The evaluation and validation of cell-mediated immunological responses for the improved detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*)

by

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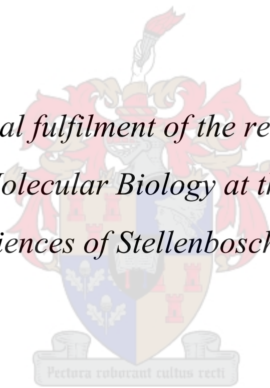

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Declaration

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This thesis includes three original papers submitted for publication in peer-reviewed journals (Chapters 4-6) and a literature review (Chapter 3). The remaining four chapters comprise the summary (Chapter 1), general introduction (Chapter 2), general discussion (Chapter 7) and conclusion (Chapter 8). The development and writing of the chapters were the principal responsibility of myself.

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Abstract

Mycobacterium bovis infection and the resulting bovine tuberculosis (bTB) disease affects a broad range of mammals including domestic animals, wildlife and humans. The presence of global wildlife maintenance hosts impedes efforts to control *M. bovis* transmission and bTB. African buffaloes (*Syncerus caffer*) are reservoir hosts of *M. bovis* in South Africa and thus pose a threat to multiple other wildlife species, adjacent livestock and their respective communities. Therefore, the efficient and early detection of *M. bovis*-infected buffaloes can help to minimize the spread of *M. bovis* and bTB disease. Considering that current standard bTB screening tools for buffaloes remain suboptimal, and in-field application presents several limitations, the validation of more optimal and approved diagnostic tools for *M. bovis* and bTB testing is required.

This project investigated three such approaches to enhancing *M. bovis* detection in buffaloes. Firstly, the tuberculin skin test (TST), the primary *ante mortem* diagnostic tool for *M. bovis* detection in South Africa, was investigated for species-specific use. Buffalo specific cut-off values were calculated for the first time and TST test performance parameters evaluated, using a well-defined negative cohort and a gold standard positive cohort. The results present evidence for current South African guidelines for TST interpretation in buffaloes, in addition to providing alternative recommendations as required for different bTB testing scenarios. Moreover, the high specificity (Sp) yet suboptimal sensitivity (Se) of the TST was demonstrated, reiterating the necessity for additional tests to increase detection of infected animals. The most widely used ancillary test to the TST is the interferon-gamma (IFN-γ) release assay (IGRA). The second section of this project validated the new commercial Mabtech ELISA^{PRO} bovine IFN-γ enzyme-linked immunosorbent assay (ELISA) that demonstrated promising test performance, with buffalo-specific cut-off values, in an IGRA using the QuantiFERON® TB-Gold Plus (QFT-Plus) stimulation platform and cohorts as described above. Furthermore, the QFT-Plus/Cattletype® IGRA, previously validated for buffalo use, was compared with the Mabtech IGRA after calculating species specific cut-off values to replace the manufacturer's cattle threshold value. This served to significantly enhance Se. Finally, as a novel approach to *in vitro* measurement of cell-mediated immune responses to *M. bovis,* a MILLIPLEX® bovine cytokine/chemokine multiplex assay was investigated for use in buffaloes. The results demonstrated the detection of all fifteen target biomarkers with

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significant differences observed between mitogen-stimulated and/or antigen-stimulated, and unstimulated plasma samples in *M. bovis*-infected buffaloes.

Overall, the results from this project reveal the importance of species-specific validation of diagnostic tests intended for field use. For wildlife species this is particularly challenging, and relevant positive and negative reference samples are not always readily available. Furthermore, the optimisation of current (and candidate) tests, as demonstrated in this project for the TST and IGRAs, should be performed in line with official validation standards (when possible). This will better enable the recognition and in-field application of enhanced and promising diagnostic tools for improved detection of *M. bovis* infection in African buffaloes.

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Opsomming

Mycobacterium bovis infeksie is verantwoordelik vir die siekte bekend as bees tuberkulose (bTB) in verskeie soogdiere soos plaasdiere- en wilde diere, asook mense. Die teenwoordigheid van 'n instandhoudingsgasheer soos die Kaapse buffel (*Syncerus caffer*) in 'n wildsisteem, ondermyn alle pogings om sulke infeksies in verskeie omgewings te beheer. Om verdere verspreiding van *M. bovis* onder soogdiere te voorkom is dit dus belangrik om *M. bovis* infeksie in 'n dier so vroeg as moontlik te identifiseer deur gebruik te maak van die mees optimale toetse. Daar word egter 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 (IFN-γ)-vrystellingstoetse ("IFN-γ release assays"; IGRAs), suboptimaal is vir die diagnose van bTB in beesverwante hoefdiere.

Die doelwit van hierdie studie was om die beskikbare toetse, soos vroeër beskryf, vir die eerste keer te optimiseer vir gebruik in buffels. Buffel spesifieke afsnypunte vir die veltoets (SICTT) was bepaal deur gebruik te maak van historiese negatiewe buffel populasies asook wel bekende positiewe populasies. Resultate verkry gedurende hierdie studie ondersteun huidige Suid-Afrikaanse veltoets riglyne vir die interpretasie van veltoets reaksies in buffels en voorsien ook addisionele gebruike en riglyne vir ander tipe buffel populasies. Die volgende komponent van hierdie studie was om 'n nuwe IFN-γ-vrystellingstoets, die 'Mabtech ELISA^{PRO} bovine' IFN-γ ensiem-gekoppelde immunosorbente toets (ELISA) te evalueer in bogenoemde negatiewe en postiewe buffel populasies en die diagnostiese potensiaal van hierdie toets te vergelyk met die meer bekende 'Cattletype' ELISA. Vir hierdie toetse is die plasma van die diere versamel nadat bloed in die QuantiFERON® TB-Gold Plus (QFT-Plus) sisteem gestimuleer was. Na buffel-spesifieke afsnypunte bepaal is vir albei ELISAs, was dit duidelik dat albei toetse se diagnostiese sensitiwiteit eenders was. Laastens, om verdere moontlike immunologiese kandidaat merkers van *M. bovis* infeksie te identifiseer vir moontlike diagnostiese gebruik in buffels, is die 'MILLIPLEX® bovine cytokine/chemokine multiplex' toets vir die eerste keer in buffels getoets. Vyftien moontlike merkers met diagnostiese potensiaal was geïdentifiseer, omdat hulle suksesvol tussen mitogeen gestimuleerde plasma, *M. bovis* antigeen-gestimuleerde plasma en ongestimuleerde plasma van *M. bovis*-geïnfekteerde buffels kon onderskei.

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Ten slotte beklemtoon, hierdie studie die belangrikheid van spesie-spesifieke optimisering en validasie van alle diagnostiese toetse wat in die veld op diere gebruik mag word. In wilde diere is hierdie tipe optimiserings en validasies aansienlik moeiliker in vergelyking met ander diere soos beeste. Verder is dit baie moeilik om bevestigde *M. bovis* negatiewe buffels te bekom. As gevolg van bogenoemde redes is hierdie studie se bevindinge waardevol vir siekte navorsing in wilde diere. Wanneer toetse gevalideer word soos in hierdie studie, moet die amptelike validasie riglyne van die land gevolg word (waar moontlik) om verbeterde diagnostiese toetse vir *M. bovis* infeksies in die Kaapse buffels te ontwikkel. Sodoende, sal toetse vinniger deur die staat aanvaar word en die gebruik daarvan in die veld grootliks ondersteun word.

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List of Publications

1. **Katrin Smith**, Netanya Bernitz, David Cooper, Tanya J. Kerr, Candice de Waal, Charlene Clarke, Samantha Goldswain, Warren McCall, Alicia McCall, Debbie Cooke, Emma Rambert, Leanie Kleynhans, Robin M. Warren, Paul van Helden, Sven D. C. Parsons, Wynand J. Goosen, Michele A. Miller. 2020. Optimisation of the tuberculin skin test for *Mycobacterium bovis* detection in African buffaloes (*Syncerus caffer*). Submitted: Preventive Veterinary Medicine.
2. **Katrin Smith**, Netanya Bernitz, Samantha Goldswain, David V. Cooper, Robin M. Warren, Wynand J. Goosen, Michele A. Miller. 2020. Improved detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using modified interferon-gamma release assays. Submitted: Veterinary Immunology and Immunopathology.
3. **Katrin Smith**, Léanie Kleynhans, Candice Snyders, Netanya Bernitz, David Cooper, Paul van Helden, Robin M. Warren, Michele A. Miller, Wynand J. Goosen. 2020. Use of the MILLIPLEX® bovine cytokine/chemokine multiplex assay to identify *Mycobacterium bovis*-infection biomarkers in African buffaloes (*Syncerus caffer*). Published: Veterinary Immunology and Immunopathology. https://doi.org/10.1016/j.vetimm.2020.110152

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List of Abbreviations

AM	alveolar macrophage
AUC	area under the curve
bTB	bovine tuberculosis
CFP-10	culture filtrate protein 10 kD
CI	confidence interval
CMI	cell-mediated immunity
CV	coefficient of variation
DAFF	Department of Agriculture, Forestry and Fisheries
DNA	deoxyribonucleic acid
DPI	days post infection
EC	early secretory antigen target 6 kD/culture filtrate protein 10 kD
ELISA	enzyme-linked immunosorbent assay
ESAT-6	early secretory antigen target 6 kD
EU	European Union
HiP	Hluhluwe-iMfolozi Park
IFN-γ	interferon-gamma
IGRA	interferon-gamma release assay
IL	interleukin
IP-10	interferon-gamma induced protein-10
IPRA	interferon-gamma induced protein-10 release assay
IR	inconclusive reactor
M. avium	*Mycobacterium avium*
M. bovis	*Mycobacterium bovis*
MCP	macrophage chemoattractant protein
MIG	monokine induced by interferon-gamma (CXCL9)
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
M. tuberculosis	*Mycobacterium tuberculosis*
OIE	World Organisation of Animal Health
PBMC	peripheral blood mononuclear cells

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PCR	polymerase chain reaction
PPD	purified protein derivative
PPD_a	*Mycobacterium avium p*urified protein derivative
PPD_b	*Mycobacterium bovis p*urified protein derivative
PHA	phytohemagglutinin
QFT	QuantiFERON®-TB Gold
QFT-Plus	QuantiFERON®-TB Gold Plus
RNA	ribonucleic acid
ROC	receiver operating characteristic
RT	room temperature
SA	South Africa
SD	standard deviation
Se	sensitivity
SFT	skinfold thickness
SICTT	single intradermal comparative tuberculin test
SITT	single intradermal tuberculin test
S/P	sample to positive control ratio
Sp	specificity
TB	tuberculosis
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper cell 17
TNF	tumour necrosis factor
TST	tuberculin skin test
UK	United Kingdom
US	United States
VEGF	vascular endothelial growth factor
VPN	Veterinary Procedural Notice

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Chapter 1: Summary

Mycobacterium bovis is the causative agent of the zoonotic disease bovine tuberculosis (bTB). This pathogen primarily infects bovids, however, has a broad host range that includes multiple domestic and wild animal species. A contribution to the continued transmission of *M. bovis* infection to animal species and humans is the global presence of wildlife maintenance hosts; in South Africa (SA) this is the African buffalo (*Syncerus* caffer). The overarching purpose of this research project was to investigate and enhance newly available and currently used cell-mediated immunological (CMI) diagnostic methods for the improved detection of *M. bovis*-infected African buffaloes.

The first research component comprised the (additional) collection and collation of buffalo tuberculin skin test (TST) data from 2015-2019. In SA, the TST is the primary approved screening test for bTB in buffaloes and domestic cattle. Species-specific cut-off values for three different TST criteria were calculated for the first time, using gold standard-defined sample cohorts. Moreover, test performance was investigated, together with an evaluation of cut-off values from international and local interpretation guidelines. The results confirmed the applicability of a 3 mm cut-off value for all three criteria (as per the contested Buffalo Veterinary Procedural Notice) which best discriminates *M. bovis*-infected from unexposed buffaloes. Moreover, the suboptimal sensitivity of the TST was demonstrated, despite applying buffalo-specific thresholds.

The second section of this study presents the validation of a standardized Mabtech bovine interferon-gamma (IFN-γ) ELISA^{PRO} kit for use in buffaloes. This was followed by the calculation of buffalo-specific cut-off values for the QuantiFERON® TB-Gold (QFT)/Mabtech IFN-γ release assay (IGRA) and commercial QFT/Cattletype® IGRA, again using representative gold standard positive and negative reference cohorts. The test performance of both IGRAs demonstrated high sensitivity and specificity for *M. bovis* detection, with the Cattletype® IGRA much improved by the application of a species-specific cut-off value in place of the recommended value for cattle.

Finally, the use of a MILLIPLEX® bovine cytokine/chemokine multiplex assay was investigated for buffalo samples. The results indicated the successful application of this platform in a wildlife species and antibody binding was demonstrated for all fifteen cytokine/chemokine targets using mitogen-stimulated buffalo whole blood plasma. Moreover, significant TB antigen-stimulated levels of six cytokines/chemokines enabled the identification of novel candidate biomarkers of *M. bovis* infection and potentially bTB disease in buffaloes.

In conclusion, the results from this project illustrate i) the limitations of the current bTB-screening standard in buffaloes (the TST), hence confirming the necessity for addition of ancillary tests to improve the detection of *M. bovis*-infected individuals; ii) the improvement of IGRA test performance (the

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primary adjunct to the TST) with the application of buffalo-specific cut-off values, in addition to a new and validated IGRA with superior test performance; and iii) the application of a popular biomarker screening tool for the simultaneous investigation of multiple cytokine/chemokine targets in a novel wildlife species. These results demonstrate the improvement of current CMI-based diagnostic methods by the calculation of species-specific cut-off values, using well-defined cohorts, previously unavailable in sufficient numbers. Moreover, new methods with promising performance or the potential for further investigation were successfully presented. Hence, progress was achieved toward enhancing *M. bovis* detection in African buffaloes.

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Chapter 2: General Introduction

This introductory chapter aims to provide an outline of the central research themes and the study aim and objectives. Moreover, this chapter will highlight the overall justification for this study that investigates novel approaches for the improved detection of *M. bovis* infection in African buffaloes (*Syncerus caffer*).

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Mycobacterium bovis

Mycobacterium bovis is a member of the *Mycobacterium tuberculosis* complex and is the primary agent responsible for bovine tuberculosis (bTB) (Bezos *et al.,* 2014). In wildlife alone, *M. bovis* has been detected in 21 species (Arnot and Michel, 2020). Additionally, *M. bovis* affects multiple other mammals including domestic animal species and humans (Sichewo *et al.,* 2019). Despite existing eradication programmes, the re-emergence of bTB and ineffectual control efforts remain a global issue due to wildlife reservoirs of *M. bovis* (Sanchez-Hidalgo *et al.,* 2017).

***Mycobacterium bovis* and African buffaloes**

The first confirmed identification of *M. bovis* in African buffaloes (*Syncerus caffer*) was in 1986 from the Hluhluwe-iMfolozi Park (HiP), presumably due to spillover from communal cattle before the park fences were erected (Hlokwe *et al.,* 2014). Four years later, infected buffalo herds were discovered in the southern area of the Kruger National Park and by 1998, the bTB prevalence in this region of the park was 38.2% (Renwick *et al.,* 2007; Arnot and Michel, 2020). Since that time, *M. bovis* has continued to spread in wildlife across South Africa (Arnot and Michel, 2020). Currently, buffaloes remain the major reservoir host of *M. bovis* in southern Africa and facilitate transmission to other wildlife species and cattle (van der Heijden *et al.,* 2016; Sichewo *et al.,* 2019). This complicates bTB management efforts in livestock, restricts domestic and wild animal translocation, and has adverse effects for wildlife industries from conservation to tourism (Renwick *et al.,* 2007). Therefore, ongoing and effective surveillance and control measures for *M. bovis* infection and bTB are critical for buffaloes. However, there is a lack of optimal diagnostic methods, primarily due to the limitations of sample collection and test validation in a wildlife species.

Single intradermal comparative tuberculin test

The primary approved screening method for bTB in cattle and buffaloes is the single intradermal comparative tuberculin test (SICTT), conceived over a century ago (Srinivasan *et al*., 2019). This version of the tuberculin skin test (TST) measures an *in vivo* reaction to injected purified protein derivative (PPD) antigens, derived from *M. bovis* and *M. avium*, with the comparison of responses used to detect cross-reactivity to environmental mycobacteria. Despite an improvement in specificity (Sp) using the comparative test, suboptimal sensitivity (Se) of the TST in bovids is still observed (Brunton *et al.,* 2018; Bernitz *et al.,* 2019a). Considering the requirement for pre-movement TST testing, false negative results (due to low Se) serve to increase transmission risks through the translocation of undetected, infected buffaloes. Furthermore, reduced Se undermines the primary objective of test-and-slaughter bTB control measures in *M. bovis*-endemic regions when infected animals remain undiagnosed and are released back into parks, particularly those bordering farming communities. The test has several other drawbacks including operator subjectivity, a requirement to handle the animals twice and PPD batch variability (Vordermeier *et al*., 2007; Duignan *et al*., 2019). The interpretation of results in buffalo is based on cattle guidelines or unvalidated guidelines developed for South African

buffalo. Hence, SICTT use in buffaloes requires validation and the calculation of species-specific thresholds to optimise its use in this species.

Cytokine release assays

The most effective methods for the detection of early *M. bovis* infection are based upon *ante mortem* measurement of the host cell-mediated immune (CMI) responses to the pathogen (Bernitz *et al.,* 2018a). Hence, the *in vitro* measurement of CMI-based cytokines, released in response to mycobacterial antigen stimulation of whole blood, is used as an adjunct test to the TST in humans, bovids and wildlife (Walzl *et al.,* 2018; Palmer *et al.,* 2020). The Bovigam® interferon-gamma (IFN-γ) release assay (IGRA) is an *in vitro* version of the SICTT that is commonly used for *M. bovis* detection in cattle and has been optimised for buffaloes by Michel *et al.* (2011). However, the Bovigam® was not used in this project due to the variable test performance reported in recent years, particularly suboptimal Se (Michel *et al.,* 2011; van der Heijden *et al.,* 2016; Bernitz *et al.,* 2018b). The stimulation platform used in this project was the QuantiFERON®-TB Gold (QFT) and QFT-Plus system (Qiagen, Venlo, Limburg, Netherlands) that has demonstrated promising test performance in cattle and buffaloes (Vordermeier *et al.,* 2001; Parsons *et al.,* 2011; Bernitz *et al.,* 2019a). Whole blood is stimulated with antigens highly specific to *M. tuberculosis* and *M. bovis,* and the platform comprises three tubes, namely i) Nil tube containing saline (unstimulated control), ii) TB antigen tube containing peptides simulating early secretory antigen target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) (antigen-stimulated) and iii) mitogen tube containing phytohemagglutinin (PHA) (positive control). The QFT and QFT-Plus platforms both use ESAT-6 and CFP-10, with the former QFT system containing an additional TB7.7 peptide (Hong *et al.,* 2019). The more recent QFT-Plus contains two TB-antigen tubes, one of which (TB2) has extra peptides for human application. Thus, in accordance with veterinary recommendations from the manufacturer, only the TB2 tube is used for African buffaloes (Parsons *et al.,* 2011; Bernitz *et al.,* 2018a; Bernitz *et al.,* 2020). Whole blood is incubated overnight, the plasma is harvested and thereafter cytokines can be measured with, for example, enzyme-linked immunosorbent assays (ELISAs). The most widely applied ancillary test to the TST is the IGRA. Parsons *et al.* (2011) demonstrated the first and promising use of the QFT assay for *M. bovis* detection in buffaloes, together with an in-house IFN-γ ELISA (Mabtech). A QFT/Cattletype® IFN-γ ELISA (Indical Biosciences) has also been validated for use in buffaloes by Bernitz *et al.* (2018a). The Cattletype® IGRA has demonstrated high Sp although variable Se. Moreover, current test interpretation is based on the manufacturer's cut-off value for cattle; therefore, test performance may be improved by species-specific optimisation with relevant cohorts (Bernitz *et al.,* 2019a; 2020). Although the QFT platform provides enhanced Sp, compromised Se is often observed; thus, additional cytokine biomarkers have been investigated. Goosen *et al.* (2015) demonstrated the applicability of IFN γ-induced protein 10 (IP-10) cytokine as a sensitive biomarker in buffaloes with a QFT/IP-10 release assay (IPRA), further validated by Bernitz *et al.* (2018b; 2019a). However, the utility of the IPRA may

be compromised by high levels of this cytokine in unstimulated controls, confounding interpretation (Bernitz *et al.,* 2019b; Palmer *et al.,* 2020).

Justification of study

Due to their role as a wildlife maintenance host of *M. bovis*, African buffaloes are included with cattle in focused efforts to control the spread of *M. bovis* and bTB. The current control strategy in South Africa (SA), applied to buffaloes and cattle alike, is based on 'test-and-slaughter' using the TST as the primary screening tool followed by the culling of animals that test positive. Affected herds are placed under quarantine and movement is restricted until bTB is eradicated (Arnot and Michel, 2020). This can have severe economic impact, from private farms with buffaloes of high monetary value to livestock owners who depend solely on this income. A further concern is posed by the risk of bTB transmission to other wildlife and domestic cattle (Hlokwe *et al.,* 2014; Sichewo *et al.,* 2020). This emphasises the need for highly sensitive, validated and practical in-field diagnostic methods. In addition, high Sp is required, both for economically valuable buffalo herds and small herds where any loss is detrimental to owners' livelihoods. Assays that utilise the antigen-specific CMI response have shown promise for *M. bovis* detection in recent years and commercially available platforms provide standardized systems that can be easily transferred between laboratories. However, the optimisation of these assays for use in buffaloes and the calculation of species-specific cut-off values is limited by the availability of reference sample cohorts. Moreover, buffalo herds across SA have varying disease prevalence, a factor that requires consideration when comprehensively assessing test performance. Therefore, with the availability of a large negative reference cohort and gold standard positive cohort, the goal of this project was to assess the performance of current promising diagnostic assays, develop strategies to further optimise these assays and to investigate new commercial platforms for the improved detection of *M. bovis* in buffaloes.

Aim

To optimise established CMI-based diagnostic tests and to investigate newly available platforms for the detection of *M. bovis*-infected African buffaloes (*Syncerus caffer*).

Objectives

1. To collate and analyse SICTT measurement data from gold standard positive (culture-confirmed) and well-characterised negative (historically *M. bovis*-unexposed) reference cohorts for the calculation of species-specific cut-off values and evaluation of test performance in buffaloes.
2. To validate the new Mabtech bovine IFN-γ ELISA^{PRO} and to calculate species specific cut-off values for the QFT/Mabtech and QFT/Cattletype® IGRAs for optimal assessment of test performance in buffaloes using highly applicable positive and negative cohorts.

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3. To investigate the application of a MILLIPLEX® bovine cytokine/chemokine multiplex assay for the identification of novel candidate biomarkers of *M. bovis* infection in buffaloes.

Ethics

Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2019-9081), and a Section 20 research permit was issued by the Department of Agriculture, Forestry and Fisheries (DAFF; 12/11/1/7/2).

References

Arnot, L.F., Michel, A., 2020. Challenges for controlling bovine tuberculosis in South Africa. Onderstepoort J. Vet. Res. https://doi.org/10.4102/ojvr.v87i1.1690

Bernitz, N., Clarke, C., Roos, E.O., Goosen, W.J., Cooper, D., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018a. Detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) tubes and the Qiagen cattletype® IFN-gamma ELISA. Vet. Immunol. Immunopathol. 196, 48–52. https://doi.org/10.1016/j.vetimm.2017.12.010

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018b. Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2018.09.004

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019a. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Bernitz, N., Kerr, T.J., Goosen, W.J., Higgitt, R.L., de Waal, C., Clarke, C., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019b. Impact of *Mycobacterium bovis*-induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 217, 109923. https://doi.org/10.1016/j.vetimm.2019.109923

Bernitz, N., Kerr, T.J., de Waal, C., Cooper, D. V., Warren, R.M., Van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2020. Test characteristics of assays to detect *Mycobacterium bovis* infection in high-prevalence African buffalo (*Syncerus caffer*) Herds. J. Wildl. Dis. https://doi.org/10.7589/2019-06-173

Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardegger, R., Raeber, A.J., López, L., Rueda, P., Domínguez, L., 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2014.04.002

Stellenbosch University https://scholar.sun.ac.za

Brunton, L.A., Prosser, A., Pfeiffer, D.U., Downs, S.H., 2018. Exploring the fate of cattle herds with inconclusive reactors to the tuberculin skin test. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00228

Duignan, A., Kenny, K., Bakker, D., Good, M., 2019. Tuberculin PPD potency assays in naturally infected tuberculous cattle as a quality control measure in the Irish bovine tuberculosis eradication programme. Front. Vet. Sci. 6. https://doi.org/10.3389/fvets.2019.00328

Goosen, W.J., Cooper, D., Miller, M.A., van Helden, P.D., Parsons, S.D.C., 2015. 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*). Clin. Vaccine Immunol. 22, 974–978. https://doi.org/10.1128/cvi.00324-15

Hlokwe, T.M., van Helden, P., Michel, A.L., 2014. Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? Prev. Vet. Med. 115, 10–17. https://doi.org/10.1016/j.prevetmed.2014.03.011

Hong, J. Y., Park, S. Y., Kim, A., Cho, S. N., & Hur, Y. G., 2019. Comparison of QFT-Plus and QFT-GIT tests for diagnosis of *M. tuberculosis* infection in immunocompetent Korean subjects. J Thorac Dis. 11(12), 5210–5217. https://doi.org/10.21037/jtd.2019.12.11

Michel, A.L., Cooper, D., Jooste, J., de Klerk, L.M., Jolles, A., 2011. Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). Prev. Vet. Med. 98, 142–151. https://doi.org/10.1016/j.prevetmed.2010.10.016

Palmer, M. V., Thacker, T.C., Rabideau, M.M., Jones, G.J., Kanipe, C., Vordermeier, H.M., Ray Waters, W., 2020. Biomarkers of cell-mediated immunity to bovine tuberculosis. Vet. Immunol. Immunopathol. 220. https://doi.org/10.1016/j.vetimm.2019.109988

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Renwick, A.R., White, P.C.L., Bengis, R.G., 2007. Bovine tuberculosis in southern African wildlife: A multi-species host-pathogen system. Epidemiol. Infect. https://doi.org/10.1017/S0950268806007205

Sanchez-Hidalgo, A., Obregón-Henao, A., Wheat, W.H., Jackson, M., Gonzalez-Juarrero, M., 2017. *Mycobacterium bovis* hosted by free-living-amoebae permits their long-term persistence survival outside of host mammalian cells and remain capable of transmitting disease to mice. Environ. Microbiol. https://doi.org/10.1111/1462-2920.13810

Sichewo, P.R., Etter, E.M.C., Michel, A.L., 2020. Wildlife-cattle interactions emerge as drivers of bovine tuberculosis in traditionally farmed cattle. Prev. Vet. Med. 174, 104847. https://doi.org/10.1016/j.prevetmed.2019.104847

Stellenbosch University https://scholar.sun.ac.za

Sichewo, P.R., Michel, A.L., Musoke, J., Etter, E.M.C., 2019. Risk factors for zoonotic tuberculosis at the wildlife–livestock–human interface in South Africa. Pathogens 8, 101. https://doi.org/10.3390/pathogens8030101

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. https://doi.org/10.1126/sciadv.aax4899

van der Heijden, E.M.D.L., Jenkins, A.O., Cooper, D. V., Rutten, V.P.M.G., Michel, A.L., 2016. Field application of immunoassays for the detection of *Mycobacterium bovis* infection in the African buffalo (*Syncerus caffer*). Vet. Immunol. Immunopathol. 169, 68–73. https://doi.org/10.1016/j.vetimm.2015.12.003

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G., 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin. Diagn. Lab. Immunol. 8, 571–578. https://doi.org/10.1128/CDLI.8.3.571-578.2001

Vordermeier, H.M., Brown, J., Cockle, P.J., Franken, W.P.J., Arend, S.M., Ottenhoff, T.H.M., Jahans, K., Hewinson, R.G., 2007. Assessment of cross-reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP-10 at the T-cell epitope level. Clin. Vaccine Immunol. 14, 1203–1209. https://doi.org/10.1128/CVI.00116-07

Walzl, G., McNerney, R., du Plessis, N., Bates, M., McHugh, T.D., Chegou, N.N., Zumla, A., 2018. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(18)30111-7

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Chapter 3: Cell-mediated immunological biomarkers and their diagnostic application in livestock and wildlife infected with *Mycobacterium bovis*

Unpublished literature review chapter

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Abstract

Mycobacterium bovis has the broadest host range of the *Mycobacterium tuberculosis* complex and infects domestic animal species, wildlife and humans. The presence of global wildlife maintenance hosts complicates *M. bovis* and bovine tuberculosis (bTB) control efforts and further threatens livestock and wildlife-related industries. Thus, it is imperative that early and accurate detection of *M. bovis* in all affected animal species is achieved. Further, an improved understanding of the complex and species-specific host immune responses to *M. bovis* could enable the development of diagnostic tests that not only identify infected animals but distinguish between infection and active disease. The primary bTB screening standard worldwide remains the tuberculin skin test (TST) that presents several test performance and logistical limitations. Hence additional tests are used, most commonly an interferon-gamma (IFN-γ) release assay (IGRA) that, similar to the TST, measures a cell-mediated immune (CMI) response to *M. bovis.* There are various cytokines and chemokines, in addition to IFN-γ, involved in the CMI component of host adaptive immunity. Due to the dominance of CMI-based responses to mycobacterial infection, cytokine and chemokine biomarkers have become a focus for diagnostic tests in livestock and wildlife. Therefore, this review describes the current understanding of host immune responses to *M. bovis* as it pertains to the development of diagnostic tools using CMI-based biomarkers in both gene expression and protein release assays, and their limitations. Although the study of CMI biomarkers has advanced fundamental understanding of the complex host-*M. bovis* interplay and bTB progression, resulting in development of several promising diagnostic assays, most of this research remains limited to cattle. Considering differences in host susceptibility, transmission and immune responses, and the wide variety of *M. bovis*-affected animal species, these knowledge gaps continue to pose some of the biggest obstacles to the improvement of *M. bovis* and bTB diagnosis.

1. Introduction

Mycobacterium bovis infection and the resulting disease, commonly referred to as bovine tuberculosis (bTB), affects a wide range of species including humans, domestic animals and wildlife (Michel, 2014; Romha *et al.,* 2018). Although *M. bovis*, as the name suggests, mainly affects bovids including cattle (*Bos taurus*), bison (*Bison bison*), African and Asian buffaloes (*Syncerus caffer* and *Bubalus bubalis*), it has been isolated from numerous other mammals, compromising animal and human health worldwide (Figure 3.1; Gagneux, 2018; Palmer *et al.,* 2020). Infection of wildlife further impacts livestock health due to the development of maintenance and spillover hosts within global wildlife populations, including badgers (*Meles meles)* in the United Kingdom (UK), African buffaloes in southern Africa, and farmed and wild cervids in the United States (US; Graham *et al.,* 2013; Hlokwe *et al.,* 2014; Tsao *et al.,* 2014). In the UK and Ireland, *M. bovis* in badgers complicates bTB control efforts and similarly in the US, New Zealand and Spain where deer, possum and wild boar, respectively, are also recognized *M. bovis* reservoirs (Brites *et al.,* 2018; Gagneux, 2018). In the Americas, there are 15 wild species reported as infected with *M. bovis* (Domingos *et al.,* 2019). In South Africa, bTB is

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endemic in two major national parks (Hlokwe *et al.,* 2014). Moreover, *M. bovis* has been identified in more than 21 wildlife species in private and public sectors (Michel *et al.,* 2010; Sichewo *et al.,* 2020).

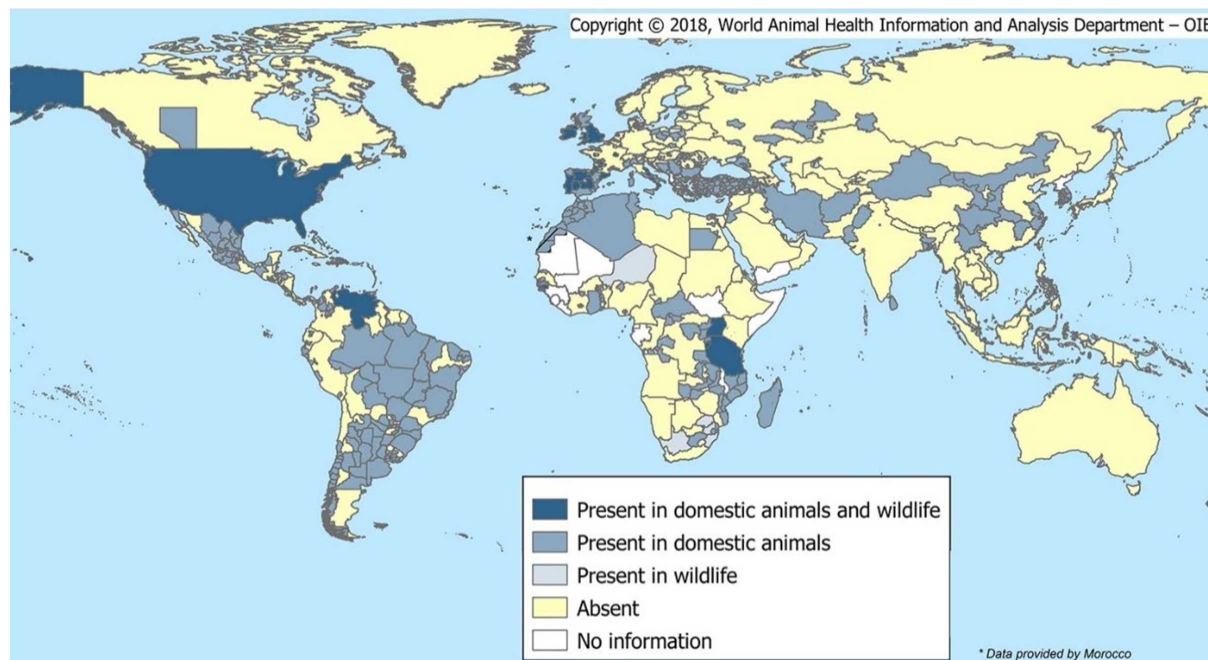

Figure 3.1. Global distribution of bTB in domestic and wild animals during 2017 and first half of 2018 (Adopted from: OIE Bulletin, 2019).

In addition to the control challenges posed by infected wildlife species, bTB negatively affects wildlife-related industries, resulting in consequences for conservation, tourism, and game sales. Infection and disease in livestock and wildlife may lead to decreased productivity, trade restrictions, impacts on food security and zoonotic transmission, resulting in significant economic losses (Palmer *et al.,* 2020; Sichewo *et al.,* 2020). Bovine tuberculosis costs the global cattle industry alone 3 billion US dollars annually (Schiller *et al.,* 2010). In addition, approximately 5 million deer are farmed worldwide and bTB is the primary health issue across multiple species in this growing economic venture (Mackintosh *et al.,* 2004; Thomas *et al.,* 2019). Despite effective bTB control measures in the US, states such as Michigan have endemic bTB in white-tailed deer (*Odocoileus virginianus*) populations that burden the hunting and wildlife industry, in addition to the regular spillback affecting cattle (Ford *et al.,* 2019; Salvador *et al.,* 2019).

