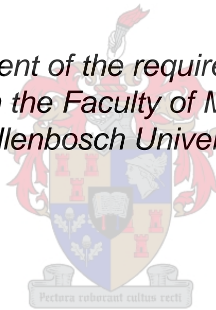
Investigation of temporal changes in immune responses to *Mycobacterium bovis* in cattle and African buffaloes (*Syncerus caffer).*

by
Charlene Clarke



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Abstract

Cattle and African buffaloes are major maintenance hosts of *Mycobacterium bovis* in South Africa and therefore serve as a potential source of infection for other animals and humans. *Mycobacterium bovis* is the causative agent of bovine tuberculosis (BTB). Identification and removal of *M. bovis*-infected animals from the herd at early stages of infection is important for effective disease management and relies on the strategic application of ante-mortem diagnostic tests. Ante-mortem diagnostic tests rely on the detection of specific immunological responses in the animal. However, these responses vary over the course of infection and therefore the accuracy of the diagnostic test may change, depending on the stage of infection. We therefore aimed to investigate the temporal changes in antigen-specific cytokine production and humoral responses to BTB in buffaloes. Interferon-gamma (IFN-ɣ) and IFN-ɣ-induced protein 10 (IP-10) production in response to mycobacterial antigens ESAT-6/CFP-10, and serum antibodies to bovine purified protein derivative (bPPD) were measured in a group of chronically test-positive, newly converted test-positive and test-negative buffaloes over one year. No consistent trends in immune responses over time were observed in any of the groups.

Cytokine release assays (CRAs) are often used in conjunction with the tuberculin skin test (TST) to improve diagnostic performance. However, many studies have shown that the TST may influence IFN-ɣ production and thereby the outcome of immunodiagnostic tests in cattle. However, these results were conflicting. A better understanding of the influence of the TST on cytokine production and the duration of this effect in a South African situation is required. We therefore aimed to investigate if the TST has an influence on IFN-ɣ and IP-10 production and test outcome in cattle and African buffaloes. IFN-ɣ and IP-10 release were measured in response to bPPD, avian PPD, ESAT-6 and CFP-10 in groups of TST-positive cattle 6, 7, 21, 41 and 78 days post-TST and in a group of TST-negative cattle 7, 21 and 78 days post-TST. IFN-ɣ and IP-10 levels in response to PPD were elevated 1-3 weeks post-TST, followed by a decrease by 41 days, suggesting immune boosting by the TST. ESAT-6/CFP-10-specific cytokine release showed conflicting results, with a group of animals showing decreased cytokine production by 41 days, whereas another group showed no change in cytokine release over time. Our findings suggest that cattle should not be

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tested with CRAs between 6 and 41 days post-TST to avoid boosting of cytokine levels and inaccurate test results.

IFN-ɣ and IP-10 release in response to PC-EC®, PC-HP®, bPPD and avian PPD were measured in a group of Bovigam®-positive and negative buffaloes at the time the TST was performed and three days later. In Bovigam®-positive buffaloes a significant decrease in cytokine production and in the proportion of test positive animals were observed three days post-TST in response to all antigens, except aPPD. Bovigam®-negative animals were not influenced by the TST. It is therefore recommended that buffaloes should be sampled pre-TST to identify all possible *M. bovis*-positive animals in this herd of high BTB incidence.

Accuracy of diagnostic assays may be affected by many factors, including the immunological stage of *M. bovis*-infection, time interval between the performance of the TST and CRA, exposure to environmental mycobacteria and BTB incidence in the herd. The correct application and interpretation of diagnostic assays of high specificity and sensitivity is required to overcome some of these factors. Therefore, information of the BTB status of the herd, exposure to environmental mycobacteria, and an understanding of the aim of the disease management plan is required.

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Opsomming

Beeste en Afrika buffels is belangrike onderhouds gaste van *Mycobacterium bovis* in Suid Afrika en dien daarom as 'n bron van infeksie vir ander diere. Hierdie patogeen is die oorsaak van bees tuberkulose (BTB) en indentifisering en verwydering van *M. bovis*-geïnfekteerde diere uit die kudde in 'n vroeë stadium van infeksie is belangrik vir effektiewe siekte beheer. Dit is afhanklik van die strategiese aanwending van ante mortem diagnostiese toetse. Daarom, vir die verbetering van toets interpretasie, was ons doel om die temporale veranderinge in antigeen-spesifieke sitokien produksie en humorale reaksies tot BTB in buffels te ondersoek. Interferon-gamma (IFN-ɣ) en IFN-ɣ-geïnduseerde proteïen 10 (IP-10) produksie in reaksie tot mikobakteriële antigene ESAT-6/CFP-10, en teenliggaam vlakke was bepaal in 'n groep chronies Bovigam®-positiewe (n = 5), pas omgeskakelde Bovigam®-positiewe (n = 5) en Bovigam®-negatiewe buffels (n = 8) oor 'n 1 jaar typerk. Daar was geen spesifieke patrone in immuun reaksies oor tyd in enige van die groepe nie, alhoewel die chronies Bovigam®-positiewe diere ESAT-6/CFP-10 positief gebly het. 'n Een jaar studie tydperk was nie genoeg om veranderinge in sitokien en teenliggaam vlakke in natuurlik-geïnfekteerde buffels te evalueer nie.

IFN-ɣ vrystellings toetse (IGRAs) word algemeen saam met die tuberkulien vel toets (TST) gebruik om die diagnostiese prestasie te verbeter. Baie studies het egter bevind dat die TST 'n effek op *in vitro* IFN-ɣ produksie in beeste het. Daarom was die doel van die studie om die effek van die TST op sitokien produksie en toets sensitiwiteit, spesifisiteit en resultaat in beeste en buffels te bepaal. Vrystelling van IFN-ɣ en IP-10 was bepaal in reaksie tot PPDs, ESAT-6 en CFP-10 in 'n groep TST-positiewe beeste 6 en 41 dae na die TST (groep 1). Soortgelyk, was sitokien vrystelling in TST-positiewe en negatiewe beeste by 7, 21 en 78 dae na die TST (groep 2) bepaal. IFN-ɣ and IP-10 vlakke in reaksie tot PPD was verhoog 1-3 weke na die TST, gevolg deur 'n afname teen 41 dae, wat dui op 'n immunologiese "boosting" deur die TST. Sitokien produksie in respons tot ESAT-6/CFP-10 in group 1 het ook betekenisvol afgeneem. Sitokien "boosting" mag egter verband hou met blootstelling aan omgewings mikobakterieë in hierdie kudde met 'n lae voorkoms van BTB. Ons resultate ondersteun die gebruik van toetse wat ESAT-6/CFP-10 gebruik om beeste rondom 41 dae na die TST te toets en so die akkuraatheid van die toets resultate te verbeter.

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IFN-ɣ en IP-10 vrystelling in respons tot PC-EC, PC-HP, bovine PPD en avian PPD was bepaal in ‘n groep Bovigam®-positiewe en negatiewe buffels op dieselfde tyd van die uitvoer van die TST en 3 dae later. Sitokien produksie en die proporsie toets-positiewe diere het betekenisvol afgeneem 3 dae na die TST in respons tot al die antigene, behalwe avian PPD, in Bovigam®-positiewe buffels. Dit word daarom aanbeveel dat bloedmonsters geneem moet word wanneer die TST uitgevoer word om al die TST-positiewe buffels te identifiseer.

Akkuraatheid van diagnostiese toetse kan deur vele faktore geaffekteer word, insluitend die immunologiese stadium van *M. bovis*-infeksie, tyd interval tussen die TST en sitokien toetse, blootstelling aan omgewings mikobakterieë, en die BTB voorkoms in die kudde. Die korrekte aanwending en interpreatsie van diagnostiese toetse met hoë sensitiwiteit en spesifisiteit word benodig om sommige van die faktore te oorkom. Inligting van the BTB voorkoms in die kudde, en die blootstelling aan omgewings mikobakterieë asook ‘n begrip van die doel van die spesifieke siekte beheer plan is daarom belangrik.

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Abbreviations

˚C	Degrees Celsius
µg	Microgram
µl	Microliter
aPPD	Avian Purified Protein Derivative
bp	Base pair
BB-BSA	Blocking buffer containing bovine serum albumin
BB-FFM	Blocking buffer containing fat free milk
BCG	Bacille Calmette Guerin
bPPD	Bovine Purified Protein Derivative
BSA	Bovine serum albumin
BTB	Bovine tuberculosis
CFP-10	Culture filtrate protein 10
CFT	Caudal fold test
CMI	Cell Mediated Immune
CI	Confidence interval
CRA	Cytokine release assay
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target 6
HiP	Hluhluwe–iMfolozi Park
HRP	Horseradish peroxidase
H_2SO_4	Sulphuric acid
IFN-ɣ	Interferon gamma
IgG	Immunoglobulin G
IGRA	Interferon gamma release assay
IL	Interleukin
IQR	Interquartile range
IP-10	Interferon gamma-induced protein 10
MGIT	Mycobacteria Growth Indicator Tube
ml	Millilitre
OD	Optical Density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
pH	Potential of Hydrogen
PPD	Purified Protein Derivative

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PWM	Pokeweed Mitogen
QFT	QuantiFERON TB Gold In-Tube
RD	Region of difference
RT	Room temperature
SFT	Skin fold thickness
SICCT	Single intradermal comparative cervical skin test
SICT	Single intradermal cervical skin test
SD	Standard deviation
TB	Tuberculosis
TMB	Tetramethylbenzidine
Th	T helper
Th1	T helper type 1
Th2	T helper type 2
TST	Tuberculin skin test
WB	Wash buffer
WHO	World Health Organisation

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Chapter 1: Introduction

Mycobacterium bovis is the causative agent of bovine tuberculosis (BTB), a chronic infectious disease that affects livestock and wildlife, and its zoonotic potential presents a risk to public health (Buddle et al., 2009; Renwick et al., 2007). *M. bovis* is a member of the *Mycobacterium tuberculosis* complex, a group of genetically related mycobacteria that cause tuberculosis (TB) in a wide range of hosts (Huard et al., 2006). In South Africa, *M. bovis* was first identified as early as 1880 in cattle, whereas the first case of BTB in wildlife was diagnosed in 1928 (Renwick et al., 2007). Maintenance hosts of *M. bovis,* such as cattle and African buffaloes, in an area is of particular concern for BTB management programs, since these animals allow persistence of infection and serve as a source of infection to many other animals, including lions, warthogs, bushbuck and kudus (Palmer et al., 2012; Renwick et al., 2007). These animals may be infected with *M. bovis* long before showing signs of disease (De Vos et al., 2001). Therefore, early diagnosis, accurate detection and removal of *M. bovis*-infected animals are important to prevent disease transmission and to support adequate disease management practices (Buddle et al., 2009; de la Rua-Domenech et al., 2006). Accurate diagnostic assays are thus essential. Ante-mortem diagnostic assays typically rely on detection of specific immunological responses, such as *in vitro* antigen-specific cytokine release or delayed-type hypersensitivity (DTH) responses for the tuberculin skin test (TST) (Welsh et al., 2005). However, immunological responses in cattle infected with *M. bovis* have been reported to change over time, which may affect the accuracy of diagnostic tests, depending on the stage of infection or disease progression (Rhodes et al., 2000a, 2000b, 2000c; Welsh et al., 2005). For this reason a greater understanding of the changes in immunological responses over the course of infection is required to interpret test results. However, this has not been thoroughly investigated in wildlife, specifically in African buffaloes. Therefore, we aimed to investigate the changes in antigen-specific cytokine production and humoral responses to BTB in *M. bovis*-infected African buffaloes over time and determine the temporal relationships of different biomarkers of BTB. This will provide information on how diagnostic tests and their interpretation may be influenced by disease progression in buffaloes. More accurate test interpretation will improve the outcome of disease management programs.

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Interferon-gamma release assays (IGRAs) are often used in conjunction with TSTs for the diagnosis of *M. bovis* infection in animals (de la Rua-Domenech et al., 2006). However, many studies have shown that interferon gamma (IFN-ɣ) production is affected by the purified protein derivative (PPD) injection used for performing TSTs in cattle. Literature has shown conflicting observations regarding IFN-ɣ release following the TST (de la Rua-Domenech et al., 2006; Gormley et al., 2006; Palmer et al., 2006; Ryan et al., 2000). In some studies, the TST has been shown to boost IFN-ɣ production to aid in identification of *M. bovis*-infected animals, especially those with low IFN-ɣ responses in early stages of disease (Palmer et al., 2006). The effect of the TST on IFN-ɣ-induced protein 10 (IP-10) production, a sensitive diagnostic biomarker with promising results in buffaloes (Goosen et al., 2015) and cattle (Parsons et al., 2016), has not been examined. Therefore, we aimed to investigate the effect of the TST on IFN-ɣ and IP-10 production in response to sensitive and specific antigens at various time points following the TST in a group of cattle originating from a low BTB prevalence herd in South African conditions. Also, since the effect of the TST on cytokine production has not been investigated in African buffaloes, we aimed to examine the changes in IFN-ɣ and IP-10 production following the TST in these animals. Furthermore, we aimed to determine whether the assay result, sensitivity, or specificity will be affected by the TST in buffaloes and cattle. A greater understanding of the effect of the TST on cytokine release assays (CRAs) will provide new information on the optimal time interval required between the performance of the TST and CRAs to improve the diagnosis of *M. bovis* infection in cattle and buffaloes.

Problem statement

Bovine TB management programs on cattle and buffaloes rely on ante-mortem diagnostic assays to identify *M. bovis*-positive animals at an early stage of infection. Removal of these animals from the herd will prevent further transmission. However, to conduct these assays at the proper time points and interpret the results correctly, a better understanding of the temporal immunological changes in *M. bovis*-infected animals are needed. More information on the changes in cell-mediated immune (CMI) and humoral responses and how these responses correlate with disease progression is required in buffaloes to identify how this may impact diagnostic tests. Furthermore,

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serial testing with TSTs followed by CRAs have been shown to influence CRA results. A better understanding of the effect of the TST and the duration of that effect is needed for buffaloes and cattle, since this may significantly influence the outcome of diagnostic tests and effective disease management. More information on the duration of the TST effect will contribute to defining the optimal amount of time required between TSTs and CRAs for serial testing in herds.

Aims and objectives

- To investigate changes in antigen-specific cytokine production over time in *M. bovis*-infected buffaloes
- To investigate the changes in serological responses to *M. bovis* antigens over time in *M. bovis*-infected buffaloes
- To determine the temporal relationship of different BTB biomarkers over time
- To determine the effect of the TST on cytokine production in *M. bovis*-infected buffaloes
- To determine the effect of the TST on cytokine production in *M. bovis*-uninfected buffaloes and cattle
- To investigate the effect of the TST on sensitivity, specificity and test outcome in *M. bovis*-infected and uninfected buffaloes and cattle

Chapter overviews

Chapter 2 is the literature review, which focuses on available diagnostic assays for BTB in animals and the effect of TSTs on these assays. Chapter 3 describes the chosen methodology for the studies and includes detailed protocols for all assay procedures. Chapter 4 sets out the results; Chapter 5 discusses the results in detail and draws conclusions and future recommendations. Chapters 3, 4 and 5 contain three separate studies; the first study investigates the temporal changes in immunological responses in naturally *M. bovis*-infected African buffaloes. The second study describes the temporal changes in antigen-induced cytokine release following the SICT in cattle, and the third study investigates the changes in antigen-induced cytokine release following the SICCT in African buffaloes. Chapter 6 presents the overall conclusion.

