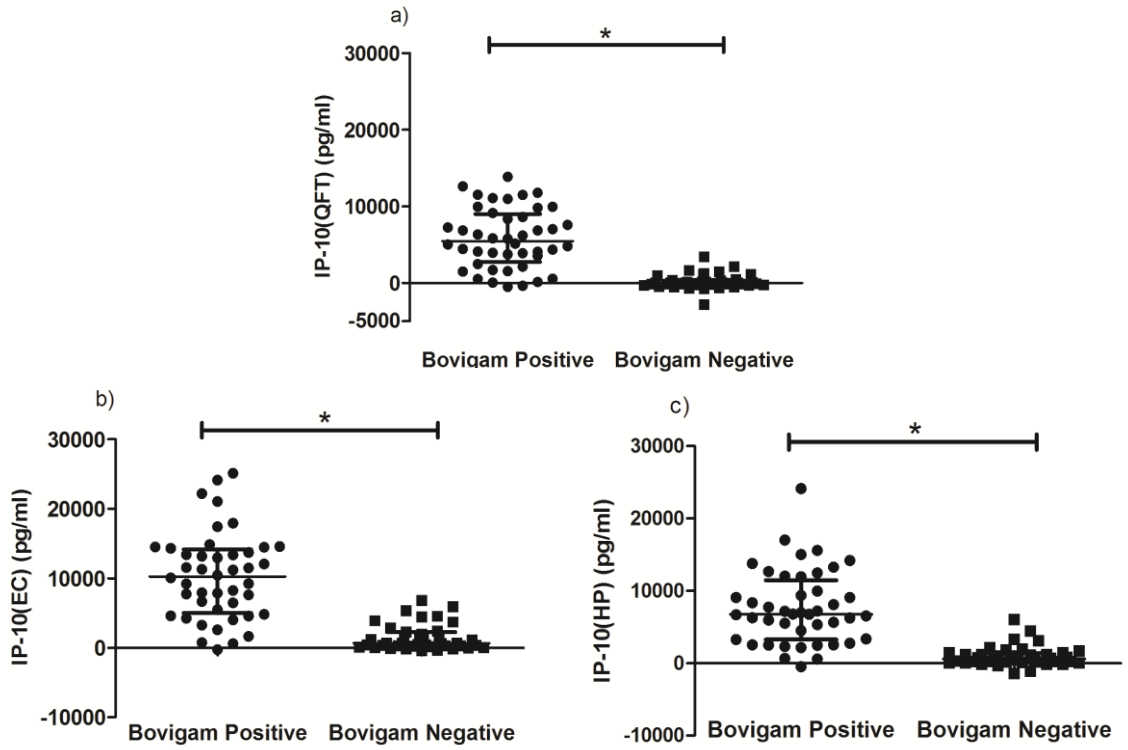

Fig. 1 IP-10 test results (*M. bovis*-specific IP-10 release) for Bovigam-positive and Bovigam-negative buffaloes following processing of whole blood with the (a) mQFT, (b) PC-EC and (c) PC-HP assays showing median and interquartile ranges. IP-10 release was significantly greater in Bovigam-positive animals. * $p < 0.001$.

IP-10 as a diagnostic biomarker

Diagnostic cut off values for the IP-10(QFT), IP-10(EC) and IP-10(HP) tests were calculated by comparing IP-10 test results for Bovigam-positive and -negative buffaloes using ROC curve analysis (Fig. 2, Table 2). A cut off value of 1486 pg/ml for the IP-10(QFT) assay detected all mQFT-positive animals and an additional 10 responders of which 7 were Bovigam-positive. A cut off value of 2155 pg/ml for the IP-10(HP) assay detected 40/42 (95%) PC-HP-positive animals and an additional 5 animals of which 2 were IP-10(EC) positive and 1 which was PC-EC-positive. The IP-10(QFT), IP-10(EC) and IP-10(HP) tests detected 86%, 82% and 93% of the 44 Bovigam-positive animals (Table 1). Of the Bovigam-negative buffaloes, 32/40 (80%) tested negative for all IP-10 assays (Table S1 under Appendix II).

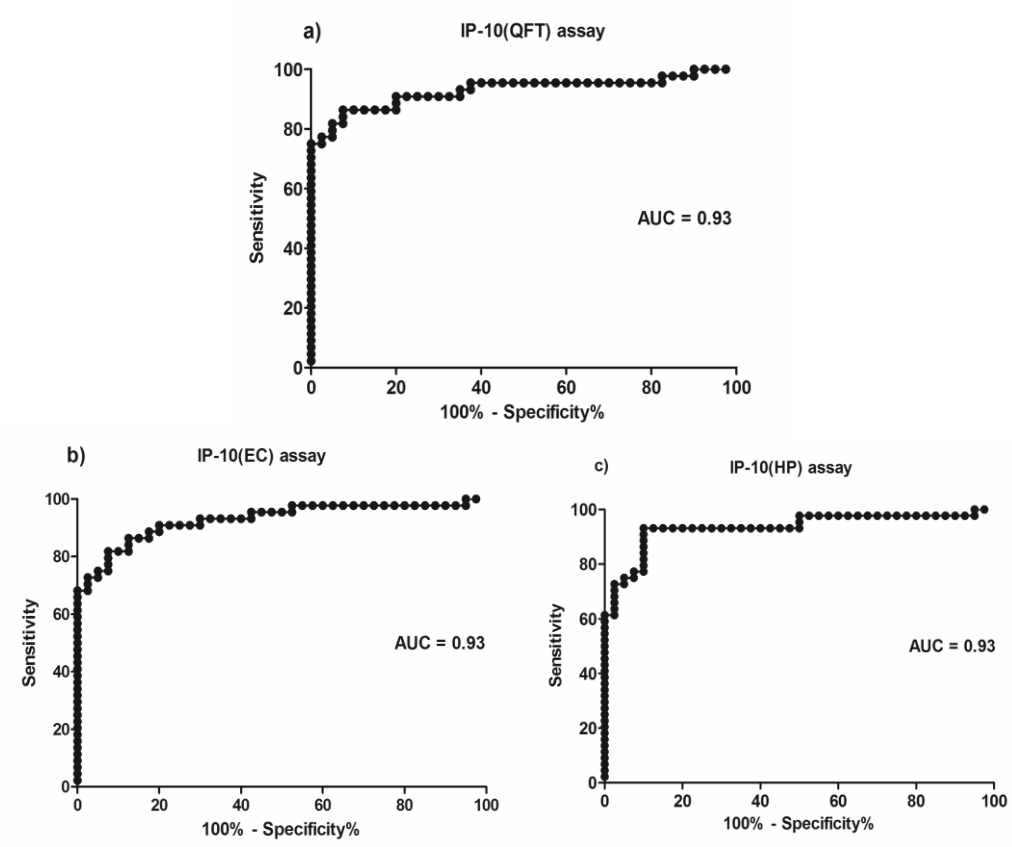

Figure 2 Receiver operating characteristic curves indicating the performance of selected cut off values in order to differentiate between Bovigam-positive and Bovigam-negative buffaloes by measurement of IP-10 in plasma obtained following processing of whole blood with (a) the mQFT, (b) the PC-EC and (c) the PC-HP assays. The high agreement between the Bovigam and IP-10 assays is indicated by the large area under the curve (AUC).

Table 2
Receiver operating characteristic curve analysis of the IP-10(QFT), IP-10(EC) and IP-10(HP) tests for discrimination between Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes[a]

IP-10 test	cut-off value (pg/ml)	% sensitivity (95% CI)	% specificity (95% CI)	AUC
mQFT	1486	86.4 (72.7-94.8)	92.5 (79.6-98.4)	0.93
PC-EC	4557	81.8 (67.3-91.8)	92.5 (79.6-98.4)	0.93
PC-HP	2155	93.2 (81.4-98.6	90 (76.4-97.2)	0.93

[a] CI, confidence interval; AUC, area under the curve..