For these reasons, it is crucial to improve the detection, diagnosis and understanding of bTB across affected species to enable development of more effective control strategies. Early and accurate diagnosis of subclinical infection can inform more efficient management of affected animals. Alternatively, if associations can be accurately established between *M. bovis* infection or bTB disease and detectable host responses, limited available resources can be focused toward removing animals or populations that pose transmission risks to preserve those with higher economic, conservation or genetic value. However, the immune responses that arise from interactions between animal hosts and *M. bovis* are typically species-specific and particularly in the case of wildlife species, less well-characterized.

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Subsequently, validated diagnostic tests based on specific host responses to *M. bovis* infection and bTB are also scarce in wildlife.

The diagnostic standard for *M. bovis* detection, still in use despite being developed over a century ago, is the TST. The TST measures a host CMI response to mycobacterial purified protein derivative (PPD) antigens, either *M. bovis*-derived (PPD_b) alone or together with *M. avium* (PPD_a) for the single intradermal comparative tuberculin test (SICTT) version (Brunton *et al.,* 2018; Srinivasan *et al.,* 2019). In cattle, a meta-analysis of TST use in the UK and Ireland reported 100% specificity (Sp), in agreement with a Great Britain study displaying almost 100% Sp for standard and severe cut-off interpretations (Goodchild *et al.,* 2015; Nunez-Garcia *et al.,* 2018). However, the sensitivity (Se) can range between 50 and 80%, although Sp and Se vary between species and cut-off values are only validated in cattle (Brunton *et al.,* 2018; Nunez-Garcia *et al.,* 2018). A major confounding factor is the exposure to environmental mycobacteria in addition to vaccine strain antigens (for cattle in limited areas) that cause cross-reactions due to homology between antigenic peptides, demonstrated at gene and protein levels (Arend *et al.,* 2002; Vordermeier *et al.,* 2007; Srinivasan *et al.,* 2019). In New Zealand, the TST displays reduced Se in deer exposed to environmental mycobacteria and is not recommended in herds with a high likelihood of *M. bovis* infection (Mackintosh *et al.,* 2004). When applied to fallow deer (*Dama dama*) in Texas (USA), the test had overall low Se and Sp, indicating minimal diagnostic value for this cervid species (Mackintosh *et al.,* 2004). There are also multiple cases of *M. bovis* crossing international borders in imported, infected deer due to negative TST results (Mackintosh *et al.,* 2004). In addition to variable test performance, the TST is subject to several limitations. The test is labour-intensive and logistically challenging in developed countries; in developing countries, this is exacerbated by restricted access to reagents and animals, veterinary capacity, and handling facilities (Michel *et al.,* 2010; Lamont *et al.,* 2014). An additional complication is management of cattle considered inconclusive reactors (IRs); in England and Wales, there were 3755 IRs in 2015 alone (Brunton *et al.,* 2018). Animals in these herds may be infected, as suggested by the report that 21% of these herds had positive reactors when re-tested. In Ireland, between 11.8% and 21.4% of IRs slaughtered before a re-test were reported to be *M. bovis*-infected (Brunton *et al.,* 2018). However, movement of such herds is not typically restricted unless there is a recent history of TB, exacerbating *M. bovis* transmission risks when using the TST alone (Brunton *et al.,* 2018).

In attempting to address drawbacks associated with the TST in bovids, at least one ancillary test is recommended (Klepp *et al.,* 2019). One of the key focus areas of bTB research that has emerged is the discovery of CMI biomarkers and consequently the development of CMI-based tests for the early detection of infection by pathogenic mycobacteria (Goosen *et al.,* 2014). Biomarker is a broad term defined as any indicator of pathogenic biological processes, either pathogen- or host-based (Walzl *et al.,* 2018; MacLean *et al.,* 2019). The most common adjunct to the TST is the CMI biomarker-based interferon-gamma (IFN-γ) release assay (IGRA) (Coad *et al.,* 2019; Srinivasan *et al.,* 2019). However, despite improvements in Se for detecting *M. bovis*-infected animals, ancillary tests can introduce extra

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costs and logistical challenges. For example, IGRAs require that whole blood samples be processed within 8-24 hours of collection, depending on the stimulation method, which is impractical if herds are located far from available laboratories (Lamont *et al.,* 2014). Moreover, IGRAs rely on a single measurement that is unable to discriminate *M. bovis* infection from bTB disease and further, may fail to diagnose early infection when blood is stimulated with more specific mycobacterial peptides instead of PPDs (Bernitz *et al.,* 2019a; Coad *et al.,* 2019). To overcome this, a combination of biomarkers may be used to improve diagnostic Se. For example, progress has been made in enhancing the detection of bTB in cattle by combining IGRAs with the simultaneous measurement of antigen-specific interleukin-1β (IL-1β) or IFN γ-induced protein 10 (IP-10; Jones *et al.,* 2010; Bernitz *et al.,* 2019a; Coad *et al.,* 2019). A systematic review by Domingos *et al.* (2019) of *M. bovis* diagnosis in wildlife species also suggested the application of at least two diagnostic methods to aid the detection of infection and potentially different disease stages.

A primary goal for the accurate *M. bovis* detection is the early identification of a maximal number of infected animals for more effective control measures. The investigation of candidate biomarkers for *M. bovis* infection and bTB is ongoing, as discussed below. However, if current biomarker research could be further advanced to differentiate infection from bTB disease, this would enable a focus on detecting stages in which the animal is potentially infectious, improving the elimination of major transmission risks or preventing infected individuals from transitioning to a diseased state. The primary *ante mortem* diagnostic tools in livestock and wildlife rely on the development of antigen-specific CMI responses to *M. bovis* (Michel *et al.,* 2011; Parsons *et al.,* 2011; Bernitz *et al.,* 2019a). Therefore, this review will focus on current understanding and knowledge gaps regarding the host response to *M. bovis* infection and the use of host CMI biomarkers for the improved understanding and diagnosis of *M. bovis* infection and bTB disease.

2. Host responses to *Mycobacterium bovis*

With the availability of bovid host and *M. bovis* genomes, the understanding of host responses to *M. bovis* has continued to develop (Lamont *et al.,* 2014). Immunity, in brief, comprises the innate and adaptive immune systems. The adaptive immune response has two distinct components, namely humoral and cell-mediated immunity (CMI), of which the latter is vital for host protection against *M. bovis* infection. All the major T lymphocyte subsets have been shown to be involved in the anti-mycobacterial immune response in cattle (Pollock *et al.,* 2005). In *M. bovis*-infected cattle, $CD4^+$ T cells produce IFN-γ for activation of macrophage anti-mycobacterial functions whereas $CD8^+$ T cells are involved in the lysis of infected cells (Pollock *et al.,* 2005). The development of a T helper type 1 (Th1)-based CMI response, resulting in production of cytokines and chemokines such as tumour necrosis factor-α (TNF-α), interleukin-12 (IL-12), IL-6 and IFN-γ by dendritic cells and macrophages, is considered essential for the control of mycobacterial infection (Welsh *et al.,* 2005). Cytokine production is activated by pathogen antigen presentation to immune cells, and disease progresses when

these pathways are disrupted (Palmer and Waters, 2006). Cytokines play a major role in determining the nature of the immune responses to infection; the balance between Th1 and T helper type 2 (Th2) functions is often studied from the perspective of their cytokine profiles, which is a dynamic process (Welsh *et al.,* 2005). Cytokine function can vary from pro-inflammatory, which promotes activation of cells to kill mycobacteria, to anti-inflammatory, which reduces the pro-inflammatory response to control and prevent tissue damage by necrosis (Widdison *et al.,* 2006). It has been demonstrated that during the early stages of mycobacterial infections, Th1-dominant cytokine responses develop (Palmer and Waters, 2006). The Th1 immune response is critical for defense against intracellular pathogens, most notably through the production of IFN-γ (Pollock *et al.,* 2005). However, a shift from Th1- to Th2-based responses is typically observed as disease develops, with a shift in CMI responses along with increasing humoral (B cell) responses (Dlugovitzky *et al.,* 2000). The Th2 response is characterized by the production of cytokines including IL-4, IL-5 and IL-10 with studies suggesting that the Th1/Th2 balance is critical for determining bTB disease progression and outcomes (Blanco *et al.,* 2009). Therefore, any changes in immune responses during the course of infection are likely to affect the diagnostic performance of immunological assays.

Although early detection of *M. bovis* infection is primarily dependent upon the measurement of CMI responses in many species, humoral responses have also been used to diagnose bTB (Welsh *et al.,* 2005; Goosen *et al.,* 2015; Lyashchenko *et al.,* 2017). In certain species, for example elephants (*Loxodonta africana* and *Elephas maximus*) and suids, anti-mycobacterial antibodies can be detected during early infection (Maas *et al.,* 2013; Roos *et al.,* 2018a). In other species such as cattle and lions (*Panthera leo*), however, antibody detection usually occurs once bTB disease progresses and the immune responses shift toward a Th2 profile (Miller *et al.,* 2012; Lyashchenko *et al.,* 2017). However, the application of serological tests requires insight into the humoral immune profile, produced by specific species, to determine the differences in immunodominant antigen recognition and response kinetics (Lesellier *et al.,* 2008; Lyashchenko *et al.,* 2020). Due to a paradigm that early immune responses are cell-mediated, this has been the primary focus of bTB research in animals to date.

In cattle and other species, a CMI-dominated early and specific response to *M. bovis* infection is observed with proliferation of antigen-specific T lymphocytes, secretion of the regulatory cytokine IL-2 and release of pro-inflammatory cytokines including IFN-γ. These responses have been reported, for example, in two studies by Corner *et al.* (2007) and Gormley and Corner (2018) that investigated CMI responses to *M. bovis* in experimentally infected badgers, one of the few relatively well-studied non-bovid species. The earliest CMI immune responses were observed 3 weeks post infection (WPI) in the animals infected with the highest *M. bovis* dose; these animals also demonstrated the most consistent CMI responses of peripheral blood mononuclear cells (PBMCs) to bovine PPD (PPD_b) stimulation. The overall CMI responses were also positively associated with pathological changes, i.e., the presence of gross lesions, detected *post mortem.* On the other hand, the humoral antibody responses in the badgers were only sporadically detected.

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Functional genomics and proteomics have enabled a better understanding of CMI responses to *M. bovis* infection (Waters *et al.,* 2011). Studies typically focus on the *in vitro* assessment of gene expression profiles in different cells or tissues, isolated after infection with *M. bovis* or related strains, and *ex vivo* analyses aimed at identifying cytokine gene expression signatures for the detection of *M. bovis*-infected animals. Wedlock *et al.* (2006) compared gene expression in primary bovine alveolar macrophages (AMs) infected with a virulent *M. bovis* strain and an attenuated version. Although both strains grew at comparable rates, the results suggested a 45% difference in gene expression between the two infection groups. Of the ten most differentially expressed genes, with the virulent strain inducing higher levels of expression, seven of these were chemokines with IL-8 and monocyte chemoattractant protein (MCP)-1 (*CCL2*) having the greatest expression. The conclusion was that AMs infected with virulent *M. bovis* displayed a more dominant pro-inflammatory gene expression profile than that of the attenuated strain. Another study using a similar method observed that lower levels of chemokines were expressed by *M. bovis*-infected AMs of cattle than *M. tuberculosis*-infected cells, highlighting a potential mechanism by which *M. bovis* circumvents the activation of the host's chemotactic response, thereby evading killing (Widdison *et al.,* 2008).

Although the host immune responses during *M. bovis* infection and disease remain less understood than for *M. tuberculosis,* recent studies have begun to shed more light on this complex pathogen-host interplay. However, susceptibility to infection, routes of infection and disease progression can vary significantly between the wide range of host species that are affected. Hence, it would be expected that immune responses and therefore, specific diagnostic biomarkers may also differ, impacting advances in host biomarker discovery and application (Palmer and Waters, 2006).

3. Host diagnostic biomarkers of pathogenic mycobacterial infection

A suitable diagnostic biomarker or multiple marker biosignature, for infection or disease, is a host- (or pathogen-) specific molecule/protein that is associated with the underlying pathological process (Wallis *et al.,* 2010; Maclean *et al.,* 2019). Identification of biomarker-based assays that can enable more accessible, affordable and efficient diagnosis has been a high priority for TB and bTB diagnostic research in recent years (MacLean *et al.,* 2019; Kelley *et al.,* 2020). Considering the World Health Organisation's target product profiles that define the performance and operational characteristics of suitable tests, it is expected that non-DNA markers are more likely to meet practical and cost targets (Yerlikaya *et al.,* 2017; MacLean *et al.,* 2019). Non-DNA methods can be performed without advanced instrumentation, utilise more easily accessible samples such as blood and serum, and are generally more affordable than DNA-based tests (Walzl *et al.,* 2018; Maclean *et al.,* 2019). Hence, the focus of current bTB diagnostic research is host biomarkers with an emphasis on cytokine/chemokine (chemotactic cytokine) proteins and antibodies, in addition to the detection of cytokine RNA expression (Chambers, 2013; Lamont *et al.,* 2014; Palmer *et al.,* 2020).

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Critical barriers to progress regarding biomarker discovery include the lack of standardization in specimen collection methods and a reliance on convenient or opportunistic samples, in addition to inadequate handling and storage of samples which can drastically alter detectable biomarker levels (Poste, 2011; Gormley and Corner, 2018). Another common limitation is suboptimal statistical power due to low sample numbers, hence, associations between biomarkers and potential disease states are challenging to investigate and prove (Poste, 2011). A biomarker should address a definitive question related to a particular state, or the prognosis, of pathogenic mycobacterial infection in individuals or populations (Wallis *et al.,* 2010). However, stages of TB are complex and include infection, active disease and latency; moreover, these states are not regarded as fixed but instead constitute a dynamic spectrum that may be affected by multiple factors, many of which remain poorly understood (Wallis *et al.,* 2010; Garcia *et al.,* 2020).

For *M. bovis* infections in animals, insight regarding the mycobacterial disease spectrum is even more limited (Palmer and Waters, 2006; Gormley and Corner, 2018; Garcia *et al.,* 2020). To date, the only method of determining an animal's true infection status is comprehensive *post mortem* examination and a battery of mycobacteriological, immunological, histopathological and molecular tests (Srinivasan *et al.,* 2019). Accurate *ante mortem,* in-field reference tests for infection and/or active bTB disease are lacking (Kelley *et al.,* 2020). Current understanding of the pathogenesis of *M. bovis* infection is also confounded by the multitude of susceptible animal hosts and the complexity of the host-pathogen interactions (Palmer and Waters, 2006; Gormley and Corner, 2018). Although naturally-infected animals are useful in the investigation of differences between infection and disease, experimental models illuminate disease progression kinetics and immunological responses starting from a fixed dose and point in time, generating the insight necessary for diagnostic test development (Lesellier *et al.,* 2009; Chambers *et al.,* 2017). A major limitation for *M. bovis* and bTB diagnostic research is the lack of clarity regarding case definitions for infection versus active disease and in particular, whether latent infections exist (de la Rua-Domenech *et al.,* 2006; Alvarez *et al.,* 2009; Gormley and Corner, 2018; Garcia *et al.,* 2020). Considering the various limitations outlined above, the fitness for purpose criteria (as defined by the World Organisation of Animal Health [OIE]) are critical for the validation and application of diagnostic bTB biomarkers in animals and should be applied on an individual species basis.

The *ante mortem* diagnosis of bTB in animals primarily relies on the detection of host CMI responses, most commonly with the use of protein and gene expression assays (Palmer *et al.,* 2020). Although the most widely used adjunct to the TST, IGRAs, in addition to the TST, can produce false negative results as the CMI responses required may diminish in advanced disease stages, while the humoral response increases (Welsh *et al.,* 2005; de la Rua-Domenech *et al.,* 2006). Furthermore, IGRAs demonstrate limited sensitivity in early infection stages and in general, bTB diagnostic assays are unable to distinguish infection from disease (Schiller *et al.,* 2010; Chambers, 2013; Bezos *et al.,* 2014; Nunez-Garcia *et al.,* 2018; Klepp *et al.,* 2019). On the other hand, a recent study by Bernitz *et al.*

(2019b) observed that levels of IFN-γ and IP-10 in incubated unstimulated whole blood were elevated in infected buffaloes with macroscopic pathological changes consistent with bTB as compared to uninfected controls. Furthermore, increased IFN-γ significantly correlated with increasing severity of pathological changes in the infected buffaloes, consistent with observations of associations between antigen-stimulated IFN-γ and bTB pathology in cattle and badgers, demonstrating the potential for these cytokines to be used as indicators of bTB disease (Vordermeier *et al.,* 2001; Tomlinson *et al.,* 2015; Bernitz *et al.,* 2019b).

A limitation of cytokine/chemokine protein assays can be the inability to detect high levels of the biomarker target due to, for example, binding saturation limits; therefore, mRNA responses and their detection may provide a more robust alternative (Palmer *et al.,* 2020). There are several studies that investigate cytokine/chemokine RNA expression to achieve diagnostic test objectives. However, another purpose of biomarkers is to improve the fundamental understanding of disease pathogenesis, which is often the focus of bTB biomarker RNA expression research, for example studies that characterise the immunological profile of *M. bovis*-infected cattle (experimental and/or natural) by measuring cytokine/chemokine mRNA expression at various time points during infection and/or disease (Welsh *et al.,* 2005; Thacker *et al.,* 2006 and 2007; Wedlock *et al.,* 2006; Blanco *et al.,* 2009; Wallis *et al.,* 2010; Palmer *et al.,* 2015). Immune response changes during bTB disease progression, particularly in terms of anti- and pro-inflammatory cytokine/chemokine profiles, can signify disease outcomes and hence play a role in the diagnosis and control of infection (Welsh *et al.,* 2005). The CMI response is the predominant immunological response to *M. bovis,* responsible for the killing and elimination defence mechanisms in addition to formation of characteristic granulomas against this intracellular pathogen (de la Rua-Domenech *et al.,* 2006). The following section will further describe some of the CMI biomarkers of infection and bTB disease in animals.

4. Cytokine/chemokine biomarkers of *Mycobacterium bovis* infection

Several promising cytokine/chemokine biomarkers of *M. bovis* infection and disease in domestic and wildlife species have been revealed in recent years, in addition to the widely recognized host biomarker IFN-γ. These include, although are not limited to, IP-10, IL-1β, IL-4, IL-8, IL-17A, *CXCL9*, IL-10 and IL-22 (Rhodes *et al.,* 2000; Widdison *et al.,* 2006; Widdison *et al.,* 2009; Aranday-Cortes *et al.,* 2012; Goosen *et al.,* 2015; Waters *et al.,* 2016; Elnaggar *et al.,* 2017; Olivier *et al.,* 2017; Gao *et al.,* 2019). Both protein and gene expression assays have been used to assess biomarker levels in different species and have facilitated identification of additional targets for diagnostic development (Palmer *et al.,* 2020). This section will describe select current and candidate CMI biomarkers of *M. bovis* infection in domestic and wild animal species.

4.1. IFN-γ

The cytokine IFN-γ is an important mediator of macrophage activation, amplifying macrophage cytokine release in response to *M. bovis* and playing a critical role in host protection and pathogen

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control (Denis *et al.,* 2005; Tomlinson *et al.,* 2015). The utility of various cytokine release assays based on IFN-γ (IGRAs) has been demonstrated in cattle, leading to the acceptance of IGRAs as an adjunct to the SICTT in European legislation, and some countries outside of the European Union (Good *et al.,* 2018; Palmer, 2018). The first bTB *in vitro* IGRA was developed in the 1980's in Australia and has since spread globally (Clegg *et al.,* 2019). Studies on *M. bovis* experimentally- and naturally-infected cattle have demonstrated the ability of IGRAs to detect a positive CMI response from as early as two weeks post-infection and often earlier than detection by the SICTT (Buddle *et al.,* 1995; Rhodes *et al.,* 2000; Pollock *et al.,* 2005; Waters *et al.,* 2010). The most commonly used platform in worldwide bTB control programmes is the Bovigam® which uses PPD_b and PPD_a stimulatory antigens (Bezos *et al.,* 2014). The Bovigam® IGRA is also effectively applied to *M. bovis* detection in goats and another PPD-based IGRA has been used in pigs (Pesciaroli *et al.,* 2012; Bezos *et al.,* 2015).

Currently, the primary ancillary method for *M. bovis* detection in African buffaloes is the IGRA, based on either PPD stimulation or the QuantiFERON® TB Gold (QFT) system that stimulates whole blood with *M. bovis/M. tuberculosis*-specific antigens early secretory antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) (EC; Michel *et al.,* 2011; Bernitz *et al.,* 2018). Thereafter, species-compatible IFN-γ enzyme-linked immunosorbent assays (ELISAs) are used to detect the biomarker. Validated IFN-γ ELISAs for buffaloes include the Bovigam® assay and Mabtech in-house, Cattletype®, and commercial Mabtech bovine ELISAs (Grobler *et al.,* 2002; De Klerk *et al.,* 2006; Parsons *et al.,* 2011; Bernitz *et al.,* 2018).

There are several other wildlife species for which IGRAs to detect *M. bovis* have been developed, as summarised in Table 3.1. Using EC (QFT)- stimulated whole blood, IFN-γ ELISAs have been modified for use in white rhinoceros (*Ceratotherium simum*) and wild dog (*Lycaon pictus*) (Higgitt *et al.,* 2018; Chileshe *et al.,* 2019). Using both PPD and EC stimulatory antigens, IGRAs have also been used in badgers, with good sensitivity and specificity, alpacas (*Vicugna pacos*), and red deer (*Cervus elaphus*) for which Rv3615c and Rv3020 antigens were also used (Dalley *et al.,* 2008; Rhodes *et al.,* 2012; Risalde *et al.,* 2017). The Cervigam™ IFN-γ ELISA (developed for red deer), used with plasma from PPD-stimulated whole blood, has also displayed promise for bTB diagnosis in white-tailed deer in addition to reindeer (*Rangifer tarandus*) and sambar deer (*Cervus unicolor*) (Palmer *et al.,* 2004).

Although IFN-γ has proved invaluable for several species as a biomarker of *M. bovis* infection, in other species, including lions and warthogs (*Phacochoerus africanus*), it does not appear to be as useful (Olivier *et al.,* 2017; Roos *et al.,* 2018b). It has also been observed that depending on the infection phase, IGRAs (and the SICTT) may fail to detect *M. bovis*-infected animals (Rodriguez-Campos *et al.,* 2014). The host immune response to mycobacteria is naturally linked to disease progression yet bTB generally presents with extended and advanced, poorly described disease stages, posing an additional challenge for the extension of IFN-γ-based diagnostics to distinguish infection from active disease (O'Hare *et al.,* 2014; Pereira *et al.,* 2020). However, evaluating associations between cytokine responses at both a gene and protein level, alongside host-specific pathological changes, can provide

more insight on the diagnostic potential of additional biomarkers (Thacker *et al.,* 2006; Garcia-Jiminez *et al.,* 2012; Silva-Miranda *et al.,* 2012; Canal *et al.,* 2017; Carrisoza-Urbina *et al.,* 2019; Palmer *et al.,* 2020).

Table 3.1. Summary of interferon-gamma (IFN-γ) release assays (IGRAs) employed in domestic and wild animal species for *M. bovis* detection and bTB diagnosis.

Species	Reference(s)	Detection method	Stimulatory antigen(s)
Buffalo (*Syncerus caffer*)	Michel *et al.* (2011) Parsons *et al.* (2011) Bernitz *et al.* (2018)	Bovigam® IGRA QFT/Mabtech ELISA QFT/Cattletype® ELISA	PPD EC EC
White rhino (*Ceratotherium simum*)	Chileshe *et al.* (2019)	QFT/Mabtech ELISA	EC
Wild dog (*Lycaon pictus)*	Higgitt *et al.* (2018)	QFT/R&D Quantikine ELISA	EC
Badger (*Meles meles*)	Dalley *et al.* (2008)		PPD/EC
Alpaca (*Vicugna pacos*)	Rhodes *et al.* (2012)	Mabtech ELISA	PPD/EC
Red deer (*Cervus elaphus*)	Risalde *et al.* (2017)	IGRA (in-house)	PPD/EC/Rv
White-tailed deer (*Odocoileus virginianus*) Reindeer (*Rangifer tarandus*) Sambar deer (*Cervus unicolor*)	Palmer *et al.* (2004)	Cervigam™ ELISA	PPD
Cattle (*Bos taurus*)	Nunez-Garcia *et al.* (2018) (Meta-analysis)	Various	PPD/EC
Goat (*Capra hircus*)	Bezos *et al.* (2015)	Bovigam® IGRA	PPD
Pig	Pesciaroli *et al.* (2012)	IGRA (in-house)	PPD

PPD: purified protein derivative (bovine/avian); EC: ESAT-6/CFP-10; Rv: Rv3615c/Rv3020; QFT: QuantiFERON®-TB Gold stimulation platform.

4.2. IP-10

The IFN *γ*-induced chemokine IP-10, expressed by lymphocytes and monocytes, is produced at high levels in humans, cattle and African buffaloes, up to 100-fold more than IFN-*γ,* following infection with tuberculous mycobacteria (Whittaker *et al.,* 2008; Parsons *et al.,* 2016; Qiu *et al.,* 2019). It has a role in delayed type hypersensitivity reactions and stimulated levels of IP-10 have shown promise for early detection of *M. bovis* infection in animals that may be negative on other tests (such as IGRAs) (Whittaker *et al.,* 2008; Roos *et al.,* 2018b; Bernitz *et al.,* 2019a; Qiu *et al.,* 2019).

Considering the potential for IP-10 to be a more sensitive marker than IFN-*γ*, there are still relatively few studies in bTB-affected species. Using a Kingfisher Biotech bovine IP-10 ELISA and QFT whole blood stimulation, a significantly higher antigen-specific IP-10 response was able to distinguish *M. bovis*-infected from culture negative warthogs (Roos *et al.,* 2018b). The same has been

shown in African buffaloes using the same platform and IP-10 has demonstrated high test sensitivity in this species (Goosen *et al.,* 2015; Bernitz *et al.,* 2019a). Furthermore, elevated levels of IP-10 in incubated samples without antigen stimulation have been correlated to the presence of *M. bovis* pathology in infected populations (Bernitz *et al.,* 2019b). Using the same assay as for buffaloes, Parsons *et al.* (2016) showed a strong correlation between IP-10 and IFN-γ release, and the robustness of IP-10 as a biomarker of *M. bovis* infection in cattle. Waters *et al.* (2012) also observed antigen-specific IP-10 mRNA responses in PBMCs from cattle, starting at 29 days after *M. bovis* challenge, that were highly correlated to IFN-γ mRNA levels and Palmer *et al.* (2020) confirmed the potential of IP-10, with mRNA isolation and protein release from whole blood, for bTB diagnosis in cattle. However, similarly to Parsons *et al.* (2016) high levels of IP-10 in unstimulated plasma from both infected and uninfected cattle was observed for some individuals. The parallel measurement of IP-10 with IFN-γ has also demonstrated the potential to maximise detection of *M. bovis*-infected cattle and buffaloes, highlighting the benefit of host biomarker signatures for enhanced bTB diagnosis (Bernitz *et al*., 2019a; Coad *et al.,* 2019).

4.3. IL-1β

IL-1β is one of the multiple cytokines secreted, primarily by innate immune cells such as monocytes and macrophages, with IFN-γ, to orchestrate an immune response toward mycobacterial infection and is viewed as one of the major pro-inflammatory cytokines (Schenk *et al.,* 2014; Elnaggar *et al.,* 2017). Jones *et al.* (2010) found IL-1β cytokine levels to be much higher in *M. bovis*-infected compared to uninfected cattle, using whole blood stimulated with either PPDs or EC. Elnaggar *et al.* (2017) found similar results when stimulating blood with EC antigens, with significant differences observed between the infected group, and both non-tuberculous mycobacteria-exposed and uninfected control cattle. Palmer *et al.* (2020) investigated IL-1β expression in whole blood stimulated with EC and Rv3615c, showing significantly higher levels in the *M. bovis*-challenged cattle, compared to uninfected controls, at 5, 8 and 12 weeks post infection (WPI). Rusk *et al.* (2017) demonstrated significant upregulation of IL-1β by *M. bovis*-specific T cells, isolated from stimulated PBMCs, by transcriptomics analysis and T cell/macrophage co-cultures using experimentally-infected calves. Finally, the study by Jones *et al.* (2010) applied IFN-γ and IL-1β assays in parallel to observe a 5% increase in sensitivity without any loss of specificity, using the EC antigens, when compared to measuring IFN-γ alone; this reiterates the utility of biomarker signatures for more efficient diagnosis.

4.4. IL-4

A characteristic indicator of the Th2 immune response to mycobacteria, IL-4 plays an anti-inflammatory role such as controlling tissue damage by down-regulating pro-inflammatory responses (Widdison *et al.,* 2006). Rhodes *et al.* (2000) investigated IL-4 cytokine expression in PBMC culture supernatants, after stimulation with PPDs or EC, using experimentally and naturally *M. bovis*-infected cattle. The overall IL-4 response, in comparison to IFN-γ, was delayed with activity peaking at

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6-8 WPI. Challenge with a low dose of *M. bovis* resulted in a reduced IFN-γ response although a specific IL-4 response remained evident. In naturally infected cattle, increased IL-4 differentiated these animals from uninfected controls with sensitivity equivalent to that of IFN-γ (Rhodes *et al.,* 2000). These findings are similar to a study by Blanco *et al.* (2009) in which IL-4 expression in PBMCs was elevated in five of nine naturally infected cattle, compared to controls.

Two studies by Thacker *et al.* (2006, 2007) on experimentally infected white-tailed deer and cattle, respectively, also found that increased IL-4 gene expression distinguished *M. bovis*-infected from uninfected animals when investigating PBMCs stimulated with PPDs or EC. The highest IL-4 levels in deer were at 12, 16 or 24 WPI; for cattle, the peak was earlier (at 4 WPI) with a decline thereafter, similar to the Rhodes *et al.* (2000) study. In the deer, *M. bovis* infection resulted in consistently more IL-4 production in animals with less pathological changes compared to the high pathology group. In cattle, however, IL-4 expression was greater in the high pathology group overall regardless of stimulus and the low pathology group was indistinguishable from the uninfected group. In contrast, Widdison *et al.* (2006) assessed infected cattle 16 WPI, when the acute infection phase was more controlled and chronic disease was starting to develop. In this study, a significant decrease in IL-4 and increase in the IFN-γ/IL-4 ratio was observed in *M. bovis*-challenged animals. Moreover, there was a significant negative correlation between IL-4 expression, and both lymph node scores and the number of mycobacteria isolated. This supports the hypothesis of a switch from a Th1- to Th2-dominated response in the first three months post *M. bovis*-infection in cattle (Thacker *et al.,* 2007).

In summary, it appears that the observed delay of the IL-4 response relative to IFN-γ corresponds to the anti-inflammatory role of IL-4 in *M. bovis* infection (Rhodes *et al.,* 2000; Widdison *et al.,* 2006). Two of the cattle infection studies demonstrated early peaks in IL-4 expression followed by rapid decreases, while IFN-γ responses remained detectable when lesion development would be expected to begin (Rhodes *et al.,* 2000; Thacker *et al.,* 2006). This suggests that IL-4 may reduce IFN-γ-induced pathology and does not compromise the protective response, although results have suggested that the switch from Th1 to Th2 responses may occur later than three months PI (Thacker *et al.,* 2007). Although not yet investigated outside bovids and cervids, the potential for IL-4 to distinguish infection states warrants further investigation.

4.5. IL-8

The precise role of the chemokine IL-8 in TB has not been fully elucidated, although studies have demonstrated IL-8 binding to tubercle bacilli and an IL-8/pathogen interaction that appears to enhance mycobactericidal properties of macrophages and neutrophils (Krupa *et al.,* 2015). Increased IL-8 is also required for granuloma formation (Gao *et al.,* 2019).

Widdison *et al.* (2009) investigated RNA expression of IL-8 in lymph node tissue from *M. bovis*-challenged cattle. The infected cohort displayed lesions at necropsy, representing a well-established infection stage and the same cohort displayed significantly elevated IL-8 expression.

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Widdison *et al.* (2009) also observed a positive correlation between IL-8 expression levels and lesion severity together with the bacterial load, in the lymph nodes examined. In contrast, Blanco *et al.* (2009) observed decreased IL-8 expression in naturally infected versus healthy cattle. The lack of IL-8 upregulation, together with the observed Th1 cytokine profile in PPD_b stimulated PBMCs, was indicative of active infection (Blanco *et al.*, 2009). In humans, low IL-8 mRNA expression, in combination with other markers, allows differentiation between active and latent TB (Wu *et al.,* 2007).

A more recent study by Gao *et al.* (2019) investigated naturally *M. bovis*-infected cattle, further characterised by a nested PCR, on *M. bovis* bacteria shed into nasal exudates, that could identify animals that posed higher transmission risks (i.e., PCR-positive and at a more advanced bTB stage). An IL-8 assay was performed with PPD_b- and EC-stimulated whole blood from cohorts of infected/PCR-negative ($bTB_{PCR\text{-}N}$), infected/PCR-positive ($bTB_{PCR\text{-}P}$), and uninfected cattle. Both stimuli resulted in significantly increased IL-8 in both *M. bovis*-infected cohorts compared to the uninfected cattle. Interestingly, unstimulated IL-8 was significantly higher in the $bTB_{PCR\text{-}N}$ cohort than both the $bTB_{PCR\text{-}P}$ and uninfected cohort. The concentrations of PPD-stimulated IL-8 were also positively correlated with IFN-γ and were higher than the levels of IFN-γ, IP-10 and IL-17A. In addition, PPD-stimulated IL-8 was superior to IP-10 and IL-17A in discriminating *M. bovis*-infected animals from uninfected animals, in good agreement with the TST and IGRA, and with a relative Se and Sp of >90% and >98%, respectively (Gao *et al.,* 2019).

Although relatively less reported, results obtained from studies of IL-8 in cattle suggest an important role of this chemokine in *M. bovis* infections (Gao *et al.,* 2019). However, further research to determine the potential of IL-8 as a bTB biomarker is still required.