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Chapter 2: Literature review

The influence of tuberculin skin tests on *in vitro* and *in vivo* immunological responses to bovine TB

2.1 Introduction

Tuberculosis is a major global infectious disease that is caused by organisms of the *Mycobacterium tuberculosis* complex. *Mycobacterium tuberculosis* and *Mycobacterium bovis* are the most important causative agents of TB in, respectively, humans and a wide range of animal species, including African buffaloes, lions, warthogs, cattle, kudu, etc. (Michel et al., 2006; Palmer et al., 2006). Tuberculosis is regarded as one of the leading causes of death in humans worldwide, resulting in 1.5 million human deaths and 9.6 million new cases in 2014 ("WHO | Global tuberculosis report 2015," n.d.). In cattle, approximately 50 million individuals were infected worldwide with *M. bovis* in 2006 (Buddle et al., 2009).

Bovine tuberculosis, which is caused by *M. bovis,* has an impact on livestock and wildlife through decreased productivity, including reduced fecundity and body condition and loss of animals due to natural death or culling as part of the test-and-cull strategy that is implemented in many countries (Jolles et al., 2005; Michel et al., 2010, 2006). Also, carcasses and other animal products with TB lesions are condemned at slaughter. All of these issues lead to major economic losses (Michel et al., 2006; Thom et al., 2006). Bovine TB is a zoonotic disease that can spread between wildlife, livestock and humans. It therefore presents a major threat of infection between multiple species and is a significant public health concern (Coad et al., 2010; Michel et al., 2010). Translocation of animals both nationally and internationally are problematic in areas with a high BTB burden, since movement of animals presents a major risk factor in the spread of *M. bovis* into BTB free areas (Michel et al., 2010, 2006; Parsons et al., 2011). Due to restricted translocation of animals between parks, conservation programs may be negatively impacted by the limited exchange of genetic material (Michel et al., 2006). Furthermore, detection of BTB on a farm or reserve will result in it being placed under quarantine, with animal sales and movement of animal products terminated, which may further influence farm revenues, and tourism and hunting in the case of wildlife (Hlokwe

et al., 2016; Michel et al., 2006). Quarantine and movement restrictions result in a decline in international and national animal and animal product trade (Michel et al., 2010, 2006; Parsons et al., 2011).

Buffaloes play an important role in ecotourism since they form part of the iconic “Big Five” animals of Africa and are also often used for farming purposes (Michel and Bengis, 2012). Furthermore, these animals are of high ecological value, since they are bulk grazers that occur in large herds and therefore open up habitats which benefits other species such as smaller animals, elective feeders and short grass feeders. Buffaloes are also a food source for many predators, such as lions (Michel et al., 2010; Michel and Bengis, 2012). Moreover, buffaloes are maintenance hosts of BTB, which allows the persistence of the disease within an area without a source of reinfection. Infected animals can live for years with reasonably good health, but be potential sources of *M. bovis* contamination to other species (De Vos et al., 2001; Michel et al., 2006). Buffaloes make especially good maintenance hosts due to their social behaviour and gregarious nature. Therefore, total eradication of BTB is extremely difficult in an area in which the infection is maintained by these hosts (De Vos et al., 2001; Palmer et al., 2012).

Effective disease management strategies are crucial to prevent the spread of BTB within a herd or to other animals, especially with translocation of livestock or wild animals (Buddle et al., 2009; Karesh et al., 2016). Management of maintenance hosts is of particular importance, since lack of effective control will result in indefinite persistence of infection and potential transmission to other species (Gormley et al., 2004; Hlokwe et al., 2016; Michel et al., 2006; Palmer et al., 2012). Accurate diagnosis of BTB is fundamental for disease management strategies to identify *M. bovis*-infected animals at an early stage and remove them from the herd before becoming a source of infection (Gormley et al., 2006; Thom et al., 2006). Ante-mortem BTB diagnostic assays with high sensitivity, i.e. the probability of correctly identifying an animal that is truly infected, and specificity, i.e. the probability of correctly identifying an animal as truly uninfected, would therefore be ideal to detect infected animals before they shed *M. bovis* bacilli into the environment (Buddle et al., 2009; de la Rua-Domenech et al., 2006; Thom et al., 2006). Typically, assays with high sensitivity would be implemented in high BTB prevalence herds to accurately identify and remove BTB-positive

individuals. In herds where BTB probability is low, assays with high specificity are required to reduce the number of false-positive animals that are identified and removed (Buddle et al., 2009; de la Rua-Domenech et al., 2006).

2.2 Currently available TB diagnostic tests

Direct detection of mycobacterial organisms provides a definitive diagnosis of TB. Mycobacterial culture is currently regarded as the gold standard for *M. bovis* infection (de la Rua-Domenech et al., 2006; de Lisle et al., 2002), although it has some limitations, including imperfect sensitivity, prolonged culturing period, difficulty of culturing some mycobacterial species and the limited ability to differentiate between different mycobacterial species (de Lisle et al., 2002; Michel et al., 2010). Speciation by polymerase chain reaction (PCR) or other genetic procedures on culture isolates provide the opportunity to identify the mycobacteria of interest and perform epidemiological studies (Michel et al., 2010; Schiller et al., 2010). PCR can also be performed directly on homogenised tissues, bodily fluids (e.g. blood, urine, respiratory or gastric lavage samples), faeces and nasal swabs, without time-consuming bacterial culturing procedures. However, this requires more sophisticated laboratory equipment and may have poorer sensitivity and specificity due to various factors, including low number of bacilli present in samples and inefficient deoxyribonucleic acid (DNA) extraction (de la Rua-Domenech et al., 2006). Also, acid-fast staining, such as Ziehl-Neelsen, can be done directly on smears taken from tuberculous lesions and examined by microscopy. This method is inexpensive and easy to perform, but lacks sensitivity due to low bacilli counts in the lesions, and specificity due to the presence of non-tuberculous mycobacteria (de Lisle et al., 2002).

Ante-mortem immunodiagnostic assays have many advantages, such as greater specificity and sensitivity, ability to detect bacilli in low numbers, time-efficiency and allowing early detection of infection (14 days post-infection) (Gormley et al., 2006). Immunodiagnostic assays measure mycobacteria-specific immune response for diagnosis of infection. Cell-mediated immune (CMI) responses involve the differentiation and proliferation of naïve T-cells into active cells (effector cells) in response to specific antigens. CD4+ effector T-cells secrete cytokines, such as IFN-ɣ, which are proteins that play a key role in the regulation of the immune response, including the

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activation of B-lymphocytes and macrophages (Janeway et al., 2001). The humoral immune response is mediated by antibodies that are produced by B-lymphocytes. Upon recognition of specific antigens, B-cells are activated and differentiate into plasma cells that can secrete specific antibodies with the aid of T-helper (Th) cells (Janeway et al., 2001). Since mycobacterial stimulation of antibody production appears to be antigen load-dependent, humoral responses are often observed only after development of disease, rather than in earlier stages of infection and increase substantially as disease progresses (Casal et al., 2014; de la Rua-Domenech et al., 2006; Janeway et al., 2001).

Assays of humoral immunity detect antibody responses to mycobacterial antigens in serum and are typically more effective in detecting animals which are at advanced stages of infection or exposed to high levels of mycobacteria (de la Rua-Domenech et al., 2006; Palmer et al., 2006). However, in some species, such as elephants and warthogs, serological assays can detect infection without significant signs of disease (Greenwald et al., 2009; M. Miller, pers. comm.). Serological assays are less costly and easy to perform, but have relatively low sensitivity in cattle and some other species (Buddle et al., 2009; de la Rua-Domenech et al., 2006). Inclusion of *M. bovis* antigens, such as immunodominant MPB83, MPB70, culture filtrate protein 10 (CFP-10) and early secretory antigenic target 6 (ESAT-6), has increased the specificity, but not the sensitivity, of serological assays (de la Rua-Domenech et al., 2006). MPB70 and MPB83 are constitutively expressed in *M. bovis,* but are hardly detected in mycobacteria with a region of difference 2 (RD2) deletion, such as Bacille Calmette Guerin (BCG) (Lyashchenko et al., 2004). There are many serological assays available that make use of these immunodominant antigens. These include immunochromatographic dual-platform VetTB assays containing MBP83, ESAT-6 and CFP-10, multi-antigen print immunoassays using 12 antigens of the *M. tuberculosis* complex (ESAT-6, CFP-10, bovine PPD, MPB70 and MPB83), IDEXX enzyme-linked immunosorbent assays (ELISAs) using MPB83 and MPB70 antigens, and an indirect ELISA using bovine PPD as capture antigen (Schiller et al., 2010; Waters et al., 2015).

Diagnostic assays of CMI can detect animals at an early stage of infection and are more sensitive than assays of humoral immunity in livestock (de la Rua-Domenech et al., 2006). The most

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commonly used CMI assay for BTB is the TST. It is based on the intradermal injection of PPD, which elicits a DTH response that is associated with the influx of sensitised T-cells and monocytes to the site of injection. Cytokines are released, which recruits more inflammatory cells (e.g. neutrophils, macrophages, monocytes) to the area, and causes vasodilation and ultimately swelling in this area which peaks about 72 h after the PPD injection (Buddle et al., 2009; de la Rua-Domenech et al., 2006). There are various ways to perform a TST, including the single intradermal cervical skin test (SICT), caudal fold test (CFT) and the single intradermal comparative cervical skin test (SICCT). The SICT and the CFT involves the intradermal injection of bPPD in the mid-cervical region and in the caudal fold, respectively (Buddle et al., 2009; Coad et al., 2010; de la Rua-Domenech et al., 2006). The change in skin fold thickness (SFT) at the site of injection after 72 h is measured with callipers and the area is examined for any cardinal signs of inflammation, i.e. heat, swelling, oedema and erythema. Cattle are regarded as test-positive if the increase in SFT is ≥ 4 mm (Buddle et al., 2009; Coad et al., 2010). The specificity of these tests are, however, reduced if the animals are exposed to non-tuberculous mycobacteria, such as bacteria from the *M. avium* complex, since exposure to these mycobacteria can result in a false-positive skin test result (Ryan et al., 2000). The SICCT involves the intradermal injection of both bPPD and avian PPD (aPPD) at two different sites in the mid-cervical region. The changes in SFT at each site are measured after 72 h and the differential response to aPPD and bPPD is determined (Buddle et al., 2009; Coad et al., 2010). Buffaloes with a change in SFT ≥ 2 mm (Parsons et al., 2011) and cattle with a change ≥ 4 mm (Buddle et al., 2009) are regarded as test-positive. The inflammatory response to aPPD is compared to the response to bPPD to distinguish *M. bovis* infections from those due to environmental mycobacteria (Coad et al., 2010; Thom et al., 2006). Therefore, fewer false-positive animals will be detected by the SICCT compared to the SICT or CFT, increasing the specificity of this test. However, the SICCT may have a lower sensitivity than the other two TSTs that only use bPPD, since a large response to aPPD may mask a response to bPPD which would lead to an increase in false-negative animals (Buddle et al., 2009; Parsons et al., 2011; Ryan et al., 2000).

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One of the most commonly used *in vitro* immunodiagnostic assays of CMI is the IGRA, which is a sensitive and specific test for detection of TB in humans and livestock. These tests can detect *M. bovis* infection earlier following infection than the TST (Gormley et al., 2006). IGRA kits are commercially available, for example the Bovigam® ELISA (ThermoFisher Scientific) for cattle (Gormley et al., 2006; Palmer et al., 2006; Sinclair et al., 2016) and QuantiFERON Gold In-Tube (QFT, Qiagen) in humans (Katial et al., 2001; Britton et al., 2005). IGRAs in animals are based on the stimulation of sensitised T-lymphocytes in the blood of *M. bovis*-infected animals with BTB antigens, such as bPPD, aPPD, ESAT-6, CFP-10 and other antigens (Buddle et al., 2009). Upon recognition of the specific antigen, T-lymphocytes release IFN-ɣ, which results in the activation of macrophages that kill mycobacteria *in vivo* (Buddle et al., 2009). IFN-ɣ has been shown to be a sensitive biomarker of T-cell activation as it correlates well with the efficacy of immune responses (Chakera et al., 2011). Therefore, measurement of IFN-ɣ by ELISA *in vitro* is a good representation of *in vivo* immune responses, with high IFN-ɣ concentrations in antigen stimulated samples correlating well with *M. bovis* infection (Chakera et al., 2011). The assay is performed by measuring the differential release of cytokines in antigen–stimulated and unstimulated whole blood samples using a sandwich ELISA (Ryan et al., 2000; Whipple et al., 2001).

Various mycobacterial-derived antigens are used for stimulation of blood samples as part of the IGRA procedure. Combinations of antigens have been used in the assay to increase sensitivity or specificity, depending on the application. These include PPDs, ESAT-6, CFP-10 and the commercially available Bovigam® PC-EC and PC-HP cocktails. Bovine PPD and aPPD are crude antigen preparations derived from heat-killed *M. bovis and M. avium,* respectively, which contain a variety of antigens (de la Rua-Domenech et al., 2006; Palmer et al., 2006). These PPDs are standardly used in the Bovigam® assay. The specificity, but not sensitivity, of the assays have been improved by utilising Bovigam® PC-HP and PC-EC antigenic cocktails, which are typically used in new generation IGRAs, for example Bovigam® 2G. In buffaloes, the sensitivity of the PPD assay is significantly greater than that of the highly specific PC-EC assay, but is not significantly greater than that of the PC-HP assay (W. Goosen, pers. comm.). PC-EC and PC-HP are derived from the *M. bovis* proteins, ESAT-6 and CFP-10, with PC-HP also utilising peptides derived from

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Rv3615 and three additional, proprietary, mycobacterial antigens (Goosen et al., 2014). Genes encoding ESAT-6 and CFP-10 are located in the genetic RD1, which are deleted from Bacille Calmette Guerin (BCG) and most non-tuberculous mycobacteria, including *M. avium*, thereby minimising cross-reactions with environmental mycobacteria and improving specificity (Buddle et al., 2009; Pollock and Andersen, 1997a; Thom et al., 2004). The QFT assay utilises ESAT-6, CFP-10 and TB7.7, which improve the specificity of the IGRA (Parsons et al., 2011). However, the QFT and Bovigam® PC-EC assays are less sensitive than the Bovigam® PC-HP assay (Goosen et al., 2015).