In order to further characterise the diagnostic utility of IP-10, agreements between the IGRAs and IP-10 tests were determined using Cohen's kappa coefficient. The agreement between the mQFT assay and the combined Bovigam assays (k = 0.69) was substantially lower than the agreement between these assays and the IP-10(QFT) test (k = 0.79) (Table 3). Furthermore, the excellent agreement between the PC-EC and PC-HP assays was retained when IP-10 was used as a diagnostic marker (Table 3).

Table 3 Agreement between the mQFT, PC-EC, and PC-HP assays and the IP-10(QFT), IP-10(EC), and IP-10(HP) tests in a cohort of Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes

Assay	Bovigam	PC-HP	IP-10(HP)	PC-EC	IP-10(EC)
mQFT	0.69 (0.55 to 0.84)	0.74 (0.60 to 0.88)	ND	0.69 (0.53 to 0.84)	ND
IP-10(QFT)	0.79 (0.65 to 0.92)	0.79 (0.65 to 0.92)	0.76 (0.63 to 0.90)	0.74 (0.59 to 0.88)	0.71 (0.56 to 0.86)
PC-HP	ND	1	0.83 (0.72 to 0.95)	0.86 (0.75 to 0.97)	0.79 (0.65 to 0.92)
IP-10(HP)	ND	0.83 (0.72 to 0.95)	1	0.79 (0.66 to 0.92)	0.81 (0.69 to 0.93)
PC-EC	ND	0.86 (0.75 to 0.97)	0.79 (0.66 to 0.92)	1	0.83 (0.71 to 0.95)
IP-10(EC)	ND	0.79 (0.65 to 0.92)	0.81 (0.69 to 0.93)	0.83 (0.71 to 0.95)	1

[a] ND, not done.

Discussion

The most sensitive immunological tests of *M. bovis* infection measure cell-mediated immune responses to pathogen-specific antigens, i.e. delayed type hypersensitivity in the case of the TST and IFN-γ release in the case of IGRAs. The latter tests depend on the presence of circulating antigen-specific memory T lymphocytes; however, if these cells are present in low numbers, IFN-γ production may be below the threshold of detection. Nonetheless, IFN-γ concentrations as low as 10 pg/ml as well as other cytokines such as TNF-α, Interleukin (IL)-12 and IL-4 can induce activation of monocytes and neutrophils resulting in secretion of IP-10 by these cells (14–16). IP-10 can therefore be used as a proxy for lymphocyte activation (9). In humans, IP-10 has been shown to be a sensitive diagnostic biomarker of antigen recognition in a whole blood system (17) and the present study indicates its diagnostic utility in buffaloes.

Firstly, IP-10 is produced in far greater amounts in whole blood in response to *M. bovis* antigen stimulation in Bovigam-positive buffaloes than in Bovigam-negative animals (Fig 1 and 2). In addition to confirming this cytokine as a sensitive biomarker of antigen-induced immune activation as previously reported in buffaloes (10), these findings indicate the diagnostic potential of this protein to distinguish between these animal groups. Similarly, in humans, IP-10 is strongly induced in whole blood from tuberculosis patients stimulated with *M. tuberculosis*-specific antigens (11).

Secondly, the diagnostic performance of IP-10 was demonstrated by ROC analysis and the calculation of relevant diagnostic cut off values for the IP-10 tests. These analyses returned AUC values greater than 0.92 for all IP-10 tests indicating the discriminatory power of this biomarker (Table 1). This finding is particularly noteworthy given the limitations of these analyses which were performed with results from a restricted number of animals. A more accurate calculation of diagnostic cut off values would require many hundreds of animals in both the test-positive and test-negative groups (18). Also, for the purposes of our analyses we

elected to use the purely objective measure of a relevant cut off value, i.e. the YI index. This method, however, does not account for the fact that, when compared to the reference assays, the IP-10 tests might reasonably be expected to correctly identify a greater number of truly infected animals. It is probable, therefore, that this approach would overestimate the appropriate cut off values and underestimate the apparent sensitivities of the IP-10 tests. The limitation of this analysis is suggested by the relatively high cut off value of 4557 pg/ml calculated in this way for the IP-10(EC) test. This value is significantly higher than that calculated for the IP-10(QFT) assay and consequently, the IP-10(EC) assay detected only 82% of Bovigam-positive animals. Conversely, the IP-10(QFT) test cut off value of 1486 pg/ml increased the detection of Bovigam-positive animals from 71% for the mQFT assay to 86% indicating the diagnostic utility of this biomarker.

Thirdly, agreement between the IP-10(QFT) test and the combination of Bovigam assays (k = 0.79) was substantially greater than that between the latter result and the mQFT assay (k = 0.69). To a large degree, this increase in agreement is as a result of the increased sensitivity of the IP-10(QFT) test (Table 1). However, in addition to being a measure of agreement between positive tests, the kappa statistic measures agreement between negative test results and as such, the increased *k*-value in this case is also highly suggestive that an optimal IP-10 cut off value might not significantly compromise diagnostic specificity. Moreover, the diagnostic value of IP-10 is further indicated given that the excellent agreement between the PC-EC and PC-HP assays (k = 0.86) is retained when using IP-10 as a biomarker (k = 0.81).

A number of limitations of this pilot-study preclude definitive conclusions on the diagnostic utility of IP-10 in buffaloes. As mentioned, the limited sample size may have influenced the calculation of appropriate IP-10 test cut off values. Moreover, the utility of IP-10 was evaluated using antigen-specific whole blood incubation assays which have been optimized for the measurement of IFN-γ and alternative incubation periods might be more appropriate for the

measurement of IP-10. Also, Bovigam-negative animals which tested positive with the IP-10 tests were not slaughtered and no further immunological investigation was possible to confirm these results.

In conclusion, while recent advancements have been made in increasing the specificity of tests of CMI for the diagnosis of *M. bovis* infection in African buffaloes (3, 8) there remains a need for increased diagnostic sensitivity. This pilot-study indicates that IP-10 shows promise as a diagnostic biomarker in this species and that its measurement in peptide-stimulated whole blood may increase the sensitivity of conventional IGRAs. Future investigations of the diagnostic utility of this protein should include the validation of optimal diagnostic cut off values, the determination of its diagnostic sensitivity and specificity and an investigation of the diagnostic performance of IP-10 in combination with IFN-γ.

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Chapter 6 – The stability of plasma IP-10 enhances its utility for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

Wynand J. Goosen [a], Paul D. van Helden [a], Robin M. Warren [a], Michele A. Miller [a], Sven D.C. Parsons [a]

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/SAMRC Centre for Tuberculosis Research/Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, South Africa

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My contribution to this research article:	Planning of project Blood collection Blood stimulation Heat treatment of plasma Storage of plasma on Protein Saver Cards Running all assays *Post mortem* examinations Tissue sample collection Mycobacterial culturing Speciation by PCR Data interpretation All statistical analysis Writing of manuscript

Abstract

The measurement of interferon gamma-induced protein 10 (IP-10) in antigen-stimulated whole blood is a sensitive biomarker of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). However, this species often occurs in remote locations and diagnostic samples must be transported to centralized laboratories for processing. In humans, plasma IP-10 is highly stable and this feature contributes to its diagnostic utility; for this reason we aimed to characterize the stability of this molecule in buffaloes. Blood from *M. bovis*-infected and -uninfected animals was incubated with pathogen-specific peptides, saline and phytohaemagglutinin, respectively. Plasma fractions were harvested and aliquots of selected samples were: i) stored at different temperatures for various times; ii) heat treated before storage at RT, and iii) stored on Protein Saver Cards (PSCs) at RT for either 2 or 8 weeks before measurement of IP-10. Incubation of plasma at 65 °C for 20 min caused no loss of IP-10 and this protein could be quantified in plasma stored on PSCs for 2 and 8 weeks. Moreover, for all storage conditions, IP-10 retained its excellent diagnostic characteristics. These features of IP-10 might allow for the heat inactivation of potentially infectious plasma which would facilitate the safe and simple transport of samples.