4.6. IL-17A

The pro-inflammatory cytokine IL-17A (IL-17), produced by Th17 lymphocytes, has been identified as a major effector cytokine required for detection and clearing of tubercle bacilli (Yoshida *et al.,* 2010). Studies of IL-17A in bTB have demonstrated its role in protective immunity, in addition to participation in granuloma formation (Vordermeier *et al.,* 2009). Blanco *et al.* (2011) studied IL-17A mRNA expression in PPD_b-stimulated PBMCs from experimentally infected cattle and found that animals with macroscopic bTB lesions developed higher IL-17A expression compared to those without lesions, with statistical significance at 60 and 90 days PI (DPI). Aranday-Cortes *et al.* (2012) also noted upregulation of IL-17A mRNA in lymph node lesions 13 WPI, in comparison to control lymph node tissue. This upregulation occurred at each granuloma stage investigated. Notably, as granulomas developed, expression decreased until there was significantly less IL-17A in stage IV compared to stage I.

In addition to increased IL-17A gene expression, increased protein production has also been observed in *M. bovis* infection. McGill *et al.* (2014) observed significantly higher levels of PPD_b/EC-stimulated IL-17A protein secreted by PBMCs from experimentally *M. bovis*-infected cattle. Increased

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numbers of antigen-specific IL-17A-secreting cells have also been found in blood from infected animals, with $CD4^{+}$ T cells discovered to be the prominent source of IL-17A following stimulation. Similar findings in cattle were also reported by Steinbach *et al.* (2016). Waters *et al.* (2016) investigated IL-17A protein in whole blood and mRNA from PBMCs of experimentally infected cattle and observed a greater than 9-fold upregulation post-infection, with correlations between gene expression and protein release, and between IL-17A and IFN-γ production. Moreover, higher IL-17A concentrations at 2.5 WPI correlated with increased lesion severity and mycobacterial burdens in cattle. Using the same tissues, Xin *et al.* (2018) also observed significantly higher PPB_b-stimulated IL-17A mRNA and protein in naturally and experimentally infected cattle compared to uninfected controls. The marked IL-17A responses elicited by *M. bovis* in cattle, combined with correlations to bTB pathology, point to the utility of IL-17A as a promising indicator of bTB disease progression.

4.7. CXCL9

The chemokine CXCL9, also known as monokine induced by IFN-γ (MIG), has been reported as a mediator of the bovine anti-mycobacterial response during bTB, with a proposed role in attracting activated T cells, and granuloma development or maintenance (Thacker *et al.,* 2007; Palmer *et al.,* 2015). Aranday-Cortes *et al.* (2013) investigated *CXCL9* expression in lymph node granulomas of experimentally *M. bovis*-infected cattle. The early stage granulomas showed significantly upregulated expression compared to the control tissue, followed by a significant decrease in *CXCL9* in granuloma stages II – IV as lesions progressed. Contrastingly, Palmer *et al.* (2015) studied pulmonary granulomas at 150 DPI and observed overall high and significantly elevated *CXCL9* expression compared to non-lesion lung tissue; however, the expression did not differ significantly between different granuloma stages. The differences in these two studies were attributed to tissue type, amongst other factors (Shu *et al.,* 2014; Palmer *et al.,* 2015). Klepp *et al.* (2019) investigated *CXCL9* expression in PBMCs from naturally infected cattle and significant differences in fold changes were observed between infected and healthy animals. Similarly, Palmer *et al.* (2020) also observed significantly elevated CXCL9 gene expression and protein levels in *M. bovis*-challenged cattle in response to EC/Rv3615c or PPD_b antigens, with detectable CXCL9 responses by 2 WPI, which remained consistently and significantly higher than that of the control group.

In lions (*Panthera leo*), EC-stimulated blood was used to assess *CXCL9* expression, and significantly increased levels of *CXCL9* enabled discrimination between *M. bovis*-infected, -exposed and uninfected lion cohorts (Olivier *et al.,* 2017; Sylvester *et al.,* 2017). Also using EC (QFT) stimulated blood, Higgitt *et al.* (2017) and Kerr *et al.* (2020) were able to detect *M. bovis* immune sensitization by upregulation of *CXCL9* in spotted hyenas (*Crocuta crocuta*) and cheetahs (*Acinonyx jubatus*), respectively. In addition, Roos *et al.* (2019) was able to show that upregulation of *CXCL9* could distinguish between *M. bovis*-infected and uninfected warthogs.

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Overall, studies on *CXCL9* have demonstrated high levels of expression in tuberculous lung and thoracic lymph nodes, in addition to stimulated whole blood, in *M. bovis*-infected cattle. The CXCL9 responses display a robustness akin to that of the IP-10 biomarker, although without the confounding effect of spontaneous production such as that of IP-10 in unstimulated samples (Palmer *et al.,* 2020).

4.8. IL-10

The Th2-associated cytokine IL-10 is a critical anti-inflammatory mediator of innate and adaptive responses to tuberculous mycobacteria (Welsh *et al.,* 2005). The function of IL-10 is to deactivate macrophages and decrease production of reactive nitrogen and oxygen species; hence, in its absence, a stronger Th1 immune response is incited, while high levels of IL-10 are associated with increased susceptibility to mycobacterial infection (Jacobs *et al.,* 2005; Palmer *et al.,* 2015).

There appears to be an inverse relationship between IL-10 and IFN-γ. Welsh *et al.* (2005) analysed PBMC cytokine mRNA of experimentally infected cattle, and reported high IL-10 levels prior to infection, which gradually declined following infection as higher *IFNG* expression was detected. However, there was a sharp increase in IL-10 at 26 WPI, with levels higher than those pre-infection, in cattle that showed the greatest severity of disease. In addition, this was correlated with decreasing CMI and increasing humoral responses (Welsh *et al*., 2005).

Similar patterns were seen in IL-10 expression in tissues. Widdison *et al.* (2006) studied IL-10 expression in lymph node tissue, noting a significant decrease in IL-10 and an increase in the IFN-γ/ IL-10 ratio in infected compared to uninfected cattle. There was also a significant negative correlation between lesion scores and mycobacterial load in lymph nodes and IL-10 expression. These results are similar to those from Thacker *et al.* (2007) who also compared IL-10 between a high and low pathology groups of *M. bovis*-infected cattle in which they discovered two-fold lower IL-10 expression in the high pathology cohort. However, these two studies were conducted at 16 and 18 WPI. Considering the role of IL-10 in limiting tissue destruction, hence smaller and less necrotic lesions displaying the highest IL-10 levels, these results were unsurprising and could explain the later peak (26 WPI) observed by Welsh *et al.* (2005). In the Widdison *et al.* study, the combined observation of supressed IL-4, IL-6, IL-10 and TNF in the infected group, together with maintenance of IL-12 and IFN-γ levels, suggested that suppression was specific and not just a general consequence of infection and necrosis from a developing chronic response.

Interestingly, Blanco *et al.* (2014) did not observe any downregulation of IL-10 in PBMCs of infected cattle with lesions, and the expression level was similar to that of infected animals without lesions. Similarly, Palmer *et al.* (2015) observed no difference in IL-10 expression between granulomas and non-lesioned lung tissue, noting that the low levels observed were expected of active granulomas. However, when combined with IL-2 and IL-17 in a predictive biomarker combination, IL-10 enhanced the classification of infected/lesion-negative animals and was hence acknowledged as a potential identifier of disease progression in herds with no clinical signs of bTB (Blanco *et al*., 2014). The ratio

IFN-γ/IL-10 has also been acknowledged as a potential indicator of *M. bovis* disease severity in red deer (Thomas *et al.,* 2017). However, most studies used a single time point to assess IL-10 expression, which may not reflect the dynamic levels of cytokines during granuloma formation. In support of this, Canal *et al.* (2017) observed significantly higher IL-10 expression in stage III and IV granulomas of lymph nodes and lung, compared to stages I and II, in naturally *M. bovis* infected cattle. Finally, lung lesions and respiratory lymph nodes from goats, experimentally infected with *M. bovis,* revealed high levels of IL-10 and highlighted the important role of this cytokine in granuloma formation (Gonzalez-Juarrero *et al.,* 2013).

4.9. IL-22

The cytokine IL-22 belongs to the IL-10 family and is produced by natural killer, mast and T cells, predominantly Th17 and Th22 (Aranday-Cortes *et al.,* 2012). Together with IL-17A, IL-22 has emerged as a major effector cytokine required for the detection and clearance of tubercle bacilli in TB studies, however, its role in bTB is less studied. It has been shown to induce protection and may inhibit mycobacterial growth inside macrophages (Mehta *et al.,* 2017).

Aranday-Cortes *et al.* (2012) investigated IL-22 mRNA in a murine bTB model, followed by a study in PBMCs from infected cattle, and observed a 74-fold upregulation of IL-22 in the lungs of infected compared to naïve mice and highly significant upregulation in the PPD_b-stimulated PBMCs of the infected cattle. The predominant source of IL-22 was CD4+ T cells, similar to IFN-γ. Waters *et al.* (2015) and Steinbach *et al.* (2016) confirmed these observations both at the gene and protein level, respectively, with the latter using naturally *M. bovis*-infected cattle. Palmer *et al.* (2020) also observed upregulated IL-22 in infected cattle, at 5, 8, 12 and 16 WPI, compared to uninfected controls.

Another study by Aranday-Cortes *et al.* (2013) examined tuberculous granulomas from infected cattle. The study reported upregulation of IL-22 in bTB lymph node lesions with a clear trend of decreasing mRNA expression from granuloma stages I to IV, indicating the potential of IL-22 as a biomarker for bTB pathology. Rusk *et al.* (2017) also noted a lack of IL-22 expression by T cells within late-stage granulomas from lung and mediastinal lymph nodes, confirmed by Palmer *et al.* (2016) who observed very low expression levels of IL-22 in lymph nodes with advanced granulomatous lesions, i.e., samples collected at ±21 WPI, with no differences in expression between granulomatous and uninfected lymph nodes. Klepp *et al.* (2019) studied IL-22 expression in PBMCs from naturally infected cattle and in addition to observing upregulation of IL-22 in the infected group, also found that IL-22 could significantly differentiate *M. bovis* infected cattle with either negative TST or IGRA results from uninfected animals. Hence, IL-22 may be useful as an ancillary biomarker for bTB detection where the results from the TST and IGRA fail to detect infected animals.

5. Discussion

This review describes host CMI biomarkers of diagnostic potential for the detection of *M. bovis* infection and bTB, with a focus on more recent research and knowledge gaps, especially as these pertain

to wildlife species. The CMI response is a vital component of host adaptive immunity to *M. bovis* (Pollock *et al.,* 2005; Welsh *et al.,* 2005). Cattle and badger studies have demonstrated the involvement of the major T lymphocyte types and production of CMI-based cytokines and chemokines during *M. bovis* immune response development, accompanied by a shift from the predominant pro-inflammatory, Th1-biased response to more anti-inflammatory, Th2 functions as infection develops (Palmer and Waters, 2006; Widdison *et al.,* 2006; Canal *et al.,* 2017; Gormley and Corner, 2018). With a focus on the observation and measurement of CMI responses, detected through changes in gene expression, protein release or both, promising diagnostic biomarker targets and their associated limitations for multi-species application have emerged.

The choice of CMI-based methods for diagnostic purposes is in part motivated by the early and specific response that is elicited in most *M. bovis* host species studied to date (Gormley and Corner, 2018). The delayed type hypersensitivity reaction, that is measured *in vivo* by the TST, is mimicked in stimulated blood cultures *in vitro* and can therefore result in a higher Se and Sp for *M. bovis* detection (Chambers *et al.,* 2013; Maas *et al.,* 2013). Additionally, the specific cytokines and chemokines, produced during the adaptive immune response, that are detected by these assays is a feature applicable to most (if not all) mammalian species, thus allowing for translational use across species. Further advantages of *in vitro* CMI assays include even earlier detection times of an immune response than the TST (as early as one week post infection), only a single immobilization due to the use of blood samples, and more potential for the standardization of tests and reagents without in-field variation and operator bias (de la Rua-Domenech *et al.,* 2006). Moreover, the cost differences between *in vivo* (TST) and *in vitro* CMI assays may be over-estimated and CMI tools could prove more cost-efficient than assumed, with further cost reductions anticipated by increased use allowing the scale-up of production, and the automation of assays (such as ELISA kits) that lower laboratory costs (Schiller *et al.,* 2010).

The most commonly used cytokine biomarker for TB diagnostic assays is IFN-γ, a critical Th1 cytokine produced upon lymphocyte activation in defense against *M. bovis* (Maas *et al.,* 2013). This cytokine is also vital for the formation and function of granulomas, a hallmark of the response to *M. bovis* in host species (Canal *et al.,* 2017; Pereira *et al.,* 2020). Compared with other cytokine assays to date, IGRAs are robust and comparatively easy to standardize, and the development of new IGRAs for multiple non-bovine species has been suggested for bTB screening and control (Schiller *et al.,* 2010; Pereira *et al.,* 2020). The increasingly widespread use of IGRAs (such as Bovigam®) in cattle, due to US- and EU-approval as a TST adjunct for parallel testing in bTB eradication programs to increase Se, may also explain interest in its use for wildlife species. Although IGRAs have been successfully applied to several domestic and wildlife species, including but not limited to cattle, goats, cervids, buffalo, white rhinoceros (*Ceratotherium simum*) and wild dog (*Lycaon pictus*), there have been limitations encountered when using them in other *M. bovis* host species (Broughan *et al.,* 2013; Maas *et al.,* 2013; Bernitz *et al.,* 2018; Higgitt *et al.,* 2018; Chileshe *et al.,* 2019). Aside from technical challenges related to the lack of available diagnostic tests and reagents for most wildlife, this may also be due to species

heterogeneity in predominant immune response pathways to *M. bovis* (Gormley and Corner *et al.,* 2018; Roos *et al.,* 2018a). Although *M. bovis* was first strongly associated with cattle, this pathogen has adapted and evolved to infect a broad range of animal host species, which may present with shared or unique characteristics in their immune responses (Brites *et al.,* 2018; Gagneux, 2018; Pereira *et al.,* 2020). Hence, more recent introductions of *M. bovis,* for example to African wildlife species, may have caused differences in the interferon protein structure, or the amount of interferon produced, between species that could result in decreased expression and thus detection of this cytokine, as observed in lions and warthogs (Olivier *et al.,* 2017; Roos *et al.,* 2018b). Another difference between species is disease susceptibility thus, particularly when disease prevalence is unknown, animals could be highly exposed or sub-clinically infected (Broughan *et al.,* 2013). The disease state will influence immune responses, as observed in experimentally infected badgers that showed consistent CMI responses with a high dose of *M. bovis* yet those with subclinical presentation had weak CMI responses although with no effects on the humoral response (Gormley and Corner, 2018). This could explain the more efficient use of alternative biomarkers or methods observed in species such as lions and warthogs, in which IFN-γ detection appeared less optimal (Miller *et al.,* 2012; Olivier *et al.,* 2017; Roos *et al.,* 2018a and 2018b).

In addition to a dominant early response, changes in IFN-γ responses have been correlated to pathological changes, although an IGRA to distinguish between *M. bovis* infection and bTB disease has not yet been developed (Xin *et al.,* 2018; Gao *et al.,* 2019). Considering the correlations observed between *IFNG* expression and granulomatous lesion development, the quantitative measurement of IFN-γ in different cohorts of defined bTB states from early infection to active disease could enable the development of an IFN-γ cytokine assay to differentiate *M. bovis*-infected from diseased animals. However, species specific validation would be critical due to observed differences in *IFNG* expression profiles, during bTB progression, between fallow deer and cattle (Garcia-Jimenez *et al.,* 2012; Palmer *et al.,* 2016; Canal *et al.,* 2017). In light of the potential drawbacks to IFN-γ detection, including sample handling that necessitates a short period between blood collection and processing and variable test performance parameters depending on species and disease stage, alternative cytokines and chemokines to IFN-γ have emerged as additional tools for *M. bovis* infection and bTB diagnosis.

Pro-inflammatory cytokines are released early after *M. bovis* infection and are thus expected to be promising candidates for prompt biomarkers of infection due to the predominant Th1 response observed in several host species. In addition to IFN-γ, two major pro-inflammatory cytokines are IL-1β and IL-6, although not much more than a role in general mycobacterial infection is known for the latter. The former, IL-1β, has demonstrated high Se for *M. bovis* detection in cattle, particularly when used in parallel with an IGRA (Jones *et al.,* 2010; Palmer *et al.,* 2020). Although not explicitly pro-inflammatory, two biomarkers (IP-10 and IL-8) have been detected at higher concentrations than IFN-γ and have shown possible correlation with bTB progression in cattle and African buffaloes (Bernitz *et al.,* 2019b; Gao *et al.,* 2019; Palmer *et al.,* 2020). Considering their robust response early after *M. bovis* infection*,* these biomarkers could be well-suited for detecting *M. bovis* infection in less

studied species. Another cytokine that demonstrates robust levels from an early infection stage is CXCL9, responsible for $CD4^+$ lymphocyte recruitment (Sylvester *et al.,* 2017; Roos *et al.,* 2019; Palmer *et al.,* 2020). There are limitations associated with CMI-based diagnostics that include reduced Se due to anergy (reduced detectable Th1-biased CMI responses as bTB progresses), and interference from co-infection and vaccination (Pereira *et al.,* 2020). However, the application of a parallel testing scheme, whereby a combination of at least two different tests able to detect slightly different sub-populations of infected animals (i.e., animals at different stages of infection), has shown promise in combatting these drawbacks, particularly in *M. bovis*-endemic settings (de la Rua-Domenech *et al.,* 2006; Bernitz *et al.,* 2018). The demonstration of two dominant pro-inflammatory cytokines, IFN-γ and IL-1β, that increased Se without compromising Sp when diagnosing *M. bovis* infection in cattle, indicates that even slight differences in cytokine pathways and functions can provide a parallel scheme to improve detection of the maximum number of infected animals. Moreover, for species in which IFN-γ detection is problematic, the detection of additional or alternative pro-inflammatory cytokines could aid the early detection of *M. bovis.* Therefore, considering the robust levels of IL-8 and its good agreement with both the TST and IGRAs, this marker could also prove a promising option for enhancing *M. bovis* detection. Another example of parallel application is the measurement of IP-10 with IFN-γ that has enhanced Se in buffaloes and cattle, with high IP-10 production having a particular advantage in very early stages of infection (Bernitz *et al.,* 2019a; Coad *et al.,* 2019). Moreover, Klepp *et al.* (2019) demonstrated the utility of IL-22 for the diagnosis of *M. bovis* infections that both the TST and IGRA failed to detect, not unexpected from a cytokine marker that stems from a separate and recently described T cell lineage, Th17.

Infection by *M. bovis* demonstrates an early Th1 bias that can tend towards inhibition as the Th2 profile increases, with this conversion between Th1 and Th2 responses showing correlations to increased pathology (Canal *et al.,* 2017). Hence, whether the objective is optimum detection of all infected animals, the detection of highly infectious individuals (i.e., animals that are shedding *M. bovis* bacilli) or the distinction between infection and active disease, it is expected that diagnosis would benefit from using a combination of pro- and anti-inflammatory biomarkers. Moreover, the immunological response to *M. bovis*, in terms of bTB disease progression, is a dynamic process and non-linear (Palmer *et al.,* 2015). Thus, different biomarker signatures could be used to identify bTB progression, as suggested by Blanco *et al.* (2014), Kelley *et al.* (2020) and Palmer *et al.* (2020). One example, shown by immunohistochemistry on granulomatous lesions of lymph nodes and lung in cattle, is the combination of pro-inflammatory IFN-γ and IL-1β with IL-10 (Canal *et al.,* 2017). The two pro-inflammatory cytokines demonstrated contrasting yet equally significant associations with granuloma development and lesioned versus non-lesioned tissue, highlighting the diversity of individual cytokine functions. The study also observed a lack of IL-10 expression in advanced granulomas that correlates with previous findings of progressively decreasing IL-10 in cattle with severe pathology (Thacker *et al.,* 2007; Canal *et al.,* 2017). The anti-inflammatory cytokines IL-4 and IL-10, which typically

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present with inverse correlations to IFN-γ over the course of infection, have shown good potential in distinguishing disease states; thus, their inclusion is suggested for biomarker signatures that cover both early and late stages of infection and bTB, with the potential to enhance diagnostic Se or Sp particularly for enhanced detection of infected animals (Rhodes *et al.,* 2000; Thacker *et al.,* 2006; Widdison *et al.,* 2006; Canal *et al.,* 2017; Thomas *et al.,* 2017). A panel of biomarkers could also provide a promising method for wildlife species in particular, due to the measurement of varied immune responses from a single sample that would provide greater confidence in the animals' disease state without the need for repeat testing, a significant challenge for most wildlife testing. Alternative potential biomarkers of bTB disease progression include IL-17A, also of the Th17 subset, and CXCL9 due to their observed increase as infection progresses with additional correlations to lesion severity (Waters *et al.,* 2016; Klepp *et al.,* 2019). This highlights the utility of adding biomarkers from different T cell subsets in a bTB testing scheme and warrants their investigation in wild species, especially considering the infeasibility and lack of standardization of the TST outside of common domestic species.

Due to the recognized role of wildlife in the maintenance of *M. bovis,* research on the development of assays for wild species (partially or fully validated) is increasing. A significant challenge in the development of CMI biomarkers for *M. bovis* in wildlife is the difficulty in obtaining sufficient reference samples to investigate and validate diagnostic assays. The lack of validated *ante mortem* tests makes diagnostic tests even more challenging to perform under field conditions, particularly in endemic settings (Srinivasan *et al.,* 2019; Kelley *et al.,* 2020). Furthermore, the procurement of gold standard reference cohorts remains a trade-off between obtaining reliable samples and the loss of valuable animals, particularly for negative cohorts, that are required to obtain data from *post mortem* examination and mycobacterial culture (Maas *et al.,* 2013). There are statistical tools that provide alternatives to the use of reference standards, such as latent-class or Bayesian approaches; however, statistical methods require large sample sizes, amongst other limitations (Bezos *et al.,* 2014; Nunez-Garcia *et al.,* 2018; Srinivasan *et al.,* 2019). In acknowledgement of these challenges, recent policy (in the form of additional chapters for the OIE Terrestrial Manual) was adopted between 2014 and 2016, including statistical approaches to validation, that extends the validation standards for diagnostic tests to wildlife (Chapter 2.2.7., OIE Terrestrial Manual 2018). Two validation pathways are provided that allow provisional recognition of a test if the complete validation process is hindered. This as in agreement with the chapter's emphasis on fit-for-purpose assays, particularly as diagnostic testing objectives may differ between domestic animal and wildlife species. The chapter also recognizes the difficulties in obtaining wildlife reference standards (particularly negative cohorts), suggesting latent-class models for performance estimates in lieu of a perfect (gold standard) reference (OIE, 2018). On the other hand, the imperfections of the gold standard requirement for mycobacterial culture have been acknowledged more recently; hence, alternative definitions for reference cohorts that improve test performance parameters may also be applicable (de la Rua-Domenech *et al.,* 2006; Schiller *et al.,* 2010; Gormley and Corner, 2018).

The successful use of IGRAs and other cytokine assays in wildlife depends on the availability (and costs) of suitable tests or reagents. However, commercial CMI biomarker detection platforms are developed and optimized using common domestic species with unknown cross reactivity to wildlife species. Therefore, researchers may need to develop novel species-specific reagents and assays, although these would have a very limited market. However, if cross reactivity can be determined using commercial reagents, this has the advantage of ready access by other researchers without having to exchange reagents. Indirect ELISAs with cross-reactive reagents have facilitated their use in related species, such as the application of bovine ELISAs to African buffaloes (Michel *et al.,* 2011; Bernitz *et al.,* 2018a) or equine ELISAs in white rhinoceros (Chileshe *et al.,* 2019). However, another viable alternative is cytokine gene expression assays that can be adapted for use in closely related species and even extended to non-related species if the sequences are conserved, which has been demonstrated for cytokine sequences in wildlife species (Landolfi *et al.,* 2010; Goosen *et al.,* 2014). Gene expression assays have been used in less common species such as warthogs, hyenas, and lions (Higgitt *et al.,* 2017; Olivier *et al*., 2017; Roos *et al.,* 2018b). The development of cytokine and chemokine multiplex assays that detect several targets simultaneously in a single sample (of small volume), with options for customisation, has also aided the development of biomarker signatures (Coad *et al.,* 2010; Schiller *et al.,* 2010). However, considering the multiple challenges faced when validating a single new CMI-based diagnostic assay, including the limited funding and research on candidate biomarkers in wildlife, the lack of progress is not surprising. Moreover, the costs and feasibility of performing these assays for a range of species should be considered when prioritizing the development of biosignatures for wildlife bTB diagnosis.

The success of management and control strategies for bTB disease is only as effective as the diagnostic assays it relies upon. The use of CMI cytokine and chemokine biomarkers has already improved insight on the comparative immunology of *M. bovis*-infected hosts, with experimental and natural infection studies conducted in cattle, badgers and cervids, amongst other species. Moreover, understanding the contribution and role of dominant profiles, such as the Th1/Th2 responses, to the development of pathology aids the identification and development of diagnostic biomarkers and biomarker panels. However, despite progress in understanding *M. bovis*-induced immune responses, the research and diagnostic biomarkers described here are still primarily restricted to cattle. In comparison to domestic species, limited resources are allocated to studies of bTB in wildlife, resulting in a paucity of information on *M. bovis* infection and disease development in other naturally infected hosts (Maas *et al.,* 2013; Gormley and Corner, 2018). Hence, more species-specific research is required, together with the development of standardized, multi-species tests and reagents. CMI-based assays may also be further improved with the addition of enhanced, immunodominant antigens for stimulation (in addition to ESAT-6 and CFP-10) to increase test Se and Sp. Additionally, advancements in techniques from multiplex biomarker detection platforms to powerful statistical approaches that estimate population characteristics when true disease status is unknown, or when logistical challenges prevent

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acquisition of gold standard reference cohorts, will further enable the validation of enhanced immunological tools in both domestic animals and wildlife.

References

Álvarez, A.H., Estrada-Chávez, C., Flores-Valdez, M.A., 2009. Molecular findings and approaches spotlighting *Mycobacterium bovis* persistence in cattle. Vet. Res. https://doi.org/10.1051/vetres/2009005

Aranday-Cortes, E., Bull, N.C., Villarreal-Ramos, B., Gough, J., Hicks, D., Ortiz-Peláez, Á., Vordermeier, H.M., Salguero, F.J., 2013. Upregulation of IL-17A, CXCL9 and CXCL10 in early-stage granulomas induced by *Mycobacterium bovis* in cattle. Transbound. Emerg. Dis. 60, 525–537. https://doi.org/10.1111/j.1865-1682.2012.01370.x

Aranday-Cortes, E., Hogarth, P.J., Kaveh, D.A., Whelan, A.O., Villarreal-Ramos, B., Lalvani, A., Vordermeier, H.M., 2012. Transcriptional profiling of disease-induced host responses in bovine tuberculosis and the identification of potential diagnostic biomarkers. PLoS One 7. https://doi.org/10.1371/journal.pone.0030626

Arend, S.M., Van Meijgaarden, K.E., De Boer, K., Cerdá De Palou, E., Van Soolingen, D., Ottenhoff, T.H.M., Van Dissel, J.T., 2002. Tuberculin skin testing and in vitro T cell responses to ESAT-6 and culture filtrate protein 10 after infection with *Mycobacterium marinum* or *M. kansasii*. J. Infect. 186, 1797-807

Arnot, L.F., Michel, A., 2020. Challenges for controlling bovine tuberculosis in South Africa. Onderstepoort J. Vet. Res. https://doi.org/10.4102/ojvr.v87i1.1690

Bernitz, N., Clarke, C., Roos, E.O., Goosen, W.J., Cooper, D., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. Detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) tubes and the Qiagen cattletype® IFN-gamma ELISA. Vet. Immunol. Immunopathol. 196, 48–52. https://doi.org/10.1016/j.vetimm.2017.12.010

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019a. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Bernitz, N., Kerr, T.J., Goosen, W.J., Higgitt, R.L., de Waal, C., Clarke, C., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019b. Impact of *Mycobacterium bovis*-induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 217, 109923. https://doi.org/10.1016/j.vetimm.2019.109923

Bezos, J., Casal, C., Díez-Delgado, I., Romero, B., Liandris, E., Álvarez, J., Sevilla, I.A., de Juan, L., Domínguez, L., Gortázar, C., 2015. Goats challenged with different members of the *Mycobacterium*

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tuberculosis complex display different clinical pictures. Vet. Immunol. Immunopathol. 167, 185–189. https://doi.org/10.1016/j.vetimm.2015.07.009

Bezos, J., Casal, C., Romero, B., Schroeder, B., Hardegger, R., Raeber, A.J., López, L., Rueda, P., Domínguez, L., 2014. Current ante-mortem techniques for diagnosis of bovine tuberculosis. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2014.04.002

Blanco, F.C., Bianco, M.V., Meikle, V., Garbaccio, S., Vagnoni, L., Forrellad, M., Klepp, L.I., Cataldi, A.A., Bigi, F., 2011. Increased IL-17 expression is associated with pathology in a bovine model of tuberculosis. Tuberculosis 91, 57–63. https://doi.org/10.1016/j.tube.2010.11.007

Blanco, F.C., Bigi, F., Soria, M.A., 2014. Identification of potential biomarkers of disease progression in bovine tuberculosis. Vet. Immunol. Immunopathol. 160, 177–183. https://doi.org/10.1016/j.vetimm.2014.04.008

Blanco, F.C., Schierloh, P., Bianco, M.V., Caimi, K., Meikle, V., Alito, A.E., Cataldi, A.A., Sasiain, M.D.C., Bigi, F., 2009. Study of the immunological profile towards *Mycobacterium bovis* antigens in naturally infected cattle. Microbiol. Immunol. 53, 460–467. https://doi.org/10.1111/j.1348-0421.2009.00141.x

Brites, D., Loiseau, C., Menardo, F., Borrell, S., Boniotti, M.B., Warren, R., Dippenaar, A., Parsons, S.D.C., Beisel, C., Behr, M.A., Fyfe, J.A., Coscolla, M., Gagneux, S., 2018. A new phylogenetic framework for the animal-adapted *Mycobacterium tuberculosis* complex. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02820

Broughan, J. M., Crawshaw, T. R., Downs, S. H., Brewer, J., Clifton-Hadley, R. S., 2013. *Mycobacterium bovis* infections in domesticated non-bovine mammalian species. Part 2: A review of diagnostic methods. Vet J. 198(2), 346-351. doi:10.1016/j.tvjl.2013.09.007

Brunton, L.A., Prosser, A., Pfeiffer, D.U., Downs, S.H., 2018. Exploring the fate of cattle herds with inconclusive reactors to the tuberculin skin test. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00228

Buddle, B.M., Keen, D., Thomson, A., Jowett, G., McCarthy, A.R., Heslop, J., De Lisle, G.W., Stanford, J.L., Aldwell, F.E., 1995. Protection of cattle from bovine tuberculosis by vaccination with BCG by the respiratory or subcutaneous route, but not by vaccination with killed *Mycobacterium* vaccae. Res. Vet. Sci. https://doi.org/10.1016/0034-5288(95)90023-3

Canal, A.M., Pezzone, N., Cataldi, A., Zumarraga, M., Larzabal, M., Garbaccio, S., Fernandez, A., Dominguez, L., Aranaz, A., Rodriguez-Bertos, A., 2017. Immunohistochemical detection of pro-inflammatory and anti-inflammatory cytokines in granulomas in cattle with natural *Mycobacterium bovis* infection. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2016.10.006

Carrisoza-Urbina, J., Morales-Salinas, E., Bedolla-Alva, M.A., Hernández-Pando, R., Gutiérrez-Pabello, J.A., 2019. Atypical granuloma formation in *Mycobacterium bovis*-infected calves. PLoS One 14. https://doi.org/10.1371/journal.pone.0218547

Stellenbosch University https://scholar.sun.ac.za

Chambers, M.A., 2013. Review of the diagnosis of tuberculosis in non-bovid wildlife species using immunological methods - An update of published work since 2009. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.12094

Chambers, M.A., Aldwell, F., Williams, G.A., Palmer, S., Gowtage, S., Ashford, R., Dalley, D.J., Davé, D., Weyer, U., Salguero, F.J., Nunez, A., Nadian, A.K., Crawshaw, T., Corner, L.A.L., Lesellier, S., 2017. The effect of oral vaccination with *Mycobacterium bovis* BCG on the development of tuberculosis in captive European badgers (*Meles meles*). Front. Cell. Infect. Microbiol. 7. https://doi.org/10.3389/fcimb.2017.00006

Chileshe, J., Roos, E.O., Goosen, W.J., Buss, P., Hausler, G., Rossouw, L., Manemela, T., van Helden, P., Warren, R., Parsons, S.D., Miller, M., 2019. An interferon-gamma release assay for the diagnosis of the *Mycobacterium bovis* infection in white rhinoceros (*Ceratotherium simum*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2019.109931

Clegg, T.A., Doyle, M., Ryan, E., More, S.J., Gormley, E., 2019. Characteristics of *Mycobacterium bovis* infected herds tested with the interferon-gamma assay. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2019.04.004

Coad, M., Clefford, D., Rhodes, S.G., Glyn Hewinson, R., Martin Vordermeier, H., Whelan, A.O., 2010. Repeat tuberculin skin testing leads to desensitisation in naturally infected tuberculous cattle which is associated with elevated interleukin-10 and decreased interleukin-1 beta responses. Vet. Res. 41. https://doi.org/10.1051/vetres/2009062

Coad, M., Doyle, M., Steinbach, S., Gormley, E., Vordermeier, M., Jones, G., 2019. Simultaneous measurement of antigen-induced CXCL10 and IFN-γ enhances test sensitivity for bovine TB detection in cattle. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2019.01.007

Corner, L.A.L., Costello, E., Lesellier, S., O'Meara, D., Sleeman, D.P., Gormley, E., 2007. Experimental tuberculosis in the European badger (*Meles meles*) after endobronchial inoculation of *Mycobacterium bovis*: I. Pathology and bacteriology. Res. Vet. Sci. 83, 53–62. https://doi.org/10.1016/j.rvsc.2006.10.016

Dalley, D., Davé, D., Lesellier, S., Palmer, S., Crawshaw, T., Hewinson, R.G., Chambers, M., 2008. Development and evaluation of a gamma-interferon assay for tuberculosis in badgers (*Meles meles*). Tuberculosis 88, 235–243. https://doi.org/10.1016/j.tube.2007.11.001

De Klerk, L., Michel, A.L., Grobler, D.G., Bengis, R.G., Bush, M., Kriek, N.P.J., Hofmeyr, M.S., Griffin, J.F.T., Mackintosh, C.G., 2006. An experimental intratonsilar infection model for bovine tuberculosis in African buffaloes, *Syncerus caffer*. Onderstepoort J. Vet. Res. 73, 293-303.

de la Fuente, J., Díez-Delgado, I., Contreras, M., Vicente, J., Cabezas-Cruz, A., Tobes, R., Manrique, M., López, V., Romero, B., Bezos, J., Dominguez, L., Sevilla, I. A., Garrido, J. M., Juste, R., Madico, G., Jones-López, E., Gortazar, C., 2015. Comparative genomics of field isolates of *Mycobacterium bovis* and *M. caprae* provides evidence for possible correlates with bacterial

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viability and virulence. PLoS Negl Trop Dis. 9(11), e0004232. doi: 10.1371/journal.pntd.0004232. PMID: 26583774; PMCID: PMC4652870.