Even though these antigen combinations improve the sensitivity and specificity of the IGRA, the assay remains suboptimal for the detection of *M. bovis* infection in wild animals. Another biomarker, IP-10, has recently been identified for use in wild animals and is showing great potential in detection of TB in both humans (Ruhwald et al., 2009), African buffaloes (Goosen et al., 2014) and cattle (Parsons et al., 2016). IP-10 is induced by IFN-ɣ and produced by monocytes to primarily facilitate attraction of immunocompetent cells to sites of inflammation (Chakera et al., 2011; Chegou et al., 2014; Ruhwald et al., 2009). IP-10 is released at significantly greater concentrations in stimulated blood samples than IFN-ɣ; approximately 100-fold higher than IFN-ɣ (Chegou et al., 2014; Goosen et al., 2015; Ruhwald et al., 2009). Therefore, if T-lymphocyte numbers are low in a sample, IFN-ɣ may be produced at levels below the threshold of detection. However, there may be sufficient IFN-ɣ to activate IP-10 production that can be detected by ELISA (Goosen et al., 2015). The sensitivity and specificity of IP-10 assays are comparable with the IGRA, although there is evidence that measurement of IP-10 may enhance sensitivity in humans, cattle and buffaloes (Chegou et al., 2014; Goosen et al., 2014; Parsons et al., 2016).

Cytokine release assays are often performed at the same time (parallel testing) or shortly after (serial testing) performing a TST. Parallel testing involves the simultaneous application of both tests, and animals are considered test-positive if they are positive on either assay (Coad et al., 2007; Ryan et al., 2006; Whipple et al., 2001). It may also involve testing only TST-negative animals on CRAs (Sinclair et al., 2016). Using the TST and CRA in parallel improves the overall sensitivity through identification of additional false-negative animals on one of the tests. Parallel

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testing is therefore useful in herds with high BTB prevalence as this would allow removal of all possible *M. bovis*-positive animals (Gormley et al., 2006; Sinclair et al., 2016). Assays with high sensitivity are inadequate for BTB diagnosis in herds with low BTB probability, as they will identify inappropriately large numbers of false-positive animals in the herd which would cause unnecessary economic losses to the farmer (Gormley et al., 2006). Therefore, serial testing is more appropriate for herds with low BTB probability, since specificity, at the expense of sensitivity, is greatly improved, thereby reducing the identification of false-positive animals in the herd. Serial testing involves testing TST-positive animals with the CRA. Animals are regarded test-positive if they are positive on both tests (Coad et al., 2007; Gormley et al., 2006; Ryan et al., 2006; Sinclair et al., 2016; Whipple et al., 2001).

However, even though CRAs and TSTs are often used in combination to improve sensitivity or specificity, many studies have shown that the performance of CRAs may be influenced by the intradermal injection of PPD during the TST (Coad et al., 2010; Gormley et al., 2004; Whipple et al., 2001).

Many other factors may also influence the performance of diagnostic assays in animals, especially when testing wild animals. These include the use of opioids as immobilisation drugs, which may result in inhibition of immune responses (Garcia et al., 2012; Sacerdote, 2006), exposure to environmental mycobacteria, time delay between blood collection and processing (Gormley et al., 2004) and stress caused by capture and captivity during the testing period. Stress may result in increased levels of glucocorticoids, such as cortisol, which may result in immune suppression and therefore significantly reduced cytokine release up to 18 weeks after capture (Cross et al., 1999; Franceschini et al., 2008; Gormley et al., 2006; Griffin and Thomson, 1998).

2.3 Effect of TST on the diagnostic performance of TB immunoassays

2.3.1 Effect of multiple TSTs on sensitivity and specificity of the TST

In many BTB testing programs, TSTs are performed on cattle followed by another TST as a confirmatory test (Coad et al., 2010). Also, in situations where BTB is identified or suspected in an area, TSTs are often performed at short intervals, approximately 60 days apart, to identify and

remove all test-positive animals as quickly and effectively as possible (Coad et al., 2010). However, repeated skin testing that is performed at short intervals may result in a reduction in the ability of an animal to respond to tuberculin, i.e. desensitisation or suppression of immune responses, which may lead to increased numbers of false-negative test results in the herd (Coad et al., 2010; de la Rua-Domenech et al., 2006; Palmer et al., 2006; Thom et al., 2004). This effect was also clearly seen in a cattle study by Ameni et al. (2007) in which SICCT-responders declined from 14% to 1% in a 1 year period after three consecutive SICCTs were performed every three months. Desensitisation was also reported in rhesus monkeys after TSTs were performed every two weeks for 14 weeks (Min et al., 2014). It has been shown that the suppression of the TST response may occur as soon as seven days after the initial TST and may remain suppressed for 50 to 60 days (Doherty et al., 1995; Palmer et al., 2006). Therefore, a minimum of 60 days between TSTs is recommended for reactivity to the TST to return to normal (Buddle et al., 2009; Coad et al., 2010; Ryan et al., 2000; Thom et al., 2006). In the USA, TSTs may only be repeated within 10 days or after 90 days of the initial TST. However, Coad et al. (2010) found that even when SICCTs were performed at 60 day intervals, desensitisation to tuberculin was observed, particularly by the third consecutive TST. Therefore, it seems that 60 days between repeated TSTs may not be sufficient (Coad et al., 2010). Interestingly, a significant decrease in the skin test response was only seen in response to bPPD, whereas the response to aPPD was not affected by repeated SICCTs (Coad et al., 2010).

Studies have demonstrated that there are multiple cytokines that may be involved in the desensitisation effect. These include interleukin (IL)-10, IL-6 and IL-1β. It has been shown that injection of PPD for the TST can lead to elevated levels of IL-10 (Coad et al., 2010); single and repeated SICCTs have resulted in significant increases in IL-10 production (Coad et al., 2010; Thom et al., 2006). IL-10 can inhibit DTH responses and therefore increased levels of this cytokine may result in desensitisation (Coad et al., 2010; Schwarz et al., 1994; Thom et al., 2006). However, increased IL-10 production post-SICCT was not seen in *M. bovis*-uninfected cattle (Coad et al., 2010). Furthermore, Thom et al. (2006) found that production of IL-6, a pro-inflammatory cytokine, decreased following repeated SICCTs which would also suppress a TST response. IL-1β

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is a mediator of the inflammatory response that is required for T-cell activation and the DTH response (Coad et al., 2010). Cattle that had single or multiple SICCTs showed a significant decrease in IL-1β production post-SICCT which would result in a decrease in the DTH response (Coad et al., 2010; Thom et al., 2006).

Animals may also develop a hypersensitivity response to tuberculin due to repeated skin testing. Hypersensitivity, or sensitisation to tuberculin, in cattle has been reported as early as 1891 (Monaghan et al., 1994) and shown to occur between three and six weeks after the first TST (Palmer et al., 2006). Sensitisation results in an increase in false-positive animals on TST (de la Rua-Domenech et al., 2006; Palmer et al., 2006); this effect is often seen in herds with low BTB prevalence (Monaghan et al., 1994).

Interestingly, TST results of *M. bovis*-uninfected cattle have not been shown to be affected by repeated skin testing (Thom et al., 2004, 2006).

2.3.2 Effect of TSTs on cytokine release assays

Cytokine release assays are often used in parallel or series with TSTs to improve diagnostic performance. However, studies have shown that performing a CRA shortly after the TST may influence the results of the CRA. There have been many conflicting findings regarding the influence of TSTs on CRAs. This may be due to various factors, including the type of TST that was performed, naturally vs. experimentally infected animals, antigens used for blood stimulation, cytokines measured, and time points at which CRAs were performed (Palmer et al., 2006).

Rothel et al. (1992) and Wood and Rothel (1994) reported a decline in IFN-ɣ release in response to PPD up to seven days post-CFT in experimentally infected cattle. This was followed by a gradual increase in IFN-ɣ release, which remained elevated for 59 days after the CFT. In contrast, other studies reported a boost in IFN-ɣ release in response to PPD, ESAT-6 and CFP-10 between three and seven days post-CFT (Palmer et al., 2006) and in response to PPD between three and 28 days post-CFT in experimentally infected cattle (Whipple et al., 2001). Similarly, an increase in IFN-ɣ release was seen shortly after the TST was performed in humans, which remained elevated four weeks post-TST (Vilaplana et al., 2008). However, ÓNualláin et al. (1997) found that the

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SICCT caused a significant decrease in IFN-ɣ production in response to bPPD between one and 28 days post-SICCT in naturally infected cattle, whereas Whelan et al. (2004) also reported a decline in IFN-ɣ in response to bPPD, aPPD, ESAT-6 and CFP-10 three days post-SICCT in experimentally infected cattle.

Other studies have found that the TST had no effect on IFN-ɣ release, and therefore on the CRA outcome. No change in IFN-ɣ release in response to PPD in naturally infected cattle following a single SICCT or multiple repeated SICCTs has been reported, measured up to seven days (Doherty et al., 1995) or 65 days post-SICCT (Coad et al., 2010; Gormley et al., 2004). Another study reported a greater IFN-ɣ response at 3 days post-SICCT compared to pre-SICCT and 10 days post-SICCT, but this was not statistically significant (Coad et al., 2007). No significant changes in IFN-ɣ release in naturally infected cattle was reported by Ryan et al. (2000) when measured between eight and 28 days post-CFT. Also, Thom et al. (2006) reported that the SICCT had no significant effect on the IGRA test outcome, but did cause a boost in IFN-ɣ production in response to aPPD, but not to bPPD, in experimentally *M. bovis*-infected cattle. Furthermore, it has been shown that the effect of the TST on subsequent IFN-ɣ release is not influenced by the number of repeated TSTs (Rangen et al., 2009).

Of note, many studies found that the CFT, rather than the SICCT, caused a boosting of IFN-ɣ in CRAs (Coad et al., 2010; Gormley et al., 2006). Coad et al. (2010) directly compared the effect of the SICCT versus that of the CFT on IFN-ɣ response in cattle and showed that CFTs, but not the SICCT, caused a significant increase in IFN-ɣ in response to PPD at three days post-CFT which returned to pre-CFT levels by 10 days post-CFT (Coad et al., 2010). Interestingly, CRAs that used bPPD and aPPD for blood stimulation, showed that the boosting of IFN-ɣ in the comparative response to aPPD and bPPD was due to a significant increase in response to bPPD in *M. bovis*-infected cattle only, since the IFN-ɣ response to aPPD was not affected by the CFT. However, Thom et al. (2006) found that the SICCT caused a greater boosting effect in aPPD compared to bPPD 1 week post-SICCT in infected cattle.

Significant boosting of IFN-ɣ levels due to the injection of PPD for the TST was only seen in *M. bovis*-infected, but not in *M. bovis*-uninfected cattle, which suggests that the boosting effect may

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actually enhance the sensitivity of the assay, since identification of *M. bovis*-positive animals could be improved without identifying more false-positive animals (Palmer et al., 2006). Moreover, IFN-ɣ release in response to ESAT-6 and CFP-10 was less affected by the TST than responses to PPD in *M. bovis*-uninfected cattle (Palmer et al., 2006).

2.3.3 Effect of TSTs on serological assays

It has been shown that TSTs may cause a boosting effect in antibody responses (Hanna et al., 1992; Lyashchenko et al., 2004; Palmer et al., 2006; Thom et al., 2006). This was specifically observed in response to the immunodominant antigens, MPB83 and MPB70 and also to ESAT-6 and bPPD in cattle (Lyashchenko et al., 2004; Palmer et al., 2006; Thom et al., 2006). MPB70 and MPB83 are major components of bPPD, which is intradermally injected for the TST and may stimulate a B-lymphocyte memory response which could explain the boosted antibody responses to these antigens (Lyashchenko et al., 2004; Miller et al., 2015). Furthermore, Thom et al. (2006) found that the expression of IL-4, cytokines that induce B-cells to proliferate and produce antibodies, increased following the SICCT, which would result in increased antibody responses. Increased antibody responses to MBP70 and MPB83 were seen as early as 2 weeks post-CFT in cattle, which decreased over the next 2 to 3 months (Waters et al., 2015). Furthermore, the CFT, followed by the SICCT 105 days later, resulted in a massive antibody boost by seven days post-SICCT in *M. bovis*-infected cattle (Waters et al., 2015). However, increased antibody responses were only observed in response to MPB70 and MPB83, but not to ESAT-6 and CFP-10 (Waters et al., 2015).The boosting effect of TST in antibody responses has also been seen in wildlife species. Specifically, a significant increase in antibody levels were seen from the day when the TST was performed to 17 days post-TST in a TB-positive black rhinoceros (Miller et al., 2015). Interestingly, Thom et al. (2006) found that cattle recently infected with *M. bovis* were easier to detect, using serological assays, after SICCTs were performed. This was probably associated with the TST boosting their limited antibody responses (Casal et al., 2014; Thom et al., 2006). In *M. bovis*-uninfected cattle, antibody levels, and therefore the test outcome, were not affected by PPD injection for the TST (Hanna et al., 1992; Palmer et al., 2006; Thom et al., 2004). For this reason, the TST could be used in conjunction with serological assays to identify *M. bovis*-infected cattle

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more accurately without increasing false-positive animals (Palmer et al., 2006; Thom et al., 2004). Therefore, since newly infected animals can be accurately identified post-TST and uninfected animals are not affected by the TST, the sensitivity of the serological assay can be significantly improved without compromising the specificity by using the TST in parallel (Palmer et al., 2006; Thom et al., 2006). In fact, Casal et al. (2014) found that the sensitivity of the serological tests increased significantly by 15 days post-TST. Interestingly, a boost in antibody responses correlates with disease severity; therefore, cases with more progressive disease showed a greater antibody boost (de la Rua-Domenech et al., 2006; Lyashchenko et al., 2004).

2.4 The influence of NTMs on TB tests

Most animals are exposed to environmental bacteria, specifically those belonging to the *M. avium* complex, throughout their lives. However, many studies have shown that exposure to and/ or infection with environmental mycobacteria may compromise IGRAs and TSTs by leading to increased numbers of false-positive or false-negative animals in a herd (Hope et al., 2005). It has been shown that animals that are aPPD-positive generally have decreased responses to bPPD (Aranaz et al., 2006). In *M. bovis* infected herds, the presence of *M. avium* results in decreased sensitivity of IGRAs and TSTs, with failure to detect *M. bovis* positive animals, despite the presence of BTB lesions (Alvarez et al., 2009; Álvarez et al., 2008; Amadori et al., 2002; Hope et al., 2005).

The type of diagnostic assay applied in a herd with *M. avium* exposure may strongly influence test outcome. For instance, assays that use both aPPD and bPPD, such as SICCT and IGRA, may not accurately identify TST-positive animals, since a high aPPD response would skew the comparative test result. However, when applying an assay that makes use of bPPD only, such as a SICT or IGRA, the number of false-positive animals may increase, since it is not possible to differentiate between a response to bPPD and aPPD (Alvarez et al., 2009; Álvarez et al., 2008).

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2.5 Conclusion

Diagnostic assays with high specificity and sensitivity are required for effective management and BTB control programs in livestock and wildlife. Combinations of assays, in series or in parallel, have significantly improved diagnostic performance by increasing sensitivity or specificity. Although TSTs are often used in combination with cytokine release assays, serological assays, or a second TST, many studies have shown that TSTs can influence the outcome of the diagnostic assays in cattle. Further studies are required to study this effect in wildlife. Moreover, care must be taken when analysing results using multiple assays, especially in herds with high environmental mycobacterial exposure.