Keywords: African buffalo, diagnosis, interferon gamma-induced protein 10; *Mycobacterium bovis*, Protein Saver Cards

Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (bTB), a zoonosis which can cause reduced productivity and death in a wide range of animals (Michel *et al*., 2006). In South Africa, African buffaloes (*Syncerus caffer*) are a major reservoir host of this pathogen and can act as a source of infection for cattle (Musoke *et al*., 2015) and other wildlife species (Olivier *et al*., 2015). The disease is therefore intensively controlled in buffaloes and this

requires the immunodiagnosis of infected animals, primarily by the detection of cell-mediated immunity to *M. bovis* antigens (Vordermeier *et al*., 2000).

One such test is the interferon gamma (IFN-γ) release assay (IGRA) which detects the secretion of this cytokine by memory lymphocytes in response to *M. bovis* purified protein derivative (PPD) or antigens such as the 6-kDa early secreted antigenic target (ESAT-6) and the 10-kDa culture filtrate protein (CFP-10) (Vordermeier *et al*., 2001; Waters *et al*., 2012). However, the applicability of this assay within the context of wildlife is confounded by the need for plasma cytokine concentrations to be measured with specialised equipment at centralised laboratories, while animals are often located in remote environments. This requires the transport of plasma under appropriately cooled or frozen conditions (Duncombe *et al*., 2013). Moreover, the movement of wildlife specimens risks the spread of highly infectious pathogens such as Foot and Mouth and African swine fever viruses (Duncombe *et al*., 2013).

As an alternative to IFN-γ, measurement of the antigen-specific release of IFN-γ-induced protein 10 (IP-10) has proven to be a strong diagnostic candidate in humans (Chakera *et al*., 2011; Ruhwald *et al*., 2007). Similarly, in buffaloes, the release of IP-10 in antigen-stimulated whole blood has been shown to be a useful marker of *M. bovis* infection (Goosen *et al*., 2014a) and to have a diagnostic sensitivity which is greater than conventional IGRAs (Goosen *et al*., 2015). In humans, plasma IP-10 is highly stable at 23 ºC for up to 2 weeks (Aabye *et al*., 2012) and dried plasma, stored at 37 ºC for 4 weeks on filter membrane Protein Saver Cards (PSCs) showed no decrease in IP-10 concentration (Aabye *et al*., 2012). This characteristic of IP-10 could have particular utility for veterinary samples as plasma stored on PSCs might easily be transported without any loss of diagnostic accuracy.

The aim of this study was, therefore, to investigate the diagnostic performance of assays measuring antigen-specific IP-10 for the diagnosis of *M. bovis* in African buffaloes following storage of plasma under various conditions.

Materials and methods

Animals

African buffaloes with known *M. bovis* exposure were captured, as previously described (Goosen *et al*., 2014b), in 2013 (Herds A13 and B13) and 2015 during a BTB test-and-slaughter program in the Hluhluwe-iMfolozi Game Reserve, South Africa. Buffaloes were tested using the TST, as previously described (Goosen *et al*., 2014b; Parsons *et al*., 2011), as well as the mQFT assay, as described below. Animals were classified as either BTB-positive (TST and mQFT-positive) or BTB-negative (TST and mQFT-negative) and assigned to two groups. Group 1 consisted of all BTB-positive buffaloes (n = 15) and 19 randomly selected BTB-negative animals tested in 2015. Group 2 consisted of all BTB-positive buffaloes (n = 17) and 20 randomly selected BTB-negative animals from Herd B13. Ethical approval for the capture and testing of these buffaloes was granted by the Stellenbosch University Animal Care and Use Committee.

Whole blood stimulation

Ten ml of heparinized whole blood was collected, by venepuncture of the jugular vein, from each animal. One ml of blood was incubated for 20 h at 37 ºC in each of the following QFT blood collection tubes (Qiagen, Venlo, Limburg, Netherlands): a Nil tube containing saline and a TB Antigen tube containing ESAT-6, CFP-10 and TB7.7 peptides. Additionally, 1 ml of blood was incubated with phytohaemagglutinin (PHA) (Sigma-Aldrich, St. Louis, Missouri, USA) at a final concentration of 10 µg/ml. Thereafter, tubes were centrifuged at 1500 x *g* for 6 min and plasma was harvested from each tube and stored at -80 ºC. Plasma IFN-γ concentrations were determined using a bovine IFN-γ enzyme linked immunosorbent assay

(ELISA) according to the manufacturer's instructions (kit 3115-1H-20; Mabtech, AB, Nacka Strand, Sweden). The mQFT assay result was defined as the IFN-γ concentration in plasma from the Nil tube subtracted from that from the TB Antigen tube. Animals with an assay result greater than 66 pg/ml were defined as mQFT-positive, as previously described (Parsons *et al*., 2011).

Heat treatment of plasma

Plasma derived from PHA-stimulated blood (activated plasma) and blood co-incubated with saline (non-activated plasma) was obtained from 8 randomly selected animals. Activated plasma was pooled, as was non-activated plasma, and aliquots of each pool were stored at -80 ºC, 4 ºC, room temperature (RT) and 37 ºC for 0, 4, 8 and 12 days. Additionally, pooled activated plasma was incubated at 65 °C for 20 min and aliquots of heat-treated as well as untreated samples were stored at RT for 0, 1, 2, 3, 4, 7 and 14 days. For animals from Group 1, QFT-processed plasma was treated as follows: one aliquot of 30 µl was incubated at 65 ºC for 20 min while a second aliquot was stored at -80 ºC.

Storage of plasma on PSC

For animals from Group 2: 25 µl aliquots of QFT-processed plasma were spotted, in duplicate, onto 903 Protein Saver^{TM} Cards (Whatmann plc, Maidstone, UK), dried at RT for 4 h, and stored at RT for 2 weeks (PSC-2w) and 8 weeks (PSC-8w), respectively. The remaining plasma was stored at -80 °C. Additionally, 5 aliquots of activated plasma (as described above) were spotted onto PSCs, as were 5 aliquots which had first been incubated at 65 ºC for 20 min, and these were stored at RT for 7 days. All plasma samples spotted onto PSCs were stored in sealed plastic bags with a desiccant.

Measurement of IP-10 by ELISA

The concentration of IP-10 in plasma samples was measured using a commercial bovine IP-10 ELISA (Kingfisher Biotech Inc., St. Paul, MN, USA), as previously described in detail (Goosen *et al*., 2015), and recorded as either the relative concentration (ELISA optical density, OD) or as the absolute concentration of IP-10 (pg/ml). For plasma spotted onto PSCs, absolute amounts of IP-10 (pg) in samples was measured using this ELISA with the following modification: the sample comprised two circular discs punched out from the centre of the spot using a standard office paper punch (Carl Mfg. Illinois, USA, Inc.) and incubated in 100 µl of blocking buffer.

Statistical analysis

For plasma samples, diagnostic IP-10 test results were defined as the plasma concentration of IP-10 in blood incubated in the QFT TB Antigen tube minus that incubated in the Nil tube. Similarly, for the PSC-2w and PSC-8w samples, test results were defined as the difference in absolute amounts of IP-10 in the sample derived from the TB Antigen and Nil tubes. Using receiver operating characteristic (ROC) curve analysis, an optimal diagnostic cut off value for plasma samples stored at -80 ºC was calculated by comparing test results for all BTB-positive (n = 32) and all BTB-negative buffaloes (n = 39). For BTB-positive and -negative animals from Group 2, cut off values were independently calculated for the PSC-2w and PSC-8w samples, respectively. In all cases, these values were calculated as the maximum value of Youden's index (Youden, 1950) and the diagnostic performance of each test was calculated as the area under the curve (AUC).

For BTB-positive animals from Group 1, the correlation between IP-10 test results derived from untreated and heat-treated plasma was described using Pearson's correlation coefficient and test results were compared using Student's t-test for paired samples. For BTB-positive buffaloes from Group 2, the correlations between the test results for the plasma IP-10, the PSC-2w and the PSC-8w assays were similarly calculated. Furthermore, PSC-2w and PSC-8w assay

results for these animals were compared using student's t-test as was the mean concentration of IP-10 in activated plasma stored on PSCs, either with or without heat treatment.