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2005.11.005

Denis, M., Buddle, B.M., 2005. Iron modulates the replication of virulent *Mycobacterium bovis* in resting and activated bovine and possum macrophages. Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2005.04.010

Dlugovitzky, D., Bay, M.L., Rateni, L., Fiorenza, G., Vietti, L., Farroni2, M.A., Bottasso, O.A., 2000. Influence of disease severity on nitrite and cytokine production by peripheral blood mononuclear cells (PBMC) from patients with pulmonary tuberculosis (TB). Clin. Exp. Immunol. 122, 343-349.

Domingos, S.C.B., Carioca Júnior, H.R., Lilenbaum, W., Santa Rosa, M.T., Pereira, C.D., Medeiros, L.S., 2019. A systematic review on the distribution of *Mycobacterium bovis* infection among wildlife in the Americas. Trop. Anim. Health Prod. https://doi.org/10.1007/s11250-019-01954-7

Elnaggar, M.M., Abdellrazeq, G.S., Elsisy, A., Mahmoud, A.H., Shyboub, A., Sester, M., Khaliel, S.A., Singh, M., Torky, H.A., Davis, W.C., 2017. Evaluation of antigen specific interleukin-1β as a biomarker to detect cattle infected with *Mycobacterium bovis*. Tuberculosis 105, 53–59. https://doi.org/10.1016/j.tube.2017.04.009

Ford, A.K., Niedringhaus, K.D., Anderson, A.N., LaCour, J.M., Nemeth, N.M., 2019. Disseminated *Mycobacterium kansasii* infection in a white-tailed deer and implications for public and livestock health. J. Vet. Diagnostic Investig. 104063871989547. https://doi.org/10.1177/1040638719895475

Gagneux, S., 2018. Ecology and evolution of *Mycobacterium tuberculosis*. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro.2018.8

Gao, X., Guo, X., Li, M., Jia, H., Lin, W., Fang, L., Jiang, Y., Zhu, H., Zhang, Z., Ding, J., Xin, T., 2019. Interleukin 8 and pentaxin (C-reactive protein) as potential new biomarkers of bovine tuberculosis. J. Clin. Microbiol. 57. https://doi.org/10.1128/JCM.00274-19

García-Jiménez, W.L., Fernández-Llario, P., Gómez, L., Benítez-Medina, J.M., García-Sánchez, A., Martínez, R., Risco, D., Gough, J., Ortiz-Peláez, A., Smith, N.H., Mendoza, J.H. de, Salguero, F.J., 2012. Histological and immunohistochemical characterisation of *Mycobacterium bovis* induced granulomas in naturally infected Fallow deer (*Dama dama*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2012.06.010

García, J.S. y, Bigi, M.M., Klepp, L.I., Blanco, F.C., Bigi, F., 2020. Does *Mycobacterium bovis* persist in cattle in a non-replicative latent state as *Mycobacterium tuberculosis* in human beings? Vet. Microbiol. 108758. https://doi.org/10.1016/j.vetmic.2020.108758

Gonzalez-Juarrero, M., Bosco-Lauth, A., Podell, B., Soffler, C., Brooks, E., Izzo, A., Sanchez-Campillo, J., Bowen, R., 2013. Experimental aerosol *Mycobacterium bovis* model of infection in goats. Tuberculosis 93, 558–564. https://doi.org/10.1016/j.tube.2013.05.006

Good, M., Bakker, D., Duignan, A., Collins, D.M., 2018. The history of in vivo tuberculin testing in bovines: Tuberculosis, a "One Health" issue. Front. Vet. Sci. https://doi.org/10.3389/fvets.2018.00059

Goodchild, A. V., Downs, S.H., Upton, P., Wood, J.L.N., De La Rua-Domenech, R., 2015. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Vet. Rec. 177, 258. https://doi.org/10.1136/vr.102961

Goosen, W.J., Cooper, D., Miller, M.A., van Helden, P.D., Parsons, S.D.C., 2015. 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*). Clin. Vaccine Immunol. 22, 974–978. https://doi.org/10.1128/cvi.00324-15

Goosen, W.J., Parsons, S.D.C., Miller, M.A., van Helden, P.D., Warren, R.M., Cooper, D., 2014. The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2014.10.008

Gormley, E., Corner, L.A.L., 2018. Pathogenesis of *Mycobacterium bovis* infection: The badger model as a paradigm for understanding tuberculosis in animals. Front. Vet. Sci. https://doi.org/10.3389/fvets.2017.00247

Graham, J., Smith, G.C., Delahay, R.J., Bailey, T., McDonald, R.A., Hodgson, D., 2013. Multi-state modelling reveals sex-dependent transmission, progression and severity of tuberculosis in wild badgers. Epidemiol. Infect. 141, 1429–1436. https://doi.org/10.1017/S0950268812003019Grobler, D.G., Michel, A.L., de Klerk, L., Bengis, R.G., 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger. Onderstepoort J. Vet. Res. 69, 221-227.

Higgit, R. L., Buss, P. E., van Helden, P. D., Miller, M. A., Parsons, S. D. C., 2017. Development of gene expression assays measuring immune responses in the spotted hyena (*Crocuta crocuta*). Afr. Zool. 52(2), 99-104. https://doi.org/10.1080/15627020.2017.1309300

Higgitt, R.L., van Schalkwyk, O.L., de Klerk-Lorist, L.M., Buss, P.E., Caldwell, P., Rossouw, L., Manamela, T., Hausler, G.A., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. An interferon gamma release assay for the detection of immune sensitization to *Mycobacterium bovis* in African wild dogs (*Lycaon pictus*). J. Wildl. Dis. https://doi.org/10.7589/2018-03-089

Hlokwe, T.M., van Helden, P., Michel, A.L., 2014. Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? Prev. Vet. Med. 115, 10–17. https://doi.org/10.1016/j.prevetmed.2014.03.011

Stellenbosch University https://scholar.sun.ac.za

Jacobs, M., Fick, L., Allie, N., Brown, N., Ryffel, B., 2005. Enhanced immune response in *Mycobacterium bovis* Bacille Calmette Guerin (BCG)-infected IL-10-deficient mice. Clin. Chem. Lab. Med. 40(9). https://doi.org/10.1515/CCLM.2002.158

Jones, G.J., Pirson, C., Hewinson, R.G., Vordermeier, H.M., 2010. Simultaneous measurement of antigen-stimulated interleukin-1β and gamma interferon production enhances test sensitivity for the detection of *Mycobacterium bovis* infection in cattle. Clin. Vaccine Immunol. 17, 1946–1951. https://doi.org/10.1128/CVI.00377-10

Kelley, H. V., Waibel, S.M., Sidiki, S., Tomatis-Souverbielle, C., Scordo, J.M., Hunt, W.G., Barr, N., Smith, R., Silwani, S.N., Averill, J.J., Baer, S., Hengesbach, J., Yildiz, V.O., Pan, X., Gebreyes, W.A., Balada-Llasat, J.M., Wang, S.H., Torrelles, J.B., 2020. Accuracy of two point-of-care tests for rapid diagnosis of bovine tuberculosis at animal level using non-invasive specimens. Sci. Rep. 10. https://doi.org/10.1038/s41598-020-62314-2

Kerr, T.J., Gumbo, R., Goosen, W.J., Rogers, P., Last, R.D., Miller, M.A., 2020. Novel techniques for detection of *Mycobacterium bovis* infection in a cheetah. Emerg. Infect. Dis. https://doi.org/10.3201/eid2603.191542

Klepp, L.I., Eirin, M.E., Garbaccio, S., Soria, M., Bigi, F., Blanco, F.C., 2019. Identification of bovine tuberculosis biomarkers to detect tuberculin skin test and IFN-γ release assay false negative cattle. Res. Vet. Sci. 122, 7–14. https://doi.org/10.1016/j.rvsc.2018.10.016

Krupa, A., Fol, M., Dziadek, B.R., Kepka, E., Wojciechowska, D., Brzostek, A., Torzewska, A., Dziadek, J., Baughman, R.P., Griffith, D., Kurdowska, A.K., 2015. Binding of CXCL8/IL-8 to *Mycobacterium tuberculosis* modulates the innate immune response. Mediators Inflamm. https://doi.org/10.1155/2015/124762

Lamont, E. A., Janagama, H. K., Ribeiro-Lima, J., Vulchanova, L., Seth, M., Yang, M., Kurmi, K., Waters, W. R., Thacker, T., Sreevatsan, S., 2014. Circulating *Mycobacterium bovis* peptides and host response proteins as biomarkers for unambiguous detection of subclinical infection. J. Clin. Microbiol. 52, 536–543. https://doi.org/10.1128/JCM.02433-13

Landolfi, J. A., Mikota, S. K., Chosy, J., Lyashchenko, K. P., Giri, K., Gairhe, K., Terio, K. A., 2010. Comparison of systemic cytokine levels in *Mycobacterium* spp. seropositive and seronegative Asian elephants (*Elephas maximus*). J. Zoo Wildlife Med. 41(3), 445-455. https://doi.org/10.1638/2009-0163.1

Lesellier, S., Corner, L., Costello, E., Sleeman, P., Lyashchenko, K., Greenwald, R., Esfandiari, J., Singh, M., Hewinson, R.G., Chambers, M., Gormley, E., 2008. Antigen specific immunological responses of badgers (*Meles meles*) experimentally infected with *Mycobacterium bovis*. Vet. Immunol. Immunopathol. 122, 35–45. https://doi.org/10.1016/j.vetimm.2007.11.005

Lesellier, S., Corner, L., Costello, E., Sleeman, P., Lyashchenko, K.P., Greenwald, R., Esfandiari, J., Glyn Hewinson, R., Chambers, M., Gormley, E., 2009. Immunological responses following experimental endobronchial infection of badgers (*Meles meles*) with different doses of

Mycobacterium bovis. Vet. Immunol. Immunopathol. 127, 174–180. https://doi.org/10.1016/ j.vetimm.2008.09.012

Lyashchenko, K.P., Grandison, A., Keskinen, K., Sikar-Gang, A., Lambotte, P., Esfandiari, J., Ireton, G.C., Vallur, A., Reed, S.G., Jones, G., Martin Vordermeier, H., Stabel, J.R., Thacker, T.C., Palmer, M. V, Waters, W.R., 2017. Identification of novel antigens recognized by serum antibodies in bovine tuberculosis. Comp. Immunol. Microb. 69. Doi.org/10.1016/j.cimid.2020.101424

Lyashchenko, K.P., Sridhara, A.A., Johnathan-Lee, A., Sikar-Gang, A., Lambotte, P., Esfandiari, J., Bernitz, N., Kerr, T.J., Miller, M.A., Waters, W.R., 2020. Differential antigen recognition by serum antibodies from three bovid hosts of *Mycobacterium bovis* infection. Comp. Immunol. Microbiol. Infect. Dis. https://doi.org/10.1016/j.cimid.2020.101424

Maas, M., Michel, A.L., Rutten, V.P.M.G., 2013. Facts and dilemmas in diagnosis of tuberculosis in wildlife. Comp. Immunol. Microbiol. Infect. Dis. https://doi.org/10.1016/j.cimid.2012.10.010

Mackintosh, C.G., De Lisle, G.W., Collins, D.M., Griffin, J.F.T., 2004. Mycobacterial diseases of deer. N. Z. Vet. J. 52, 163–174. https://doi.org/10.1080/00480169.2004.36424

MacLean, E., Broger, T., Yerliyaka, S., Fernandez-Carballo, B.L., Pai, M., Denkinger, C.M., 2019. A systematic review of biomarkers to detect active tuberculosis. Nat. Microbiol. https://doi.org/ 10.1038/s41564-019-0380-2

McGill, J.L., Sacco, R.E., Baldwin, C.L., Telfer, J.C., Palmer, M. V., Ray Waters, W., 2014. The role of gamma delta T cells in immunity to *Mycobacterium bovis* infection in cattle. Vet. Immunol. Immunopathol. 159, 133–143. https://doi.org/10.1016/j.vetimm.2014.02.010

Mehta, P.K., Dharra, R., Thakur, Z., 2017. Cattle as experimental model to study immunopathogenesis of tuberculosis. Mycobact. Dis. 07. https://doi.org/10.4172/2161-1068.1000248

Michel, A.L., 2014. Improving specific disease outcomes through a One Health approach-tuberculosis, Rev. sci. tech. Off. int. Epiz. 33, 2, 583-592.

Michel, A.L., Cooper, D., Jooste, J., de Klerk, L.M., Jolles, A., 2011. Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). Prev. Vet. Med. 98, 142–151. https://doi.org/10.1016/j.prevetmed.2010.10.016

Michel, A.L., Müller, B., van Helden, P.D., 2010. *Mycobacterium bovis* at the animal-human interface: A problem, or not? Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2009.08.029

Miller, M., Joubert, J., Mathebula, N., De Klerk-Lorist, L. M., Lyashchenko, K. P., Bengis, R., van Helden, P., Hofmeyr, M., Olea-Popelka, F., Greenwald, R., Esfandiari, J., Buss, P., 2012. Detection of antibodies to tuberculosis antigens in free-ranging lions (*Panthera leo*) infected with *Mycobacterium bovis* in Kruger National Park, South Africa. J Zoo Wildl Med. 43(2), 317-323. doi:10.1638/2011-0171.1

Nuñez-Garcia, J., Downs, S.H., Parry, J.E., Abernethy, D.A., Broughan, J.M., Cameron, A.R., Cook, A.J., de la Rua-Domenech, R., Goodchild, A. V., Gunn, J., More, S.J., Rhodes, S., Rolfe, S., Sharp, M., Upton, P.A., Vordermeier, H.M., Watson, E., Welsh, M., Whelan, A.O., Woolliams, J.A.,

Clifton-Hadley, R.S., Greiner, M., 2018. Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. Prev. Vet. Med. 153, 94–107. https://doi.org/10.1016/j.prevetmed.2017.02.017

O'Hare, A., Orton, R.J., Bessell, P.R., Kao, R.R., 2014. Estimating epidemiological parameters for bovine tuberculosis in British cattle using a Bayesian partial-likelihood approach. Proc. R. Soc. B Biol. Sci. 281. https://doi.org/10.1098/rspb.2014.0248

OIE (World Organisation of Animal Health)., 2018 (8th Edition). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual). Available from: https://www.oie.int/standard-setting/terrestrial-manual/access-online/

Okamoto Yoshida, Y., Umemura, M., Yahagi, A., O'Brien, R.L., Ikuta, K., Kishihara, K., Hara, H., Nakae, S., Iwakura, Y., Matsuzaki, G., 2010. Essential role of IL-17A in the formation of a mycobacterial infection-induced granuloma in the lung. J. Immunol. 184, 4414–4422. https://doi.org/10.4049/jimmunol.0903332

Olivier, T.T., Viljoen, I.M., Hofmeyr, J., Hausler, G.A., Goosen, W.J., Tordiffe, A.S.W., Buss, P., Loxton, A.G., Warren, R.M., Miller, M.A., van Helden, P.D., Parsons, S.D.C., 2017. Development of a gene expression assay for the diagnosis of *Mycobacterium bovis* infection in African lions (*Panthera leo*). Transbound. Emerg. Dis. 64, 774–781. https://doi.org/10.1111/tbed.12436

Palmer, M. V., 2018. Emerging understanding of tuberculosis and the granuloma by comparative analysis in humans, cattle, zebrafish, and nonhuman primates. Vet. Pathol. https://doi.org/10.1177/0300985817712795

Palmer, M. V., Thacker, T.C., Rabideau, M.M., Jones, G.J., Kanipe, C., Vordermeier, H.M., Ray Waters, W., 2020. Biomarkers of cell-mediated immunity to bovine tuberculosis. Vet. Immunol. Immunopathol. 220. https://doi.org/10.1016/j.vetimm.2019.109988

Palmer, M. V., Thacker, T.C., Waters, W.R., 2016. Differential cytokine gene expression in granulomas from lungs and lymph nodes of cattle experimentally infected with aerosolized *Mycobacterium bovis*. PLoS One 11. https://doi.org/10.1371/journal.pone.0167471

Palmer, M. V., Thacker, T.C., Waters, W.R., 2015. Analysis of cytokine gene expression using a novel chromogenic in-situ hybridization method in pulmonary granulomas of cattle infected experimentally by aerosolized *Mycobacterium bovis*. J. Comp. Pathol. 153, 150–159. https://doi.org/10.1016/j.jcpa.2015.06.004

Palmer, M. V., Waters, W.R., 2006. Advances in bovine tuberculosis diagnosis and pathogenesis: What policy makers need to know. Vet. Microbiol. 112, 181–190. https://doi.org/10.1016/j.vetmic.2005.11.028

Palmer, M. V, Waters, W.R., Whipple, D.L., Slaughter, R.E., Jones, S.L., 2004. Evaluation of an *in vitro* blood-based assay to detect production of interferon-gamma by *Mycobacterium bovis*-infected white-tailed deer (*Odocoileus virginianus*). J. Vet. Diagn. Invest. 16, 17-21.

Palmer, M. V., Waters, W. R., Thacker, T. C., 2007. Lesion development and immunohistochemical changes in granulomas from cattle experimentally infected with *Mycobacterium bovis*. Vet Pathol. 44(6), 863-874. doi:10.1354/vp.44-6-863

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Parsons, S.D.C., McGill, K., Doyle, M.B., Goosen, W.J., Van Helden, P.D., Gormley, E., 2016. Antigen-specific IP-10 release is a sensitive biomarker of *Mycobacterium bovis* infection in cattle. PLoS One. https://doi.org/10.1371/journal.pone.0155440

Pereira, A.C., Reis, A.C., Ramos, B., Cunha, M. V., 2020. Animal tuberculosis: Impact of disease heterogeneity in transmission, diagnosis and control. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.13539

Pesciaroli, M., Russo, M., Mazzone, P., Aronica, V., Fiasconaro, M., Boniotti, M. B., Corneli, S., Cagiola, M., Pacciarini, M., Di Marco, V., Pasquali, P., 2012. Evaluation of the interferon-gamma (IFN-γ) assay to diagnose *Mycobacterium bovis* infection in pigs. Vet Immunol Immunopathol. 148(3-4), 369-372. doi:10.1016/j.vetimm.2012.06.020

Pollock, J.M., Welsh, M.D., McNair, J., 2005. Immune responses in bovine tuberculosis: Towards new strategies for the diagnosis and control of disease, Vet. Immunol. Immunop. https://doi.org/10.1016/j.vetimm.2005.08.012

Poste, G., 2011. Bring on the biomarkers. Nature 469, 156-157. https://doi.org/10.1038/469156a

Qiu, X., Tang, Y., Yue, Y., Zeng, Y., Li, W., Qu, Y., Mu, D., 2019. Accuracy of interferon-γ-induced protein 10 for diagnosing latent tuberculosis infection: a systematic review and meta-analysis. Clin. Microbiol. Infect. https://doi.org/10.1016/j.cmi.2018.12.006

Ray Waters, W., Palmer, M. V., 2015. *Mycobacterium bovis* infection of cattle and white-tailed deer: Translational research of relevance to human tuberculosis. ILAR J. 56, 26–43. https://doi.org/10.1093/ilar/ilv001

Rhodes, S., Holder, T., Clifford, D., Dexter, I., Brewer, J., Smith, N., Waring, L., Crawshaw, T., Gillgan, S., Lyashchenko, K., Lawrence, J., Clarke, J., De La Rua-Domenech, R., Vordermeier, M., 2012. Evaluation of gamma interferon and antibody tuberculosis tests in alpacas. Clin. Vaccine Immunol. 19, 1677–1683. https://doi.org/10.1128/CVI.00405-12

Rhodes, S.G., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2000. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology 99, 195-202.

Risalde, M.Á., Thomas, J., Sevilla, I., Serrano, M., Ortíz, J.A., Garrido, J., Domínguez, M., Domínguez, L., Gortázar, C., Ruíz-Fons, J.F., 2017. Development and evaluation of an interferon gamma assay for the diagnosis of tuberculosis in red deer experimentally infected with *Mycobacterium bovis*. BMC Vet. Res. 13. https://doi.org/10.1186/s12917-017-1262-6

Stellenbosch University https://scholar.sun.ac.za

Rodriguez-Campos, S., Smith, N.H., Boniotti, M.B., Aranaz, A., 2014. Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: Implications for diagnostics and legislation of bovine tuberculosis. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2014.02.009

Romha, G., Gebru, G., Asefa, A., Mamo, G., 2018. Epidemiology of *Mycobacterium bovis* and *Mycobacterium tuberculosis* in animals: Transmission dynamics and control challenges of zoonotic TB in Ethiopia. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2018.06.012

Roos, E.O., Olea-Popelka, F., Buss, P., de Klerk-Lorist, L.M., Cooper, D., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018a. Seroprevalence of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*) in bovine tuberculosis-endemic regions of South Africa. Transbound. Emerg. Dis. 65, 1182–1189. https://doi.org/10.1111/tbed.12856

Roos, E.O., Olea-Popelka, F., Buss, P., de Klerk-Lorist, L.M., Cooper, D., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018b. IP-10: A potential biomarker for detection of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*). Vet. Immunol. Immunopathol. 201, 43–48. https://doi.org/10.1016/j.vetimm.2018.05.007

Roos, E.O., Scott, L.A., Ndou, S., Olea-Popelka, F., Buss, P.E., de Klerk-Lorist, L.M., Warren, R.M., van Helden, P.D., Sylvester, T.T., Miller, M.A., Parsons, S.D.C., 2019. Cytokine gene expression assay as a diagnostic tool for detection of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*). Sci. Rep. 9. https://doi.org/10.1038/s41598-019-53045-0

Rusk, R.A., Palmer, M. V., Waters, W.R., McGill, J.L., 2017. Measuring bovine γδ T cell function at the site of *Mycobacterium bovis* infection. Vet. Immunol. Immunopathol. 193–194, 38–49. https://doi.org/10.1016/j.vetimm.2017.10.004

Salvador, L.C.M., O'Brien, D.J., Cosgrove, M.K., Stuber, T.P., Schooley, A., Crispell, J., Church, S., Grohn, Y.T., Robbe-Austerman, S., Kao, R.R., 2019. Disease management at the wildlife-livestock interface: using whole-genome sequencing to study the role of elk in *Mycobacterium bovis* transmission in Michigan, USA. https://doi.org/10.1111/mec.15061

Schenk, M., Fabri, M., Krutzik, S.R., Lee, D.J., Vu, D.M., Sieling, P.A., Montoya, D., Liu, P.T., Modlin, R.L., 2014. Interleukin-1β triggers the differentiation of macrophages with enhanced capacity to present mycobacterial antigen to T cells. Immunology 141, 174–180. https://doi.org/10.1111/imm.12167

Schiller, I., Oesch, B., Vordermeier, H.M., Palmer, M. V., Harris, B.N., Orloski, K.A., Buddle, B.M., Thacker, T.C., Lyashchenko, K.P., Waters, W.R., 2010. Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound. Emerg. Dis. https://doi.org/10.1111/j.1865-1682.2010.01148.x

Shu, D., Heiser, A., Neil Wedlock, D., Luo, D., de Lisle, G.W., Buddle, B.M., 2014. Comparison of gene expression of immune mediators in lung and pulmonary lymph node granulomas from cattle experimentally infected with *Mycobacterium bovis*. Vet. Immunol. Immunopathol. 160, 81–89. https://doi.org/10.1016/j.vetimm.2014.03.017

Stellenbosch University https://scholar.sun.ac.za

Sichewo, P.R., Michel, A.L., Musoke, J., Etter, E.M.C., 2019. Risk factors for zoonotic tuberculosis at the wildlife–livestock–human interface in South Africa. Pathogens 8, 101. https://doi.org/10.3390/pathogens8030101

Sichewo, P.R., Vander Kelen, C., Thys, S., Michel, A.L., 2020. Risk practices for bovine tuberculosis transmission to cattle and livestock farming communities living at wildlife-livestock-human interface in northern Kwazulu Natal, South Africa. PLoS Negl. Trop. Dis. 14. https://doi.org/10.1371/journal.pntd.0007618

Silva Miranda, M., Breiman, A., Allain, S., Deknuydt, F., Altare, F., 2012. The tuberculous granuloma: An unsuccessful host defence mechanism providing a safety shelter for the bacteria? Clin. Dev. Immunol. https://doi.org/10.1155/2012/139127

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. https://doi.org/10.1126/sciadv.aax4899

Steinbach, S., Vordermeier, H.M., Jones, G.J., 2016. CD4+ and πσT Cells are the main producers of IL-22 and IL-17A in lymphocytes from *Mycobacterium bovis*-infected cattle. Sci. Rep. 6. https://doi.org/10.1038/srep29990

Sylvester, T. T., Martin, L. E., Buss, P., Loxton, A. G., Hausler, G. A., Rossouw, L., van Helden, P., Parsons, S. D., Olea-Popelka, F., Miller, M. A., 2017. Prevalence and risk factors for *Mycobacterium bovis* infection in African lions (*Panthera leo*) in the Kruger National Park. J Wildl Dis. 53(2), 372-376. doi:10.7589/2016-07-159

Thacker, T.C., Palmer, M. V., Waters, W.R., 2007. Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2007.05.009

Thacker, T.C., Palmer, M. V., Waters, W.R., 2006. Correlation of cytokine gene expression with pathology in white-tailed deer (*Odocoileus virginianus*) infected with *Mycobacterium bovis*. Clin. Vaccine Immunol. 13, 640–647. https://doi.org/10.1128/CVI.00024-06

Thomas, J., Infantes-Lorenzo, J.A., Moreno, I., Romero, B., Garrido, J.M., Juste, R., Domínguez, M., Domínguez, L., Gortazar, C., Risalde, M.A., 2019. A new test to detect antibodies against *Mycobacterium tuberculosis* complex in red deer serum. Vet. J. https://doi.org/10.1016/j.tvjl.2018.12.021

Thomas, J., Risalde, M.Á., Serrano, M., Sevilla, I., Geijo, M., Ortíz, J.A., Fuertes, M., Ruíz-Fons, J.F., de la Fuente, J., Domínguez, L., Juste, R., Garrido, J., Gortázar, C., 2017. The response of red deer to oral administration of heat-inactivated *Mycobacterium bovis* and challenge with a field strain. Vet. Microbiol. 208, 195–202. https://doi.org/10.1016/j.vetmic.2017.08.007

Tomlinson, A.J., Chambers, M.A., Mcdonald, R.A., Delahay, R.J., 2015. Association of quantitative interferon-γ responses with the progression of naturally acquired *Mycobacterium bovis* infection in

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wild European badgers (*Meles meles*). Immunology 144, 263–270. https://doi.org/10.1111/imm.12369

Tsao, K., Robbe-Austerman, S., Miller, R.S., Portacci, K., Grear, D.A., Webb, C., 2014. Sources of bovine tuberculosis in the United States. Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2014.09.025

Vordermeier, H. M., Cockle, P. J., Whelan, A. O., Rhodes, S., Hewinson, R. G., 2000. Toward the development of diagnostic assays to discriminate between *Mycobacterium bovis* infection and bacille Calmette-Guérin vaccination in cattle. Clin Infect Dis. 30 Suppl 3, S291-S298. doi:10.1086/313877

Vordermeier, H.M., Brown, J., Cockle, P.J., Franken, W.P.J., Arend, S.M., Ottenhoff, T.H.M., Jahans, K., Hewinson, R.G., 2007. Assessment of cross-reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP-10 at the T-cell epitope level. Clin. Vaccine Immunol. 14, 1203–1209. https://doi.org/10.1128/CVI.00116-07

Vordermeier, H.M., Villarreal-Ramos, B., Cockle, P.J., McAulay, M., Rhodes, S.G., Thacker, T., Gilbert, S.C., McShane, H., Hill, A.V.S., Xing, Z., Hewinson, R.G., 2009. Viral booster vaccines improve *Mycobacterium bovis* BCG-induced protection against bovine tuberculosis. Infect. Immun. 77, 3364–3373. https://doi.org/10.1128/IAI.00287-09

Vordermeier, H.M., Whelan, A., Cockle, P.J., Farrant, L., Palmer, N., Hewinson, R.G., 2001. Use of synthetic peptides derived from the antigens ESAT-6 and CFP-10 for differential diagnosis of bovine tuberculosis in cattle. Clin. Diagn. Lab. Immunol. 8, 571–578. https://doi.org/10.1128/CDLI.8.3.571-578.2001

Wallis, R. S., Pai, M., Menzies, D., Doherty, T. M., Walzl, G., Perkins, M. D., Zumla, A., 2010. Biomarkers and diagnostics for tuberculosis: progress, needs, and translation into practice. Lancet. 375(9729), 1920-1937. doi:10.1016/S0140-6736(10)60359-5

Walzl, G., McNerney, R., du Plessis, N., Bates, M., McHugh, T.D., Chegou, N.N., Zumla, A., 2018. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(18)30111-7

Waters, W.R., Maggioli, M.F., Palmer, M. V., Thacker, T.C., McGill, J.L., Vordermeier, H.M., Berney-Meyer, L., Jacobs, W.R., Larsen, M.H., 2016. Interleukin-17A as a biomarker for bovine tuberculosis. Clin. Vaccine Immunol. 23, 168–180. https://doi.org/10.1128/CVI.00637-15

Waters, W.R., Palmer, M. V., Thacker, T.C., Davis, W.C., Sreevatsan, S., Coussens, P., Meade, K.G., Hope, J.C., Estes, D.M., 2011. Tuberculosis immunity: Opportunities from studies with cattle. Clin. Dev. Immunol. https://doi.org/10.1155/2011/768542

Waters, W.R., Thacker, T.C., Nonnecke, B.J., Palmer, M. V., Schiller, I., Oesch, B., Vordermeier, H.M., Silva, E., Estes, D.M., 2012. Evaluation of gamma interferon (IFN-γ)-induced protein 10 responses for detection of cattle infected with *Mycobacterium bovis*: Comparisons to IFN-γ responses. Clin. Vaccine Immunol. 19, 346–351. https://doi.org/10.1128/CVI.05657-11

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Waters, W.R., Whelan, A.O., Lyashchenko, K.P., Greenwald, R., Palmer, M. V., Harris, B.N., Hewinson, R.G., Vordermeier, H.M., 2010. Immune responses in cattle inoculated with *Mycobacterium bovis, Mycobacterium tuberculosis*, or *Mycobacterium kansasii*. Clin. Vaccine Immunol. 17, 247–252. https://doi.org/10.1128/CVI.00442-09

Wedlock, D.N., Kawakami, R.P., Koach, J., Buddle, B.M., Collins, D.M., 2006. Differences of gene expression in bovine alveolar macrophages infected with virulent and attenuated isogenic strains of *Mycobacterium bovis*. Int. Immunopharmacol. https://doi.org/10.1016/j.intimp.2006.01.003

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G., Pollock, J.M., 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology 114, 101–111. https://doi.org/10.1111/j.1365-2567.2004.02003.x

Whittaker, E., Gordon, A., Kampmann, B., 2008. Is IP-10 a better biomarker for active and latent tuberculosis in children than IFN-γ? PLoS One 3. https://doi.org/10.1371/journal.pone.0003901

Widdison, S., Schreuder, L.J., Villarreal-Ramos, B., Howard, C.J., Watson, M., Coffey, T.J., 2006. Cytokine expression profiles of bovine lymph nodes: Effects of *Mycobacterium bovis* infection and Bacille Calmette-Guérin vaccination. Clin. Exp. Immunol. 144, 281–289. https://doi.org/10.1111/j.1365-2249.2006.03053.x

Widdison, S., Watson, M., Coffey, T.J., 2009. Correlation between lymph node pathology and chemokine expression during bovine tuberculosis. Tuberculosis. https://doi.org/10.1016/j.tube.2009.09.003

Widdison, S., Watson, M., Piercy, J., Howard, C., Coffey, T.J., 2008. Granulocyte chemotactic properties of *M. tuberculosis* versus *M. bovis*-infected bovine alveolar macrophages. Mol. Immunol. https://doi.org/10.1016/j.molimm.2007.06.357

Wu, B., Huang, C., Kato-Maeda, M., Hopewell, P.C., Daley, C.L., Krensky, A.M., Clayberger, C., 2007. Messenger RNA expression of IL-8, FOXP3, and IL-12β differentiates latent tuberculosis infection from disease. J. Immunol. 178(6), 3688-3694.

Xin, T., Gao, X., Yang, H., Li, P., Liang, Q., Hou, S., Sui, X., Guo, X., Yuan, W., Zhu, H., Ding, J., Jia, H., 2018. Limitations of using IL-17A and IFN-γ-induced protein 10 to detect bovine tuberculosis. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00028

Yerlikaya, S., Broger, T., MacLean, E., Pai, M., Denkinger, C.M., 2017. A tuberculosis biomarker database: the key to novel TB diagnostics. Int. J. Infect. Dis. https://doi.org/10.1016/j.ijid.2017.01.025

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Chapter 4: Optimisation of the tuberculin skin test for *Mycobacterium bovis* detection in African buffaloes (*Syncerus caffer*)

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Abstract

Effective screening methods are critical for preventing the spread of bovine tuberculosis (bTB) among livestock and wildlife species. The tuberculin skin test (TST) remains the primary test for bTB diagnosis globally, although performance is suboptimal. African buffaloes (*Syncerus caffer*) are a maintenance host of *Mycobacterium bovis* in South Africa, tested using the single intradermal tuberculin test (SITT) or comparative test (SICTT). The interpretation of these tests has been based on cattle thresholds due to the lack of species-specific cut-off values for African buffaloes. Therefore, the aims of this study were to calculate buffalo-specific thresholds for different TST criteria (SITT, SICTT, and $SICTT_{72h}$ that calculates the differential change between *M. bovis* and *M. avium* reactions at 72h only to limit measurement error) and compare performances using these cut-off values. The results confirm that 3 mm best discriminates *M. bovis*-infected from unexposed control buffaloes with sensitivities of 69% (95% CI 60-78; SITT and SICTT) and 76% (95% CI 65-83; $SICTT_{72h}$), and specificities of 86% (95% CI 80-90; SITT), 96% (95% CI 92-98; $SICTT_{72h}$) and 97% (95% CI 93-99; SICTT), respectively. A comparison between TST criteria using buffalo-specific thresholds demonstrates that the comparative TST performs better than the SITT, although sensitivity remains suboptimal. Therefore, further research and the addition of ancillary tests, such as cytokine release assays, are necessary to improve *M. bovis* detection in African buffaloes.