Chapter 3: Materials and methods

3.1 Animals and sample processing

3.1.1 Kinetics of immunological responses in naturally *M. bovis*-infected African buffaloes

Between May 2015 and May 2016, African buffaloes (n = 18) were captured every 2 to 3 months for a total of 6 captures from a camp (approximately 9 hectare) at Satara, Kruger National Park, South Africa. Buffaloes were chemically immobilised as previously described (Parsons et al., 2011) and whole blood was collected by venipuncture of the jugular vein into heparinised blood tubes and serum tubes. On each sampling occasion, animals were tested using QFT and Bovigam® PPD ELISAs, as described below. However, due to logistical challenges, not all animals could be reliably captured at each time point. Buffaloes were divided into groups according to their Bovigam® result and were included in the present study if they were Bovigam®-positive on two consecutive captures. Group 1 consisted of chronically Bovigam®-positive animals (n = 5) that were tested for the full study period; group 2 consisted of newly converted Bovigam®-positive animals (n = 5) that were included in the study from capture 4 – 6. Additionally, group 3 consisted of Bovigam®-negative animals (n = 8) that were kept in the same camp as the animals from groups 1 and 2 and were tested sporadically at different captures.

3.1.2 Temporal changes in antigen-induced cytokine release in cattle

Holstein cattle were sourced from a dairy farm in the Western Cape, South Africa, which has been under BTB quarantine for approximately 10 years. During this time, the SICT, as described below, was initially performed every 3 to 4 months. However, since no macroscopic lesions were found and no animals were *M. bovis* culture positive, the SICT was performed annually during the last few years. The study utilised two cohorts of cattle in the same herd from which heparinised whole blood was collected by venipuncture of the coccygeal vein. Group 1 consisted of 29 SICT-positive animals that were sampled 6 days and 41 days post-SICT and group 2 included 16 SICT-positive and 17 SICT-negative animals that were sampled at seven, 21 and 78 days post-SICT. These time

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points were opportunistic samples determined by the availability of the cattle and veterinarian. Additionally, animals in group 2 were tested using the SICCT, as described below, at the time of blood collection 78 days post-SICT, prior to the TST. On each sampling occasion, animals were tested using the Bovigam® PPD ELISA and QFT assays, as described below.

Animals from group 1 were slaughtered at circa 70 days post-SICT at a commercial abattoir and inspected for evidence of BTB lesions. Tissue samples were collected for mycobacterial culture, as described below.

3.1.3 Changes in antigen-induced cytokine release in African buffaloes following the SICCT

In July 2015, 286 African buffaloes were captured as part of an annual BTB control program in the Hluhluwe-iMfolozi Park (HiP), KwaZulu-Natal, South Africa, and held in temporary bomas before being chemically immobilised as previously described (Parsons et al., 2011). Whole blood was collected from each animal into heparinised blood tubes and serum tubes (BD Pharmingen, New Jersey, USA) by venipuncture of the jugular vein, and SICCTs were performed at this time (pre-SICCT). Bovigam® PPD, PC-EC and PC-HP ELISAs were performed to measure IFN-ɣ and IP-10 levels for each animal, as described below. All animals that tested positive to any Bovigam® ELISA (group 1; n = 55), a cohort of animals that were Bovigam® and SICCT-negative (group 2; n = 21) and animals that were SICCT-positive, but Bovigam®-negative (group 3; n = 5), were immobilised (as before) three days after the SICCT was performed (post-SICCT). The number of animals tested on each assay was subjected to the availability of stimulated blood for each animal. Blood was collected, as above, and the Bovigam® ELISAs were repeated for these animals. All Bovigam® and SICCT-positive animals were sacrificed and post-mortem examinations and mycobacterial culture were performed, as described below.

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3.2 Ethical approval

Ethical approval was obtained for the various studies from the University of Stellenbosch Animal Ethics committee. The ethics reference numbers were SU-ACUM13-00016 and SU-ACUD15-00111. Also, Section 20 approvals for the Satara buffalo study and Hluhluwe-iMfolozi buffalo study were granted by the Department of Agriculture, Forestry and Fisheries, South Africa.

3.3 Tuberculin skin tests

3.3.1 Single intradermal cervical skin test

Following measurement of the SFT at the site of injection, 0.1 ml bPPD (WDT, Hoyerhagen, Germany; 30 000 IU/ml) was intradermally injected in the neck. Three days hereafter, the SFT was measured again and examined for evidence of inflammation, including redness, oedema, necrosis and heat. Cattle were regarded as SICT-positive if the SFT showed an increase of more than 4 mm (Buddle et al., 2009).

3.3.2 Single intradermal comparative cervical skin test

Single intradermal comparative cervical skin tests were performed by injecting 0.1 ml bPPD and 0.1 ml aPPD (WDT) into the skin on the left and right side of the neck, respectively, following the measurement of the SFT at the sites of injection. After three days, the SFT was measured and the area examined for evidence of inflammation, as above. Buffaloes were considered SICCT-positive if the increase in skin fold thickness at the bPPD site of injection was 2 mm more than that at the aPPD injection site (Parsons et al., 2011); whereas for the cattle the difference was more than 4 mm to be regarded as SICCT-positive (Buddle et al., 2009).

3.4 Cytokine release assays

3.4.1 Bovigam® ELISA

For all animals, aliquots of whole blood (250 µl) were incubated with 25 µl phosphate buffered saline (PBS); 25 µl bPPD, prepared according to the manufacturer's instructions; 25 µl aPPD (both

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Prionics, Schlieren, Switzerland), similarly prepared; and 25 µl pokeweed mitogen (PWM; Sigma-Aldrich, St. Louis, Missouri, United States) solution in PBS (final concentration 5 µg/ml), respectively. In addition, for the HiP buffaloes, 250 µl whole blood aliquots were incubated with 25 µl PC-HP peptide solution and 25 µl PC-EC peptide solution (both Prionics), respectively. Following incubation at 37˚C for 20 h, plasma was harvested and stored at -80˚C. In all studies, blood processing was conducted within six hours after sample collection. IFN-ɣ release in the plasma samples was determined using the Bovigam® ELISA kit (Prionics) as follows. Equal volumes of plasma diluent buffer and plasma (50 µl each) were incubated in wells of pre-coated 96-well microplates for 1h at room temperature (RT). All subsequent steps were also performed at RT. Positive and negative controls were added to appropriate wells in duplicate. Plates were washed by hand 5 times with wash buffer and 100 µl of horseradish peroxidase (HRP)-labelled anti-bovine IFN-ɣ in conjugate diluent buffer (1:100) was added to all wells. After 1 h incubation, plates were washed as before. A chromogen solution containing tetramethylbenzidine (TMB) in enzyme substrate buffer (1:100) was added to all wells (100 µl/ well) and incubated for 30 min in the dark. Thereafter 50 µl/ well of enzyme stopping solution (0.5 M H_2SO_4) was added to stop the colour reaction and the absorbance of each sample was read at 450 and 630 nm using a LT-4000 Microplate Reader (Labtech, Uckfield, UK). For the assay to be valid, the mean absorbance (optical density; OD) of the negative controls must be < 0.13 and that of the positive control > 0.7. Also, the differential OD value between the response to PWM and Nil ($OD^{PWM-Nil}$) must be ≥ 0.5.

For each animal, the OD values measured in samples co-incubated with aPPD (OD^{aPPD}) and PBS (OD^{Nil}) were subtracted, respectively, from samples stimulated with bPPD (OD^{bPPD}). An animal was regarded as PPD positive if $OD^{bPPD} - OD^{Nil} \geq 0.1$ and $OD^{bPPD} - OD^{aPPD} \geq 0.1$ and PPD negative if $OD^{bPPD} - OD^{Nil} < 0.1$, or $OD^{bPPD} - OD^{aPPD} < 0.1$. Moreover, OD^{Nil} was subtracted from OD values from samples co-incubated with PC-HP (OD^{PC-HP}) and PC-EC (OD^{PC-EC}), respectively. Animals were regarded PC-HP-positive if $OD^{PC-HP} - OD^{Nil} \geq 0.1$ and PC-EC-positive if $OD^{PC-EC} – OD^{Nil} \geq 0.1$, and those with OD differential values < 0.1 were test negative.

3.4.2 QuantiFERON® -TB Gold In-Tube assay

For all cattle and the Satara buffaloes, 1 ml heparinised whole blood was transferred to QuantiFERON® -TB Gold In-Tube (QFT) assay blood collection tubes (Qiagen, Venlo, Limburg, Netherlands). These included a Nil tube containing saline and a TB antigen tube containing a peptide cocktail simulating ESAT-6, CFP-10 and TB 7.7. Furthermore, aliquots of whole blood (250 µl) were incubated with 25 µl PWM (final concentration: 5µg/ml) to serve as a positive control for each sample. The tubes were shaken vigorously, incubated for 20 h at 37˚C and centrifuged at 3000 x g for 10 min after which the plasma fraction was harvested and stored at -80˚C. In all studies, blood processing was conducted within six hours after sample collection. The IFN-ɣ and IP-10 concentrations were determined using a bovine IFN-ɣ ELISA (Mabtech, AB, Nacka Strand, Sweden) and a bovine IP-10 ELISA (Kingfisher Biotech Inc., St. Paul, MN, USA), respectively, as described below. Animals were regarded QFT-positive if the IFN-ɣ concentration in the TB antigen stimulated sample, i.e. [QFT^{TB}] minus that in the Nil sample, i.e. [QFT^{Nil}], was equal to or greater than 66 pg/ml (Parsons et al., 2011). For the IP-10 ELISA, an animal was regarded as test-positive if the IP-10 concentration in the TB antigen stimulated sample minus that in the Nil sample was equal to or greater than 1486 pg/ml (Goosen et al., 2015).

3.4.3 Mabtech IFN-ɣ ELISA

The IFN-ɣ concentration in selected plasma samples was determined as follows. Anti-bovine IFN-ɣ capture antibody (Kit 3119-1H-20; Mabtech) in PBS (2 ug/ml) was used to coat wells of 96-well MaxiSorp polystyrene ELISA plates (Thermo Fisher Scientific, Massachusetts, USA) which were incubated at 4˚C overnight. All subsequent steps were performed at RT. Plates were washed by hand with wash buffer (WB) consisting of 0.05% Tween-20 (Sigma-Aldrich, Missouri, USA) in PBS and all wells were blocked with 200 µl blocking buffer comprising 0.1% bovine serum albumin (Roche, Basel, Switzerland) in WB (BB-BSA). After 1 h incubation, plates were washed again and 50 µl of selected plasma samples with 50 µl BB-BSA were added to individual wells, in duplicate. A dilution series of bovine recombinant IFN-ɣ protein (Mabtech), ranging from 0 – 1000 pg/ml was added to individual wells, in duplicate, and plates were incubated for 2 h. Plates were then washed and incubated for 1 h with a 0.25 µg/ml solution of biotinylated anti-bovine IFN-ɣ antibody in BB-

BSA. After washing, plates were incubated for an additional 1 h with a 1:1000 solution of streptavidin-HRP (Mabtech) in BB-BSA. After a final wash step, plates were incubated for 20 min with TMB substrate solution (BD Pharmingen, New Jersey, USA) after which sulphuric acid (2M; 50 µl/well) was added to stop the colour reaction. The optical density (OD) of each well was measured at 450 nm and 630 nm. The OD_{630} was subtracted from OD_{450} and the IFN-ɣ concentration for each sample was derived from the standard curve using GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, USA).

3.4.4 IP-10 ELISA

The IP-10 concentration in selected plasma samples was determined using a protocol similar to the IFN-ɣ ELISA with the following differences. A 0.5 µg/ml solution of anti-bovine IP-10 capture antibody (catalogue no. PB0385B-100; Kingfisher Biotech Inc., St. Paul, MN, USA) in PBS was used to coat the wells of 96-well plates. After the blocking step, each well was incubated with 25 µl plasma sample and 75 µl BB-BSA and a dilution series of recombinant bovine IP-10 protein (100 µl/well; catalogue no. RP0079B-005; Kingfisher), ranging from 0 - 5000 pg/ml. Plates were then incubated with a 0.2 µg/ml solution of biotinylated anti-bovine IP-10 antibody (catalogue no. PBB0393B-050; Kingfisher) in BB-BSA and thereafter with streptavidin-HRP (R&D systems, Minnesota, USA) diluted 1:200 in BB-BSA. Finally, plates were incubated with TMB substrate solution after which sulphuric acid (2M; 50 µl/well) was added to stop the colour reaction. The OD of each well was measured as before and the IP-10 concentration in each well was derived from the standard curve as above.

3.5 Serological assays

Serological assays were performed with the Satara buffalo serum samples. 96-well MaxiSorp polystyrene ELISA plates (Thermo Fisher Scientific, Massachusetts, USA) were coated with 100 µl of a 10 µg/ml bPPD solution (Prionics) in 0.05 M carbonate-bicarbonate buffer (pH 9.6; Sigma Aldrich). For every well coated with antigen, a control well was blocked with blocking buffer which consisted of 20% fat free milk in PBS and 0.05% Tween-20 (BB-FFM). Plates were incubated for 1 h at 37˚C. All subsequent steps were performed at RT. Plates were washed 5 times by hand with

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WB and all wells were blocked with a 200 µl volume BB-FFM for 1 h. Then, plates were washed as above and serum aliquots from each animal were diluted 1:200 in BB-FFM and added to appropriate test and control wells. After a 1 h incubation period, plates were washed and 100 µl/well of HRP-conjugated recombinant protein A/G (Thermo Scientific, MA, USA) diluted 1: 20 000 in PBS was added to the wells and incubated for 1 h. After a final wash step, plates were incubated 15 min in the dark with TMB substrate solution, after which sulphuric acid (2M; 50 µl/well) was added to stop the colour reaction. The OD of each well was measured at 450 nm and 630 nm and an assay result for all animals was calculated as the mean $OD_{450-630}$ value of the PPD-coated wells minus that of the no-antigen control wells (Δ OD).

3.6 Cortisol ELISA

For the HiP buffaloes, blood cortisol levels were measured in pre- and post-SICCT serum samples using a commercial competitive cortisol ELISA (Kit no. ab108665, Abcam, Cambridge, UK) to determine whether stress associated with capture changed between the two sampling time points, which may ultimately influence the cytokine assay results. Serum samples, ready-to-use standards (0, 10, 50, 150, 500 ng/ml) and negative controls (20 µl each), that were included in the ELISA kit, were added in duplicate to their respective wells in an anti-cortisol IgG coated 96-well microplate. Cortisol-HRP conjugate (200 µl/well) was added to all wells except to the negative control wells. After 1 h incubation at 37˚C, the plates were washed 3 times by hand with a wash buffer and 100 µl TMB substrate solution was added to all wells. Plates were incubated for 15 min in the dark and 100 µl stop solution was added. The OD of all wells was measured at 450 nm. The mean OD value of the negative control was subtracted from each point of the standard curve and each sample. The cortisol concentrations of each sample were determined from the standard curve as described above. The cortisol ELISA was only performed on 18 buffaloes that showed the greatest increase in IFN-ɣ release (group A) from pre- to post-SICCT and 18 animals that showed the greatest decrease in IFN-ɣ release (group B), as measured by Bovigam®.