The agreement between selected diagnostic results were calculated as Cohen's kappa coefficient (*k*) using freely available online software (http://graphpad.com/quickcalcs/kappa1/) and all other analysis was done using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results and Discussion

Determination of a diagnostic cut off value for plasma IP-10

An optimal diagnostic cut off value of 1420 pg/ml (Fig. 1) for the plasma IP-10 assay resulted in a test sensitivity of 94 % (95% CI: 79.2-99.2) and test specificity of 92% (95% CI: 79.1-98.4). This corresponds closely with an optimal IP-10 cut off value for QFT-processed samples from buffaloes which has previously been calculated as 1486 pg/ml (Goosen *et al*., 2015).

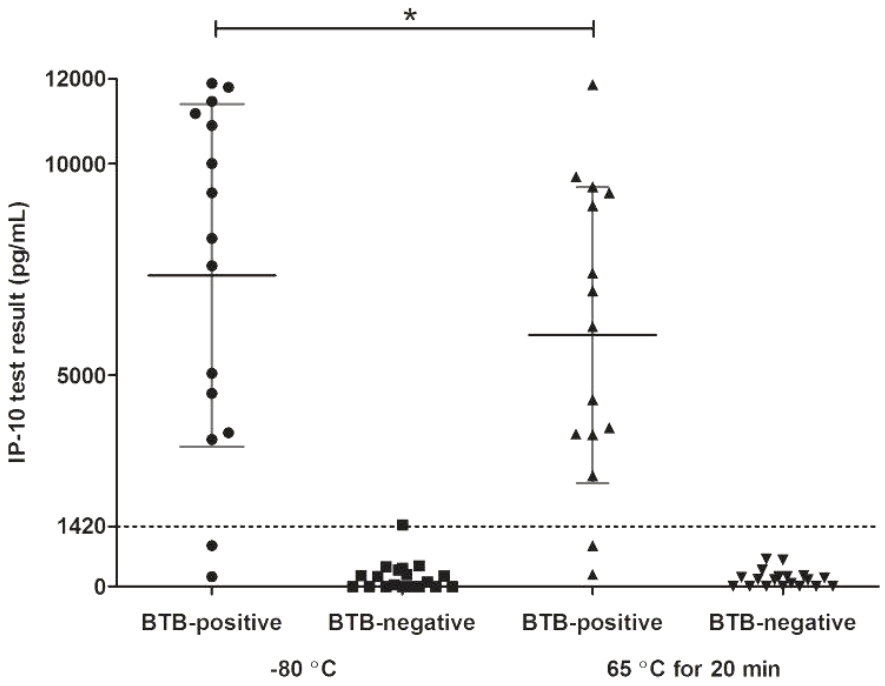

Fig. 1. Heat treatment of plasma does not compromise the detection of antigen-induced IP-10 release. Whole blood from BTB-positive and -negative buffaloes was incubated in QuantiFERON blood collection tubes and test results were calculated as the *M. bovis* peptide-specific release of IP-10 in plasma. For plasma stored at−80◦C, results for each group were compared and an optimal assay cut-off value of1420 pg/ml was determined by ROC analysis. For BTB-positive animals, IP-10 assay results obtained using plasma treated at 65◦C for 20 min were significantly lower than those obtained using untreated plasma. *, $p \leq 0.001$, Student's t-test.

Thermal stability of plasma IP-10

The relative concentration of IP-10 in non-activated plasma, measured as the ELISA optical density, was similar in samples stored at -80 ºC, 4 ºC, RT and 37 ºC for 12 days, indicating the thermal stability of this protein (Table 1). These findings are similar to those from a human study which showed plasma IP-10 to be stable for up to 14 days at RT (Aabye *et al.*, 2013). However, for activated plasma from buffaloes, while the concentration of IP-10 remained stable for 12 days at -80 °C, it was markedly decreased with an increase in storage temperature (Table 1). More importantly, IP-10 was undetected in activated plasma stored at either RT or 37 °C for 4, 8 and 12 days (Table 1). We postulate that this temperature-dependant loss of IP-

10 in PHA-stimulated plasma may be indicative of enzymatic degradation of this protein as chemokines are commonly subjected to post-translational proteolysis (Wolf *et al*., 2008). This hypothesis is supported by the fact that activated leukocytes produce several kinds of proteases, including CD26 (dipeptidylpeptidase IV) and numerous matrix metalloproteases (MMPs), many of which degrade chemokines (Lee *et al*., 2001; Moser *et al*., 2004).

Table 1

The concentration of IP-10 in samples of pooled plasma from 8 buffaloes, stored at different temperatures for between 0 and 12 days.

		Plasma IP-10 concentration (optical density)			
Plasma	Storage	Day 0	Day 4	Day 8	Day 12
Non-activated[a]	-80 ºC	0.31	0,28	0,34	0,28
	4 ºC	n.d.[b]	0,29	0,34	0,28
	RT[c]	n.d.	0,27	0,31	0,28
	37 ºC	n.d.	0,25	0,27	0,26
Activated[d]	-80 ºC	0.46	0,44	0,53	0,43
	4 ºC	n.d.	0,44	0,37	0,15
	RT	n.d.	0,08	0,08	0,09
	37 ºC	n.d.	0,08	0,08	0,09

[a] Collected and pooled following overnight incubation of whole blood with saline.
[b] Not done.
[c] Room temperature.
[d] Collected and pooled following overnight incubation of whole blood with phytohaemagglutinin (10 µg/ml).

Heat treatment of activated plasma at 65 °C for 20 min abrogated the rapid drop in IP-10 concentration in activated plasma stored at RT (Table 2) further suggesting that this phenomenon is mediated by a proteolytic enzyme which is susceptible to thermal denaturation. Importantly, this treatment resulted in a marginal loss of IP-10 and confirmed the thermal stability of this chemokine (Table 2).

Table 2
The concentration of IP-10 in samples of pooled plasma from 8 buffaloes, harvested following incubation of whole blood with phytohaemagglutinin, and either heated at 65 °C for 20 min or maintained at -80 °C before storage at room temperature for between 0 and 14 days.

	Plasma IP-10 concentration (optical density)						
Treatment	Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	Day 14
-80 °C	0,43	0,44	0,13	0,02	0,01	0,01	0,00
65 °C for 20 min	0,38	0,40	0,40	0,39	0,37	0,31	0,29

Similarly, for BTB-positive animals, IP-10 assay results obtained using plasma treated at 65 °C for 20 min were modestly but significantly lower than those obtained using untreated plasma (Fig 1); however, these results were highly correlated ($P < 0.0001$). Notably, using the cut off of 1420 pg/ml, as calculated above, diagnostic IP-10 assay results obtained using untreated plasma and plasma treated at 65 °C for 20 min showed extremely high agreement ($k = 0.94$, 95% CI: 0.82 to 1.00).

Stability of IP-10 on PSCs

The optimal diagnostic cut off value for the PSC-2w assay was 1328 pg, which resulted in a test sensitivity of 88% (95% CI: 64% - 99%) and a test specificity of 95 % (95% CI: 75% - 100%) (Fig 2). A cut off value of 589 pg for the PSC-8w assay resulted in a test sensitivity of 83% (95% CI: 56.6-96.2) and test specificity of 95% (95% CI: 75.1-100) (Fig 2). For both assays, ROC analysis returned AUC values of approximately 0.97, indicating their excellent diagnostic performance. Plasma IP-10 test results for BTB-positive animals from Group 2 were significantly correlated with those of the PSC-2w assay ($p < 0.0005$) and PSC-8w assay ($p < 0.0001$), demonstrating the stability of buffalo plasma IP-10 stored on PSCs. These findings agree with results from a human study which also showed a highly significant correlation between IP-10 detected in plasma and after storage on PSCs (Aabye *et al*., 2013, 2012). However, the magnitude of the PSC-8w assay results were significantly lower than those of the PSC-2w assay ($p < 0.001$), indicating a gradual loss of IP-10 stored on the PSCs at RT (Fig 2).