Introduction

Mycobacterium bovis infects a wide range of species, including humans, and has a global impact on animal health (Gagneux*,* 2018; Palmer *et al.,* 2020). The existence of *M. bovis* maintenance hosts within global wildlife populations, including badgers (*Meles meles*) in the United Kingdom and African buffaloes (*Syncerus caffer*) in southern Africa, complicates efforts to manage and control bovine tuberculosis (bTB) in livestock (Hlokwe *et al.,* 2014; Tsao *et al.,* 2014). In addition, *M. bovis* affects wildlife-related industries with adverse consequences for conservation, tourism, and game sales due to spillover of infection to multiple wildlife species, restricted movement of animals, and culling of infected animals (Renwick *et al,* 2007; Arnot and Michel, 2020).

In South Africa (SA), regulatory bTB control efforts are focused toward both domestic cattle and African buffaloes (Arnot and Michel, 2020). The success of these measures primarily relies on effective *ante mortem* techniques to accurately screen for *M. bovis* infection (Brites *et al.,* 2018; Bernitz *et al.,* 2019). The most common screening test for bTB is the tuberculin skin test (TST), which has been in use for over 100 years (Srinivasan *et al.,* 2019). One TST approach is the single intradermal tuberculin test (SITT) that measures the host's *in vivo* cell-mediated immune (CMI) responses to injected *M. bovis* purified protein derivative (PPD) (Srinivasan *et al.,* 2019). The SITT has several drawbacks including compromised specificity (Sp) due to PPD cross-reactions with environmental mycobacteria (Vordermeier *et al.,* 2007; Bernitz *et al.,* 2019; Duignan *et al.,* 2019). To improve the test's discriminative ability, the single intradermal comparative tuberculin test (SICTT) was developed to

include the measurement of a differential response to *M. bovis*- and *M. avium*-derived PPD antigens (Srinivasan *et al.,* 2019). Even though this modification has demonstrated improved Sp, reduced sensitivity (Se) has also been observed (Brunton *et al.,* 2018).

Despite limitations, the TST remains the standard test for screening African buffaloes for *M. bovis* infection with the interpretation of results based on extrapolation from cattle interpretation guidelines. However, the TST has not been validated in African buffaloes, and the calculation of species-specific cut-off values may improve test performance (Arnot and Michel, 2020). Current OIE guidelines (World Organization of Animal Health, 2018) advocate a 4 mm skinfold thickness (SFT) increase at the bovine PPD (PPD_b) injection site over the avian PPD (PPD_a) site as a bTB-positive test result for terrestrial species. In SA, a 4 mm increase (0h measurement subtracted from measurement at 72h post-injection) at the PPD_b injection site for the SITT, or a 4 mm differential increase between the PPD_b (72-0h) and PPD_a (72-0h) injection sites, are used for interpreting results when testing historically bTB-negative or suspect cattle herds (bTB Manual, 2016). However, for known *M. bovis*-endemic cattle herds, it is recommended that this threshold be lowered to 3 mm. The most recent Buffalo Veterinary Procedural Notice (VPN, 2017, SA) states that a minimum SFT increase of 3 mm at the PPD_b injection site compared to the SFT at the PPD_a site (SICTT), or a 3 mm differential increase in SFT at the PPD_b (72-0h) injection site only (SITT), be considered a positive TST result. However, the 2017 VPN guidelines have recently been contested and are currently being revised.

To determine the diagnostic performance of the TST in buffaloes, calculation and comparison of buffalo-specific cut-off values in relevant cohorts are required. Therefore, this study aimed to: i) calculate buffalo-specific cut-off values for three TST criteria (SITT, SICTT, $SICTT_{72h}$) using *M. bovis*-infected and unexposed buffalo cohorts, and ii) compare TST test performance and criteria using calculated buffalo-specific and currently used cut-off values in cohorts with known *M. bovis* infection status. Results from this study provide data to inform cut-off values and interpretation guidelines that may improve detection of *M. bovis* infection when using the TST in African buffalo herds.

Materials and methods

Ethics

Ethical approval for this project was granted by the Stellenbosch University Animal Care and Use Committee (ACU-2019-9081), and a Section 20 research permit was issued by the Department of Agriculture, Forestry and Fisheries (DAFF; 12/11/1/7/2).

M. bovis-infected buffaloes

Between 2015 and 2019, buffaloes were mass captured during the annual bTB test-and-cull programs in Hluhluwe-iMfolozi Park (HiP; KwaZuluNatal, SA), a known *M. bovis*-endemic area. Buffaloes were chemically immobilized and the SICTT performed as described below. All buffaloes from HiP suspected to be *M. bovis*-infected, based on any positive test result (TST or either of two cytokine release assays, interferon-gamma [IFN-γ] or IFN γ-induced protein 10), were culled and

underwent *post mortem* examination. Mycobacterial cultures were performed on frozen tissue samples and genetic speciation by PCR of cultured isolates was used to confirm the presence of *M. bovis*, as previously described (Warren *et al*., 2006; Goosen *et al*., 2014). The SFT measurements from culture-confirmed, *M. bovis*-infected buffaloes were used in this study.

M. bovis-unexposed buffaloes

In 2019, 201 buffaloes from 10 private wildlife farms in SA with no history of *M. bovis* (unexposed) were immobilized for bTB screening using the SICTT. Due to their high monetary value, none of these animals were culled and therefore, no tissue samples were collected for mycobacterial culture. However, based on history and prior testing to support an uninfected status, SFT measurements recorded during testing of these herds were used as results from a *M. bovis* negative cohort for this study. The buffaloes from each setting (different private farms and HiP) are maintained under a variety of conditions. The test results used in this study hence reflect a spectrum of realistic influences and not a controlled experiment.

SICTT

The SICTT was performed in both the *M. bovis*-infected and unexposed buffalo cohorts, as previously described (Parsons *et al.,* 2011). Briefly, a mid-cervical area on each side of the buffaloes' neck was shaved and the baseline SFT was measured using Hauptner clock dial calipers (Kyron Laboratories, Benrose, South Africa) prior to PPD administration. Intradermal injections of 0.1 mL of *M. bovis* PPD (PPD_b; 3000 IU; WDT) and 0.1 mL of *M. avium* PPD (PPD_a; 2500 IU) were administered by experienced registered veterinarians and animal health technicians using two McLintock® tuberculosis test syringes (Kyron Laboratories) in the left and right side of the neck, respectively. After approximately 72h, buffaloes were immobilized and the SFT at each injection site was measured again by the same operator that performed the initial measurements.

Data analyses

Data analyses were conducted in GraphPad Prism Version 7.04 (GraphPad Software Inc., San Diego, CA, USA). An unpaired t-test was used to assess differences between the means of the two cohorts, for each of three TST criteria outlined below (SITT, $SICTT_{72h}$, SICTT). Receiver operator characteristic (ROC) curve analyses were performed to determine buffalo-specific diagnostic cut-off values for the TST criteria, based on the highest Youden's index, using results from confirmed *M. bovis*-infected and unexposed buffaloes (Youden, 1950). Alternative cut-off values were calculated as the mean + 2 standard deviation (SD), using the *M. bovis*-unexposed cohort alone (Sharma and Jain, 2014).

The test results for the three different TST criteria, using the SFT measurements (in mm), were calculated as follows: SITT - the PPD_b differential value, i.e., the 0h SFT measurement at the PPD_b site subtracted from the 72h measurement (72-0h); $SICTT_{72h}$ - the difference between the PPD_b and PPD_a SFT measurements at 72h only (i.e., PPD_a site measurement at 72h subtracted from the PPD_b site measurement at 72h); and SICTT - the difference calculated as the change in SFT (72-0h) at the PPD_a site subtracted from the change at the PPD_b site. For all criteria, if either the PPD_a (72-0h), PPD_b (72-

0h) or final SICTT calculation resulted in a negative value (due to presumed dehydration), the actual values were used rather than rounding to zero. However, cut-off values were also calculated after correcting any negative final values to zero (to assess the effects of such corrections).

Test performance was calculated for the buffalo-specific cut-off values in addition to cut-off values of 2 mm, 3 mm and 4 mm, for each TST criterium, to simulate currently used TST thresholds. Test Se was calculated as the number of true positives, divided by true positives plus false negatives, using results from the *M. bovis*-infected buffalo cohort; Sp was determined by the number of true negatives divided by true negatives and false positives using results from the unexposed buffalo cohort (Lalkhen and McCluskey, 2008). The 95% confidence intervals (95% CI) were calculated for Se and Sp values. To compare Se and Sp between different TST criteria and cut-off values, proportions of TST-positive and -negative buffaloes were calculated using both study cohorts and compared using McNemar's chi-squared test for association of paired counts (available at: https://epitools.ausvet.com.au/mcnemar). For all analyses, results were considered significant if $p<0.01$ to increase the stringency of the comparisons.

Results

The distributions of differential SFT measurements calculated from the responses at the PPD_b and PPD_a injection sites of 107 culture-confirmed *M. bovis*-infected and 201 *M. bovis*-unexposed African buffaloes are displayed in Figure 4.1. Comparisons of the means for all three TST criteria (SITT, $SICTT_{72h}$, SICTT) significantly distinguished between the *M. bovis*-infected and unexposed buffalo cohorts ($p<0.001$).

Optimal buffalo-specific cut-off values, calculated for each TST criterium using ROC curve analyses, were 3.2 mm for the SITT, 2.5 mm for the $SICTT_{72h}$, and 1.1 mm and 2.8 mm for the SICTT; the ROC curve analysis yielded two cut-off values with identical Youden's indices (YI = 1.66) for the SICTT approach. For practical in-field use, calculated cut-off values were rounded to the nearest integer prior to comparing test performances using the two buffalo cohorts. The 2.5 mm ($SICTT_{72h}$) cut-off value was rounded to 3 mm since no significant differences in Se and Sp were observed between these two values ($p=0.13$; $p=0.25$, respectively). Similarly, the cut-off values 2.8 mm and 1.1 mm for the SICTT were rounded to 3 mm and 1 mm, with no observed differences in Se and Sp between each pair of values ($p=0.48$; $p=1.0$ and $p=1.0$; $p=0.25$, respectively). However, the calculated cut-off value for the SITT (3.2 mm) was not rounded off due to a significant difference in Sp ($p=0.004$) between this cut-off and the buffalo VPN recommended cut-off value of 3 mm, despite no observed difference in Se ($p=1.0$). All cut-off values and their respective Se and Sp are displayed in Table 1.

Alternative buffalo-specific cut-off values were calculated using the mean + 2 SD method and SFT measurements from the *M. bovis*-unexposed buffalo cohorts, for all three TST criteria (Table 4.1). These cut-off values were 4.9 mm for the SITT, 3.8 mm for the $SICTT_{72h}$ and 3.5 mm for the SICTT. Again, for practical in-field reasons, these values were rounded off (after confirming that there were no significant differences in Se and Sp between the calculated and rounded-off values) to 5 mm for the

SITT (p=1.0), and 4 mm (the cattle recommended cut-off) for both the $SICTT_{72h}$ and SICTT (p=1.0; p=0.25 and p=1.0, respectively). Cut-off values were re-calculated using the same approach, however, negative values for PPD_b (72-0h), PPD_b - PPD_a (72h), or ΔPPD_b - ΔPPD_a (SICTT) were corrected to zero. The resulting values (rounded off as above for practicality) were 5 mm for the SITT (the same as for the previous calculation), and 3 mm for both the $SICTT_{72h}$ and SICTT which are equivalent to both the ROC curve analysis-derived (and buffalo VPN recommended) cut-off values.

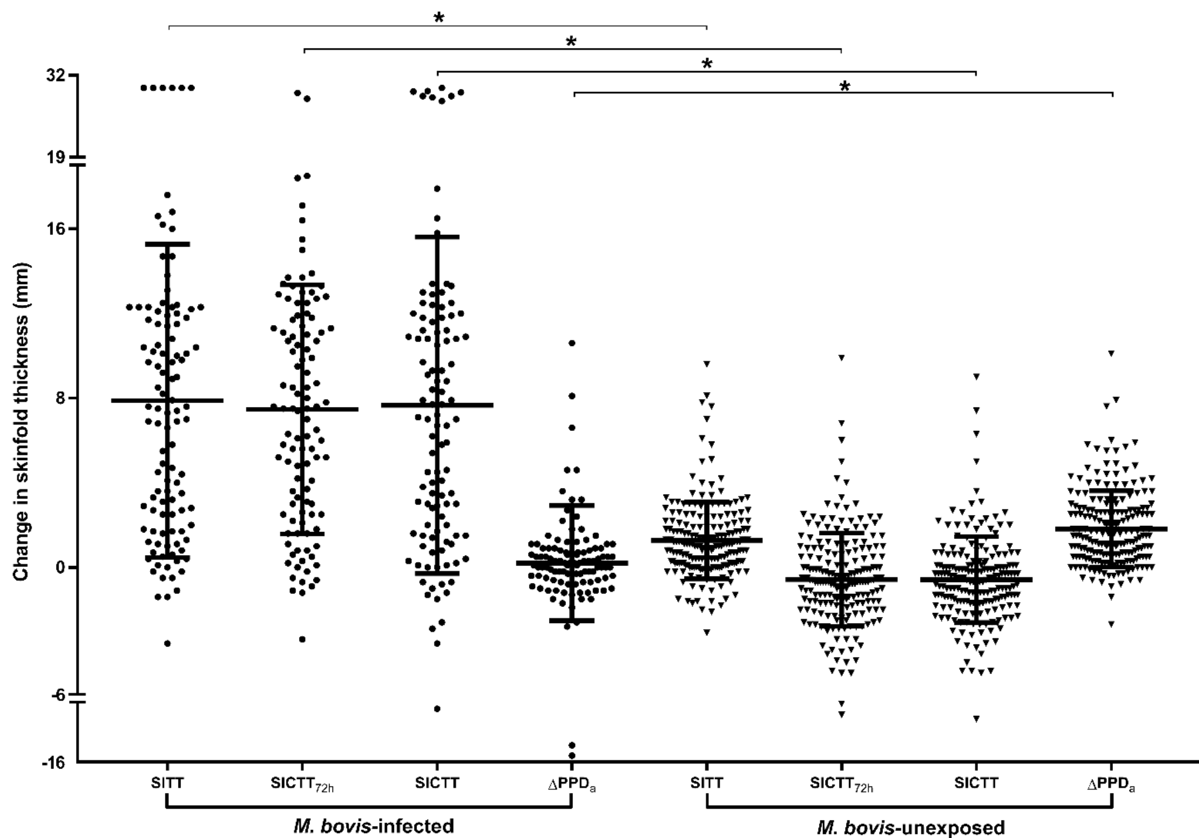

Figure 4.1. Distribution of changes in skinfold thickness measured (in mm) at the bovine purified protein derivative (PPD_b) and avian purified protein derivative (PPD_a) injection sites, of *M. bovis* -infected (n=107) and -unexposed (n=201) buffalo cohorts. The horizontal bars represent the mean and standard deviation. SITT: PPD_b (72h) – PPD_b (0h); $SICTT_{72h}$: PPD_b (72h) – PPD_a (72h); SICTT: ΔPPD_b (72-0h) - ΔPPD_a (72-0h); ΔPPD_a: PPD_a (72h) – PPD_a (0h). *: p<0.001.

In comparison to the ROC curve analysis-derived cut-off values, the Se values for the alternative buffalo-specific cut-offs were not significantly lower for the $SICTT_{72h}$ and SICTT at 75.7% (p=0.04) and 69.2% (p=0.02), respectively. However, the Se of the SITT (68.2%) was significantly lower when the alternative cut-off value was used (p=0.004). The Sp values for the three criteria were higher, although again not significantly (p=0.25) with regard to the $SICTT_{72h}$ (97%) and SICTT (98%). However, the Sp of the SITT alternative cut-off (95.5%) was significantly higher than the Sp using the ROC curve analysis cut-off (p=0.004; McNemar's chi-squared test).

Table 4.1. Summary of calculated and currently used cut-off values for three tuberculin skin test criteria and respective test performance, using skinfold thickness measurements from *M. bovis*-infected (n=107) and unexposed (n=201) buffaloes.

	Cut-off (≥ mm)	**Se% (95% CI)**	**TST-positive/ *M. bovis*-infected (/107)**	**Sp% (95% CI)**	**TST-negative/ *M. bovis*-unexposed (/201)**
	2	75.7 (66.5-83.5)	81	74.1 (67.5-80)	149
SITT	3	69.2 (59.5-77.7)	74	85.6 (79.9-90.1)	172
SFT (bov) at 72h –	**3.2**[a]	**68.2 (58.5-76.9)**	**73**	**90.5 (85.6-94.2)**	**182**
SFT (bov) at 0h	4	64.5 (54.6-73.5)	69	94 (89.8-96.9)	189
	5[b]	58.9 (49-68.3)	63	95.5 (91.7-97.9)	192
SICTT$_{72h}$	2	81.3 (71.6-87.4)	87	88.6 (83.3-92.6)	178
SFT (bov) at 72h –	**3**[a]	**75.7 (65.4-82.7)**	**81**	**95.5 (91.7-97.9)**	**192**
SFT (av) at 72h	4[b]	70.1 (60.5-78.6)	75	97 (93.6-98.9)	195
	1[a]	82.2 (73.7-89)	88	84.6 (78.8-89.3)	170
SICTT	2	73.8 (64.4-81.9)	79	90 (85.1-93.8)	181
ΔSFT (bov 72-0h) –	**3**[a]	**69.2 (59.5-77.7)**	**74**	**96.5 (93-98.6)**	**194**
ΔSFT (av 72-0h)	4[b]	62.6 (51.8-70.9)	67	98 (95-99.5)	197

Se - sensitivity; CI – confidence interval; Sp – specificity; SFT – skinfold thickness; bov – bovine PPD injection site; av – avian PPD site; [a]ROC curve analysis-derived cut-off values; [b]mean + 2 SD-derived cut-off values.

The area under the curve (AUC) results for the three ROC curve analysis-derived TST cut-off values were 0.82, 0.92 and 0.89, respectively (Figure 4.2). The largest AUC (0.92) was for the $SICTT_{72h}$, although a comparison of test performances (with the ROC curve analysis-derived cut-off values) indicated that the Se of the $SICTT_{72h}$ (75.7%) was not significantly higher than for the SITT (p=0.02) or SICTT (p=0.01). However, the Sp of both the $SICTT_{72h}$ (95.5%) and SICTT (96.5%) was significantly higher than the SITT (p=0.004 and p=0.001, respectively).

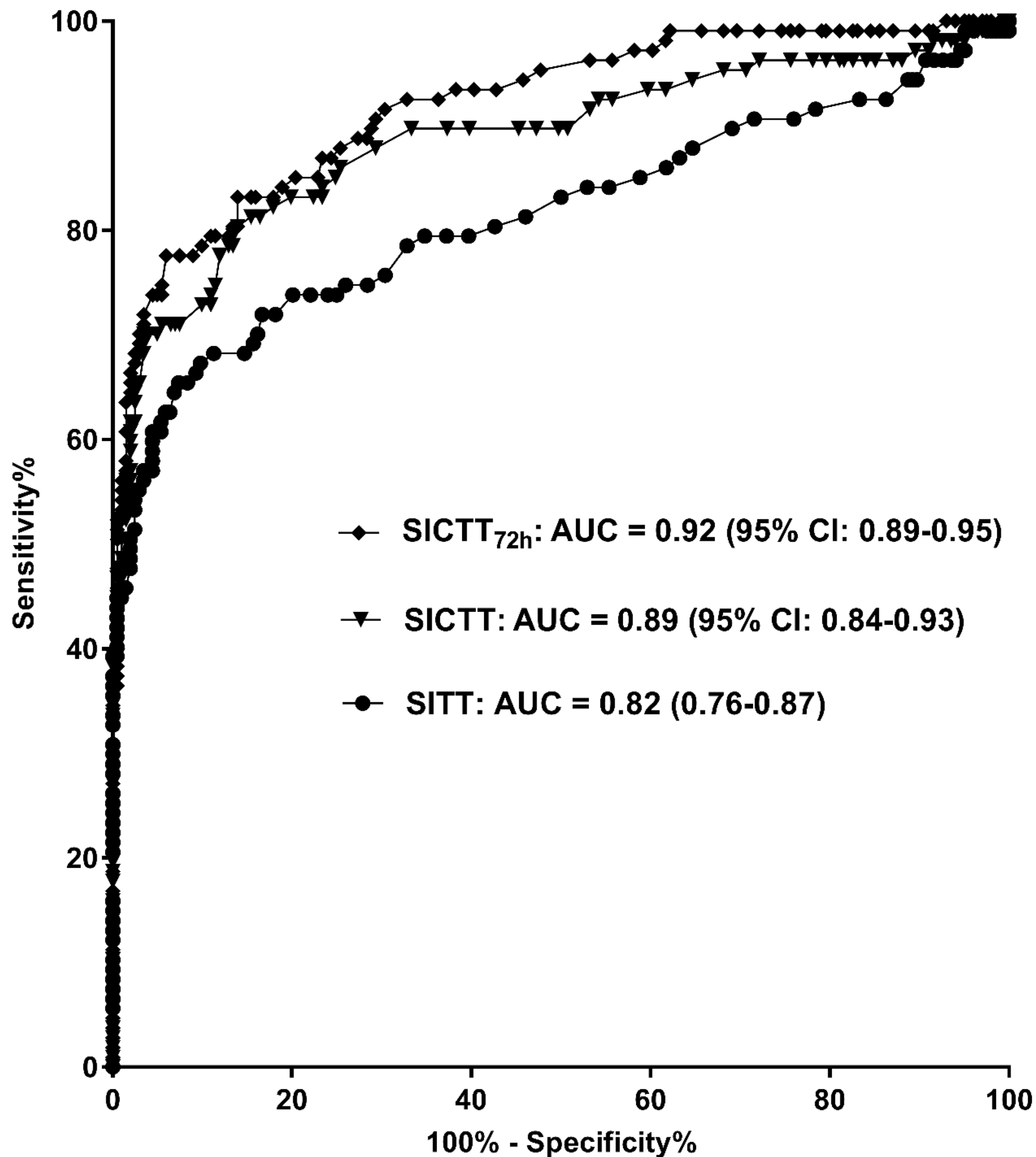

Figure 4.2. Receiver operator characteristic (ROC) curve analyses performed using results from three different tuberculin skin test criteria, based on skinfold thickness measurements (mm), using buffaloes from *M. bovis*-infected (n=107) and unexposed (n=201) cohorts. $SICTT_{72h}$: PPD_b (72h) – PPD_a (72h); SICTT: ΔPPD_b (72-0h) - ΔPPD_a (72-0h); SITT: PPD_b (72h) – PPD_b (0h); AUC: area under the curve; CI: confidence interval.

Discussion

In this study, the SFT measurements from gold-standard positive (culture-confirmed *M. bovis*-infected, n=107) and negative (historically known unexposed, n=201) African buffaloes were used to evaluate TST criteria for validation of this screening test for bTB in this species. Based on comparisons using the same reference cohorts, the best test performance was observed for the $SICTT_{72h}$ using a buffalo-specific cut-off value of 3 mm. The high AUC (0.92) of this TST criterium, together with calculations of moderate sensitivity (75.7%) and high specificity (95.5%) for the 3 mm cut-off value, support the provisional validation of this test when using OIE-validated techniques (i.e., PPD doses and administration method) for African buffaloes in SA.

As stated by the OIE, validation of a diagnostic test for wildlife requires that the test be "fit for purpose" (OIE Terrestrial Manual, 2018). In the case of the TST, it has been validated for the detection of bTB for international trade (Terrestrial Manual, 2018). The purpose of the TST in buffaloes in SA is also to certify freedom from infection for translocation, hence the fitness of the test has already been determined for this purpose by the OIE. In addition, the requirements for provisional recognition of the TST in buffaloes were met in this study by calculating diagnostic Se and Sp, determining cut-off values and including the description of well-characterized reference samples (comprising a minimum of 10 animals each for the positive and negative cohorts; Validation Guideline 2.2.7., Terrestrial Manual, 2018). A comparison of buffalo-specific cut-off values for three TST criteria and their test performances provide an objective evaluation on which to base recommendations for the use and interpretation of the TST in buffalo herds in SA.

In this study, the ROC curve analysis-based buffalo-specific cut-off values, rounded to 3 mm for practical in-field purposes for both SICTT criteria, showed the highest test performance values overall. Interestingly, 3 mm is the (contested) recommended cut-off for buffaloes in South Africa (VPN, 2017). Studies in cattle have reported a range of cut-off values for TST use, which appear to vary by different geographical regions or countries. Awah-Ndukum *et al.* (2016) found that 3 mm and 3.5 mm were the best-performing cut-off values for the SICTT in cattle in Cameroon. However, Ameni *et al.* (2008) observed 2 mm as the optimal cut-off value for Central Ethiopian cattle, affirming the OIE statement that a more stringent cut-off may be required depending on geographical area, disease prevalence and the objectives of the testing program (de la Rua-Domenech *et al*., 2006; Terrestrial Manual, 2018). The performance of the TST in buffaloes has also been shown to be influenced by *M. bovis* prevalence (van der Heijden *et al.,* 2016). However, the results in this study confirm the applicability of a 3 mm cut-off for buffaloes in SA, particularly for herds with unknown bTB status.

Although the calculation of cut-off values is part of test validation, varied testing purposes may necessitate application of different cut-off values. This study assessed test performances of alternate cut-off values for cases in which a more stringent or lenient interpretation may be preferred, such as the screening of herds with defined disease status. For example, when testing herds from *M. bovis*-endemic regions, the objective may be to maximize detection of all infected buffaloes; therefore, lower cut-off

values of 2 mm (for the SITT and $SICTT_{72h}$) or 1 mm (SICTT) are recommended to increase Se (76-82%). However, for regions or herds with no known history of *M. bovis* infections, higher cut-off values of 4 mm ($SICTT_{72h}$ and SICTT) or 5 mm (SITT) will provide increased Sp (96-98%), reducing the chance of false positive results in economically valuable herds. Therefore, no single cut-off value for the TST will be optimal for all bTB-testing scenarios in buffaloes.

The test performances of the three different TST criteria were compared to determine which method should be recommended for use in buffaloes. Both comparative versions of the TST ($SICTT_{72}$ and SICTT) displayed overall better test performance than the SITT. When applying the 3 mm cut-off value, the two SICTT interpretations displayed significantly improved Sp compared to the SITT. Interestingly, the largest AUC value (indicative of test performance) was observed for the $SICTT_{72h}$, which excludes potential changes between 0h and 72h. These changes are considered important in buffaloes due to the common occurrence of dehydration in buffaloes restricted to bomas for 3 days, which may cause decreases in SFT measurements. However, considering the current findings, it appears that either SICTT method is suitable for bTB screening in buffaloes.

It is important to recognize that there is a balance between Se and Sp for any test and achieving high concurrent values may not be possible, therefore, appropriate policies should be implemented according to disease prevalence and risk factors (Nunez-Garcia *et al.,* 2018; Terrestrial Manual, 2018). Although buffalo-specific cut-off values were evaluated for the TST using clearly defined positive and negative reference cohorts in SA, the results of this study agree with current global findings in cattle and other species regarding the high Sp albeit suboptimal Se of the TST (Mackintosh *et al.,* 2004; de la Rua-Domenech *et al.,* 2006; Goodchild *et al.,* 2015; Bernitz *et al.,* 2018 and 2019). A meta- and latent class-analysis by Nunez-Garcia *et al.* (2018) revealed a median Se for the SICTT of 50% in cattle, based on data from 1934-2015 in the UK and Ireland. Reasons for the compromised Se of the TST include the imperfect standardization of the tuberculin antigens, interference from concurrent infection or immunosuppression of the host and the presence of anergy in animals with advanced disease, and the poorly-defined TST antigenic components (Smith *et al.,* 2006; Nunez-Garcia *et al.,* 2018; Srinivasan *et al.,* 2019). In addition to reduced Se, TST use in buffaloes is subject to confounding factors such as operator technique and bias, and the standardization of calliper calibration. Since different personnel performed the TST in HiP and the various private reserves, this was an unavoidable limitation of this study. Due to these limitations, the TST has been used in conjunction with ancillary tests, the most common being an IFN-γ release assay (IGRA) that has demonstrated improved detection of *M. bovis*-infected animals (de la Rua-Domenech *et al.,* 2006; Gormley *et al.,* 2006; Vordermeier *et al.,* 2006; Bernitz *et al.,* 2019).

In summary, this study demonstrates that the currently recommended cut-off value of 3 mm for buffaloes in SA is appropriate, for either the SITT or SICTT. Moreover, evidence is presented to support the use of alternative cut-off values where a more sensitive or specific approach is required, when the disease status of a buffalo herd is known or depending on the testing objective. Finally, the results

indicate that the test performance of the TST is not ideal for detecting *M. bovis*-infected animals, due to the relatively low Se, and should therefore be combined with additional tests to improve detection and bTB control outcomes in African buffaloes.

References

Ameni, G., Hewinson, G., Aseffa, A., Young, D., Vordermeier, M., 2008. Appraisal of interpretation criteria for the comparative intradermal tuberculin test for diagnosis of tuberculosis in cattle in central Ethiopia. Clin. Vaccine Immunol. 15, 1272–1276. https://doi.org/10.1128/CVI.00114-08

Awah-Ndukum, J., Temwa, J., Ngwa, V.N., Mouiche, M.M., Iyawa, D., Zoli, P.A., 2016. Interpretation criteria for comparative intradermal tuberculin test for diagnosis of bovine tuberculosis in cattle in Maroua Area of Cameroon. Vet. Med. Int. 2016. https://doi.org/10.1155/2016/4834851

Arnot, L.F., Michel, A., 2020. Challenges for controlling bovine tuberculosis in South Africa. Onderstepoort J. Vet. Res. https://doi.org/10.4102/ojvr.v87i1.1690

Bernitz, N., Goosen, W.J., Clarke, C., Kerr, T.J., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 204, 40–43. https://doi.org/10.1016/j.vetimm.2018.09.004

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Brites, D., Loiseau, C., Menardo, F., Borrell, S., Boniotti, M.B., Warren, R., Dippenaar, A., Parsons, S.D.C., Beisel, C., Behr, M.A., Fyfe, J.A., Coscolla, M., Gagneux, S., 2018. A new phylogenetic framework for the animal-adapted *Mycobacterium tuberculosis* complex. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02820

Brunton, L.A., Prosser, A., Pfeiffer, D.U., Downs, S.H., 2018. Exploring the fate of cattle herds with inconclusive reactors to the tuberculin skin test. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00228

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. Ante mortem diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2005.11.005

Department of Agriculture, Forestry and Fisheries of SA (DAFF), 2016, Bovine tuberculosis manual. Available from: https://www.nda.agric.za/vetweb/pamphlets&Information/Policy/Tuberculosis%20in%20Cattle%20 Interim%20Manual%20for%20the%20Veterinarian%20&%20AHT%20-%20Sept2....pdf

Stellenbosch University https://scholar.sun.ac.za

Department of Agriculture, Forestry and Fisheries of SA (DAFF), 2017, Veterinary procedural notice for buffalo disease management. Available from https://www.daff.gov.za/vetweb/pamphlets &Information/Policy/Buffalo%20Disease%20Risk%20Management%20VPN_Signed%202017-02-17.pdf.

Duignan, A., Kenny, K., Bakker, D., Good, M., 2019. Tuberculin PPD potency assays in naturally infected tuberculous cattle as a quality control measure in the Irish bovine tuberculosis eradication programme. Front. Vet. Sci. 6. https://doi.org/10.3389/fvets.2019.00328

Gagneux, S., 2018. Ecology and evolution of *Mycobacterium tuberculosis*. Nat. Rev. Microbiol. https://doi.org/10.1038/nrmicro.2018.8

Goodchild, A. V., Downs, S.H., Upton, P., Wood, J.L.N., De La Rua-Domenech, R., 2015. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Vet. Rec. 177, 258. https://doi.org/10.1136/vr.102961

Goosen, W.J., Parsons, S.D.C., Miller, M.A., van Helden, P.D., Warren, R.M., Cooper, D., 2014. The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2014.10.008

Gormley, E., Doyle, M.B., Fitzsimons, T., McGill, K., Collins, J.D., 2006. Diagnosis of *Mycobacterium bovis* infection in cattle by use of the gamma-interferon (Bovigam®) assay. Vet. Microbiol. 112, 171–179. https://doi.org/10.1016/j.vetmic.2005.11.029

Hlokwe, T.M., van Helden, P., Michel, A.L., 2014. Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? Prev. Vet. Med. 115, 10–17. https://doi.org/10.1016/j.prevetmed.2014.03.011

Lalkhen, A.G., McCluskey, A., 2008. Clinical tests: Sensitivity and specificity. Contin. Educ. Anaesthesia, Crit. Care Pain 8, 221–223. https://doi.org/10.1093/bjaceaccp/mkn041

Mackintosh, C.G., De Lisle, G.W., Collins, D.M., Griffin, J.F.T., 2004. Mycobacterial diseases of deer. N. Z. Vet. J. 52, 163–174. https://doi.org/10.1080/00480169.2004.36424

Nuñez-Garcia, J., Downs, S.H., Parry, J.E., Abernethy, D.A., Broughan, J.M., Cameron, A.R., Cook, A.J., de la Rua-Domenech, R., Goodchild, A. V., Gunn, J., More, S.J., Rhodes, S., Rolfe, S., Sharp, M., Upton, P.A., Vordermeier, H.M., Watson, E., Welsh, M., Whelan, A.O., Woolliams, J.A., Clifton-Hadley, R.S., Greiner, M., 2018. Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. Prev. Vet. Med. 153, 94–107. https://doi.org/10.1016/j.prevetmed.2017.02.01

OIE (World Organisation of Animal Health)., 2018 (8th Edition). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual). Available from: https://www.oie.int/standard-setting/terrestrial-manual/access-online/

Palmer, M. V., Thacker, T.C., Rabideau, M.M., Jones, G.J., Kanipe, C., Vordermeier, H.M., Ray Waters, W., 2020. Biomarkers of cell-mediated immunity to bovine tuberculosis. Vet. Immunol.

Stellenbosch University https://scholar.sun.ac.za

Immunopathol. 220. https://doi.org/10.1016/j.vetimm.2019.109988

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Renwick, A.R., White, P.C.L., Bengis, R.G., 2007. Bovine tuberculosis in southern African wildlife: A multi-species host-pathogen system. Epidemiol. Infect. https://doi.org/10.1017/S0950268806007205

Sharma, B., Jain, R., 2014. Right choice of a method for determination of cut-off values: A statistical tool for a diagnostic test. Asian J. Med. Sci. 11, 2. https://doi.org/10.3126/ajms.v5i3.9296

Smith, N., Gordon, S., de la Rua-Domenech, R., Clifton-Hadley, R., Hewinson, R.G., 2006. Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis.* Nat. Rev. Microbiol. 4, 670–681. https://doi.org/10.1038/nrmicro1472

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. https://doi.org/10.1126/sciadv.aax4899

Tsao, K., Robbe-Austerman, S., Miller, R.S., Portacci, K., Grear, D.A., Webb, C., 2014. Sources of bovine tuberculosis in the United States. Infect. Genet. Evol. https://doi.org/10.1016/j.meegid.2014.09.025

van der Heijden, E.M.D.L., Jenkins, A.O., Cooper, D. V., Rutten, V.P.M.G., Michel, A.L., 2016. Field application of immunoassays for the detection of *Mycobacterium bovis* infection in the African buffalo (*Syncerus caffer*). Vet. Immunol. Immunopathol. 169, 68–73. https://doi.org/10.1016/j.vetimm.2015.12.003

Vordermeier, H.M., Whelan, A.O., Ewer, K., Goodchild, T., Clifotn-Hadley, R., Williams, J., Hewinson, R.G., 2006. The Bovigam© assay as ancillary to the tuberculin skin test. Gov. Vet. J. 16, 72-80.