3.7 Post-mortem examination, mycobacterial culture and speciation

Animals were inspected for visible BTB lesions at post-mortem examination. Retropharyngeal, parotid, tracheobronchial, mediastinal, mesenteric, and mandibular lymph nodes and tonsils were collected from the HiP buffaloes. Retropharyngeal, tracheobronchial and mediastinal lymph nodes were collected from cattle. Tissues were sliced in 2-4 mm sections and examined for the presence of BTB lesions. Tissues with visible BTB lesions were cultured separately while tissues with no visible lesions were pooled for mycobacterial culture.

Mycobacterial culture took place in a biosafety level 3 laboratory at Stellenbosch University, Tygerberg. Approximately 1cm^3 of tissue in 2 ml PBS was homogenised for 15 min using metal beads (4 mm) in a bullet blender (Next Advance, Averill Park, NY, USA). Thereafter, tissue was decontaminated for 15 min with approximately 5 ml MycoPrep^{TM} NACL-NaOH (Becton Dickinson, Franklin Lakes, NJ, USA) with frequent vortexing. Following the addition of approximately 10 ml PBS to neutralise the samples, the mixture was centrifuged at 3000 x g for 15 min. The supernatant was decanted and 500 µl of the pellet and 800 µl BBL MGIT PANTA antibiotic mixture was added to a Mycobacteria Growth Indicator Tube (MGIT), and incubated in a BACTEC MGIT^{TM} 960 Mycobacterial Detection System (all Becton Dickinson) for 42 days. An aliquot from each culture positive sample was taken from the MGIT tubes and fixed on microscope slides, using serum albumin (NHLS, Johannesburg, SA) and heated for 2 h at 80˚C. Ziehl-Neelsen staining for acid-fast bacilli was performed as follows. Slides were flooded with carbol fuchsin (DMP, Johannesburg, SA), heated with a bunsen flame until steaming and left for 5 min. After repeating this step, slides were rinsed with water before being flooded with acid-alcohol (5%) for 2 min. The slides were rinsed again and covered with methylene blue (DMP) for 2 min. Thereafter, slides were rinsed and air dried overnight before being examined under a light microscope for acid-fast bacteria. Aliquots (200 µl) of ZN-positive culture samples were boiled for 30 min at 100˚C before being removed from the biosafety lab. Speciation polymerase chain reactions (PCRs) were conducted, as previously described (Warren et al., 2006), to determine whether the samples were *M. bovis* positive. In short, each 25 µl PCR reaction contained four primer sets containing forward, internal and reverse primers targeting RD1, RD4, RD9 and RD12. Furthermore, Q-buffer (5 µl),

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DNA template (1 µl), 10x buffer (2.5 µl), $MgCl_2$ (2 µl), deoxynucleotides (200 µM each) and HotStarTaq polymerase (0.125 µl) were added to the reaction. PCR cycling were initiated at 95˚C for 15 min, followed by 45 cycles at 94˚C for 1 min, 62˚C for 1 min and 72˚C for 1 min. Thereafter, samples were incubated at 72˚C for 10 min, after which gel electrophoresis was performed on the PCR amplification products at 100 V on a 1.5% agarose gel (Lonza, Montague Gardens, Cape Town, SA) in sodium tetraborate buffer (Sigma Aldrich). Ethidium bromide was used for gel staining and 2 µl 6x loading dye per 5 µl amplicon were used for sample loading. A GeneRuler 100bp plus DNA ladder (Thermo Fisher Scientific) was added to the gel for band size determination. *M. bovis* positive samples had four bands of the following sizes: 108 base pairs (bp), 146 bp, 268 bp and 306 bp.

3.8 Statistical analysis

Data were tested for normality with the D'Agostino & Pearson omnibus normality test. Depending on which 2 groups were compared, a paired t-test or unpaired t-test was performed on data that were normally distributed. For data that were not assumed to follow a Gaussian distribution, the Mann Whitney U-test was performed on unpaired groups and the Wilcoxon signed rank test on those with paired observations. One-way ANOVAs were performed when comparing multiple groups. Data were analysed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, USA). Data were presented as median and interquartile range, since these values are not affected by outliers and non-normal data and also provide a measure of variability for the tested herds.

The diagnostic test evaluation calculator (Medcalc Software, Ostend, Belgium) was used to calculate the confidence intervals of the specificity of diagnostic tests and combinations thereof. McNemar's test (GraphPad Software) were used to calculate the statistical difference between groups of paired observations, whereas Fishers's exact test (GraphPad Software) was used to calculate the difference between independent groups. The Spearman Rank test was used to determine the correlation between assays and Cohen's kappa was used to determine test agreement.

Chapter 4: Results

4.1 Kinetics of immunological responses in naturally *M. bovis*-infected African buffaloes

4.1.1 Temporal changes in IP-10 and IFN-ɣ production

IP-10 and IFN-ɣ concentrations in response to whole blood stimulation in QFT tubes [QFT^{TB} – QFT^{Nil}] were measured by ELISA at various time points to investigate the temporal changes in these cytokines from 3 cohorts of buffaloes, i.e. group 1, chronically Bovigam® positive animals (n = 5); group 2, newly converted Bovigam®-positive animals with 2 consistent positive findings prior to inclusion in the study (n = 5); and group 3, Bovigam®-negative animals that originated from a high BTB prevalence area and were tested at random (n = 8). The median and interquartile range (IQR) of IFN-ɣ levels (pg/ml) from animals in groups 1 to 3 at all captures are shown in Table 1 and Figure 1 and the medians and IQR of IP-10 levels (pg/ml) are shown in Table 2 and Figure 1.

Table 1: Medians and interquartile range (IQR) of IFN-ɣ release (pg/ml) in response to blood stimulation in QFT tubes [QFT^{TB} – QFT^{Nil}].

	Group 1		Group 2		Group 3	
Captures	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
1	455.4	252.9 to 571.8			47.1	19.61 to 230.5
2	590.6	260.8 to 952.2				
3	373	161.5 to 1387				
4	292.5	269.2 to 2081	6.3	0.001 to 239.5		
5	897.4	645.5 to 2299	0.9	-18.8 to 1.5	24.8	3.2 to 777.3
6	637	168.8 to 1299	1.0	-0.8 to 7.6	10.4	-2.5 to 1715

Not done

Table 2: Medians and interquartile range (IQR) of IP-10 release (pg/ml) in response to blood stimulation in QFT tubes [QFT^{TB} – QFT^{Nil}].

	Group 1		Group 2		Group 3	
Captures	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
1	14967	12988 to 17293			7060	2479 to 10098
2	8830	5482 to 16917				
3	7433	4002 to 12033				
4	13501	6282 to 14761	960.6	-111.6 to 1433		
5	12813	7748 to 16166	378.2	-121.4 to 1692	9296	1943 to 18057
6	8025	7636 to 15319	54.34	-858.6 to 312.4	1922	-205.9 to 18901

Not done

In group 1, there were no significant differences ($p > 0.05$; Kruskal-Wallis) in the median IP-10 or IFN-ɣ concentrations [QFT^{TB} – QFT^{Nil}] between any of the 6 captures. All five buffaloes from Group 1 (Table 3) were consistently test-positive at all 6 captures on both the QFT IFN-ɣ (Fig 1a) and QFT IP-10 assays (Fig 1d) which agreed with the positive Bovigam® results for all the animals from this group. There was perfect agreement between all assays (Cohen's kappa = 1.0).

In group 2, there were no significant differences ($p > 0.05$; Kruskal-Wallis) in the median IP-10 or IFN-ɣ concentrations [QFT^{TB} – QFT^{Nil}] between captures 4, 5 or 6. Two of the 5 buffaloes were consistently negative at captures 4 – 6 on both the QFT IFN-ɣ (Fig 1b) and QFT IP-10 (Fig 1e) assays; a third animal (animal # C15) was negative at all 3 time points on the IFN-ɣ assay and at 2 time points on the IP-10 assay (Table 3). Agreement between these 2 assays for group 2 was good (Cohen's kappa = 0.6). However, the overall negative test outcome for group 2 was contradictory to the Bovigam® results, which were consistently positive from 2 captures before their inclusion in the study.

In group 3, there were no significant differences ($p > 0.05$; Kruskal-Wallis) in the median IFN-ɣ (Fig 1c) and IP-10 (Fig 1f) concentrations [QFT^{TB} – QFT^{Nil}] between captures 1, 5 and 6, respectively. The *M. bovis* exposed buffaloes from group 3 were all Bovigam®-negative. However, the test

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outcomes on the QFT IP-10 and QFT IFN-ɣ assays for the 8 animals in this group were inconsistent between captures and between individuals (Table 3). One of the group 3 buffaloes (# 53, Table 3) was euthanised due to severe respiratory compromise and a post-mortem examination was performed. Lesions characteristic of BTB were found and the animal had severe aspiration pneumonia. This animal was consistently QFT IFN-ɣ and IP-10 positive. Agreement between the QFT IP-10 and QFT IFN-ɣ assays for group 3 was poor (Cohen's kappa = 0.2).

The median concentrations of, respectively, IP-10 and IFN-ɣ [QFT^{TB} – QFT^{Nil}] were compared between the 3 groups. There was significantly greater ($p \leq 0.004$; Mann-Whitney U test) IP-10 release in group 1 compared to group 3 at capture 1, and group 1 compared to group 2 at captures 4 and 5; no significant difference ($p > 0.05$; Kruskal-Wallis) was observed at capture 6 between any of the 3 groups. However, on the QFT IFN-ɣ assay, no significant differences ($p > 0.05$; Mann-Whitney U test/ Kruskal-Wallis) in IFN-ɣ release were observed between any of the 3 groups at captures 1, 4, 5, or 6.

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Table 3: Test outcome of the QFT IFN-ɣ and IP-10 assays for animals that were chronically Bovigam®-positive (group 1), newly converted Bovigam®-positive (group 2) and Bovigam®-negative (group 3).

		QFT IP-10 Capture						QFT IFN-ɣ Capture					
	Animal #	1	2	3	4	5	6	1	2	3	4	5	6
Group 1	1	+	+	+	+	+	+	+	+	+	+	+	+
	62	+	+	+		+		+	+	+		+	
	38	+	+	+		+	+	+	+	+		+	+
	57		+	+	+	+	+		+	+	+	+	+
	20	+	+	+	+	+	+	+	+	+	+	+	+
Group 2	26				-						-		
	63				+						+		
	15				-	-	-				-	-	-
	27				-	-	-				-	-	-
	C15				-	+	-				-	-	-
Group 3	61	+						+					
	50	+						-					
	39	+				+		-				-	
	48	-						-					
	47	+				+	-	-				-	-
	59	+						-					
	53	+				+	+	+				+	+
	52	+				-	+	+				-	-

Not done; "+", Test-positive; "-", Test-negative

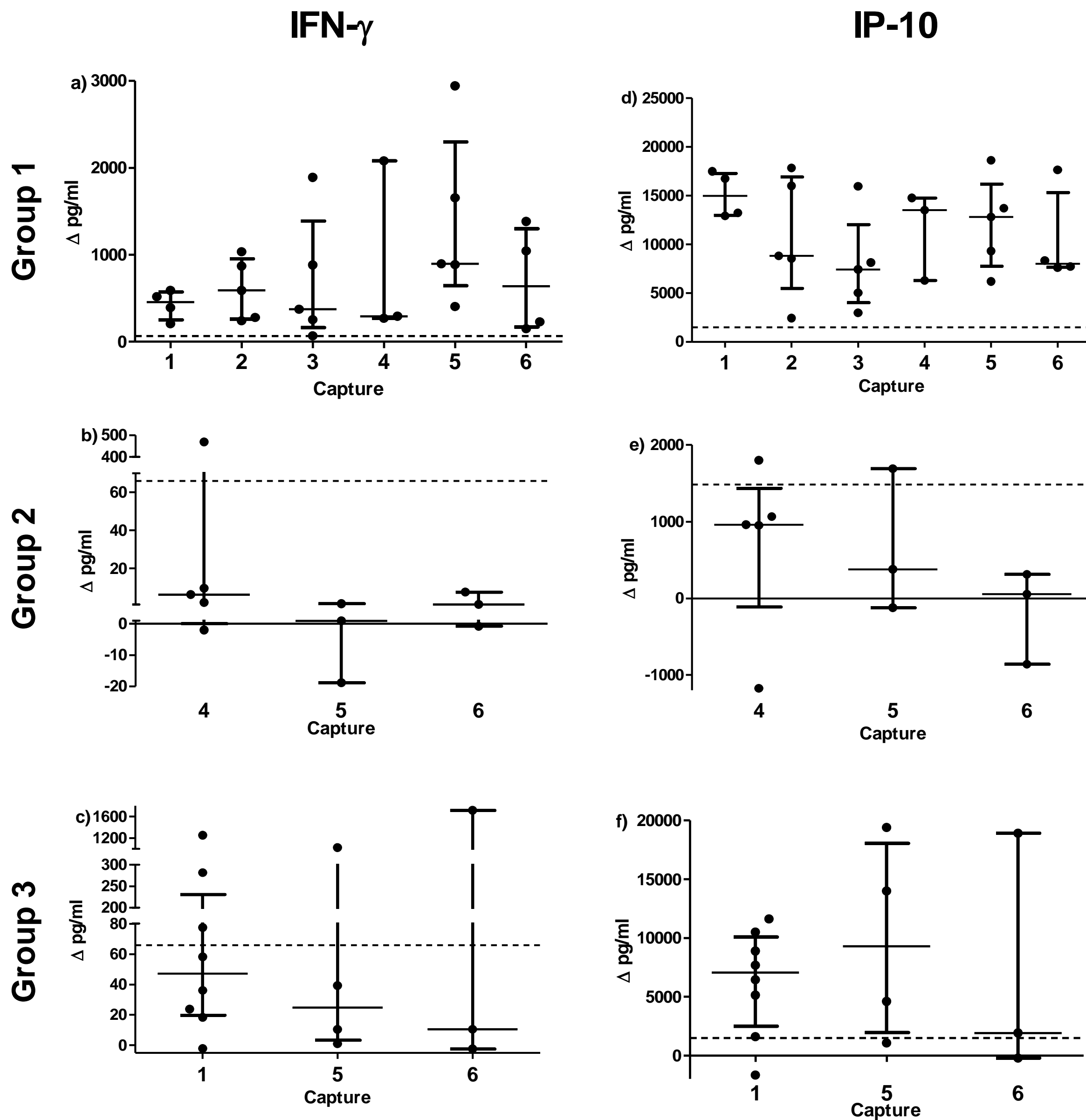

Figure 1: Temporal changes in IFN-ɣ (a, b, c) and IP-10 (d, e, f) concentrations (pg/ml) in response to whole blood incubated in QFT tubes [QFT^{TB} – QFT^{Nil}] for animals from group 1 (a, d), i.e. chronically Bovigam®-positive animals (n = 5), group 2 (b, e), i.e. newly converted Bovigam®-positive animals (n = 5) and group 3, Bovigam®-negative animals (n = 8). Diagnostic cut-off values are indicated as dotted lines. Medians and interquartile ranges are indicated by horizontal bars.