In contrast, human IP-10 has been shown to be highly stable on PSCs stored at RT over a period of 4 weeks (Aabye *et al*., 2013). However, the stability of this protein stored in this manner was not evaluated over a longer period and human plasma stored on PSCs at temperatures of 37 °C and 50 °C showed a loss of IP-10 over time which was similar to that seen in the present study (Aabye *et al*., 2012). This phenomenon did not preclude interpretation of the PSC-2w and PSC-8w assays; however, it was associated with a slight loss in assay sensitivity over time (Fig 2). Nonetheless, the plasma IP-10 assay showed very good agreement with both the PSC-2w and PSC-8w assays, respectively (k = 0.84, 95% CI: 0.67 to 1.00 and k = 0.79, 95% CI: 0.59 to 0.98). There was no significant difference between the mean IP-10 concentration from heat treated plasma samples (5368 pg/ml) and untreated plasma samples (5118 pg/ml) blotted on PSCs and left at RT for 1 week (p = 0.2829).

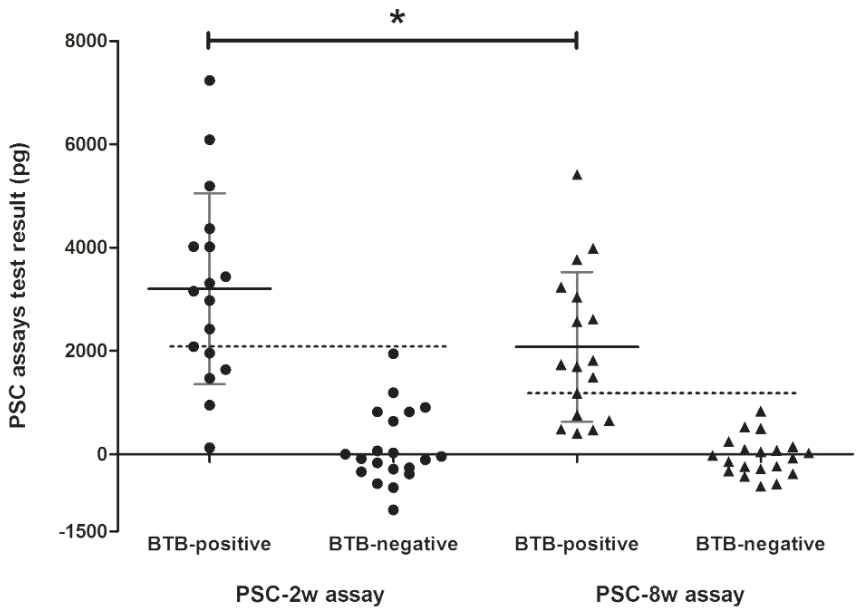

Fig. 2. The sensitivity of the IP-10 assay is decreased after storage of plasma on Protein Saver Cards (PSCs). Whole blood from BTB-positive and -negative buffaloes was incubated in QuantiFERON blood collection tubes and test results were calculated as the *M. bovis* peptide-specific release of IP-10 in plasma. Results for BTB-positive animals derived using plasma stored on PSCs for 2 weeks at room temperature (PSC-2wassay) were significantly greater than those using plasma stored for 8 weeks (PSC-8w assay). Optimal cut-off values of 1328 pg and 589 pg, and assay sensitivities of 88% and 83%, respectively, were calculated by ROC analysis. *, p < 0.01, Student's t-test.

Conclusion

The diagnostic cut off value calculated for the IP-10 plasma assay in this study is comparable with that previously reported for buffaloes (Goosen *et al*., 2015), indicating that this cytokine is a robust biomarker of *M. bovis* infection in this species. As for humans, buffalo IP-10 can be measured in plasma stored on PSCs. However, the diagnostic sensitivity of tests utilizing such samples is reduced with increased storage time and this technique might best be used for short term storage of plasma, i.e. during transport. Particularly noteworthy is the thermal stability of plasma IP-10 and samples can be heated at 65 °C for 20 min with no degradation of this protein. This characteristic of IP-10 might allow for heat-inactivation of plasma pathogens and for the safe transport of diagnostic samples.

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Chapter 7 – General Conclusion

African buffaloes (*Syncerus caffer*) are one of the most important wildlife maintenance hosts of bTB on the African continent. Spill-over of this pathogen to other wildlife species or domestic cattle may not only result in reduced productivity or death of infected animals, but also poses a serious zoonotic risk (1). Current control strategies in South Africa strive to effectively manage and prevent the spread of bTB by controlling it in livestock and buffaloes, using a test and slaughter program. However, diagnostic assays used during these programs, such as the SICTT and commercially available IGRAs, are still believed to be sub-optimal for the diagnosis of bTB in bovids (2). A potential approach to improving detection of *M. bovis* infection could be the use of novel diagnostic antigens or the identification of alternative or ancillary biomarkers to IFN-γ (3–6). Therefore, the studies presented in this dissertation aimed to identify and develop novel approaches for improving the detection of *M. bovis* infection in African buffaloes.

Assays of cell mediated immunity

The modified QuantiFERON TB-Gold assay (mQFT), a human TB test, has been previously described for the diagnosis of bTB in buffaloes (7). Additional investigation has shown that plasma collected from the QFT tubes prior to centrifugation could be reliably utilized to detect interferon-gamma from buffalo whole blood (8). Moreover, increasing the blood incubation time from 20h to 30h improved the mQFT assay's sensitivity. However, in this study, both the Bovigam® PC-EC assay and the Bovigam® PC-HP assay were more sensitive than either the SICTT or mQFT assay in its current format. Nonetheless, the application of the mQFT assay remains practical for bTB diagnosis in buffaloes under field conditions compared to any Bovigam assay and improvements, as described above, may improve its sensitivity.

In an additional study, both the Bovigam PPD assay and modified Bovigam PPD assay displayed greatest sensitivity for the detection of *M. bovis*-infected buffaloes (Goosen, unpublished data). The SICTT detected additional IGRA-negative animals and maximum sensitivity was attained when the SCITT and Bovigam PPD assays were used in combination. These findings are supported by previous cattle studies that advocate the parallel use of the SICTT and the Bovigam PPD assay (9,10). Evidence-based diagnostic test algorithms will improve detection and inform policy decision in the bTB control programs for buffalo in South Africa.

IP-10 as biomarker of cell-mediated immunity

The studies presented here have also shown that 1) IP-10 is a useful marker of immune activation in buffaloes when using a commercially available IP-10 bovine ELISA (11); 2) measurement of antigen-specific IP-10 can accurately distinguish between uninfected and *M. bovis*-infected buffaloes and addition of the IP-10 assay increases the sensitivity over conventional IGRAs (12); 3) the calculated cut-off value for IP-10 was consistent in two independent studies, suggesting its reliability as a potential diagnostic assay in buffaloes (12,13); 4) IP-10 can be measured in plasma stored on Protein Saver Cards (PSCs); however the sensitivity of tests utilizing such samples is reduced with increased storage (13); and lastly, 5) plasma samples can be heated to 65 ºC for 20 min with no degradation of IP-10, demonstrating the thermal stability of this cytokine (13).

These findings have highlighted the potential application of IP-10 for the diagnosis of bTB in African buffaloes. Improved sensitivity of *M. bovis*-specific IGRAs is a significant advantage of using IP-10 as a preferred biomarker in this species. These results suggest that IP-10 may be a useful biomarker of immunological activation for detection of other diseases in buffalo.

Other advantages of IP-10 are its thermal tolerance and stability on PSCs. These characteristics facilitate movement of diagnostic samples by permitting heat-inactivation of potential pathogens in plasma, and transport of samples by conventional methods, respectively. Storage, transport and biosafety are important issues when developing a diagnostic test for wildlife which live in remote areas, away from veterinary laboratories. Therefore, IP-10 as a diagnostic biomarker may facilitate testing of buffalo herds.