Vordermeier, H.M., Brown, J., Cockle, P.J., Franken, W.P.J., Arend, S.M., Ottenhoff, T.H.M., Jahans, K., Hewinson, R.G., 2007. Assessment of cross-reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP-10 at the T-cell epitope level. Clin. Vaccine Immunol. 14, 1203–1209. https://doi.org/10.1128/CVI.00116-07

Warren, R.M., Gey Van Pittius, N.C., Barnard, M., Hesseling, A., Engelke, E., De Kock, M., Gutierrez, M.C., Chege, G.K., Victor, T.C., Hoal, E.G., Van Helden, P.D., 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. Int. J. Tuberc. Lung Dis. 10, 818–822.

Whelan, A.O., Clifford, D., Upadhyay, B., Breadon, E.L., McNair, J., Hewinson, G.R., Vordermeier, M.H., 2010. Development of a skin test for bovine tuberculosis for differentiating infected from

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vaccinated animals. J. Clin. Microbiol. 48, 3176–3181. https://doi.org/10.1128/JCM.00420-10
Youden, W.J., 1950. Index for rating diagnostic tests. J. Cancer 3, 32-35.

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Chapter 5: Improved detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using modified interferon-gamma release assays

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Abstract

The African buffalo (*Syncerus caffer*) is an economically and ecologically important wildlife species in South Africa; it is also a primary wildlife maintenance host of *Mycobacterium bovis.* Accurate and early detection of *M. bovis* infection in buffaloes is important for controlling transmission. Assays that detect cell-mediated immune responses to *M. bovis* in buffaloes have been developed although these often display suboptimal sensitivity or specificity. Therefore, the aim of this study was to evaluate the newly available Mabtech bovine interferon-gamma (IFN-γ) ELISA^{PRO} kit for enhanced detection of buffalo IFN-γ in whole blood samples stimulated with the QuantiFERON® TB Gold Plus platform. Additionally, the test performance of the Mabtech IFN-γ release assay (IGRA) was compared to the currently used Cattletype® IGRA by determining buffalo-specific cut-off values for the two IGRAs and using gold standard-positive (*M. bovis* culture-confirmed) and *M. bovis*-unexposed negative cohorts. Validation of the Mabtech ELISA revealed negligible matrix interference and a linear and parallel response for recombinant bovine and native buffalo IFN-γ in the range 1.95 to 250 pg/mL. Intra- and inter-assay reproducibility produced coefficients of variation <5.5% and <6.1%, respectively, with a limit of detection at 3.2 pg/mL. Using receiver operator characteristic curve analyses, buffalo-specific cut-off values were calculated as 8 pg/mL for the Mabtech IGRA and 5% (signal to positive control ratio) for the Cattletype® IGRA. The sensitivities were 89% and 83% for the Mabtech and Cattletype IGRAs with specificities of 94% and 97%, respectively. Although the species-specific cut-off values require further evaluation in a relevant test group, the results suggest that the Mabtech IGRA is a promising, sensitive and specific diagnostic tool for *M. bovis* detection in African buffaloes.

Introduction

Mycobacterium bovis infection and the resulting disease, commonly referred to as bovine tuberculosis (bTB), affects a wide range of species including humans, domestic animals and wildlife (Michel, 2014; Romha *et al.,* 2018). Infection and disease may lead to decreased livestock productivity, trade restrictions and impacts on food security, resulting in significant economic losses (Sichewo *et al.,* 2019; Palmer *et al.,* 2020). Moreover, the infection of wildlife negatively affects the respective industries, resulting in consequences for conservation, tourism and game sales (Renwick *et al.,* 2007). Importantly, the presence of wildlife maintenance hosts complicates control of *M. bovis* (Brites *et al.,* 2018). This is demonstrated in the United Kingdom by ongoing outbreaks in cattle due to infected European badgers (*Meles meles*) and in South Africa due to spillover from African buffaloes (*Syncerus caffer*) to livestock and other wildlife species (Hlokwe *et al.,* 2014).

Effective control of bTB requires accurate and early detection of *M. bovis* infection; however, there are limited species-specific diagnostic tests available for wildlife. For bTB, diagnosis depends on the detection of host cell-mediated immune (CMI) responses (Welsh *et al.,* 2005). The current diagnostic standard, in cattle and buffaloes, is the *in vivo* single intradermal comparative tuberculin test (SICTT) that measures a delayed type hypersensitivity response to bovine and avian purified protein derivatives

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(PPDs) (Brunton *et al.,* 2018; Srinivasan *et al.,* 2019). However, the application of this test in wildlife requires two immobilizations to perform the test, three days apart (Bernitz *et al.,* 2019a). Therefore, alternative *in vitro* blood-based tests, such as the Bovigam® interferon-gamma (IFN-γ) cytokine release assay (IGRA), have been investigated in African buffaloes (Goosen *et al.,* 2015). Although this IGRA has high sensitivity (Se), compromised specificity (Sp) is often observed due to cross-reactivity of PPDs with environmental non-tuberculous mycobacteria (Michel *et al.,* 2011; Bernitz *et al.,* 2018b, 2019a). To enhance Sp, cytokine release assays have been modified by using the QuantiFERON® TB Gold, and more recently Gold Plus (QFT-Plus), stimulation platforms for whole blood (Parsons *et al.,* 2011; Goosen *et al.,* 2014). This assay uses specific mycobacterial peptides in place of PPDs as stimulating antigens, namely the early secretory antigen target 6 kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) (Parsons *et al.,* 2011). Thereafter, cytokine concentrations are measured in QFT-plasma samples using tests such as a modified in-house bovine IFN-γ enzyme linked immunosorbent assay (ELISA; Mabtech, Nacka Strand, Sweden) or more recently, the commercially available Cattletype® IFN-γ ELISA (Indical Bioscience, Leipzig, Germany). Both IGRAs have been used for the detection of *M. bovis* in buffaloes, however, the in-house Mabtech IGRA cut-off value was calculated without distinct gold standard positive or negative cohorts, and buffalo-specific cut-off values have not yet been calculated for the Cattletype® IGRA (Parsons *et al.,* 2011; Bernitz *et al.,* 2018a). Considering that bTB prevalence influences test performance, the evaluation of cut-off values using gold standard reference cohorts, when possible, is critical for optimizing IGRA performance (Coad *et al.,* 2019; van der Heijden *et al.,* 2020). However, the challenges in obtaining gold standard reference cohorts and sufficiently large sample sizes in wildlife are widely acknowledged, particularly if culture results from a negative cohort are required since this would involve culling economically valuable animals (Good *et al.,* 2018; OIE Terrestrial Manual, 2018).

Recently, a new standardized, fully quantitative Mabtech bovine IFN-γ ELISA^{PRO} kit became available. This ELISA is validated for use in cattle and provides concentrations of IFN-γ, in contrast to the relative signal to positive ratio (S/P%) measurements for the Cattletype® ELISA. Therefore, the aim of this study was to validate and investigate the combined use of the QFT-Plus platform and Mabtech ELISA^{PRO} using a culture-confirmed, *M. bovis*-infected cohort and a historically *M. bovis*-unexposed buffalo cohort, previously unavailable with sufficient sample numbers. An additional aim was to compare test performance to the currently used QFT-Plus/Cattletype® ELISA, using calculated buffalo-specific cut-off values for the first time, for potential enhancement of *M. bovis* detection in African buffaloes.

Materials and methods

Animals and sampling

An overview of the study design for the Mabtech IGRA is depicted in Figure 5.1. Archived plasma from whole blood samples stimulated using the QFT-Plus platform (Qiagen, Venlo, The Netherlands),

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as previously described, were selected for this study (Parsons *et al.,* 2011; Bernitz *et al.,* 2018a). The positive cohort consisted of 54 buffaloes that were confirmed to be *M. bovis*-infected by mycobacterial culture and speciation (Warren *et al.,* 2006; Goosen *et al.,* 2014); these served as the gold standard reference samples. Archived plasma from 70, randomly selected, *M. bovis*-unexposed buffaloes was used as the negative reference cohort. These buffaloes originated from tested, known *M. bovis*-negative private farms, whose history was used in lieu of gold standard negative mycobacterial culture results since buffaloes were not culled. Results from the Cattletype® IFN-γ ELISA (Indical Biosciences), previously conducted on all buffaloes as described by Bernitz *et al.* (2019a), were used for comparison to the Mabtech bovine IFN-γ ELISA^PRO results, as determined in this study.

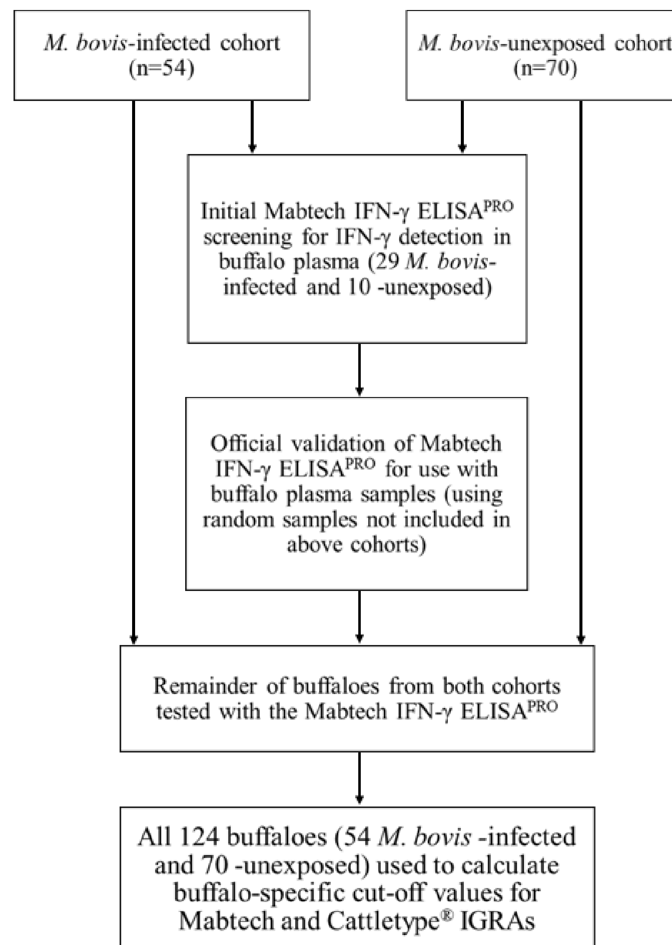

Figure 5.1. Overall study design for validation and cut-off calculation for the Mabtech IFN-γ ELISA^PRO and comparison to the Cattletype® IFN-γ ELISA using archived plasma from QFT-stimulated whole blood from known *M. bovis*-infected and uninfected buffaloes.

Screening of the Mabtech bovine IFN-γ ELISA^PRO

To determine if the commercially available Mabtech bovine IFN-γ ELISA^PRO kit (Mabtech; Product: 3119-1HP-10) could be used to detect African buffalo IFN-γ, a single pre-coated 96-well ELISA plate was used to screen plasma from 39 buffaloes' QFT nil and TB2 antigen-stimulated whole blood samples, as shown in Figure 5.1.

The Mabtech bovine IFN-γ ELISA^{PRO} was used according to the manufacturer's instructions, except where otherwise described. Briefly, wells precoated with anti-bovine IFN-γ monoclonal capture antibody (mAb MT17.1) were washed three times with ELISA wash buffer. A stock solution of bovine recombinant IFN-γ (rIFN-γ) was reconstituted at 0.5 µg/mL and serial dilutions made in ELISA diluent to produce a standard curve with concentrations ranging from 1 – 1000 pg/mL. The assay background control consisted of ELISA diluent only. The standard curve dilutions were done in duplicate, and samples diluted 1:2 in ELISA diluent were then added to the wells (100 µl per well). Duplicate blank wells, an additional control to the assay background control, were left empty. After a two-hour incubation at room temperature (RT; 21.4˚C), the plate was washed five times and 100 µL biotinylated anti-bovine IFN-γ detection antibody (mAb MT307), at a working concentration of 0.25 µg/mL, was added to each well. After a one-hour incubation at RT, wells were washed five times and 100 µL/well streptavidin-horseradish peroxidase (SA-HRP), diluted 1:1000 in SA-HRP diluent, was added. Plates were incubated for one hour at RT, washed five times and then 100 µL tetramethylbenzidine (TMB) substrate was added to each well. Plates were incubated for 15 min in the dark at RT. Thereafter, 100 µL/well Stop solution was added. The optical densities (OD) of the standard curve and sample wells were measured at 450nm and 630nm using an iMark™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Results were calculated as $OD_{450\text{-}630nm}$. After subtracting the blank OD from the ODs of samples, standards and the background control, IFN-γ concentrations were calculated based on a four-parameter logistic (4PL) regression analysis using the rIFN-γ standard curve, as described previously (Cox *et al.,* 2012) using GraphPad Prism 7 software (GraphPad Prism 7, San Diego, CA, USA).

Validation of the Mabtech IFN-γ ELISA^{PRO}

Assay linearity and parallelism

Assay linearity and parallelism were evaluated for IFN-γ concentrations between 0 and 500 pg/mL. The relative IFN-γ concentration of a pooled QFT mitogen plasma sample from three randomly selected buffaloes was determined using the Cattletype® IGRA and bovine rIFN-γ for quantification, using a standard curve. The pooled buffalo sample was then diluted in Mabtech ELISA diluent to obtain a starting concentration of ~500 pg/mL IFN-γ. This sample was serially diluted 1:2 in ELISA diluent to form an 11-point concentration series. The same concentration series was prepared for rIFN-γ from a 0.5 µg/mL stock. Each dilution of rIFN-γ and pooled plasma was plated in duplicate using the Mabtech IFN-γ ELISA, and IFN-γ concentrations calculated as described above. The results for both dilution series were analyzed by 4PL regression analysis (GraphPad Prism 7). The ELISA linearity was characterized as the correlation coefficient (R^2) of the rIFN-γ and buffalo IFN-γ dilution series. The assay working range was calculated after determining linearity (%) for each concentration point by dividing the measured IFN-γ concentrations by the expected concentrations and multiplying by 100%. Linearity values between 80-120% were considered acceptable (Valentin *et al.,* 2011). To

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calculate parallelism, the slopes of the rIFN-γ and buffalo IFN-γ were compared using an F-test (GraphPad Prism 7).

IFN-γ recovery

Recovery of bovine rIFN-γ in buffalo plasma matrix was determined by preparing a pooled sample consisting of QFT nil (unstimulated) plasma (OD range 0.01 – 0.03) from sixteen buffaloes tested with the Cattletype® IGRA. The pooled plasma was prepared as 100%, 50%, 25%, and 0% matrix solutions using the Mabtech ELISA diluent. Thereafter, reconstituted bovine rIFN-γ was spiked into the four matrix dilutions (100%, 50%, 25% and 0%) at 5% of the total volume, resulting in a final concentration of 75 pg/mL. A blank (unspiked) plasma sample for each matrix dilution was also included. All plasma matrix samples, in addition to the ELISA's standard curve, were tested in triplicate using the Mabtech ELISA^{PRO} and OD readings were measured as described above. The concentrations of rIFN-γ ([rIFN-γ]) were calculated based on the standard curve, as previously described. The recovery (%) of spiked rIFN-γ from the plasma matrices was calculated as: ([rIFN-γ] in spiked sample - [IFN-γ] in blank sample) ÷ [rIFN-γ] in reference sample} × 100%.

Assay repeatability and reproducibility

Assay repeatability and reproducibility were determined using mitogen plasma from three buffaloes. These were selected, based on previously measured extrapolated concentrations using the Cattletype® IFN-γ ELISA, to lie within the Mabtech ELISA's working range as previously determined. For each sample, nine aliquots were prepared and stored at 4˚C, representative of field and transport storage conditions, during the testing period. The plasma samples were each tested in triplicate and the Mabtech ELISA was repeated over three consecutive days. Mean IFN-γ concentrations were determined for all samples and intra-assay precision (repeatability) was calculated as the coefficient of variation (CV) of triplicate sample concentrations measured on the same day. Inter-assay precision (reproducibility) was calculated as the CV of each sample concentration measured for each ELISA over the three days.

Limit of detection and limit of quantification

To determine the limit of detection (LOD) and limit of quantification (LOQ) of the Mabtech bovine IFN-γ ELISA, OD values of 24 replicates of ELISA diluent and a standard curve were measured. The mean OD value and standard deviation (SD) of the diluent replicates were calculated. The LOD (OD) was calculated as the mean + 3 SD and the LOQ (OD) was calculated as the mean + 10 SD (Shrivastava and Gupta, 2011). The OD values were then converted to IFN-γ concentrations using the standard curve.

Calculation of diagnostic cut-off value for the Mabtech IGRA

To determine a buffalo-specific diagnostic cut-off value for the Mabtech IGRA using all 124 buffaloes in this study, QFT nil and QFT TB2 antigen-stimulated buffalo whole blood plasma from an additional 85 buffaloes (the remaining 25 from the *M. bovis*-infected cohort and the 60 from the unexposed cohort) were tested using the Mabtech bovine IFN-γ ELISA^{PRO} (Figure 1). A standard curve, prepared as described above and run in duplicate, was included on each plate.

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Data analyses

ELISA data were captured using Bio-Rad Microplate Manager® software (Bio-Rad Laboratories, Inc.) and exported to Microsoft Excel (2002) worksheets (Microsoft Corporation, Redmond, WA, USA). Data analyses were conducted in GraphPad Prism Version 7.04 (GraphPad Software Inc.). ELISA standard curves were analysed using a 4PL regression analysis and sample concentrations were interpolated. A Wilcoxon matched pairs signed rank test was used to compare IFN-γ concentrations in QFT nil and QFT TB2 antigen samples from *M. bovis*-infected buffaloes. A Mann-Whitney test was used to compare the TB2 antigen-specific Mabtech IGRA results of *M. bovis*-infected and unexposed buffalo cohorts. Receiver operator characteristic (ROC) curve analyses were performed to determine the buffalo-specific diagnostic cut-off values for the Mabtech and Cattletype® IGRAs, using the same 124 animals from the two described cohorts, based on the highest Youden's index (Youden, 1950). Alternate cut-off values for the Mabtech and Cattletype® IGRAs were calculated as the mean + 2 SDs using the unexposed control cohort (Sharma and Jain, 2014). Test Se was calculated as true positives, confirmed by mycobacterial culture, divided by true positives plus false negatives; Sp was determined by the number of true negatives (herds with no known history of *M. bovis* used as culture-negative proxy) divided by true negatives and false positives (Lalkhen and McCluskey, 2008). To compare diagnostic performance between the two IGRAs, the proportions of test positive buffaloes were examined using a Z-score calculator for two population proportions (Available at: https://www.socscistatistics.com/tests/ztest/default2.aspx); performances using both the calculated cut-off value and the manufacturer's cattle cut-off for the Cattletype® IGRA were compared to the Mabtech IGRA. For all analyses, a p-value<0.05 was considered statistically significant.

Results

The IFN-γ concentrations measured by the Mabtech bovine IFN-γ ELISA^{PRO} in the initial screening were significantly higher in the QFT TB2 antigen compared to nil plasma samples from the *M. bovis*-infected cohort (median and 95% confidence interval [CI]: TB2 - 73 pg/mL, 21-250 pg/mL; nil - 7 pg/mL, 2-42 pg/mL; p<0.0001). When antigen-specific IFN-γ concentrations were compared, those from the *M. bovis*-infected cohort were significantly greater than in the unexposed buffalo group (median and 95% CI: 63 pg/mL, 20-196 pg/mL; 2 pg/mL, 0-4 pg/mL, respectively; p<0.0001). Based on these preliminary results, further validation of the Mabtech bovine IFN-γ ELISA^{PRO} was performed with buffalo QFT-Plus stimulated archived samples.

Validation of the Mabtech IFN-γ ELISA^{PRO}

Using the Mabtech bovine IFN-γ ELISA^{PRO}, a linear response was observed for both bovine rIFN-γ and buffalo IFN-γ for concentrations between 1.95 and 250 pg/mL (R^2>0.976) (Figure 5.2). The slope of the concentration curve for rIFN-γ was not significantly different from that of the buffalo IFN-γ samples (F = 0.07, p = 0.8) (Figure 5.2).

The mean IFN-γ concentration of the pooled unstimulated buffalo plasma samples used to prepare the recovery matrices was <5 pg/mL (data not shown). Mean recoveries of spiked rIFN-γ in buffalo plasma were 74% in 100% matrix, 83% in 50% matrix, and 96% in the 25% matrix, respectively (Table 5.1). The recovery values increased as the plasma matrix was diluted, with recoveries in 50% matrix and lower within the acceptable range (80-120%). Therefore, further experiments used buffalo plasma samples diluted 1:2 in ELISA diluent.

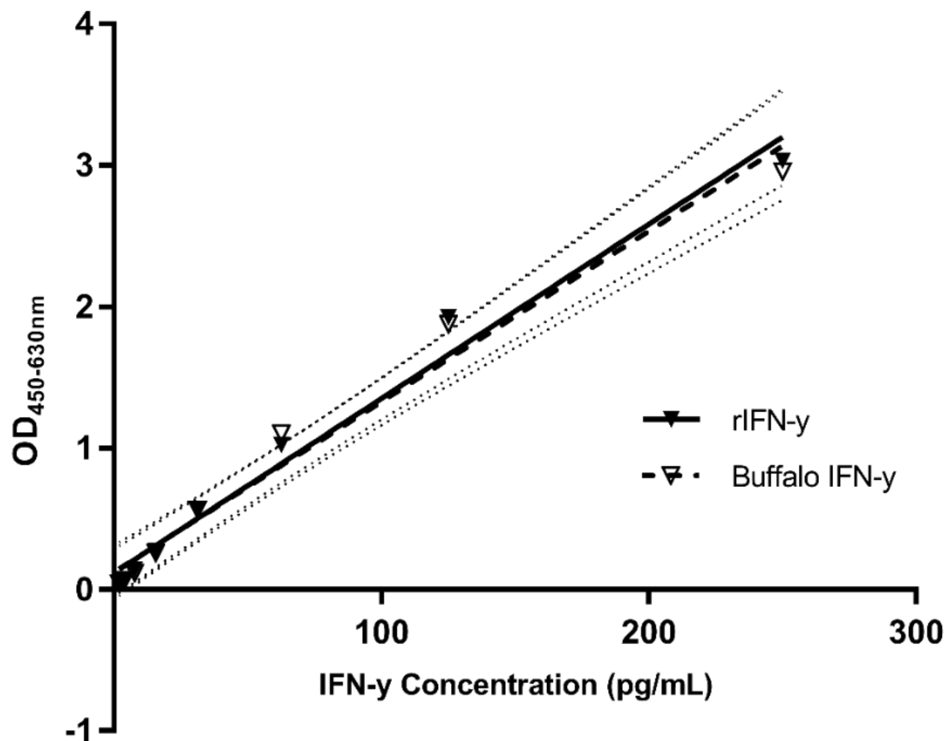

Figure 5.2. Linear regression analysis of a dilution series of recombinant bovine interferon gamma (IFN-γ) and buffalo IFN-γ in the concentration range of 1.95 – 250 pg/mL, measured using the Mabtech bovine IFN-γ ELISA^{PRO} kit as described by the manufacturer. The dotted lines indicate the standard error. The assay displayed a linear response (R2>0.9) and there was no significant difference between slopes ($F = 0.07$; $p = 0.8$). OD: optical density.

Table 5.1. Recovery of 75 pg/mL recombinant bovine interferon gamma (rIFN-γ) in pooled buffalo plasma matrix using the Mabtech bovine IFN-γ ELISA^{PRO} kit assay.

	Sample Recovery (%)			
	100% plasma	50% plasma	25% plasma	0% plasma
Replicate 1	76	87	101	99
Replicate 2	79	85	97	105
Replicate 3	67	77	90	96
Mean	74	83	96	100
SD	6.0	5.7	5.6	4.3

SD – standard deviation.

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The coefficients of variation for assay repeatability and reproducibility, using QFT mitogen samples from three buffaloes, were 1.4–5.5% and 5.4-6.1%, respectively (Table 5.2). The LOD and LOQ were calculated as 2.3 and 3.7 pg/mL; however, the nearest empirical values of 3.2 and 10 pg/mL were determined to be the LOD and LOQ.

Table 5.2. Intra- and inter- assay variability of the Mabtech bovine IFN-γ ELISA^{PRO} kit assay as determined by the coefficient of variation (CV%) based on QFT mitogen-stimulated whole blood from three buffaloes, measured every day in triplicate for three days.

Buffalo	Intra-assay precision			Inter-assay precision		
	Mean (pg/mL)	SD	CV (%)	Mean (pg/mL)	SD	CV (%)
1	103.0	1.4	1.4	98.4	5.6	5.7
2	142.0	0.9	0.7	137.2	7.4	5.4
3	207.0	11.3	5.5	196.1	11.9	6.1

SD – standard deviation; CV – coefficient of variation.

Calculation of diagnostic cut-off value for Mabtech IGRA

The ROC curve analysis for the Mabtech IGRA using samples from 124 *M. bovis*-infected and unexposed buffaloes yielded a cut-off value of 8 pg/mL (Se = 89%; Sp = 94%) and an area under the curve (AUC) of 0.94 (Table 5.3, Figure 5.3). There was a significant difference between infected and unexposed buffalo cohorts in antigen-specific IFN-γ concentrations (TB2-nil) with mean values of 146 pg/mL and 2 pg/mL, respectively ($p<0.0001$; Figure 5.3). Applying the mean + 2 SD approach, an alternative cut-off value of 9 pg/mL (Se = 87%; Sp = 94%) was calculated.

Table 5.3. Test Se and Sp of the QFT Plus -Mabtech and -Cattletype® IGRAs using 124 buffaloes from two cohorts; i) *Mycobacterium bovis*-positive, confirmed by mycobacterial culture (n=54); and ii) *M. bovis*-unexposed buffaloes from farms with no known history of bovine tuberculosis (n=70). Cut-off values were determined by receiver operator characteristic (ROC) curve analyses based on both cohorts, and secondly by determination of the mean + 2 SD using the unexposed cohort. For the Cattletype® IGRA, the manufacturer's cut-off value was also assessed.

	Mabtech IGRA Cut-offs		Cattletype® IGRA Cut-offs		
	ROC	Mean + 2 SD	ROC	Mean + 2 SD	Bovine[a]
Se (%)	89 (77-96[b])	87 (75-95)	83 (71-92)	80 (67-89)	41 (28-55)
	48/54[c]	47/54	45/54	43/54	22/54
Sp (%)	94 (86-98)	94 (86-98)	97 (90-100)	97 (90-100)	100 (95-100)
	66/70[d]	66/70	68/70	68/70	70/70

Se – sensitivity; Sp - specificity; SD – standard deviation; [a]cattle cut-off (manufacturer); [b]95% confidence intervals; [c]correctly-identified positive buffaloes; [d]correctly-identified negative buffaloes.

Using the same sample set and approach, the ROC curve analysis of the Cattletype® IGRA results yielded an optimal cut-off value of S/P = 5% (Se = 83%; Sp = 97%; AUC=0.93) and an alternate cut-off value of 6% (Se = 80%; Sp = 97%). Similarly, the Cattletype® IGRA showed significantly higher mean antigen-specific IFN-γ values in the *M. bovis*-infected cohort (59% S/P) compared to the unexposed buffaloes (0% S/P) ($p<0.0001$; Figure 5.3).

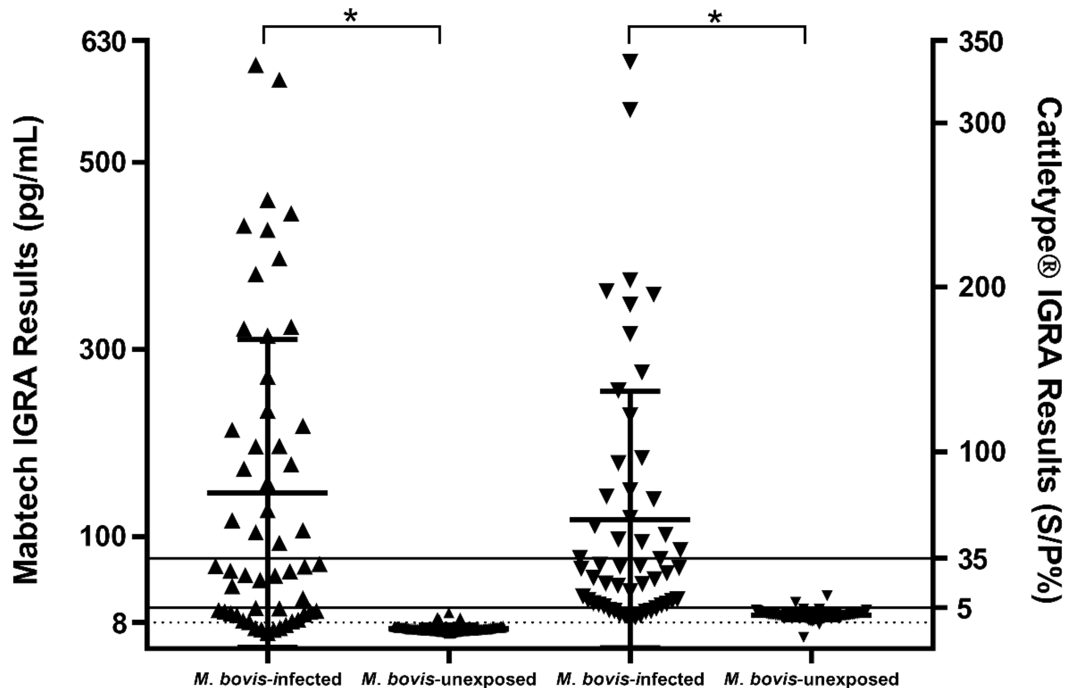

Figure 5.3. Comparison of antigen-specific IFN-γ test results for *M. bovis*-infected buffaloes (n=54), confirmed by mycobacterial culture, and *M. bovis*-unexposed buffaloes from historically *M. bovis*-negative farms (n=70), using the QFT Plus -Mabtech bovine IFN-γ ELISA^{PRO} kit (▲) and -Cattletype® IFN-γ IGRA (▼); * = $p<0.0001$ (unpaired t-test). The mean and standard deviation are indicated for each cohort. The dotted line represents the Mabtech IGRA buffalo-specific cut-off (8 pg/mL); solid lines indicate the Cattletype® IGRA buffalo- and cattle-specific cut-offs (5% and 35% S/P, respectively).

Comparing the diagnostic performance between the Mabtech IGRA (using the buffalo cut-off 8 pg/mL) and the Cattletype® IGRA (using the manufacturer cattle cut-off of 35%) resulted in a significant difference in test Se; the latter displayed an increase in Se from 41% to 83% when the buffalo cut-off was applied (Table 5.3). However, when applying the buffalo-specific cut-off value (5%) to the Cattletype® IGRA, no significant differences in test performance parameters were observed between the Mabtech and Cattletype® IGRAs.

Discussion

Interferon-gamma release assays for detection of bTB in buffaloes have been previously described although with reported suboptimal Se, particularly when specific mycobacterial peptides are used for

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stimulation such as for the QFT-Plus platform (Bernitz *et al.*, 2020). Neither the Bovigam® nor the Cattletype® IGRA has applied buffalo-specific cut-off values for interpretation which, together with other factors, influences test performance (Michel *et al.,* 2011; Bernitz *et al.,* 2019a). Therefore, in this study, a new standardized ELISA, the Mabtech bovine IFN-γ ELISA^{PRO}, was screened to determine if detection of *M. bovis*-infected buffaloes could be improved. In addition, a species-specific cut-off value for the Cattletype® IGRA was calculated, preceding a comparison of test performance between the two IGRAs using well-described reference cohorts. Using archived samples of QFT-stimulated whole blood from known *M. bovis*-infected and unexposed buffaloes, the Mabtech IGRA demonstrated accurate detection of buffalo IFN-γ with improved Se (89%) and high Sp (94%) using buffalo-specific cut-off values. Due to the assay's potential as a diagnostic tool for bTB in buffaloes, further ELISA validation was performed.

The Mabtech ELISA^{PRO} was able to detect buffalo IFN-γ with similar characteristics to that observed with bovine recombinant IFN-γ. The Mabtech ELISA displayed good linearity and parallelism, indicating the suitability of the ELISA^{PRO} for the measurement of buffalo IFN-γ. This was anticipated due to the high degree of homology between several cattle and African buffalo cytokine sequences and furthermore, the utility of alternative commercial bovine ELISA assays for the detection of IFN-γ and other biomarkers in buffalo plasma (Parsons *et al.,* 2011; Goosen *et al.,* 2014; Goosen *et al.,* 2015).

The Mabtech ELISA protocol provided by the manufacturer was suitable for use with buffalo plasma samples without major modifications. Recoveries of bovine IFN-γ from buffalo plasma matrix were within acceptable range (Andreasson *et al.*, 2015) and confirmed the use of a 1:2 dilution as per the manufacturer's instructions. Assay repeatability and reproducibility were well below the recommended maximum CV of 10%, again supporting the use of this ELISA as a diagnostic test for buffaloes.

To validate a new diagnostic test for wildlife, cut-off values need to be calculated using defined positive and negative reference cohorts totalling at least 60 samples (OIE Terrestrial Manual, 2018). This study used a gold standard positive cohort and a historically *M. bovis*-unexposed cohort. This is considered preferable to a culture-negative cohort from an *M. bovis*-endemic herd, typically the only culture-negative animals available, due to the imperfections of the current culturing method (de la Rua-Domenech *et al.,* 2006; Bernitz *et al.,* 2018a).

In this study, the buffalo-specific cut-off values for the QFT-Plus/Mabtech bovine IFN-γ ELISA^{PRO}, as determined by two methods, were very similar at 8 and 9 pg/mL, increasing confidence in these results. These values are both much lower than the buffalo cut-off value of 66 pg/mL calculated for a QFT/in-house Mabtech IFN-γ ELISA (Parsons *et al.,* 2011). An IGRA cut-off value is expected to vary between species and assays, although typically remaining within comparable range. However, the higher cut-off value calculated by Parsons *et al.,* (2011) is not unexpected considering that the negative cohort originated from an *M. bovis*-endemic herd. Hence, buffaloes that were exposed and

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truly infected, yet *M. bovis* culture-negative, may have been included due to the suboptimal Se of mycobacterial culture, further compromised by potential clearing of infection by immunocompetent animals causing low infection doses (de la Rua-Domenech *et al.,* 2006). In the current study, the historically negative cohort is preferable to a gold standard definition and therefore results in a more relevant diagnostic cut-off value for screening African buffaloes in South Africa, especially when testing herds with unknown disease prevalence. The differences in cut-off values between these two studies confirm the importance of evaluating assays using relevant and well-described cohorts when available (van der Heijden *et al.,* 2020). However, the performance of the Mabtech and Cattletype® IGRAs and their respective cut-off values still requires further investigation using a comprehensive test group, representative of the varied bTB prevalence observed in South African buffalo herds.