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4.1.2 Temporal changes in the humoral response to *M. bovis*

Antigen-specific antibody responses were measured by ELISA in the serum samples from the 3 groups of buffaloes to determine if there were any changes over time. The median OD values of circulating antibodies of group 1 at captures 1 – 6, of group 2 at captures 4 – 6, and of group 3 at captures 1, 5 and 6 are shown in Table 4 and Figure 2. There were no significant differences ($p > 0.05$, Kruskal-Wallis) in circulating antibody levels between captures in any of the 3 groups (Fig 2). Also, no significant differences ($p > 0.05$, Mann-Whitney U test/ Kruskal-Wallis) in antibody levels were observed between any of the 3 groups at captures 1, 4, 5 or 6.

Table 4: Anti-PPD antibody levels (OD) in serum samples of animals from groups 1, 2 and 3 at 6 captures over a 1 year period.

	Group 1		**Group 2**		**Group 3**	
Captures	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
1	0.5	0.4 to 0.6			0.4	0.3 to 0.8
2	0.5	0.3 to 0.6				
3	0.5	0.3 to 0.54				
4	0.6	0.5 to 0.8	0.6	0.4 to 0.9		
5	0.3	0.3 to 0.5	0.4	0.2 to 0.6	0.5	0.4 to 0.6
6	0.6	0.3 to 0.7	0.4	0.3 to 0.5	0.5	0.3 to 0.7

Not done

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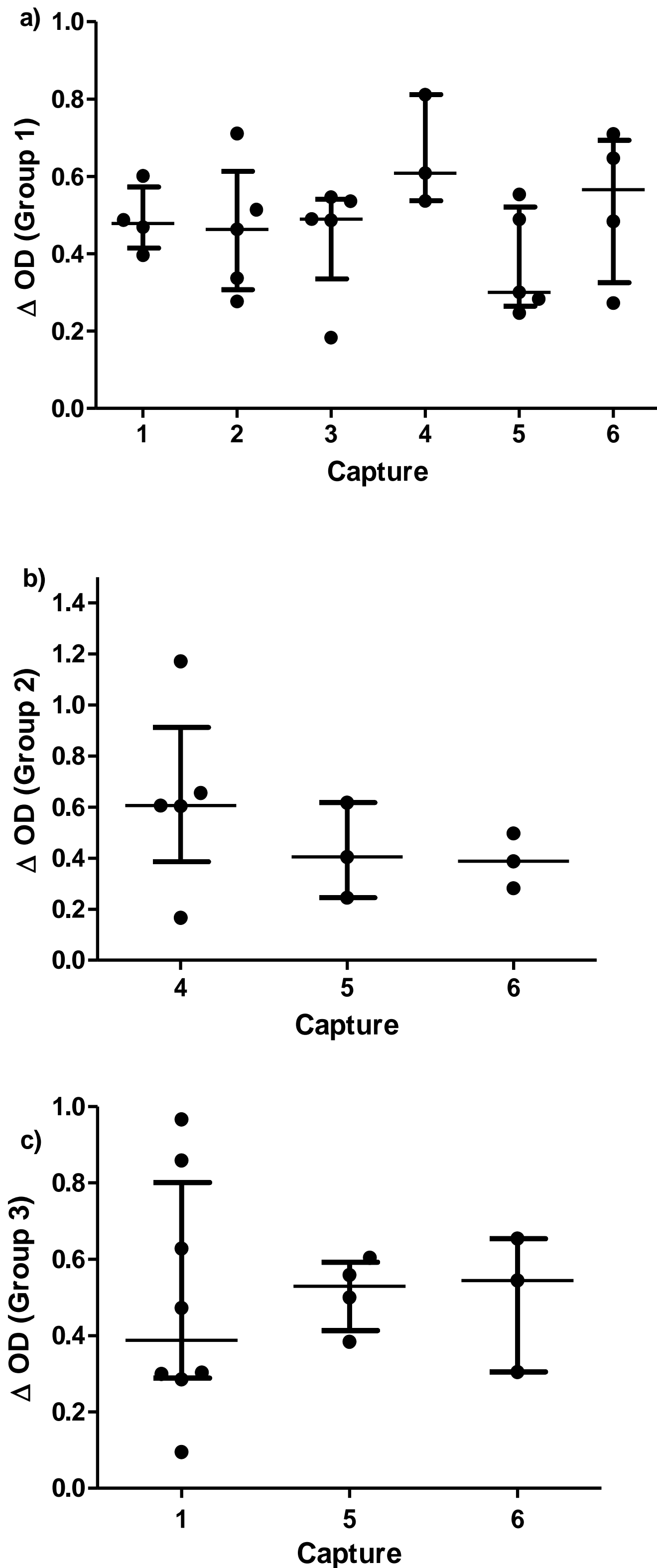

Figure 2: Anti-bPPD serum antibodies measured by ELISA (ΔOD, $OD^{bPPD} - OD^{Negative\ control}$) in buffaloes from a) group 1, b) group 2 and c) group 3 at specific time points. Medians with interquartile range are indicated by horizontal bars.

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4.1.3 Temporal changes in host response to *M. bovis* in chronically infected buffaloes

In order to assess potential changes in antigen-specific responses in buffaloes that were suspected to be chronically infected (group 1), cytokine and antibody responses were evaluated over a 1 year period. Figure 3 shows a comparison of the temporal changes in QFT IFN-ɣ and IP-10 responses (pg/ml) and levels of anti-bovine PPD serum antibodies (Δ OD, $OD^{bPPD} – OD^{Negative\ control}$) for individual animals from group 1 over the study period. Although all 5 buffaloes from this group were positive on the QFT IFN-ɣ, QFT IP-10 and Bovigam® assays at all 6 captures, IP-10 and IFN-ɣ concentrations fluctuated and no clear patterns of cytokine release over time or between animals were observed. Furthermore, antibody levels also fluctuated between captures and animals and no patterns of temporal changes were observed, although 2 animals showed a general increase (Fig 3d, e), 1 showed a decrease (Fig 3c) and 2 animals showed very little change over time (Fig 3a, b). Nevertheless, there was no significant correlation between any of the 3 assays over time ($p > 0.05$; Spearman Rank Test). IP-10 concentrations were much higher than IFN-ɣ concentrations for all animals.

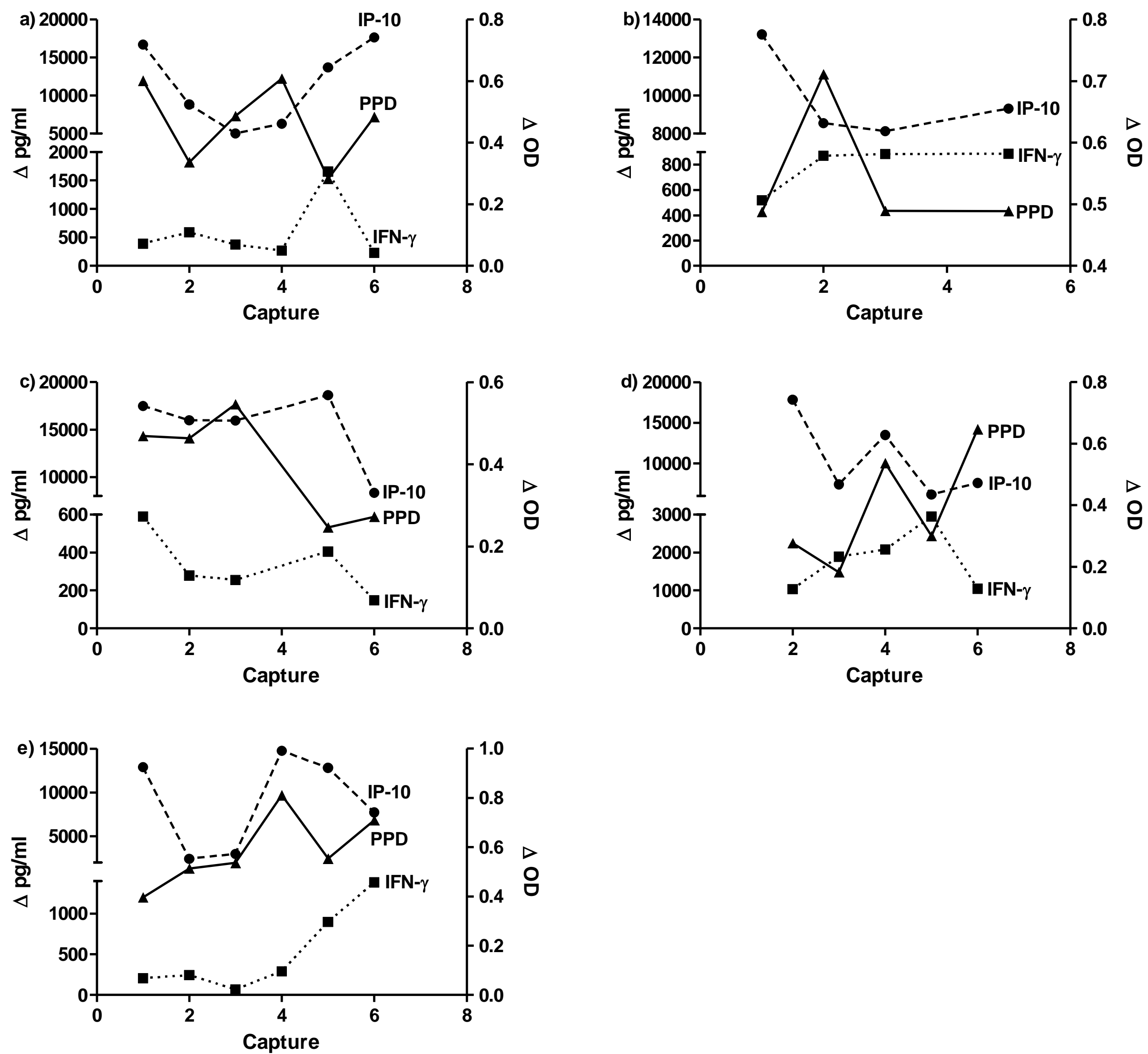

Figure 3: Antigen-specific immune responses over time. IFN-ɣ and IP-10 production [QFT^{TB} – QFT^{Nil}] (pg/ml) and antibody levels to bPPD (Δ OD) in individual animals, i.e. animals # 1 (a), # 62 (b), # 38 (c), # 57 (d) and # 20 (e), from group 1 at captures conducted over a 1 year period are indicated

4.2 Temporal changes in antigen-induced cytokine release in cattle

4.2.1 Group 1: SICT-positive cattle

4.2.1.1 Temporal changes in IFN-ɣ release in response to PPD (Bovigam®) post-SICT

The Bovigam® assay was used to measure the antigen-specific IFN-ɣ release in whole blood from skin test positive cattle at 6 and 41 days post-SICT in response to bPPD and aPPD. At 6 days post-SICT, the median $OD^{bPPD} – OD^{Nil}$ was 2.7 (IQR: 2.4 to 2.8), that of $OD^{aPPD} – OD^{Nil}$ was 2.2 (IQR: 1.2 to 2.6) and of $OD^{bPPD} – OD^{aPPD}$ was 0.5 (IQR: 0.1 to 1.0). At 41 days post-SICT, the medians of $OD^{bPPD} – OD^{Nil}$, $OD^{aPPD} – OD^{Nil}$ and $OD^{bPPD} – OD^{aPPD}$ were 0.6 (IQR: 0.2 to 1.1), 0.2 (IQR: 0.1 to 0.4) and 0.3 (IQR: 0.1 to 0.7), respectively (Figure 4).

The release of IFN-ɣ in response to bPPD ($OD^{bPPD} – OD^{Nil}$; Fig 4a) and aPPD ($OD^{aPPD} – OD^{Nil}$; Fig 4b) was significantly lower ($p < 0.0001$; Wilcoxon matched pairs test) at 41 days post-SICT than at 6 days post-SICT. However, there was no significant difference ($p = 0.14$; Wilcoxon matched pairs test) in the differential response to these antigens ($OD^{bPPD} – OD^{aPPD}$) between the 6 and 41 day time points (Fig 4c). Twenty-four out of 29 (82.7%) animals were Bovigam®-positive at 6 days post-SICT, while 17 of the initial 24, and 5 additional animals (total 22; 75.9%) were positive at 41 days post-SICT. There was no significant difference ($p = 0.77$; McNemar's test) in the proportion of test-positive cattle at 6 days compared to 41 days post-SICT.

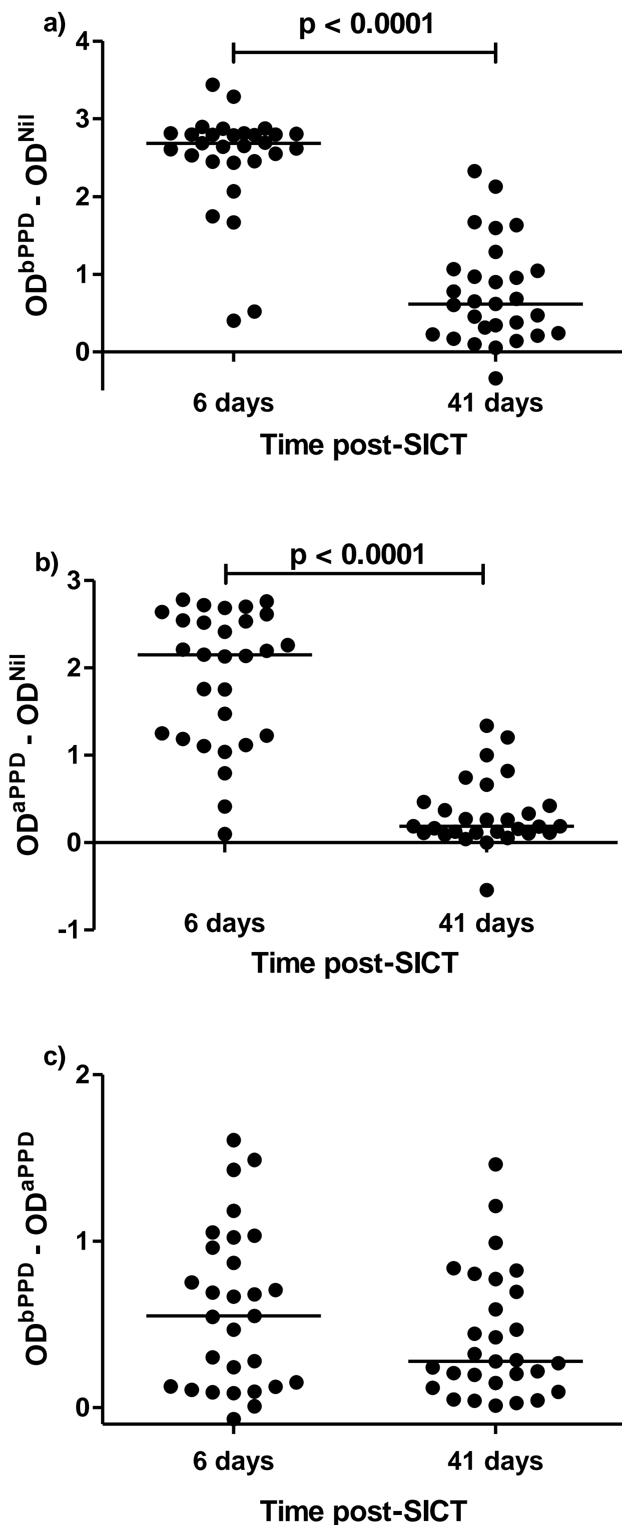

Figure 4: IFN-ɣ release at 6 and 41 days post-SICT in response to bPPD, aPPD and saline (Nil) in SICT-positive cattle (n = 29). There was a significant decrease ($p < 0.0001$; Wilcoxon signed rank test) in IFN-ɣ release for a) $OD^{bPPD} – OD^{Nil}$ and b) $OD^{aPPD} – OD^{Nil}$, but no significant difference between IFN-ɣ release at 6 days and 41 days post-SICT for c) $OD^{bPPD} – OD^{aPPD}$. Horizontal bars represent medians.