Future studies

To successfully advance this research, the performance of IGRAs, the SICTT and the bovine IP-10 ELISA must be further investigated in buffalo populations with varying bTB prevalence to determine factors influencing the performance of these diagnostic tests. Additional studies should be conducted to address the logistical constraints of cytokine release assays under field conditions by evaluating commercially prepared *M. bovis*-antigen tubes. Given the thermal and storage stability characteristics of IP-10, further research is needed to thoroughly evaluate its diagnostic performance in *M. bovis*-antigen stimulated plasma samples that are stored and transported under expected field conditions from buffaloes with variable stages of *M. bovis* infection and disease to determine whether these provide alternative methods to address current logistical limitations. Lastly, since IP-10 appears to be produced in significant abundance in antigen-stimulated buffalo whole blood, additional research should investigate the use of this biomarker for other diagnostic applications.

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Appendix I

Supplementary Table 1

Test results for African buffaloes with discordant bovine tuberculosis test outcomes.

	BEC [a]		BHP [b]		mQFT20 [c]		mQFT30 [d]		SICTT [e]	*M.bovis* culture [f]
Animal	Result	ΔOD [g] (450nm)	Result	ΔOD (450nm)	Result	ΔIFN-γ [h] (pg/ml)	Result	ΔIFN-γ (pg/ml)		
1	+ [i]	0.158	+	0.131	- [j]	16	-	57	-	n.d. [k]
2	+	2.839	+	2.564	+	841	+	1352	+	-
3	-	0.000	-	0.027	-	0	-	3	-	n.d.
4	-	0.051	-	0.055	-	0	-	3	-	n.d.
5	-	0.022	-	0.029	-	0	-	0	-	n.d.
6	-	0.005	-	0	-	13	-	55	-	n.d.
7	-	0.017	-	0	-	0	-	5	-	n.d.
8	-	0.050	+	0.170	-	0	-	17	-	n.d.
9	+	1.228	+	1.109	+	226	+	767	+	-
10	-	0	-	0	-	0	-	0	-	n.d.
11	-	0.001	-	0.023	-	0	-	0	-	n.d.
12	-	0.024	-	0.025	-	0	-	7	-	n.d.
13	-	0.054	-	0.068	-	23	-	14	-	n.d.
14	+	0.549	+	0.586	+	137	+	783	+	*M.bovis*
15	-	0.000	-	0.000	-	0	-	2	-	n.d.
16	+	2.717	+	3.654	+	837	+	1258	+	*M.bovis*
17	-	0.040	-	0	-	40	-	40	-	n.d.
18	+	2.530	+	2.414	+	435	+	1080	+	-
19	+	2.477	+	2.033	+	712	+	882	+	*M.bovis*
20	+	2.757	+	2.650	+	1293	+	1582	+	*M.bovis*
21	-	0.002	-	0.003	-	0	-	0	-	n.d.
22	-	0	-	0	-	0	-	0	-	n.d.
23	+	1.864	+	1.566	+	216	+	893	+	*M.bovis*
24	-	0.000	-	0.000	-	0	-	0	-	n.d.
25	-	0	-	0	-	3	-	0	-	n.d.
26	+	2.262	+	2.192	+	1192	+	846	+	*M.bovis*
27	-	0.043	-	0.057	-	0	-	19	-	n.d.
28	+	0.318	+	0.283	+	122	+	290	+	*M.bovis*
29	-	0	-	0	-	0	-	6	-	n.d.
30	-	0.042	-	0.091	-	46	-	35	+	n.v.l. [l]
31	-	0	-	0.062	-	0	-	0	-	n.d.

32	+	0.150	+	0.206	-	0	-	0	-	n.d.
33	+	0.260	+	0.165	-	0	-	11	+	n.d.
34	+	0.095	+	0.098	-	0	-	20	-	n.d.
35	-	0.001	-	0	-	0	-	0	-	n.d.
36	+	0.168	+	0.191	-	46	+	236	+	*M.bovis*
37	+	2.777	+	2.599	+	367	+	915	+	*M.bovis*
38	-	0	-	0	-	0	-	5	-	n.d.
39	-	0.001	-	0.077	-	0	-	0	-	n.d.
40	-	0.005	-	0.025	-	0	-	0	-	n.d.
41	+	0.643	-	0.000	-	14	-	0	-	n.d.
42	+	0.253	+	0.184	-	36	+	74	+	-
43	+	0.254	+	0.226	-	26	+	108	+	*M.bovis*
44	+	2.080	+	2.159	+	397	+	1330	+	*M.bovis*
45	-	0	-	0	-	7	-	20	-	n.d.
46	-	0.000	-	0.000	-	0	-	0	-	n.d.
47	-	0.000	-	0.039	-	0	-	0	-	n.d.
48	-	0.065	-	0.057	-	44	-	3	+	*M.bovis*
49	+	0.111	-	0.083	-	12	-	27	+	*M.bovis*
50	+	0.393	+	0.410	-	1	-	2	-	n.d.
51	+	0.618	+	0.578	+	190	+	409	+	n.d.
52	-	0.085	-	0.073	-	45	-	41	+	-
53	+	0.816	+	0.919	+	177	+	383	+	*M.bovis*
54	+	1.817	+	1.854	+	444	+	934	+	*M.bovis*
65	+	2.462	+	2.433	+	734	+	875	+	n.d.
66	-	0.000	-	0.022	-	0	-	0	-	n.d.
67	+	0.191	+	0.212	-	22	-	162	-	*M.bovis*
68	-	0	+	0.288	+	191	+	549	+	*M.bovis*
69	-	0.023	-	0.024	-	17	-	0	-	n.d.
70	-	0.067	+	0.103	-	26	+	68	-	n.d.
71	+	3.019	+	2.741	+	1387	+	1347	+	*M.bovis*
72	-	0.005	-	0	-	0	-	34	-	n.d.
73	+	1.427	+	0.984	+	584	+	663	+	*M.bovis*

74	+	2.715	+	3.084	+	1001	+	72	+	*M.bovis*
75	-	0.088	+	0.154	+	129	+	142	-	n.d.
76	-	0	-	0	-	0	-	1	-	n.d.
77	+	0.179	+	0.295	+	120	+	99	+	*M.bovis*
78	+	0.841	+	0.843	+	280	+	503	+	n.d.
79	+	0.931	+	1.031	+	450	+	661	+	*M.bovis*
80	+	2.653	+	2.509	+	890	+	638	+	*M.bovis*
81	+	2.064	+	2.136	+	1036	+	528	+	*M.bovis*
82	+	0.286	+	0.258	+	137	+	218	+	*M.bovis*
83	-	0.000	-	0.019	-	34	-	60	-	n.d.
84	+	2.205	+	2.155	+	307	+	789	+	*M.bovis*
85	+	2.923	+	2.566	+	669	+	592	+	*M.bovis*
86	-	0.000	-	0.000	-	0	-	4	-	n.d.
87	+	2.134	+	2.047	+	1312	+	1180	+	*M.bovis*
88	-	0.000	-	0.000	-	0	-	4	-	n.d.
89	+	1.903	+	2.381	+	947	+	1303	+	n.d.
90	-	0.082	-	0.074	-	0	-	11	-	n.d.
91	-	0	-	0.043	-	22	-	15	-	-
92	-	0	-	0	-	0	-	7	-	n.d.
93	-	0.004	-	0.000	-	0	-	9	-	n.d.
94	-	0.075	-	0.075	-	36	+	121	-	n.d.

[a] Bovigam® assay utilising the ESAT-6 and CFP-10 proteins
[b] Bovigam® assay utilising a hypothetical protein
[c] modified QuantiFERON® TB-Gold assay (20 h blood incubation)
[d] modified QuantiFERON® TB-Gold assay (30 h blood incubation)
[e] single intradermal comparative tuberculin test
[f] All culture-positive samples was tested by Polymerase Chain Reaction to determine if M.bovis. (Warren et al., 2006)
[g] Optical density of the peptide stimulated sample minus that of the Nil sample.
[h] IFN-γ concentration in the TB Antigen-stimulated sample minus that in the Nil sample
[I] positive
[j] negative
[k] not done = n.d
[l] no visible lesion = n.v.l.