The importance of applying species-specific diagnostic cut-off values is demonstrated for the Cattletype® IGRA by the differences observed in Se and Sp when applying the manufacturer's cattle cut-off compared to the buffalo values calculated in this study. Suboptimal Se (38%) of this assay has been reported in buffaloes in a previous study (Bernitz *et al.,* 2020). This result prompted the evaluation of the new Mabtech IGRA and a comparison of test performance with the currently used Cattletype® IGRA. Comparing results using the same defined buffalo cohorts, the Mabtech IGRA had significantly higher Se than the Cattletype® IGRA. However, this was based on the manufacturer cut-off value for the Cattletype® IGRA (35% S/P). Since the buffalo cut-off value was significantly lower than the cattle cut-off value at 5% versus 35% S/P, this was expected to impact test performance. When the performance of the two IGRAs was compared using the buffalo-specific cut-off values, there was no significant difference observed between these assays. Furthermore, the Se of the Cattletype® IGRA improved significantly, from 41% to 83%, without a compromise in Sp, after determination of a buffalo-specific cut-off value. These results highlight the importance of evaluating test performance parameters using a fitness for purpose approach, particularly for novel species.

The Mabtech and Cattletype® IGRAs had high Se (89 and 83%, respectively) and Sp (94 and 97%, respectively) using the buffalo diagnostic values. A study by Michel *et al.* (2011) of the Bovigam® IGRA in buffaloes observed higher Se (92%), although Sp was reduced (68%). This is not uncommon due to the more complex nature of the PPD antigens leading to cross-reactions with environmental mycobacteria (Michel *et al.,* 2011). Reduced Sp is particularly problematic for uninfected buffalo herds due to the burden of false positives buffaloes with high economic value. However, the same study identified alternative test interpretations for the Bovigam® that resulted in an increase in Sp (92%), with a consequent decrease in Se (87%). A more recent study in buffaloes also identified 92% Sp for the Bovigam® IGRA, although Se was lower at 73% (Bernitz *et al.,* 2020). Typically, the use of mycobacterial peptides rather than PPDs for whole blood stimulation decreases the Se (Bernitz *et al.,* 2018b); however, this study demonstrates comparable if not enhanced test performance of the Mabtech and Cattletype® IGRAs to that of the Bovigam®. In combination with a field-friendly format and favorable test parameters, the results suggest that the Mabtech assay could aid *M. bovis* testing in

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African buffaloes. Although these results appear promising, further research for the Mabtech and Cattletype® IGRA is required to confirm Se, Sp and predictive values by applying the calculated buffalo cut-off values to a larger, randomly selected test population.

In conclusion, the new Mabtech bovine IFN-γ ELISA^PRO when used with QFT-Plus stimulated whole blood samples, is a sensitive and specific assay for differentiating *M. bovis* -infected from -unexposed buffaloes. By determining buffalo-specific diagnostic cut-off values for both the Mabtech and Cattletype® IGRAs, similar test performance between the two IGRAs was achieved, in addition to the Cattletype® showing significant improvement in Se. Moreover, the ability of the Mabtech IGRA to fully quantify IFN-γ may signify further potential of this assay for the investigation of disease states. This study provides evidence for the use of these two IFN-γ ELISAs together with the QFT-Plus stimulation platform for the enhanced detection of *M. bovis* infection in African buffaloes.

References

Andreasson, U., Perret-Liaudet, A., van Waalwijk van Doorn, L.J.C., Blennow, K., Chiasserini, D., Engelborghs, S., Fladby, T., Genc, S., Kruse, N., Kuiperij, H.B., Kulic, L., Lewczuk, P., Mollenhauer, B., Mroczko, B., Parnetti, L., Vanmechelen, E., Verbeek, M.M., Winblad, B., Zetterberg, H., Koel-Simmelink, M., Teunissen, C.E., 2015. A practical guide to immunoassay method validation. Front. Neurol. 6. https://doi.org/10.3389/fneur.2015.00179

Bernitz, N., Clarke, C., Roos, E.O., Goosen, W.J., Cooper, D., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018a. Detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) tubes and the Qiagen Cattletype® IFN-gamma ELISA. Vet. Immunol. Immunopathol. 196, 48–52. https://doi.org/10.1016/j.vetimm.2017.12.010

Bernitz, N., Goosen, W.J., Clarke, C., Kerr, T.J., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018b. Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 204, 40–43. https://doi.org/10.1016/j.vetimm.2018.09.004

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019a. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Bernitz, N., Kerr, T.J., Goosen, W.J., Higgitt, R.L., de Waal, C., Clarke, C., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019b. Impact of *Mycobacterium bovis*-induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 217, 109923. https://doi.org/10.1016/j.vetimm.2019.109923

Bernitz, N., Kerr, T.J., de Waal, C., Cooper, D. V., Warren, R.M., Van Helden, P.D., Parsons, S.D.C.,

Stellenbosch University https://scholar.sun.ac.za

Miller, M.A., 2020. Test characteristics of assays to detect *Mycobacterium bovis* infection in high-prevalence African buffalo (*Syncerus caffer)* herds. J. Wildl. Dis. https://doi.org/10.7589/2019-06-173

Brites, D., Loiseau, C., Menardo, F., Borrell, S., Boniotti, M.B., Warren, R., Dippenaar, A., Parsons, S.D.C., Beisel, C., Behr, M.A., Fyfe, J.A., Coscolla, M., Gagneux, S., 2018. A new phylogenetic framework for the animal-adapted *Mycobacterium tuberculosis* complex. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.02820

Brunton, L.A., Prosser, A., Pfeiffer, D.U., Downs, S.H., 2018. Exploring the fate of cattle herds with inconclusive reactors to the tuberculin skin test. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00228

Chegou, N.N., Black, G.F., Loxton, A.G., Stanley, K., Essone, P.N., Klein, M.R., Parida, S.K., Kaufmann, S.H.E., Doherty, T.M., Friggen, A.H., Franken, K.L., Ottenhoff, T.H., Walzl, G., 2012. Potential of novel *Mycobacterium tuberculosis* infection phase-dependent antigens in the diagnosis of TB disease in a high burden setting. BMC Infect. Dis. 12. https://doi.org/10.1186/1471-2334-12-10

Chileshe, J., Roos, E.O., Goosen, W.J., Buss, P., Hausler, G., Rossouw, L., Manemela, T., van Helden, P., Warren, R., Parsons, S.D., Miller, M., 2019. An interferon-gamma release assay for the diagnosis of the *Mycobacterium bovis* infection in white rhinoceros (*Ceratotherium simum*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2019.109931

Coad, M., Doyle, M., Steinbach, S., Gormley, E., Vordermeier, M., Jones, G., 2019. Simultaneous measurement of antigen-induced CXCL10 and IFN-γ enhances test sensitivity for bovine TB detection in cattle. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2019.01.007

Cox, K.L., Devanarayan, V., Kriauciunas, A., Manetta, J., Montrose, C., Sittampalam, S. Immunoassay methods. In: Sittampalam, G.S., Grossman, A., Brimacombe, K., et al., editors. 2012. Assay Guidance Manual [internet]. Available from: https://www.ncbi.nlm.nih.gov/books/NBK92434/

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. *Ante mortem* diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2005.11.005

Gaur, R.L., Pai, M., Banaei, N., 2013. Impact of blood volume, tube shaking, and incubation time on reproducibility of QuantiFERON-TB Gold in-tube assay. J. Clin. Microbiol. 51, 3521–3526. https://doi.org/10.1128/JCM.01627-13

Good, M., Bakker, D., Duignan, A., Collins, D.M., 2018. The history of *in vivo* tuberculin testing in bovines: Tuberculosis, a "One Health" issue. Front. Vet. Sci. https://doi.org/10.3389/fvets.2018.00059

Goosen, W.J., Cooper, D., Miller, M.A., van Helden, P.D., Parsons, S.D.C., 2015. IP-10 is a sensitive biomarker of antigen recognition in whole-blood stimulation assays used for the diagnosis of

Stellenbosch University https://scholar.sun.ac.za

Mycobacterium bovis infection in African buffaloes (*Syncerus caffer*). Clin. Vaccine Immunol. 22, 974–978. https://doi.org/10.1128/cvi.00324-15

Goosen, W.J., Parsons, S.D.C., Miller, M.A., van Helden, P.D., Warren, R.M., Cooper, D., 2014. The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2014.10.008

Higgitt, R.L., van Schalkwyk, O.L., deKlerk-Lorist, L.-M., Buss, P.E., Caldwell, P., Rossouw, L., Manamela, T., Hausler, G.A., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. An interferon gamma release assay for the detection of immune sensitization to *Mycobacterium bovis* in African wild dogs (*Lycaon pictus*) . J. Wildl. Dis. https://doi.org/10.7589/2018-03-089

Hlokwe, T.M., van Helden, P., Michel, A.L., 2014. Evidence of increasing intra and inter-species transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? Prev. Vet. Med. 115, 10–17. https://doi.org/10.1016/j.prevetmed.2014.03.011

Lalkhen, A.G., McCluskey, A., 2008. Clinical tests: Sensitivity and specificity. Contin. Educ. Anaesthesia, Crit. Care Pain 8, 221–223. https://doi.org/10.1093/bjaceaccp/mkn041

Michel, A.L., 2014. Improving specific disease outcomes through a One Health approach-tuberculosis. Rev. sci. tech. Off. int. Epiz. 33, 2, 583-592.

Michel, A.L., Cooper, D., Jooste, J., de Klerk, L.M., Jolles, A., 2011. Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). Prev. Vet. Med. 98, 142–151. https://doi.org/10.1016/j.prevetmed.2010.10.016

Moura, E.P., Toledo, V., Oliveira, M., Spíndola-De-Miranda, S., Andrade, H.M., Guimarães, T., 2004. Pulmonary tuberculosis: evaluation of interferon-g levels as an immunological healing marker based on the response to the Bacillus Calmette-Guerin. Mem. Inst. Oswaldo Cruz 99, 3. https://doi.org/10.1590/S0074-02762004000300008

OIE (World Organisation for Animal Health), 2018. Terrestrial Manual 2018. Chapter 2.2.7 – Principles and methods for the validation of diagnostic tests for infectious diseases applicable to wildlife. https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.07_WILDLIFE.pdf

Palmer, M. V., Thacker, T.C., Rabideau, M.M., Jones, G.J., Kanipe, C., Vordermeier, H.M., Waters, R., 2020. Biomarkers of cell-mediated immunity to bovine tuberculosis. Vet. Immunol. Immunopathol. 220. https://doi.org/10.1016/j.vetimm.2019.109988

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Renwick, A.R., White, P.C.L., Bengis, R.G., 2007. Bovine tuberculosis in southern African wildlife: A multi-species host-pathogen system. Epidemiol. Infect. https://doi.org/10.1017/S0950268806007205

Stellenbosch University https://scholar.sun.ac.za

Romha, G., Gebru, G., Asefa, A., Mamo, G., 2018. Epidemiology of *Mycobacterium bovis* and *Mycobacterium tuberculosis* in animals: Transmission dynamics and control challenges of zoonotic TB in Ethiopia. Prev. Vet. Med. https://doi.org/10.1016/j.prevetmed.2018.06.012

Sharma, B., Jain, R., 2014. Right choice of a method for determination of cut-off values: A statistical tool for a diagnostic test. Asian J. Med. Sci. 11, 2. https://doi.org/10.3126/ajms.v5i3.9296

Shrivastava, A., Gupta, V., 2011. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chronicles Young Sci. 2, 21. https://doi.org/10.4103/2229-5186.79345

Sichewo, P.R., Michel, A.L., Musoke, J., Etter, E.M.C., 2019. Risk factors for zoonotic tuberculosis at the wildlife–livestock–human interface in South Africa. Pathogens 8, 101. https://doi.org/10.3390/pathogens8030101

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. https://doi.org/10.1126/sciadv.aax4899

Valentin, M.A., Ma, S., Zhao, A., Legay, F., Avrameas, A., 2011. Validation of immunoassay for protein biomarkers: Bioanalytical study plan implementation to support pre-clinical and clinical studies. J. Pharm. Biomed. Anal. https://doi.org/10.1016/j.jpba.2011.03.033

van der Heijden, E.M.D.L., Jenkins, A.O., Cooper, D. V., Rutten, V.P.M.G., Michel, A.L., 2016. Field application of immunoassays for the detection of *Mycobacterium bovis* infection in the African buffalo (*Syncerus caffer*). Vet. Immunol. Immunopathol. 169, 68–73. https://doi.org/10.1016/j.vetimm.2015.12.003

van der Heijden, E.M.D.L., Cooper, D. V., Rutten, V.P.M.G., Michel, A.L., 2020. *Mycobacterium bovis* prevalence affects the performance of a commercial serological assay for bovine tuberculosis in African buffaloes. Comp. Immunol. Microbiol. Infect. Dis. 70. https://doi.org/10.1016/j.cimid.2019.101369

Warren, R.M., Gey Van Pittius, N.C., Barnard, M., Hesseling, A., Engelke, E., De Kock, M., Gutierrez, M.C., Chege, G.K., Victor, T.C., Hoal, E.G., Van Helden, P.D., 2006. Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. Int. J. Tuberc. Lung Dis. 10, 818–822.

Welsh, M.D., Cunningham, R.T., Corbett, D.M., Girvin, R.M., McNair, J., Skuce, R.A., Bryson, D.G., Pollock, J.M., 2005. Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. Immunology 114, 101–111. https://doi.org/10.1111/j.1365-2567.2004.02003.x

Youden, W.J., 1950. Index for rating diagnostic tests. J. Cancer 3, 32–35.

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Chapter 6: Use of the MILLIPLEX® bovine cytokine/chemokine multiplex assay to identify *Mycobacterium bovis*-infection biomarkers in African buffaloes (*Syncerus caffer*)

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Abstract

As a recognized *Mycobacterium bovis* maintenance host, the African buffalo (*Syncerus caffer*) poses transmission risks to livestock, humans and other wildlife. Early detection of *M. bovis* infection is critical for limiting its spread. Currently, tests detecting cell-mediated immune responses are used for diagnosis in buffaloes. However, these may have suboptimal sensitivity or specificity, depending on the blood stimulation method. Recent evidence suggests that assays using combinations of host cytokine biomarkers may increase diagnostic performance. Therefore, this study aimed to investigate the application of a MILLIPLEX® bovine cytokine/chemokine multiplex assay to identify candidate biomarkers of *M. bovis* infection in buffaloes. Whole blood from twelve culture-confirmed *M. bovis*-infected buffaloes, stimulated with the QuantiFERON® TB Gold Plus in-tube system, was tested using the MILLIPLEX® platform. Results indicated binding of bovine antibodies to fifteen buffalo cytokine/chemokine targets. Moreover, there was a significant difference in concentrations between unstimulated and TB antigen-stimulated buffalo samples for seven cytokines/chemokines included in the kit. Although these preliminary results require further investigation in larger sample sets and a comparison between *M. bovis*-infected and uninfected cohorts, the utility of the MILLIPLEX® platform in a novel species was demonstrated, in addition to identifying potential African buffalo cytokines for future research.

Introduction

Mycobacterium bovis is the primary causative agent of bovine tuberculosis (bTB) in bovids, in addition to possessing zoonotic potential (Rodriguez-Campos *et al.,* 2014; Zimpel *et al.,* 2020). Although cattle are considered the major host of *M. bovis,* several wildlife species are also recognized as reservoirs, including African buffaloes (*Syncerus caffer*) in South Africa (Pereira *et al.,* 2020).

The current most sensitive methods for early detection of *M. bovis* in cattle and buffaloes are based on the host's adaptive cell-mediated immune (CMI) response toward *M. bovis* antigens (Goosen *et al.,* 2015; Coad *et al.,* 2019). Examples include the *in vivo* single intradermal comparative tuberculin test (SICTT) and *in vitro* interferon-gamma (IFN-γ) release assays (IGRAs) (Bernitz *et al.,* 2018). The latter measure IFN-γ after stimulation of peripheral blood lymphocytes with *M. bovis* purified protein derivatives (PPDs) or mycobacteria-specific peptides early secretory antigenic target 6kDa (ESAT-6) and culture filtrate protein 10 kDa (CFP-10) (Goosen *et al.,* 2014). The QuantiFERON® TB Gold (QFT) and Gold Plus (QFT-Plus) platforms both use ESAT-6 and CFP-10, with the former QFT system containing an additional TB7.7 peptide (Hong *et al.,* 2019). The more recent QFT-Plus contains two TB-antigen tubes, one of which (TB2) has extra peptides for human application (Hong *et al.,* 2019). Hence, in accordance with recommendations for the off-label use from the manufacturer (Qiagen, Venlo, The Netherlands), only the TB2 tube is used for the detection of *M. bovis* infection in African buffaloes (Parsons *et al.,* 2011; Bernitz *et al.,* 2018; Bernitz *et al.,* 2020). Compared to an assay such as the Bovigam®, the QFT system has several advantages for field application including direct collection

of blood in closed, sterile antigen test tubes (reducing possible contamination), automated whole blood plasma separation following centrifugation that allows for easier plasma storage or immediate testing for multiple biomarkers, which contribute to the ease-of-use for untrained operators. However, use of this platform can lead to lower sensitivity (Se) than for PPD-based assays (Bernitz *et al.,* 2019a). Consequently, the utility of diagnostic assays that incorporate the QFT platform would be enhanced if Se could be improved while retaining specificity. One possibility is the detection of additional immunological biomarkers of *M. bovis* infection. Recently, the parallel measurement of IFN-γ and IFN γ-induced protein 10 (IP-10) has demonstrated improved detection of *M. bovis* infection in cattle and buffaloes while maintaining high specificity (Sp) (Goosen *et al*., 2015; Bernitz *et al.,* 2019a; Coad *et al.,* 2019).

In humans, the use of cytokine biomarker signatures has provided greater confidence in TB diagnostic results and shown increased ability to distinguish latent infection from active disease (Walzl *et al.,* 2018). A popular screening method for host signatures of TB in humans is the multiplex microbead-based Luminex® technology that enables the simultaneous assessment of multiple cytokines and/or chemokines (Chegou *et al.,* 2009; La Manna *et al.,* 2018). However, the application of a Luminex cytokine array to investigate *M. bovis* in wildlife appears limited to badgers (*Meles meles*) (Strain *et al.,* 2011). Therefore, the aim of this study was to evaluate the use of the 15-plex Luminex/MILLIPLEX® bovine cytokine/chemokine assay as a potential tool for the identification of novel biomarkers of *M. bovis* infection in African buffaloes.

Materials and methods

Animals and sample processing

During 2018 and 2019, lithium heparinized whole blood was collected from 559 chemically immobilized, ear-tagged African buffaloes as part of annual *M. bovis* test-and-slaughter programs in the *M. bovis*-endemic Hluhluwe-iMfolozi Park (KwaZulu-Natal, South Africa), as previously described (Parsons *et al*., 2011). Within 4-6 hours of collection, whole blood was transported out of direct sunlight at ambient temperature to the field laboratory and stimulated using the QFT-Plus assay (Qiagen, Venlo, The Netherlands) upon arrival, as previously described (Parsons *et al*., 2011; Bernitz *et al*., 2018). Briefly, 1 mL aliquots were transferred to each QFT tube (Nil, TB2 and Mitogen), inverted 10 times and incubated for 20 h at 37˚C. Thereafter, tubes were centrifuged for 10 min at 3000 x g and the plasma harvested and stored at 4°C. The IFN-γ and IP-10 release assays (IGRA; IPRA) were performed within two days of collection and the remaining plasma was stored at -20°C for future testing (Bernitz *et al*., 2019a). All test-positive and -suspect (SICTT, IPRA or IGRA) buffaloes were separated and culled within 2-3 weeks of testing. Mycobacterial culture was performed on tissues collected during *post mortem* examination, followed by speciation of cultured isolates by PCR to confirm *M. bovis* infection status (Warren *et al.,* 2006; Bernitz *et al.,* 2019b; Bernitz *et al.,* 2020). Briefly, a heat-inactivated (20 min, 95°C) aliquot of culture from the BACTEC™ MGIT™ 960 Mycobacterial Detection System

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(Becton Dickinson, Franklin Lakes, New Jersey, USA) was collected and DNA amplified using regions of difference (RD) primers (RD1, RD4, RD9 and RD12), as previously described (Warren *et al*., 2006). Amplification products were visualized by 2% agarose gel electrophoresis and compared to *M. bovis* and *M. tuberculosis* (H37RV) DNA positive controls.

MILLIPLEX® Bovine Cytokine/Chemokine Magnetic Bead Panel Multiplex Assay

A MILLIPLEX® Bovine Cytokine/Chemokine 15-plex kit (BCYT1-33K; EMD Millipore Corporation, Billerica, MA, USA) utilizing antibodies to bovine IFN-γ, interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, macrophage inflammatory protein (MIP)-1α, IL-36 receptor antagonist (RA), IP-10, monocyte chemoattractant protein (MCP)-1, MIP-1β, tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF)-A was used to screen stored buffalo plasma, harvested from whole blood previously stimulated using the QFT-Plus assay. Twelve *M. bovis*-infected buffaloes, confirmed by mycobacterial culture and with at least one lesion consistent with bTB at *post mortem*, were randomly selected from the *M. bovis*-endemic cohort described above. The selection of samples from buffaloes with confirmed disease was to maximize detection of cytokine differences between unstimulated and antigen-specific responses, in this pilot study to evaluate a range of responses to *M. bovis* infection. For each buffalo, plasma from the QFT-Plus nil, TB2 antigen- and mitogen-stimulated whole blood samples was used for the assay. The assay was performed according to the manufacturer's instructions. Briefly, plasma samples were diluted 1:2 in assay buffer before adding 25 μL of standards, quality controls and samples to the plate in duplicate. This was followed by addition of 25 μL magnetic beads. The plate was sealed, covered with foil and incubated overnight on a plate shaker at 4˚C. The plate was washed three times and 25 μL of detection antibody was added to each well. After incubating the plate at room temperature (RT) for 1 h, 25 μL streptavidin-phycoerythrin (PE) was added per well. The plate was sealed, covered and incubated for a further 30 min at RT. The plate underwent a final series of washes before 150 μL of drive fluid was added. Concentrations of markers were measured on the Luminex® MAGPIX® instrument (Luminex Corporation, Austin, TX, USA) and data was acquired and analyzed using the Bio-Plex® Manager™ software (Version 6.2; Bio-Rad, Hercules, CA, USA). . Quality control values for each marker were consistently within the range indicated by the manufacturer.

Data analyses

The data were exported to a Microsoft Excel (2002) worksheet (Microsoft Corporation, Redmond, WA, USA). For concentrations that were outside the quantifiable range, values were extrapolated by assigning the highest or lowest detectable concentration for that analyte, plus or minus 10%, respectively. Data analyses were conducted in GraphPad Prism Version 7.04 (GraphPad Software Inc., San Diego, CA, USA). Column statistics were performed per stimulation condition for the 15 biomarkers to calculate the median, 95% confidence interval and data distribution. Significant differences in protein concentrations between results from QFT-Plus nil and mitogen-stimulated samples, and nil and TB2 antigen-stimulated samples, were determined using a Friedman test followed

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by Dunn's multiple-comparison test for each cytokine and chemokine. For all analyses, a p-value ≤ 0.05 was considered statistically significant.

Results and Discussion

Overall, measurable concentrations (pg/mL) were observed for all fifteen cytokines and chemokines in QFT-Plus stimulated buffalo whole blood plasma measured using the MILLIPLEX® bovine cytokine/chemokine multiplex assay (Table 6.1). There were significant differences in concentrations between unstimulated and mitogen-stimulated samples for twelve of the cytokines/chemokines (Table 6.1). Of these, six cytokines/chemokines (IP-10, IL-6, IL-17A, MIP-1β, MIP-1α and IFN-γ) also showed significant concentration differences between TB2-antigen plasma and unstimulated samples, in addition to mitogen and unstimulated samples, from *M. bovis*-infected buffaloes; a seventh cytokine, MCP-1, displayed a significant TB-antigen response only (Table 6.1, Figure 6.1). These findings suggest that the MILLIPLEX® bovine cytokine/chemokine multiplex assay can detect African buffalo plasma cytokines/chemokines.

The six cytokines/chemokines that displayed both significant ESAT-6/CFP-10 antigen-specific and mitogen responses in *M. bovis*-infected buffaloes suggested their potential as diagnostic biomarkers. The IFN-γ and IP-10 results were anticipated considering the demonstrated utility of bovine IGRAs and IPRAs in African buffaloes (Goosen *et al.,* 2015; Bernitz *et al.,* 2018, 2019a; Palmer *et al.,* 2018).

Both IL-6 and IL-17A are pro-inflammatory cytokines. The former is known for its role in general mycobacterial infections, although research on both cytokines in bTB specifically is limited (Elnaggar *et al.,* 2017). Interestingly, Widdison *et al.* (2006) observed suppressed IL-6 expression when RNA in unstimulated samples was isolated from experimentally-infected cattle, compared to healthy animals, in *M. bovis* challenge studies. In contrast, human macrophages infected with *M. tuberculosis* and *M. smegmatis* displayed significantly increased IL-6 production (Singh and Goyal, 2013). Cattle studies have demonstrated the potential of IL-17A as a biomarker of bTB. Aranday-Cortes *et al.* (2012) showed the upregulation of IL-17A during bTB granuloma development and a study by Waters *et al.* (2016) reported at least 9-fold upregulation of IL-17A and other Th17-associated cytokine genes in response to *M. bovis* PPD stimulation in infected cattle. Moreover, Xin *et al.* (2018) observed significantly higher mRNA and subsequent protein concentrations of bovine antigen-stimulated IL-17A in naturally and experimentally *M. bovis*-infected cattle compared to controls. To further evaluate whether the concentrations of these two cytokines change in *M. bovis*-infected buffaloes, future studies should be considered using an uninfected cohort of animals to determine if IL-6 and IL-17A are potential biomarkers of bTB.

There are limited studies on the role of chemokines MIP-1α and MIP-1β as biomarkers of mycobacterial infection in cattle and humans. Widdison *et al.* (2009) reported a significant increase in MIP-1α gene expression in experimentally *M. bovis*-infected cattle as compared to uninfected animals.

A positive correlation between MIP-1α expression and bTB pathology within lymph nodes was also observed, however, further investigation into the earlier stages of infection is required (Widdison *et al.,* 2009). Saukkonen *et al.* (2002) also showed that MIP-1α and MIP-1β were the major chemokines produced in response to *M. tuberculosis* infection of human alveolar macrophages. Therefore, these chemokines may provide additional targets for further investigation of *M. bovis* infections in buffaloes.

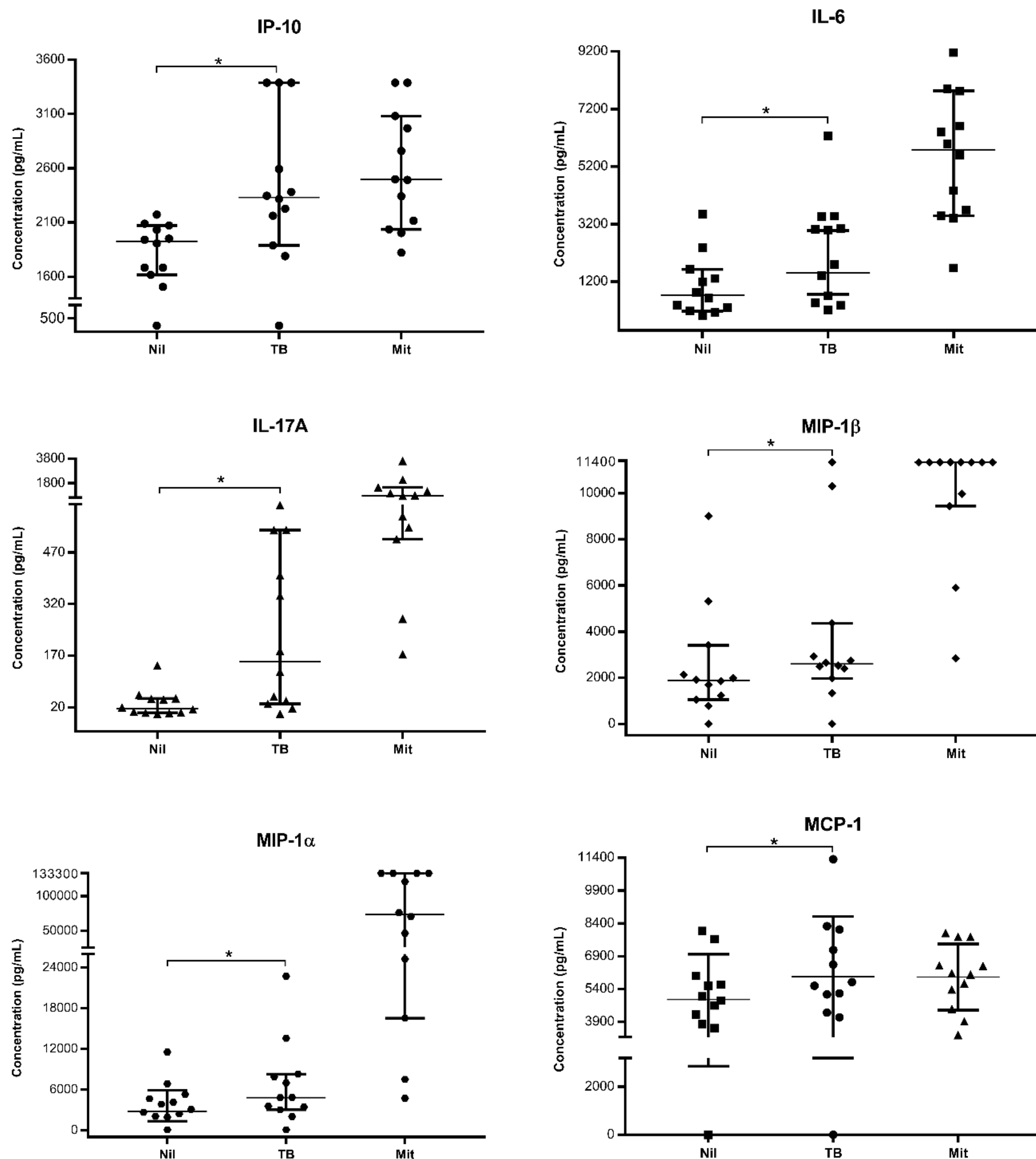

Figure 6.1. Concentrations (pg/ml) of six selected cytokines/chemokines with significant TB-specific differential responses in plasma from QuantiFERON® TB Gold Plus unstimulated (Nil), TB2-antigen stimulated (TB) and mitogen-stimulated (Mit) whole blood samples from *M. bovis* infected African buffaloes (n=12) determined by the MILLIPLEX® bovine cytokine/chemokine multiplex assay (*p < 0.05). Horizontal bars represent medians with 95% confidence intervals.

Although MCP-1 displayed a significant TB-specific response, the mitogen response was suboptimal and the median nil concentration was high, thus potentially decreasing its potential as a biomarker. These results are consistent with a study by Goosen *et al*. (2014) in which MCP-1 was detected at high levels in both nil and TB antigen-stimulated buffalo samples, with no significant differences between the two.

There are some limitations to this study. All twelve buffaloes in this study had gross pathological lesions consistent with bTB disease, therefore, only samples from *M. bovis*-diseased buffalo were screened with the MILLIPLEX® assay. Thus, it is challenging to explain the lack of significant mitogen responses observed for three of the biomarkers without including known uninfected control animals. For MCP-1, IL-4 and IL-36RA, the median mitogen response was elevated compared to the nil and TB-specific response. However, the relatively high unstimulated levels of MCP-1 and IL-36RA detected could either indicate circulating levels of this cytokine, or cross-reactivity or non-specific binding of buffalo plasma constituents to the assay antibodies. Alternatively, considering that most mitogen responses in this assay were significantly increased thus indicating successful stimulation of whole blood samples and detection of native buffalo cytokine proteins by bovine antibodies, these three biomarkers may be produced at lower levels or the PHA mitogen is not an optimal buffalo whole blood stimulation agent. Therefore, in order to confirm whether these cytokines/chemokines have potential as biomarkers of *M. bovis*, future studies will include uninfected and early infected buffaloes.

The two cytokines IL-4 and IL-10 did not show an antigen-specific response in this study, although they have shown promise as biomarkers of TB; this may be associated with their anti-inflammatory function. A study by Widdison *et al.* (2006) showed decreased IL-4 and IL-10 expression in *M. bovis*-infected cattle in comparison to healthy animals. Moreover, other studies (Rhodes *et al.,* 2000; Thacker *et al.,* 2007) have reported that IL-4 peaked early during infection and then returned to undetectable levels as infection progressed. Therefore, changes in antigen-specific IL-4 and IL-10 should be investigated in buffaloes that have early and chronic *M. bovis* infections and these results compared to uninfected animals. The limitations of this study highlight the necessity of understanding cytokine function in the context of *M. bovis* infection and how this compares to cytokine production in healthy animals; this can be investigated using the MILLIPLEX® platform.

The absence of a significant TB-specific whole blood response for the pro-inflammatory cytokine IL-8 in this bTB-diseased cohort is consistent with observations by Blanco *et al.* (2009), where lower IL-8 mRNA expression was observed associated with active disease compared to early infection in cattle. A high level of unstimulated IL-8 (median approx. 9000 pg/mL), also responsible for reducing the antigen-specific result in this study, has also been observed in a more recent study in cattle (Gao *et al.,* 2019). Moreover, overall levels of IL-8 (unstimulated and stimulated) were shown to be higher than IFN-γ, IP-10 and IL-17A, as seen in this study. The cohort for this study consisted of buffaloes with active disease only. Therefore, uninfected and early infected animals, would be required to determine if IL-8 can diagnose *M. bovis* infection and/or distinguish between bTB states in buffaloes.

Table 6.1. Median plasma cytokine/chemokine concentrations (pg/mL) measured in QuantiFERON® TB Gold Plus-stimulated whole blood plasma from known *M. bovis*-infected African buffaloes (n=12) using the MILLIPLEX® bovine cytokine/chemokine multiplex assay.