4.2.1.2 Temporal changes in QFT test outcomes (IFN-ɣ and IP-10)

IFN-ɣ and IP-10 concentrations (pg/ml) were measured at 6 and 41 days post-SICT in 29 SICT-positive cattle after whole blood stimulation in QFT tubes. The median values for IFN-ɣ [QFT^{TB} – QFT^{Nil}] at 6 and 41 days post-SICT (Fig 5a), were 25.7 pg/ml (IQR: 7.2 to 131.6 pg/ml) and 2.9 pg/ml (IQR: -3.0 to 8.7 pg/ml), respectively. The median concentrations of IP-10 [QFT^{TB} – QFT^{Nil}] (Fig 5b) were 2115 pg/ml (IQR: 345.7 to 6227 pg/ml) and 380.7 pg/ml (IQR: 28.8 to 1273 pg/ml) at 6 and 41 days post-SICT, respectively. Using the Wilcoxon signed rank test, significant decreases in IFN-ɣ ($p < 0.0001$) and IP-10 ($p = 0.0002$) release were observed from 6 to 41 days post-SICT.

Using the QFT IFN-ɣ assay, 10 of 29 (34.5%) cattle tested positive at 6 days post-SICT, whereas none tested positive at 41 days post-SICT (Fig 5a); 7 of the 10 animals that were QFT IFN-ɣ positive, were also Bovigam® positive at the 6 day time point. The difference in the proportion of QFT IFN-ɣ positive animals between 6 and 41 days was significant (0.0044; McNemar's test). Seventeen out of 29 (58.6%) cattle tested positive with the QFT IP-10 assay at 6 days post-SICT whereas 4 of the initial 17 and 2 additional animals (total 6; 20.7%) were positive at 41 days post-SICT (Fig 5b). The decrease in the proportion of IP-10 positive animals between 6 and 41 days was significant ($p = 0.0098$; McNemar's test). The majority of IP-10 positive cattle (14/17 from day 6 and 5/6 from day 41) were also Bovigam® positive. Moreover, 10 animals were positive at 6 days post-SICT on both the QFT IP-10 and QFT IFN-ɣ assays, although none were positive on both tests at day 41.

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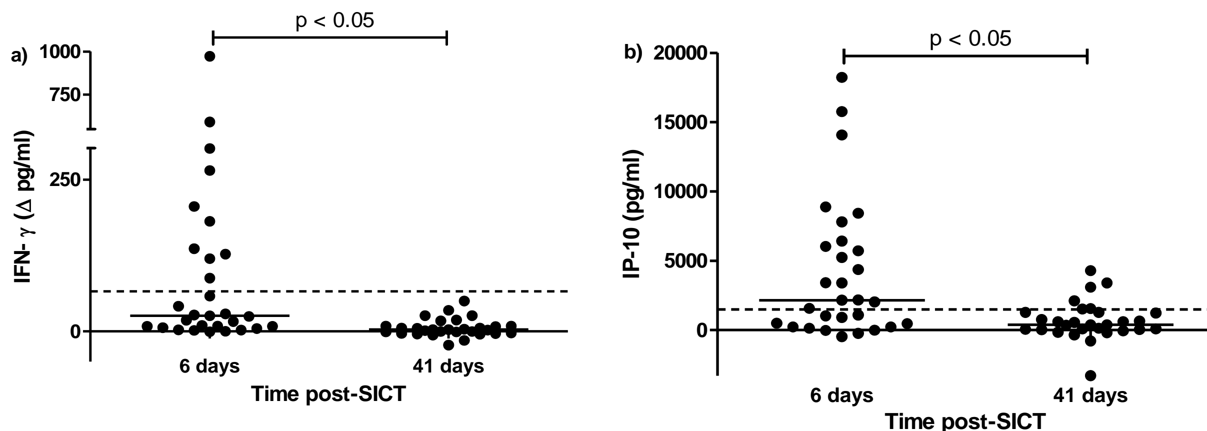

Figure 5: Release of a) IFN-ɣ and b) IP-10 in whole blood from cattle (n = 29) stimulated in QFT tubes [QFT^{TB} – QFT^{Nil}] at 6 and 41 days post-SICT. Horizontal bars represent medians. Diagnostic cut-off values are illustrated as dotted lines. Significant differences are indicated with a bracket and p-value.

4.2.1.3 Post-mortem examination

All 29 of the tested animals were sent to the abattoir. No visible lesions were observed at post-mortem examination; head and thoracic lymph node samples collected for mycobacterial culture were *M. bovis*-culture negative.

4.2.1.4 Diagnostic test specificity

Specificities $\left(\frac{\text{Truly negative}}{\text{Truly negative + False positive}}\right)$ were determined for the 3 assays, i.e. Bovigam®, QFT IFN-ɣ and QFT IP-10, and for combinations thereof (Table 5) using the 29 cattle which were all confirmed *M. bovis*-negative. The number of false positive plus truly negative animals was 29 (all cattle tested). In order to maximise specificity, animals were defined as test-negative if they were negative on 1 or more tests, and test-positive if they were positive on all tests used in the particular combination. At 6 days post-SICT, the combination of all 3 assays and the combination of Bovigam® and QFT IFN-ɣ assays had the highest specificity, but these were not significantly greater (p = 0.25) than the specificity of the QFT IFN-ɣ assay used alone or in combination with the QFT IP-10 assay. These tests, however, had a significantly greater (p < 0.05) specificity than all other assays or assay combinations. At 41 days post-SICT, the QFT IFN-ɣ assay used alone or in combination with any of the other assays had a significantly greater specificity than the QFT IP-10

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(p = 0.04) and Bovigam® (p = 0.0001) assays, but was not significantly greater than that for the combination of Bovigam® and QFT IP-10 assays. Moreover, apart from the Bovigam® assay, all assays and assay combinations showed significantly greater ($p < 0.05$) specificity at 41 days post-SICT compared to 6 days post-SICT. Differences between diagnostic assays were calculated with McNemar’s test (GraphPad Software) and confidence intervals were determined with the diagnostic test evaluation calculator (Medcalc Software).

Table 5: Specificity and 95% CI of diagnostic assays alone or in combination as calculated from group 1 *M. bovis* uninfected cattle (n = 29) at 6 days and 41 days post-SICT.

	6 days post-SICT		**41 days post-SICT**	
Diagnostic assay	**Specificity**	**95% CI**	**Specificity**	**95% CI**
QFT IFN-ɣ	66%	46 - 82%	100%	88 - 100%
QFT IP-10	41%	24 - 61%	79%	60 - 92%
Bovigam®	17%	6 - 36%	24%	10 - 44%
Bovigam® + QFT IP-10	52%	33 - 71%	83%	64 - 94%
Bovigam® + QFT IFN-ɣ	76%	56 - 90%	100%	88 - 100%
QFT IP-10 + QFT IFN-ɣ	66%	46 - 82%	100%	88 - 100%
Bovigam® + QFT IP-10 + QFT IFN-ɣ	76%	56 - 90%	100%	88 - 100%

CI; Confidence interval

4.2.2 Group 2: SICT-positive and SICT-negative cattle cohorts

4.2.2.1 Temporal changes in IFN-ɣ release in response to PPD (Bovigam®) post-SICT

A second cohort of animals from the same herd were included in the study to introduce a SICT-negative subset of animals to examine the effect of the SICT on cytokine release in SICT-negative cattle. The Bovigam® assay was used to measure the IFN-ɣ levels in response to bPPD and aPPD in the SICT-positive and –negative cohorts at 7, 21 and 78 days post-SICT. The median values and IQR for $OD^{bPPD} – OD^{Nil}$, $OD^{aPPD} – OD^{Nil}$ and $OD^{bPPD} – OD^{aPPD}$ at 7, 21 and 78 days post-SICT of SICT-positive cattle (n = 16) are shown in Table 6 and of SICT-negative cattle (n = 17) are shown in Table 7.

Table 6: Median OD values and interquartile range (IQR) of IFN-ɣ levels in response to bPPD, aPPD and the differential response to these antigens for SICT-positive animals at 7, 21 and 78 days post-SICT.

	7 days post-SICT		**21 days post-SICT**		**78 days post-SICT**	
Condition	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
$OD^{bPPD} – OD^{Nil}$	2.2	0.8 to 2.6	2.4	1.8 to 2.9	0.7	0.4 to 1.8
$OD^{aPPD} – OD^{Nil}$	1.4	0.6 to 2.3	1.6	1.0 to 2.7	0.5	0.3 to 1.2
$OD^{bPPD} – OD^{aPPD}$	0.3	-0.03 to 0.9	0.2	-0.006 to 1.1	0.2	0.1 to 0.4

Table 7: Median OD values and interquartile range (IQR) of IFN-ɣ levels in response to bPPD, aPPD and the differential response to these antigens for SICT-negative animals at 7, 21 and 78 days post-SICT.

	7 days post-SICT		**21 days post-SICT**		**78 days post-SICT**	
Antigen	**Median**	**IQR**	**Median**	**IQR**	**Median**	**IQR**
$OD^{bPPD} – OD^{Nil}$	0.5	0.2 to 1.1	0.3	0.07 to 0.9	0.2	0.07 to 0.6
$OD^{aPPD} – OD^{Nil}$	0.5	0.2 to 1.4	0.3	0.08 to 0.9	0.2	0.06 to 0.7
$OD^{bPPD} – OD^{aPPD}$	0.003	-0.1 to 0.13	0.004	-0.2 to 0.04	-0.007	-0.08 to 0.02

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The release of IFN-ɣ in response to bPPD (Fig 6a and b) and in the differential response to bPPD and aPPD (Fig 6e and f), was significantly greater ($p < 0.05$; Mann Whitney U test) in SICT-positive cattle than in SICT-negative cattle at 7, 21 and 78 days post-SICT. However, IFN-ɣ release in response to aPPD only showed a significant difference ($p = 0.003$; Mann-Whitney U test) between the 2 groups at 21 days post-SICT, not at 7 or 78 days post-SICT (Fig 6c and d). Furthermore, using the Wilcoxon signed rank test, there was significantly lower IFN-ɣ release in response to bPPD at 78 days post-SICT compared to 7 ($p = 0.02$) and 21 ($p = 0.009$) days post-SICT in the SICT-positive group, but no significant difference between 7 and 21 days post-SICT (Fig 6a). Similarly, there was significantly ($p = 0.04$) lower IFN-ɣ release in response to aPPD in the SICT-positive cattle at 78 days post-SICT compared to 21 days post-SICT; however, no significant differences were observed between the other time points (Fig 6b). Also, there was no significant difference in IFN-ɣ levels in the differential response to bPPD and aPPD in the SICT-positive cattle between any time points and no significant differences ($p > 0.05$; Wilcoxon matched pair test) were observed in IFN-ɣ release in the SICT-negative groups at any time point.

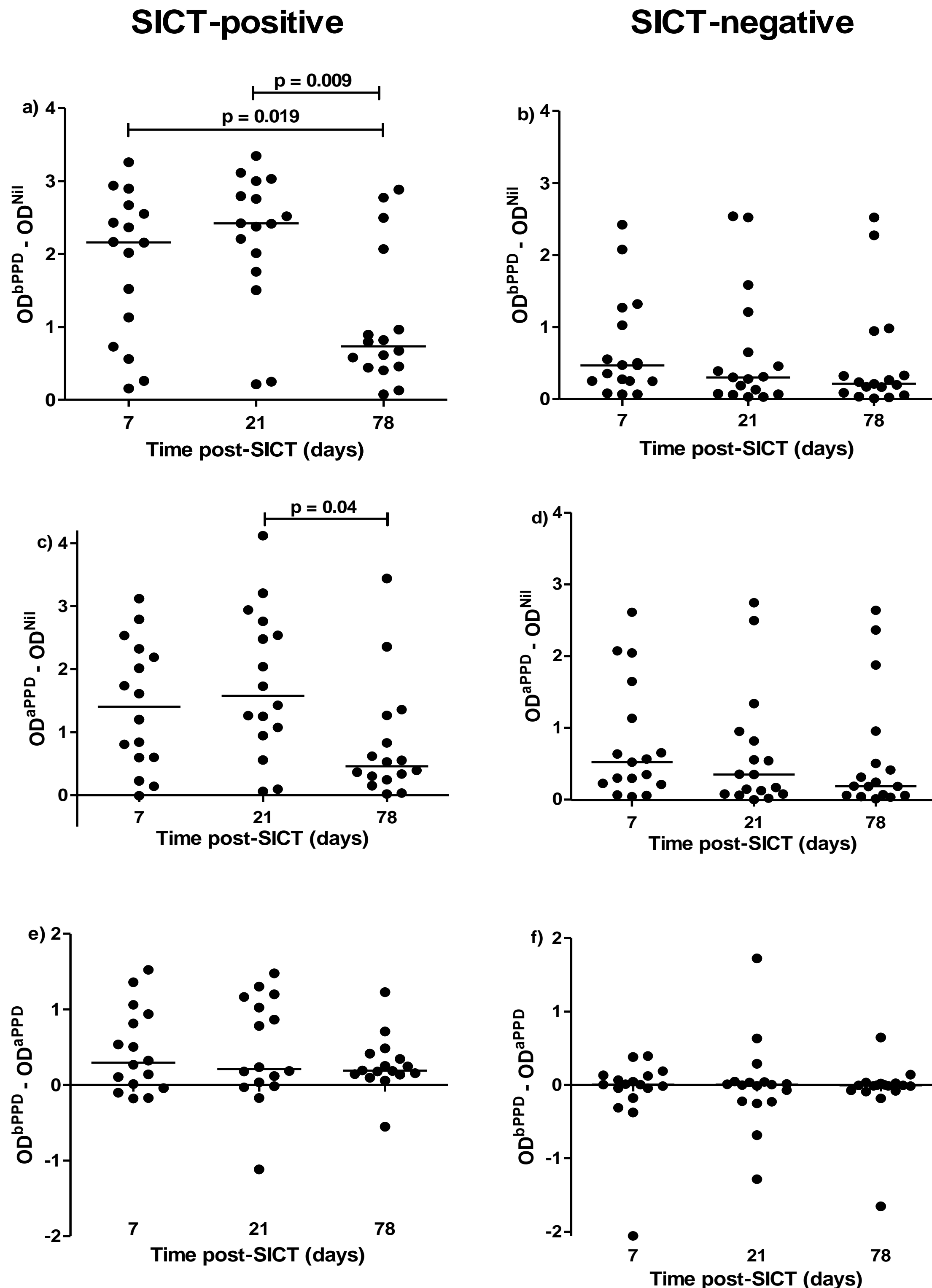

Figure 6: IFN-ɣ release measured with Bovigam® at 7, 21 and 78 days post-SICT in response to whole blood stimulation with bPPD, aPPD and saline (Nil). Graphs present $OD^{bPPD} – OD^{Nil}$ in SICT-positive (a) and –negative groups (b); $OD^{aPPD} – OD^{Nil}$ in SICT-positive (c) and –negative groups (d); and $OD^{bPPD} – OD^{aPPD}$ in SICT-positive (e) and –negative groups (f). There were significant differences ($p < 0.05$; Wilcoxon matched pair test) between groups represented with a bracket and p-value. Horizontal bars represent medians.