Appendix II

Table S1

Antigen-specific cytokine release and test results for the mQFT, PC-EC and PC-HP assays and the IP-10(QFT), IP-10(EC) and IP-10(HP) tests in a cohort of Bovigam-positive (n = 44) and Bovigam-negative (n = 40) buffaloes.

	PC-EC(1)		PC-HP(1)		mQFT(1)		IP-10(EC)		IP-10(HP)		IP-10(QFT)	
Animal	Result	ΔOD[a] (450nm)	Result	ΔOD (450nm)	Result	ΔIFN-γ (pg/ml)	Result	ΔIP-10 (pg/ml)	Result	ΔIP-10 (pg/ml)	Result	ΔIP-10 (pg/ml)
1	+[b]	0.158	+	0.131	-[c]	16	-	4250	+	2456	-	116
2	+	2.839	+	2.564	+	841	+	10079	+	6688	+	3896
3	-	0	-	0.027	-	0	-	503	-	1488	-	0
4	-	0.051	-	0.055	-	0	-	217	-	114	-	0
5	-	0.022	-	0.029	-	0	-	2890	-	1173	-	67
6	-	0.005	-	0	-	13	-	0	-	0	-	129
7	-	0.017	-	0	-	0	-	381	-	241	-	0
8	-	0.05	+	0.17	-	0	-	0	-	0	-	58
9	+	1.228	+	1.109	+	226	+	8242	+	7172	+	2123
10	-	0	-	0	-	0	-	38	-	0	-	29
11	-	0.001	-	0.023	-	0	-	0	-	0	-	8
12	-	0.024	-	0.025	-	0	-	412	-	1023	-	0
13	-	0.054	-	0.068	-	23	-	3924	+	3332	-	1122
14	+	0.549	+	0.586	+	137	+	7871	+	3351	+	2458
15	-	0	-	0	-	0	-	276	-	95	-	42
16	+	2.717	+	3.654	+	837	+	14849	+	6536	+	6187
17	-	0.04	-	0	-	40	-	2314	-	204	-	9
18	+	2.53	+	2.414	+	435	+	11217	+	8101	+	4064
19	+	2.477	+	2.033	+	712	+	9227	+	7746	+	3751
20	+	2.757	+	2.65	+	1293	+	14469	+	8349	+	4100

21	-	0.002	-	0.003	-	0	-	1	-	226	-	0
22	-	0	-	0	-	0	-	710	-	313	-	0
23	+	1.864	+	1.566	+	216	+	17424	+	13790	+	8595
24	-	0	-	0	-	0	-	121	-	0	-	0
25	-	0	-	0	-	3	-	15	-	1194	-	0
26	+	2.262	+	2.192	+	1192	+	17943	+	12492	+	6319
27	-	0.043	-	0.057	-	0	+	6817	+	4484	-	1260
28	+	0.318	+	0.283	+	122	+	14317	+	9372	+	7587
29	-	0	-	0	-	0	-	0	-	0	-	0
30	-	0.042	-	0.091	-	46	-	4467	-	738	+	2131
31	-	0	-	0.062	-	0	-	453	-	87	-	49
32	+	0.15	+	0.206	-	0	-	601	-	580	-	0
33	+	0.26	+	0.165	-	0	+	4564	+	4503	+	3552
34	+	0.095	+	0.098	-	0	+	4836	+	2487	-	506
35	-	0.001	-	0	-	0	-	435	-	782	-	322
36	+	0.168	+	0.191	-	46	+	4610	+	2540	+	3934
37	+	2.777	+	2.599	+	367	+	25117	+	17035	+	9929
38	-	0	-	0	-	0	-	690	-	1451	-	0
39	-	0.001	-	0.077	-	0	+	5372	-	276	-	0
40	-	0.005	-	0.025	-	0	-	350	-	449	-	0
41	+	0.643	-	0	-	14	-	762	-	646	-	0
42	+	0.253	+	0.184	-	36	+	13413	+	6777	+	5823
43	+	0.254	+	0.226	-	26	+	11507	+	13281	+	6860
44	+	2.08	+	2.159	+	397	+	24131	+	24131	+	12615
45	-	0	-	0	-	7	-	1999	-	2141	-	467
46	-	0	-	0	-	0	-	1121	-	1224	-	0
47	-	0	-	0.039	-	0	-	0	-	244	-	0
48	-	0.065	-	0.057	-	44	-	4551	+	3124	-	964
49	+	0.111	-	0.083	-	12	-	4033	+	2544	+	1499
50	+	0.393	+	0.41	-	1	+	7618	+	6247	-	544

51	+	0.618	+	0.578	+	190	+	13172	+	12676	+	5011
52	-	0.085	-	0.073	-	45	+	5963	+	6069	-	1472
53	+	0.816	+	0.919	+	177	+	12062	+	12043	+	11539
54	+	1.817	+	1.854	+	444	+	21058	+	15605	+	9817
65	+	2.462	+	2.433	+	734	+	6468	+	5509	+	5133
66	-	0	-	0.022	-	0	-	1437	-	1979	-	0
67	+	0.191	+	0.212	-	22	+	5498	+	3254	+	4365
68	-	0	+	0.288	+	191	-	3277	+	2761	+	4447
69	-	0.023	-	0.024	-	17	-	1233	-	435	-	0
70	-	0.067	+	0.103	-	26	-	2585	+	2324	+	1533
71	+	3.019	+	2.741	+	1387	+	6677	+	6292	+	7252
72	-	0.005	-	0	-	0	-	315	-	491	-	0
73	+	1.427	+	0.984	+	584	+	7937	+	5331	+	8345
74	+	2.715	+	3.084	+	1001	+	11288	+	6986	+	11075
75	-	0.088	+	0.154	+	129	-	1642	+	2168	+	1679
76	-	0	-	0	-	0	-	0	-	0	-	0
77	+	0.179	+	0.295	+	120	+	13721	+	14188	+	4793
78	+	0.841	+	0.843	+	280	+	13375	+	5651	+	6849
79	+	0.931	+	1.031	+	450	+	11557	+	9063	+	9956
80	+	2.653	+	2.509	+	890	+	14559	+	11934	+	11511
81	+	2.064	+	2.136	+	1036	+	9191	+	6769	+	9143
82	+	0.286	+	0.258	+	137	+	7734	+	5972	+	5784
83	-	0	-	0.019	-	34	-	1650	-	1753	+	1669
84	+	2.205	+	2.155	+	307	+	14510	+	9983	+	10983
85	+	2.923	+	2.566	+	669	+	22194	+	15026	+	7024
86	-	0	-	0	-	0	-	298	-	61	-	162
87	+	2.134	+	2.047	+	1312	+	10459	+	7201	+	11788
88	-	0	-	0	-	0	-	1409	-	1034	-	393
89	+	1.903	+	2.381	+	947	+	12951	+	9063	+	13874
90	-	0.082	-	0.074	-	0	-	2397	-	730	-	1129

91	-	0	-	0.043	-	22	-	710	-	231	-	0
92	-	0	-	0	-	0	-	651	-	1190	-	0
93	-	0.004	-	0	-	0	-	1253	-	858	-	153
94	-	0.075	-	0.075	-	36	-	3747	-	1839	+	3446

[a] Optical density of the peptide stimulated sample minus that of the unstimulated sample
[b] positive
[c] negative

1. **Goosen WJ, Miller MA, Chegou NN, Cooper D, Warren RM, van Helden PD, Parsons SDC**. 2014. Agreement between assays of cell-mediated immunity utilizing *Mycobacterium bovis*-specific antigens for the diagnosis of tuberculosis in African buffaloes (*Syncerus caffer*). Vet Immunol Immunopathol **160**:133–138.

Appendix III

Table S1

Test results for all African buffaloes positive for any test.