Cytokine/	**Median (25% - 75% percentiles) – pg/mL**				**Dunnet's multiple comparisons test (p-value)[b]**	
chemokine[a]	**Nil[c]**	**TB[c]**	**Mitogen[c]**	**TB minus Nil**	**Nil vs TB**	**Nil vs Mit**
IP-10	1923 (1633-2061)	2330 (1957-3189)	2495 (2056-3052)	500 (160-1157)	**0.005**	**0.004**
IL-6	725 (202-1544)	2395 (518-3352)	5783 (3539-7525)	1121 (173-2119)	**0.005**	**<0.0001**
IL-17A	16.6 (4.19-45.1)	153 (31.9-502)	771 (518-1370)	125 (17-445)	**0.013**	**0.005**
MIP-1β	1882 (1096-3095)	2595 (2078-4004)	11333 (9576-11333)	724 (443-1244)	**0.040**	**<0.0001**
MIP-1α	3470 (2149-5153)	4834 (3134-8201)	73586 (18710-133210)	1268 (356-3341)	**0.049**	**0.001**
MCP-1	4965 (3914-5902)	5629 (4535-7454)	6092 (4713-7454)	422 (24-2459)	**0.049**	0.195
TNF-α	1433 (523-9998)	2089 (974-10969)	5426 (2911-13936)	381 (58-815)	0.116	**0.004**
IFN-y	16.9 (7.92-32.2)	109 (45.9-249)	316 (101-939)	96 (43-206)	0.126	**0.022**
IL-8	3080 (2043-3456)	2817 (2599-4968)	4032 (3292-4807)	97 (075-488)	0.197	0.175
IL-1β	42.8 (16.6-219)	65.5 (34.8-380)	980 (720-3391)	16 (-10-63)	0.316	**0.014**
IL-1α	18.7 (5.77-89.2)	25.7 (7.81-92.6)	123 (59.6-311)	2 (-2-11)	0.378	**0.018**
IL-4	10.3 (10.3-10.3)	10.3 (10.3-10.3)	140 (25.9-445)	0 (0-0)	0.523	0.110
VEGF-A	186 (100-363)	255 (80.6-372)	118 (69.1-236)	1 (-5-23)	0.703	0.075
IL-10	117 (47.9-755)	114 (46.2-728)	737 (391-1513)	0.3 (02-11)	0.716	**0.004**
IL-36RA	190 (105-377)	191 (99.9-372)	267 (115-501)	0.3 (-17-34)	0.982	0.490

[a]cell-mediated immunological markers measured by the 15-plex assay; [b]post hoc test comparing TB- and Mitogen-stimulations to the Nil (control) group; [c]whole blood QFT-Plus stimulations including unstimulated (Nil), TB-specific antigen (ESAT-6/CFP-10)-stimulated (TB) and PHA-stimulated (Mitogen) conditions.

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The concentrations of IP-10 detected in this study were distinctly lower than obtained for the initial IPRA. Both the IPRA and IGRA previously reported for *M. bovis* detection in African buffaloes contain anti-bovine cytokine antibodies, similar to the MILLIPLEX® bovine assay. Hence, successful cross-reactivity with native buffalo cytokines was anticipated due to a high degree of homology (>95%) previously reported between cattle and African buffalo cytokine/chemokine mRNA and inferred amino acid sequences (Parsons *et al.,* 2011; Goosen *et al.,* 2014). However, the lower concentrations measured in this study may indicate suboptimal binding efficiency between this specific platform's antibodies and endogenous buffalo proteins, emphasizing the importance of antibody selection with regard to different species-specific targets, as previously reported (Goosen *et al.,* 2014; Parsons *et al.,* 2016).

In summary, the MILLIPLEX® assay demonstrated applicability as an immunological biomarker screening tool for cytokines and chemokines in African buffalo QFT-stimulated whole blood plasma. These findings support the further investigation of the multiplex assay in larger buffalo cohorts, notably with the inclusion of uninfected animals. Thereafter, any promising candidate biomarkers identified by this platform could be selected for individual ELISA optimizations, validation and test performance assessment as potential ancillary diagnostic cytokine release assays for the early detection of *M. bovis* infection in African buffaloes. The advantages of minimal sample volume (±25 µL/plasma sample) combined with time- and potential cost-savings, due to the testing of multiple CMI biomarkers in a single run, make the MILLIPLEX® an appealing screening platform for the development of diagnostic signatures to improve detection of *M. bovis* infection and accurate diagnosis of bTB in African buffaloes.

References

Aranday-Cortes, E., Hogarth, P.J., Kaveh, D.A., Whelan, A.O., Villarreal-Ramos, B., Lalvani, A., Vordermeier, H.M., 2012. Transcriptional profiling of disease-induced host responses in bovine tuberculosis and the identification of potential diagnostic biomarkers. PLoS One 7. https://doi.org/10.1371/journal.pone.0030626

Bernitz, N., Clarke, C., Roos, E.O., Goosen, W.J., Cooper, D., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. Detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*) using QuantiFERON®-TB Gold (QFT) tubes and the Qiagen Cattletype® IFN-gamma ELISA. Vet. Immunol. Immunopathol. 196, 48–52. https://doi.org/10.1016/j.vetimm.2017.12.010

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019a. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT) plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Bernitz, N., Kerr, T.J., Goosen, W.J., Higgitt, R.L., de Waal, C., Clarke, C., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019b. Impact of *Mycobacterium bovis*-

induced pathology on interpretation of QuantiFERON®-TB Gold assay results in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 217, 109923. https://doi.org/10.1016/j.vetimm.2019.109923

Bernitz, N., Kerr, T.J., de Waal, C., Cooper, D. V., Warren, R.M., Van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2020. Test characteristics of assays to detect *Mycobacterium bovis* infection in high-prevalence African buffalo (*Syncerus caffer*) herds . J. Wildl. Dis. https://doi.org/10.7589/2019-06-173

Blanco, F.C., Schierloh, P., Bianco, M.V., Caimi, K., Meikle, V., Alito, A.E., Cataldi, A.A., Sasiain, M.D.C., Bigi, F., 2009. Study of the immunological profile towards *Mycobacterium bovis* antigens in naturally infected cattle. Microbiol. Immunol. 53, 460–467. https://doi.org/10.1111/j.1348-0421.2009.00141.x

Chegou, N.N., Black, G.F., Kidd, M., van Helden, P.D., Walzl, G., 2009. Host markers in Quantiferon supernatants differentiate active TB from latent TB infection: Preliminary report. BMC Pulm. Med. 9. https://doi.org/10.1186/1471-2466-9-21

Coad, M., Doyle, M., Steinbach, S., Gormley, E., Vordermeier, M., Jones, G., 2019. Simultaneous measurement of antigen-induced CXCL10 and IFN-γ enhances test sensitivity for bovine TB detection in cattle. Vet. Microbiol. https://doi.org/10.1016/j.vetmic.2019.01.007

Elnaggar, M.M., Abdellrazeq, G.S., Elsisy, A., Mahmoud, A.H., Shyboub, A., Sester, M., Khaliel, S.A., Singh, M., Torky, H.A., Davis, W.C., 2017. Evaluation of antigen specific interleukin-1β as a biomarker to detect cattle infected with *Mycobacterium bovis*. Tuberculosis. https://doi.org/10.1016/j.tube.2017.04.009

Gao, X., Guo, X., Li, M., Jia, H., Lin, W., Fang, L., Jiang, Y., Zhu, H., Zhang, Z., Ding, J., Xin, T., 2019. Interleukin 8 and pentaxin (C-reactive protein) as potential new biomarkers of bovine tuberculosis. J. Clin. Microbiol. 57. https://doi.org/10.1128/JCM.00274-19

Goosen, W.J., Cooper, D., Miller, M.A., van Helden, P.D., Parsons, S.D.C., 2015. 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*). Clin. Vaccine Immunol. 22, 974–978. https://doi.org/10.1128/cvi.00324-15

Goosen, W.J., Parsons, S.D.C., Miller, M.A., van Helden, P.D., Warren, R.M., Cooper, D., 2014. The evaluation of candidate biomarkers of cell-mediated immunity for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2014.10.008

Hong, J. Y., Park, S. Y., Kim, A., Cho, S. N., & Hur, Y. G., 2019. Comparison of QFT-Plus and QFT-GIT tests for diagnosis of *M. tuberculosis* infection in immunocompetent Korean subjects. J Thorac Dis. 11(12), 5210–5217. https://doi.org/10.21037/jtd.2019.12.11

La Manna, M.P., Orlando, V., Li Donni, P., Sireci, G., Di Carlo, P., Cascio, A., Dieli, F., Caccamo, N., 2018. Identification of plasma biomarkers for discrimination between tuberculosis infection/disease

Stellenbosch University https://scholar.sun.ac.za

and pulmonary non tuberculosis disease. PLoS One 13. https://doi.org/10.1371/journal.pone.0192664

Palmer, M. V., 2018. Emerging understanding of tuberculosis and the granuloma by comparative analysis in humans, cattle, zebrafish, and nonhuman primates. Vet. Pathol. https://doi.org/10.1177/0300985817712795

Parsons, S.D.C., McGill, K., Doyle, M.B., Goosen, W.J., Van Helden, P.D., Gormley, E., 2016. Antigen-specific IP-10 release is a sensitive biomarker of *Mycobacterium bovis* infection in cattle. PLoS One. https://doi.org/10.1371/journal.pone.0155440

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Pereira, A.C., Reis, A.C., Ramos, B., Cunha, M. V., 2020. Animal tuberculosis: Impact of disease heterogeneity in transmission, diagnosis and control. Transbound. Emerg. Dis. https://doi.org/10.1111/tbed.13539

Rhodes, S.G., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2000. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology 99, 195-202.

Rodriguez-Campos, S., Smith, N.H., Boniotti, M.B., Aranaz, A., 2014. Overview and phylogeny of *Mycobacterium tuberculosis* complex organisms: Implications for diagnostics and legislation of bovine tuberculosis. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2014.02.009

Saukkonen, J.J., Bazydlo, B., Thomas, M., Strieter, R.M., Keane, J., Kornfeld, H., 2002. β-chemokines are induced by *Mycobacterium tuberculosis* and inhibit its growth. Infect. Immun. 70, 1684–1693. https://doi.org/10.1128/IAI.70.4.1684-1693.2002

Singh, P.P., Goyal, A., 2013. Interleukin-6: a potent biomarker of mycobacterial infection. Springerplus 2, 686. http://www.springerplus.com/content/2/1/686

Strain, S.A.J., Mcnair, J., Mcdowell, S.W.J., Branch, B., 2011. Bovine tuberculosis: a review of diagnostic tests for *M. bovis* infection in badgers. Bacteriology Branch, Veterinary Sciences Division, Agri-Food and Biosciences Inst. Available at: https://www.daera-ni.gov.uk/sites/default/files/publications/dard/afbi-literature-review-tb-review-diagnostic-tests-badgers.pdf

Thacker, T.C., Palmer, M. V., Waters, W.R., 2007. Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2007.05.009

Walzl, G., McNerney, R., du Plessis, N., Bates, M., McHugh, T.D., Chegou, N.N., Zumla, A., 2018. Tuberculosis: advances and challenges in development of new diagnostics and biomarkers. Lancet Infect. Dis. https://doi.org/10.1016/S1473-3099(18)30111-7

Warren, R.M., Gey Van Pittius, N.C., Barnard, M., Hesseling, A., Engelke, E., De Kock, M., Gutierrez, M.C., Chege, G.K., Victor, T.C., Hoal, E.G., Van Helden, P.D., 2006. Differentiation of

Stellenbosch University https://scholar.sun.ac.za

Mycobacterium tuberculosis complex by PCR amplification of genomic regions of difference. Int. J. Tuberc. Lung Dis. 10, 818–822.

Waters, W.R., Maggioli, M.F., Palmer, M. V., Thacker, T.C., McGill, J.L., Vordermeier, H.M., Berney-Meyer, L., Jacobs, W.R., Larsen, M.H., 2016. Interleukin-17A as a biomarker for bovine tuberculosis. Clin. Vaccine Immunol. 23, 168–180. https://doi.org/10.1128/CVI.00637-15

Widdison, S., Schreuder, L.J., Villarreal-Ramos, B., Howard, C.J., Watson, M., Coffey, T.J., 2006. Cytokine expression profiles of bovine lymph nodes: Effects of *Mycobacterium bovis* infection and bacille Calmette-Guérin vaccination. Clin. Exp. Immunol. 144, 281–289. https://doi.org/10.1111/j.1365-2249.2006.03053.x

Widdison, S., Watson, M., Coffey, T.J., 2009. Correlation between lymph node pathology and chemokine expression during bovine tuberculosis. Tuberculosis. https://doi.org/10.1016/j.tube.2009.09.003

Xin, T., Gao, X., Yang, H., Li, P., Liang, Q., Hou, S., Sui, X., Guo, X., Yuan, W., Zhu, H., Ding, J., Jia, H., 2018. Limitations of using IL-17A and IFN-γ-induced protein 10 to detect bovine tuberculosis. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00028

Zimpel, C.K., Salvatore Patané, J.L., Coelho Proença Guedes, A., de, R.F., Pereira-Silva, T.T., Soler Camargo, N.C., de Souza Filho, A.F., Soares Ferreira Neto, J., Carlos Setubal, J., Bryan Heinemann, M., Marcia de Sá, A., 2020. Global distribution and evolution of *Mycobacterium bovis* lineages. Front. Microbiol. https://doi.org/10.3389/fmicb.2020.00843

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Chapter 7: General Discussion

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

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This thesis describes the optimisation of current CMI diagnostic tests and the investigation and validation of new CMI response-based platforms for the detection of *M. bovis* in buffaloes. African buffaloes are a recognised reservoir host of *M. bovis* in SA, posing a transmission risk and hindering control efforts to eradicate bTB in livestock, wildlife and humans. The performance of CMI-associated methods for the detection of *M. bovis* infection in buffaloes, despite remaining the primary diagnostic tools, still requires improvement. Existing tests, including the *in vivo* SICTT and *in vitro* cytokine (IFN-γ) release assays, benefit significantly from species-specific validation and optimisation when relevant samples are available. In this project, buffalo-specific optimisation of the TST provided the first objective evidence for test interpretation guidelines and test performance, affirming the high Sp yet low Se of the TST and the requirement for adjunct tests. Current and new IGRAs were assessed and compared after calculation of buffalo-specific cut-off values and when these values were applied, the assays displayed improved test performance. However, to further improve detection, approaches that include the combined application of different tests, or novel assays such as the MILLIPLEX® multiplex assay (investigated in Chapter 6), should be evaluated to create diagnostic biosignatures.

The TST remains the approved standard for bTB screening in buffaloes in SA, yet has variable and suboptimal Se (Bernitz *et al.,* 2018, 2019). However, prior to this research, there have been no reports of scientifically validated species-specific evidence for cut-off values and test performance. The availability of appropriate reference cohorts of buffalo is an acknowledged challenge and may significantly influence the determination of test performance (Michel *et al.,* 2011). For example, cut-off values calculated in this study and previously (Parsons *et al.,* 2011) for an IGRA using the same company's platforms generated vastly different values when testing buffaloes. This can be explained by the earlier study's inclusion of a negative cohort originating from an *M. bovis*-endemic herd that, although culture-negative, were exposed and may have been infected due to the low Se of mycobacterial culture (Schiller *et al.,* 2010). In accordance with the OIE acknowledgement that appropriate policies for TST interpretation should be adopted depending on bTB prevalence and risk factors (i.e., presence of wildlife reservoirs), cattle studies have investigated interpretation criteria in different regions (Terrestrial Manual, 2018). In Ethiopia, a 2 mm cut-off increased Se while Sp remained identical, while a study in Cameroon revealed 3 mm and 3.5 mm to be the optimal cut-off values (Ameni *et al.,* 2008; Awah-Ndukum *et al.,* 2016). Although the OIE guideline is 4 mm, the recommendation for a more stringent interpretation, particularly for populations with higher risk or known *M. bovis* exposure, is included. For example, Great Britain recognises an alternative 'severe' TST interpretation of 2 mm, typically applied to herds in which bTB lesions or *M. bovis* are detected in a TST reactor (Goodchild *et al.,* 2015). This highlights the effect of disease prevalence on test peformance, and consequently the selection of an appropriate cut-off value, also observed recently in bTB-affected buffalo herds (van der Heijden *et al.,* 2020). Hence, in this project, buffalo-specific cut-off values were calculated and the optimal application of the TST was demonstrated using a negative reference cohort from different farms

with no history of *M. bovis*, from which TST data were collated for the first time in sufficient numbers. The positive reference cohort was chosen based on the gold standard criterium of mycobacterial culture. The comparative TST (SICTT), the version most commonly applied in buffaloes, demonstrated good test performance in distinguishing *M. bovis*-infected from uninfected buffaloes. The cut-off values returned by ROC curve analysis were similar or equal to the contested buffalo recommendations, confirming the applicability of the SA guidelines. TST performance was optimal when using these cut-off values with almost perfect Sp, however, a maximum Se of only 76%. This confirms the favourable Sp yet suboptimal Se of the TST, observed for both cattle and buffaloes (Nunez-Garcia *et al.,* 2018; Bernitz *et al.,* 2018, 2019). Considering the requirement for TST screening in all bTB testing scenarios for buffaloes, low Se is of particular concern for the several *M. bovis*-endemic herds and regions in SA for which highly sensitive diagnostics are a priority. It has been noted that, in bTB-endemic countries with high or unknown levels of environmental mycobacteria, the SITT (that uses bovine PPD stimulations only) can result in high false positive rates due to sensitisation with these mycobacteria (de la Rua-Domenech *et al.,* 2006; Good and Duignan, 2011). On the other hand, the SICTT version can cause high levels of false negatives by concealing PPD_b responses in animals sensitized with environmental mycbacteria, as observed in this study that used a cohort from one of the *M. bovis*-endemic national parks. There have been recent efforts toward better defining the stimulatory antigens used in the TST with the aim of increasing both Se and Sp (Srinivasan *et al.,* 2019). However, improving the performance of the TST remains a challenge worldwide. Hence, it is strongly recommended that at least one ancillary test be used together with the TST. However, especially with the use of higher cut-off values for which buffalo-specific evidence was provided in this project, the TST remains a viable option for herds with no known exposure to *M. bovis*, particularly to restrict false positives due to the high economic value of these buffaloes.

Considering the requirement for adjunct tests to the TST and the promising performance of IGRAs to date, the newly available Mabtech bovine IFN-γ ELISA^{PRO} was assessed as a standardised platform for *M. bovis* diagnosis in buffaloes. Moreover, species-specific cut-off values were calculated for the Mabtech IGRA and the commercial Cattletype® IGRA, previously demonstrated as promising in buffaloes (Bernitz *et al.,* 2018 and 2019). As mentioned, this project utilised a large negative reference cohort for the first time. Although the gold standard requirement remains mycobacterial culture, it is acknowledged that culture is an imperfect method hence a well-characterised alternative (such as the cohort used in this study) may be more suitable when determining test performance (de la Rua-Domenech *et al.,* 2006; Schiller *et al.,* 2010). The Mabtech IGRA demonstrated both high Se and Sp and considering test performance overall, was superior in comparison to the Cattletype® IGRA. The Se of the Cattletype® IGRA, however, was significantly improved when using the calculated buffalo cut-off in place of the manufacturer (bovine) cut-off value. This emphasizes the importance of calculating cut-off values and assessing test performance not only for the specific target species but also

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with the use of cohorts representative of the populations or regions for which the test is intended. However, sample availability is an unavoidable limitation in wildlife studies. Considering the results obtained for the Mabtech and Cattletype® IGRAs, pending the remaining test performance validation steps, these provide a promising adjunct option to the TST for *M. bovis* detection in buffaloes. Moreover, when considering optimal parallel application, an IGRA such as the two investigated in this project that combines the QFT-Plus stimulation platform with an IFN-γ ELISAs may be more applicable than the Bovigam® system with its similarities to the TST tuberculin antigens. Another advantage of the new Mabtech IGRA, compared to the Cattletype® IGRA and Bovigam® IGRA, is that it quantifies IFN-γ concentrations (as pg/mL). This could provide an opportunity for further investigation of temporal changes in this cytokine marker in buffalo plasma, considering an observed correlation to bTB disease severity in cattle and deer (Thacker *et al.,* 2006; Garcia-Jimenez *et al.,* 2012; Canal *et al.,* 2017; Gao *et al.,* 2019).

Finally, due to the recent availability of a bovine multiplex platform and evidence pointing to the improved detection of *M. bovis* infection when using combinations of more than one host biomarker, the MILLIPLEX® cytokine/chemokine assay was investigated for use in buffaloes. The binding of bovine antibodies to 15 buffalo cyokine/chemokine targets was demonstrated, in addition to significantly increased antigen-specific levels of 6 biomarkers in comparison to unstimulated plasma. Moreover, the detection of several biomarker targets with significant differences between mitogen-stimulated and unstimulated concentrations was achieved. Some of these biomarkers, including IL-4, IL-8, IL-10 and IL-17A, have shown promise (from bTB studies in cattle and cervids) for both *M. bovis* detection and the potential to distinguish early infection from active disease (Rhodes *et al.,* 2000; Widdison *et al.,* 2006; Thacker *et al.,* 2007; Blanco *et al.,* 2009; Waters *et al.,* 2016; Xin *et al.,* 2018). These results indicated the potential for a popular biomarker screening tool to be applied to buffaloes. This can aid the identification of cytokine/chemokine targets for individual optimisation if cross-reactive antibodies are available, which may be a more cost-effective follow-up than repeated multiplex testing. Alternatively, considering more recent options for customization of these assays, cytokine and chemokine biomarkers that have indicated promise in cattle or other animals studies could be incorporated in an adapted MILLIPLEX® assay for buffalo screening. Ideally, both *M. bovis*-infected and uninfected buffalo sample cohorts should be included in the assay to efficiently identify biomarkers of infection and/or disease. However, if infeasible, the simultaneous screening of multiple candidate biomarkers in a single sample of small volume remains a major advantage, particularly for wildlife due to the difficulties in obtaining good reference samples.

There are some important factors to consider, alongside test performance parameters, for the application of current and candidate diagnostic tests, namely: i) the guidelines for diagnostic test recognition (provisional or full i.e., OIE recognised); ii) the costs incurred by any ancillary test to the SICTT, due to its mandatory inclusion in SA; and iii) in-field variability with regard to testing objectives

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(i.e., requirement for either high Se or Sp), *M. bovis* or bTB prevalence, and the setting (i.e., private farms with high-value buffaloes and the economic means to support optimal diagnostics versus national parks with a lack of financial resources and less valuable buffalo herds yet a requirement for conservation of all wildlife species therein). The results obtained in the project emphasize and allude to these critical considerations for future *M. bovis* detection and bTB diagnosis in African buffaloes.

References

Ameni, G., Hewinson, G., Aseffa, A., Young, D., Vordermeier, M., 2008. Appraisal of interpretation criteria for the comparative intradermal tuberculin test for diagnosis of tuberculosis in cattle in central Ethiopia. Clin. Vaccine Immunol. 15, 1272–1276. https://doi.org/10.1128/CVI.00114-08

Awah-Ndukum, J., Temwa, J., Ngwa, V.N., Mouiche, M.M., Iyawa, D., Zoli, P.A., 2016. Interpretation criteria for comparative intradermal tuberculin test for diagnosis of bovine tuberculosis in cattle in Maroua area of Cameroon. Vet. Med. Int. 2016. https://doi.org/10.1155/2016/4834851

Bernitz, N., Goosen, W.J., Clarke, C., Kerr, T.J., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2018. Parallel testing increases detection of *Mycobacterium bovis*-infected African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. 204, 40–43. https://doi.org/10.1016/j.vetimm.2018.09.004

Bernitz, N., Kerr, T.J., Goosen, W.J., Clarke, C., Higgitt, R., Roos, E.O., Cooper, D. V., Warren, R.M., van Helden, P.D., Parsons, S.D.C., Miller, M.A., 2019. Parallel measurement of IFN-γ and IP-10 in QuantiFERON®-TB Gold (QFT)plasma improves the detection of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Prev. Vet. Med. 169. https://doi.org/10.1016/j.prevetmed.2019.104700

Blanco, F.C., Schierloh, P., Bianco, M.V., Caimi, K., Meikle, V., Alito, A.E., Cataldi, A.A., Sasiain, M.D.C., Bigi, F., 2009. Study of the immunological profile towards *Mycobacterium bovis* antigens in naturally infected cattle. Microbiol. Immunol. 53, 460–467. https://doi.org/10.1111/j.1348-0421.2009.00141.x

Canal, A.M., Pezzone, N., Cataldi, A., Zumarraga, M., Larzabal, M., Garbaccio, S., Fernandez, A., Dominguez, L., Aranaz, A., Rodriguez-Bertos, A., 2017. Immunohistochemical detection of pro-inflammatory and anti-inflammatory cytokines in granulomas in cattle with natural *Mycobacterium bovis* infection. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2016.10.006

de la Rua-Domenech, R., Goodchild, A.T., Vordermeier, H.M., Hewinson, R.G., Christiansen, K.H., Clifton-Hadley, R.S., 2006. *Ante mortem* diagnosis of tuberculosis in cattle: A review of the tuberculin tests, γ-interferon assay and other ancillary diagnostic techniques. Res. Vet. Sci. https://doi.org/10.1016/j.rvsc.2005.11.005

Gao, X., Guo, X., Li, M., Jia, H., Lin, W., Fang, L., Jiang, Y., Zhu, H., Zhang, Z., Ding, J., Xin, T., 2019. Interleukin 8 and pentaxin (C-reactive protein) as potential new biomarkers of bovine tuberculosis. J. Clin. Microbiol. 57. https://doi.org/10.1128/JCM.00274-19

García-Jiménez, W.L., Fernández-Llario, P., Gómez, L., Benítez-Medina, J.M., García-Sánchez, A., Martínez, R., Risco, D., Gough, J., Ortiz-Peláez, A., Smith, N.H., Mendoza, J.H. de, Salguero, F.J., 2012. Histological and immunohistochemical characterisation of *Mycobacterium bovis* induced granulomas in naturally infected Fallow deer (*Dama dama*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2012.06.010

Goodchild, A. V., Downs, S.H., Upton, P., Wood, J.L.N., De La Rua-Domenech, R., 2015. Specificity of the comparative skin test for bovine tuberculosis in Great Britain. Vet. Rec. 177, 258. https://doi.org/10.1136/vr.102961

Good, M., Duignan, A., 2011. Perspectives on the history of bovine TB and the role of tuberculin in bovine TB eradication. Vet. Med. Int. 2011. https://doi.org/10.4061/2011/410470

Michel, A.L., Cooper, D., Jooste, J., de Klerk, L.M., Jolles, A., 2011. Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). Prev. Vet. Med. 98, 142–151. https://doi.org/10.1016/j.prevetmed.2010.10.016

Nuñez-Garcia, J., Downs, S.H., Parry, J.E., Abernethy, D.A., Broughan, J.M., Cameron, A.R., Cook, A.J., de la Rua-Domenech, R., Goodchild, A. V., Gunn, J., More, S.J., Rhodes, S., Rolfe, S., Sharp, M., Upton, P.A., Vordermeier, H.M., Watson, E., Welsh, M., Whelan, A.O., Woolliams, J.A., Clifton-Hadley, R.S., Greiner, M., 2018. Meta-analyses of the sensitivity and specificity of ante-mortem and post-mortem diagnostic tests for bovine tuberculosis in the UK and Ireland. Prev. Vet. Med. 153, 94–107. https://doi.org/10.1016/j.prevetmed.2017.02.017

OIE (World Organisation of Animal Health)., 2018 (8th Edition). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Terrestrial Manual). Available from: https://www.oie.int/ standard-setting/terrestrial-manual/access-online/

Parsons, S.D.C., Cooper, D., McCall, A.J., McCall, W.A., Streicher, E.M., le Maitre, N.C., Müller, A., Gey van Pittius, N.C., Warren, R.M., van Helden, P.D., 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2011.04.006

Rhodes, S.G., Buddle, B.M., Hewinson, R.G., Vordermeier, H.M., 2000. Bovine tuberculosis: immune responses in the peripheral blood and at the site of active disease. Immunology 99, 195-202.

Schiller, I., Oesch, B., Vordermeier, H.M., Palmer, M. V., Harris, B.N., Orloski, K.A., Buddle, B.M., Thacker, T.C., Lyashchenko, K.P., Waters, W.R., 2010. Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. Transbound. Emerg. Dis. https://doi.org/10.1111/j.1865-1682.2010.01148.x

Srinivasan, S., Jones, G., Veerasami, M., Steinbach, S., Holder, T., Zewude, A., Fromsa, A., Ameni, G., Easterling, L., Bakker, D., Juleff, N., Gifford, G., Hewinson, R.G., Vordermeier, H.M., Kapur, V., 2019. A defined antigen skin test for the diagnosis of bovine tuberculosis. Sci. Adv. 5, eaax4899. https://doi.org/10.1126/sciadv.aax4899

Stellenbosch University https://scholar.sun.ac.za

Thacker, T.C., Palmer, M. V., Waters, W.R., 2007. Associations between cytokine gene expression and pathology in *Mycobacterium bovis* infected cattle. Vet. Immunol. Immunopathol. https://doi.org/10.1016/j.vetimm.2007.05.009

Thacker, T.C., Palmer, M. V., Waters, W.R., 2006. Correlation of cytokine gene expression with pathology in white-tailed deer (*Odocoileus virginianus*) infected with *Mycobacterium bovis*. Clin. Vaccine Immunol. 13, 640–647. https://doi.org/10.1128/CVI.00024-06

van der Heijden, E.M.D.L., Cooper, D. V., Rutten, V.P.M.G., Michel, A.L., 2020. *Mycobacterium bovis* prevalence affects the performance of a commercial serological assay for bovine tuberculosis in African buffaloes. Comp. Immunol. Microbiol. Infect. Dis. 70. https://doi.org/10.1016/j.cimid.2019.101369

Waters, W.R., Maggioli, M.F., Palmer, M. V., Thacker, T.C., McGill, J.L., Vordermeier, H.M., Berney-Meyer, L., Jacobs, W.R., Larsen, M.H., 2016. Interleukin-17A as a biomarker for bovine tuberculosis. Clin. Vaccine Immunol. 23, 168–180. https://doi.org/10.1128/CVI.00637-15

Widdison, S., Schreuder, L.J., Villarreal-Ramos, B., Howard, C.J., Watson, M., Coffey, T.J., 2006. Cytokine expression profiles of bovine lymph nodes: Effects of *Mycobacterium bovis* infection and Bacille Calmette-Guérin vaccination. Clin. Exp. Immunol. 144, 281–289. https://doi.org/10.1111/j.1365-2249.2006.03053.x

Xin, T., Gao, X., Yang, H., Li, P., Liang, Q., Hou, S., Sui, X., Guo, X., Yuan, W., Zhu, H., Ding, J., Jia, H., 2018. Limitations of using IL-17A and IFN-γ-induced protein 10 to detect bovine tuberculosis. Front. Vet. Sci. 5. https://doi.org/10.3389/fvets.2018.00028

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Chapter 8: Conclusion

The overarching aim to optimise current CMI-based methods and investigate new platforms for enhanced *M. bovis* detection in buffaloes was achieved in this project through a number of objectives. The TST will most likely remain the primary approved bTB screening standard for buffaloes in SA in the near future. Thus, the calculation of species-specific cut-off values and demonstration of test performance thereof, for the first time, can aid and inform optimal, evidence-based application of the TST in varied testing scenarios or regions in SA. Moreover, throughout this project, the OIE validation requirements for provisional recognition were used for guidance in test evaluation. This should be an important consideration for diagnostic research, not only for tests such as the TST that are already approved (albeit require further species-specific optimisation), so that tangible, applicable (i.e., in-field) goals may be achieved. Another obstacle to improving wildlife diagnostic tools is cost. Due to the suboptimal Se of the TST, despite the use of buffalo-specific thresholds, an ancillary test is recommended. However, the mandatory TST already incurs high expenses due to the requirement for capturing buffaloes, retaining them for three days and immobilizing the animals twice. Hence, the addition of an adjunct test and the extra expense must be carefully considered in relation to the testing objective, logistics of sample processing and access to laboratories, and the herd or regional *M. bovis* infection or bTB prevalence, if known. In addition, *M. bovis* prevalence, management of buffaloes, and testing goals will influence the selection of test cut-off value and performance.

The commercial, standardized Mabtech bovine IFN-γ ELISA^{PRO}, used with the QFT-Plus assay and validated in this project, demonstrated both high Se and Sp for the detection of *M. bovis*-infected buffaloes. Moreover, test performance appeared superior to the established QFT/Cattletype® IGRA and the results of Mabtech IGRA are expressed as concentrations of IFN-γ, which provides quantitative results that can be compared in serial testing. However, the Se of the Cattletype® IGRA was significantly improved after a buffalo-specific cut-off value was calculated and applied. The results from the TST and IGRA research emphasize two points, namely i) the importance of using well-characterized, applicable reference cohorts, and ii) the relevance of species-specific validation and optimisation for diagnostic tests. The differences in test performance between hosts (even those between closely-related species such as cattle and buffaloes) was demonstrated by the calculation of buffalo cut-off values that varied from those calculated for cattle for the same assay. These aspects will always require attention for the best possible application of diagnostic tests in wildlife.

The final objective was achieved by demonstrating the successful application of a bovine multiplex platform, the MILLIPLEX® cytokine/chemokine assay, to buffalo. The assay included 15 biomarker targets, several of which have shown promise in cattle studies. Considering the popularity and

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advantages of current and emerging multiplex screening tools, the simultaneous identification of biomarker targets in African buffaloes is a promising approach for future research on *M. bovis* infection and bTB disease in this species.

Future research

Although the cohorts used in this study were relevant and generally unbiased, an ideal selection for further investigation of diagnostic assays intended for field use, including the TST and IGRAs, would include buffalo herds with varying, yet known, disease prevalence. This would more optimally represent the variation in bTB prevalence across different buffalo herds in SA, thus broadening the applicability of these diagnostic tests even further. The use of culture-positive buffaloes from an *M. bovis*-endemic herd for the positive reference cohort, despite this being the gold standard requirement, could result in an overestimation of Se due to some unavoidable selection bias. Therefore, a herd with high prevalence as the only selection criterium would be more ideal than the selection of culture-positive animals only, as the latter can result in the exclusion of subclinical or early-infected individuals due to the low Se of mycobacterial culture. The buffalo-specific cut-off values calculated in this project for the TST, Mabtech IGRA and Cattletype® IGRA require further evaluation in an independent and applicable test group of buffaloes. This will provide the necessary performance parameters, including negative and positive predictive values, required for full (OIE) diagnostic test recognition. Lastly, the promising and novel CMI response-based biomarkers detected preliminarily in buffaloes with the MILLIPLEX® platform warrant further research, alone and in combinations, for the enhancement of *M. bovis* detection and potential differentiation between bTB disease states in this species. Additionally, candidate biomarkers should be investigated in alternative samples types, i.e., serum, due to the practical, in-field and diagnostic potential of circulating responses. Considering recent advances in understanding individual cytokine and chemokine function in host responses to *M. bovis,* and the increasing development of CMI-related tests and reagents, the validation of biosignatures to better diagnose or differentiate infection and disease provides an important avenue for future diagnostic research in African buffaloes.