Animal	Bovigam PPD[a]			modified PPD[b]		PC-EC[c]		PC-HP[d]		SICTT[e]	*M. bovis* isolation[f]	PM examination[g]
	Result	OD[h](Bov)-OD(Nil)	OD(Bov) - OD(Av)	Result	OD(Fort) - OD(Nil)	Result	OD(EC) - OD(Nil)	Result	OD(HP) - OD(Nil)			
A1	+[i]	1,625	1,562	+	0,119	+	1,143	+	1,310	+	+	2[j]-R; 1-Tr; 1-M; 2-To; 3-L
A6	+	0,333	0,302	+	0,013	-[k]	0,003	-	0,060	+	+	3-R; 1-Tr
A12	+	1,725	1,580	+	0,022	+	0,620	+	0,750	+	+	2-R
A14	-	-0,053	0,071	-	-0,206	+	0,108	+	0,606	-	+	2-R
A24	+	0,538	0,535	+	0,018	+	0,213	+	0,351	+	+	SUS-R
A25	+	2,400	2,324	+	0,049	+	1,114	+	1,230	+	+	3-R
A35	-	0,082	0,065	-	0,020	-	0,028	-	0,032	+	-	1-R
A49	+	0,426	0,401	+	0,005	+	0,154	+	0,188	+	+	3-R
A51	-	-0,003	0,202	-	-0,129	-	0,060	+	0,174	-	-	0
A54	+	0,396	0,356	+	0,018	+	0,180	+	0,234	+	+	0
A59	-	0,028	0,024	-	0,006	-	0,030	-	0,024	+	+	0
A61	+	0,174	0,117	+	0,059	-	0,040	-	0,082	+	+	3-Tr
A62	+	0,245	0,234	+	0,016	+	0,113	+	0,107	+	-	1-R; 1- Tr
A66	+	0,436	0,421	+	0,013	+	0,142	+	0,197	+	+	3-R; 3-To
A78	+	0,767	0,762	+	0,007	+	0,474	+	0,586	+	+	2-R; 1-To
A82	+	0,976	0,901	+	0,022	+	0,513	+	0,649	+	+	2-R
A93	+	0,103	0,095	+	0,004	-	0,053	+	0,099	+	+	2-R
A99	+	0,988	0,932	+	0,013	+	0,454	+	0,568	+	+	2-R
A101	+	1,618	1,408	+	0,045	+	0,451	+	0,522	+	+	3-R; 2-To
A107	-	0,092	0,087	-	0,011	-	0,033	-	0,045	+	+	2-R

B3	+	1,726	1,512	+	0,196	+	0,743	+	0,793	+	+	2-To
B5	-	0,043	0,047	-	-0,017	+	0,361	-	-0,023	+	-	0
B15	+	0,438	0,292	+	0,140	-	-0,001	-	0,017	-	-	0
B16	+	0,332	0,309	+	0,016	+	0,192	+	0,212	-	-	0
B19	+	1,858	1,685	+	0,535	+	1,104	+	1,296	+	+	2-P; 1-S
B24	+	0,943	0,935	+	0,057	+	0,403	+	0,710	+	-	0
B26	+	1,676	1,621	+	0,035	+	0,981	+	0,959	+	+	1-Tr; 2-L
B28	+	0,197	0,125	+	0,050	-	0,007	-	0,011	-	-	1-R; 1-Pa
B29	+	2,269	2,132	+	0,055	+	0,972	+	0,790	+	+	3-M; 3-L
B30	+	1,888	1,794	+	0,024	+	0,890	+	0,919	+	+	3-Tr; 3-M; 1-P; 3-L
B31	+	0,268	0,275	+	0,023	-	0,066	+	0,095	+	+	0
B39	+	2,599	2,102	+	0,155	+	2,930	+	2,607	+	+	3-R
B40	+	0,159	0,113	-	0,159	-	-0,060	-	0,019	-	-	2-R; 1-Tr; 1-M
B45	-	0,088	-1,027	-	0,071	+	0,689	-	-0,008	-	-	0
B47	-	0,034	0,004	-	0,029	+	0,114	-	0,039	-	-	0
B49	-	0,228	0,077	-	0,071	-	0,063	-	0,083	+	-	0
B50	+	0,885	0,761	+	0,045	-	0,007	-	0,006	-	-	0
B51	-	0,053	0,005	-	0,005	+	0,137	-	0,008	-	-	1-Tr; SUS-L
B52	+	2,855	1,129	+	0,197	+	2,706	+	2,563	-	+	2-L
B53	+	0,456	0,424	+	0,121	+	0,297	+	0,294	+	+	2-L
B59	+	2,881	2,273	+	0,073	+	2,527	+	2,812	+	-	2-Tr
B63	+	2,029	0,833	+	2,181	-	0,036	+	0,142	+	-	2-Pa
B65	+	1,955	1,844	+	0,125	+	0,780	+	1,073	+	-	1-R
B66	+	3,433	3,008	+	0,077	+	2,616	+	2,594	-	+	3-P; 3-L
B67	+	1,134	1,089	+	-0,048	+	0,661	+	0,842	+	+	3-R; 3-Tr; 3-M;3-L
B68	+	0,163	0,123	+	0,115	-	-0,023	-	-0,012	-	-	0
B69	-	0,261	0,084	-	0,031	-	0,008	-	-0,008	+	-	0
B75	+	3,017	2,923	+	0,099	+	2,244	+	2,561	+	-	2-L;

B78	+	0,164	0,108	+	0,049	-	0,009	-	0,032	-	-	2-retro
B80	+	0,981	0,895	+	0,082	+	0,126	+	0,179	-	+	3-retro
B82	+	0,312	0,268	+	0,087	-	0,042	-	0,076	-	-	0
B89	+	0,170	0,133	+	0,048	-	0,046	-	0,056	+	+	2-L
B92	+	0,665	0,664	+	-0,121	+	0,180	+	0,257	-	+	2-R; 1-M; 3-L
C18	+	1,190	1,043	+	0,123	+	0,370	+	0,497	+	+	3-R
C28	-	0,212	-0,153	-	0,606	+	0,108	+	0,118	-	-	0
C29	+	1,155	1,003	+	0,079	+	0,440	+	0,569	+	-	1-R
C47	+	2,901	2,488	+	0,148	+	2,675	+	2,755	+	+	3-S; 2-Tr; 2-M; 1-To; 3-Pa
C52	+	2,764	2,306	+	0,558	+	2,831	+	2,949	+	n.d.[l]	3-R; 2-To
C63	+	1,561	1,320	+	0,143	+	0,748	+	1,085	+	+	2-R
C69	+	0,145	0,095	+	0,037	-	-0,007	-	-0,008	+	-	0
C70	-	0,051	-0,168	-	0,180	+	0,135	+	0,189	-	-	0
C72	+	0,786	0,747	+	0,056	+	0,520	+	0,332	+	+	3-R
C80	+	1,308	1,160	+	0,197	+	0,954	+	0,843	+	-	1-To

[a] Bovigam® assay utilizing *M. bovis* purified protein derivatives (PPD) and *M. avium* PPD.
[b] Modified Bovigam® assay utilizing *M. bovis*, *M. avium* and *M. fortuitum* PPDs.
[c] Bovigam® assay utilizing peptides derived from ESAT-6 and CFP-10.
[d] Bovigam® assay utilizing peptides derived from ESAT-6, CFP-10, Rv3615 and 3 additional mycobacterial antigens.
[e] Single intradermal comparative tuberculin test.
[f] Warren *et al*. (2006)
[g] *Post-mortem* examination of the: Retropharyngeal lymph nodes (R), Tracheobronchial lymph nodes (Tr), Mediastinal or mediastinal lymph nodes (M), Tonsils (To), Lungs (L), Submandibular lymph nodes (S), Prescapular lymph nodes (P) and Parotid lymph nodes (Pa).
[h] Optical density of the antigen stimulated sample minus that of the Nil sample.
[i] Positive.
[j] Lesion scores: 0-no visible lesions; 1-small focal lesion; 2-several small foci or single large lesion and 3-multifocal or confluent lesions.
[k] Negative.
[l] not done.