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4.2.2.2 Temporal changes in QFT test outcomes (IFN-ɣ and IP-10)

IFN-ɣ and IP-10 concentrations were measured by ELISA after whole blood stimulation in QFT tubes at 7, 21 and 78 days post-SICT. The median IFN-ɣ concentrations [QFT^{TB} – QFT^{Nil}] of SICT-positive cattle at 7, 21 and 78 days were 22.8 pg/ml (IQR: 10.9 to 39.7 pg/ml), 38.18 pg/ml (IQR: 4.5 to 83.5 pg/ml) and 18.9 pg/ml (IQR: 7.1 to 78.2 pg/ml), respectively, and for the SICT-negative cattle, the medians were 28.7 pg/ml (IQR: 4.6 to 219 pg/ml), 18.3 pg/ml (IQR: 5.4 to 115.1 pg/ml) and 41.8 pg/ml (IQR: 4.4 to 371.5 pg/ml), respectively. The median IP-10 concentrations [QFT^{TB} – QFT^{Nil}] of the SICT-positive animals at 7, 21 and 78 days post-SICT were 238.5 pg/ml (IQR: -8.1 to 1366 pg/ml), 936.3 pg/ml (IQR: 41.4 to 2202 pg/ml) and 823.9 pg/ml (IQR: 98.2 to 2288 pg/ml), respectively. There were no significant differences ($p > 0.05$; Wilcoxon matched pair test) in IFN-ɣ or IP-10 release between 7, 21 and 78 days in the SICT-negative and SICT-positive groups, respectively. Also, no significant differences ($p > 0.05$; Mann-Whitney U test) were observed between the SICT-positive and –negative groups at any time point. Moreover, there were a number of responders from both the SICT-positive and SICT-negative groups in the QFT IFN-ɣ and IP-10 assays (Fig 7).

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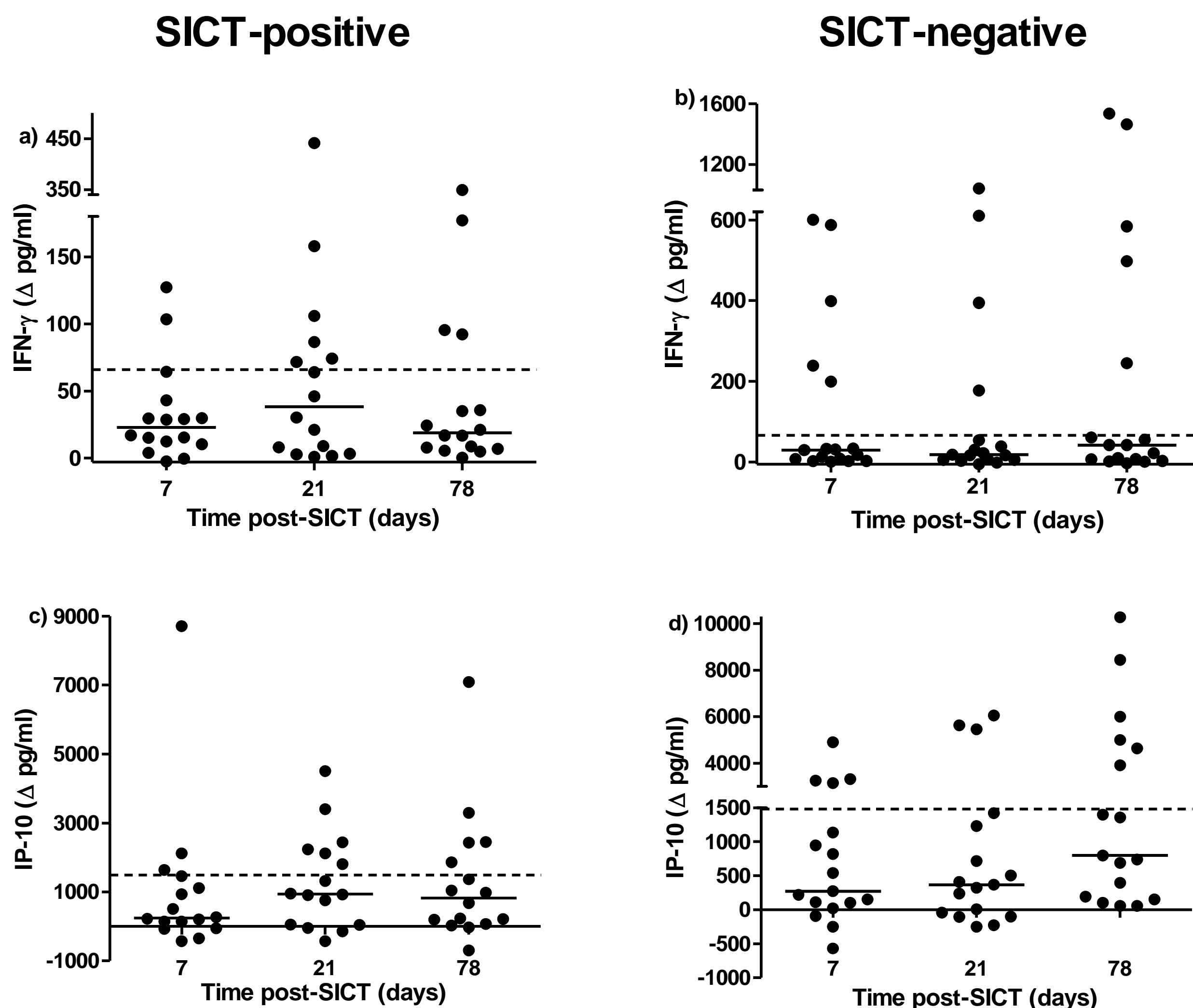

Figure 7: Release of IFN-ɣ in the SICT-positive (a) and –negative (b) cattle, and IP-10 in SICT-positive (c) and –negative (d) animals, in whole blood stimulated in QFT tubes [QFT^{TB} – QFT^{Nil}] at 7, 21 and 78 days post-SICT. Both SICT-negative and SICT-positive groups had responder animals. Diagnostic cut-off values are indicated as dotted lines. Horizontal bars represent median values.

Since the time between 7 and 21 days post-SICT was of limited duration and there was no significant difference in cytokine release between these time points, results from days 7 and 21 were combined to compare these with results from day 78. Animals were defined as QFT-positive if they tested positive at either 7 or 21 days; and QFT-negative if they tested negative at both time points. Of the 33 animals in group 2, 12 (36.4%) cattle tested QFT IFN-ɣ positive at either day 7 or 21; of these 12, only 5 animals tested positive at 78 days post-SICT (Table 8). There was no

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significant difference (p = 0.55) in the proportion of QFT IFN-ɣ positive animals from 7 or 21 days (36.4%) and 78 days post-SICT (27.3%), as determined by McNemars's test. Also, 21 out of 33 (63.6%) animals tested QFT IFN-ɣ-negative at both 7 and 21 days post-SICT and of these, 17 (81%) tested negative at 78 days post-SICT (Table 8). The majority of QFT IFN-ɣ negative cattle at 7 and 21 days remained negative at 78 days post-SICT (81%), providing confidence in the negative result. However, agreement between the 7/ 21 day and 78 day time points was poor (Cohen's kappa = 0.15).

Using the IP-10 assay, 12 of 33 (36.4%) animals tested QFT-positive at 7 or 21 days post-SICT. Of these, only 7 cattle tested positive at 78 days post-SICT (Table 8). Also, similar to the QFT IFN-ɣ results, there was no significant difference (p = 1.0, McNemar's test) in the proportion of QFT IP-10 positive animals from 7 or 21 days (36.4%) and 78 days post-SICT (33.3%). Furthermore, 21 of 33 animals (63.6%) were IP-10 negative at 7 and 21 days post-SICT. Of these, 17 animals (81%) were test-negative at 78 days post-SICT, similar to QFT IFN-ɣ assay results. Agreement between the 7/ 21 day and 78 day time points was low (Cohen's kappa = 0.32).

Table 8: QFT IFN-ɣ and QFT IP-10 test outcomes at 7 or 21 days and 78 days post-SICT of cattle that were test positive at any time point on either QFT IFN-ɣ or IP-10 assays

Animal #		QFT IFN-ɣ 7/21 days	QFT IFN-ɣ 78 days	QFT IP-10 7/21 days	QFT IP-10 78 days
1	SICT-positive	+	-	-	-
2	SICT-positive	+	-	+	+
3	SICT-positive	+	-	-	-
4	SICT-positive	+	-	+	-
5	SICT-positive	+	+	+	+
6	SICT-positive	+	+	+	+
7	SICT-positive	-	+	-	-
8	SICT-positive	-	+	-	-
9	SICT-positive	-	-	+	-
10	SICT-positive	-	-	-	+
11	SICT-positive	-	-	+	+
12	SICT-positive	-	-	+	-
13	SICT-positive	-	-	+	-
14	SICT-negative	+	+	+	+
15	SICT-negative	+	+	+	+
16	SICT-negative	+	+	+	+
17	SICT-negative	+	-	-	-
18	SICT-negative	+	-	+	-
19	SICT-negative	+	-	-	-
20	SICT-negative	-	+	-	+
21	SICT-negative	-	+	-	+
22	SICT-negative	-	-	-	+

"+", Test-positive; "-", Test-negative

4.2.2.3 SICCT outcome

Single intradermal comparative cervical tuberculin tests (SICCTs) were performed at 78 days post-SICT on both the SICT-positive and SICT-negative cattle groups at the time of blood collection. Only 4 of 16 animals from the original SICT-positive group were SICCT-positive (25%) and none from the SICT-negative group. The number of SICCT-positive animals was significantly greater ($p = 0.04$; Fisher's exact test) in the SICT-positive group than in the SICT-negative group. Furthermore, there were significantly greater changes in SFT (Δ SFT) in the SICT-positive group compared to the SICT-negative group (Fig 8a) at the bPPD ($p < 0.0001$; Mann-Whitney U test) and aPPD ($p = 0.004$; Mann-Whitney U test) sites, respectively. Also, the median SFT at the bPPD site

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(median = 4.8 mm; IQR: 3.3 to 6.1) was significantly greater ($p = 0.0005$; Wilcoxon matched pair test) than at the aPPD site (median = 1.9 mm; IQR: 0.9 to 3.2) in the SICT-positive group. Moreover, the differential increase in SFT between the bPPD and aPPD sites ($SFT^{bPPD} - SFT^{aPPD}$) (Fig 8b) was significantly greater ($p < 0.0001$) in the SICT-positive group (median = 2.55 mm; IQR: 1.5 to 4.3) than in the SICT-negative group (median = 0 mm; IQR: -0.7 to 1.2), as determined by the Mann-Whitney U test.

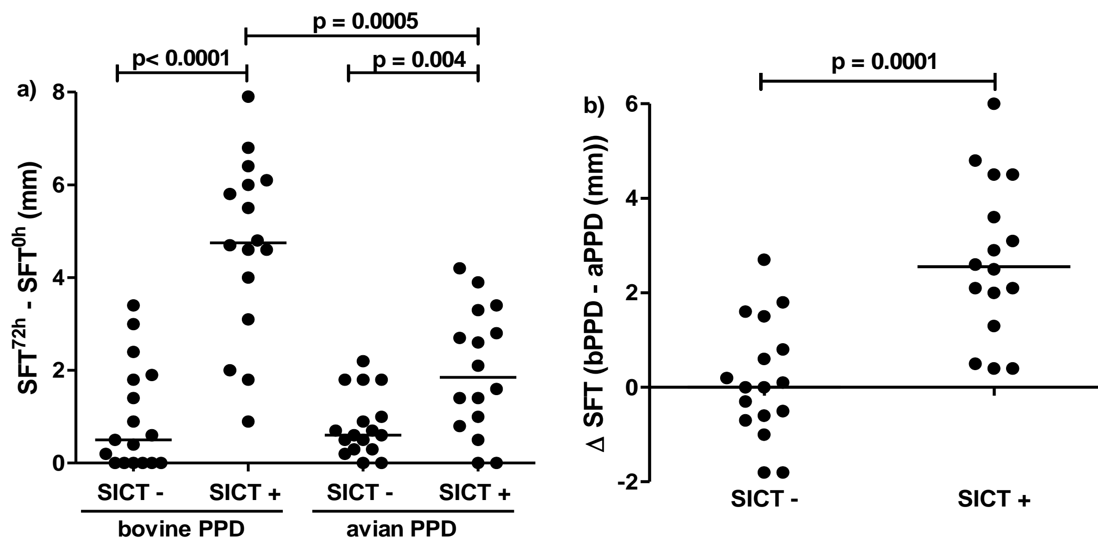

Figure 8: Change in skin fold thickness (SFT; mm) between 0 to 3 days post-SICCT in response to bPPD and aPPD in SICT-positive and SICT-negative cattle (a). Significant differences, as determined by Mann-Whitney U test, are indicated by bars and p-values. Significant differences ($p < 0.05$; Wilcoxon signed rank test) were observed in the differential response to bPPD and aPPD ($SFT^{bPPD} - SFT^{aPPD}$) (b). Horizontal bars represent medians.

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4.3 Changes in antigen-induced cytokine release in African buffaloes following the SICCT

4.3.1 Animals

The release of IP-10 and IFN-ɣ in response to Bovigam® antigens was measured pre-SICCT (day 0) and post-SICCT (day 3) to investigate the effect of the SICCT on cytokine production and test outcome of *in vitro* diagnostic assays in buffaloes (n = 286). Animals were divided in groups according to their day 0 test outcome. Group 1 consisted of 55 buffaloes that tested positive on any Bovigam® ELISA, i.e. the PPD, PC-HP or PC-EC assays, regardless of the SICCT result. Group 2 served as a negative control group and consisted of 21 buffaloes that tested negative on all Bovigam® assays and on SICCT. Group 3 consisted of five buffaloes that were SICCT-positive, but Bovigam®-negative.

All buffaloes from groups 1 and 3 were slaughtered, tissue samples were cultured and speciation was performed. Thirty of 55 group 1 buffaloes (55%) and 2 of 5 group 3 buffaloes (40%) were *M. bovis*-positive on culture. Due to limited stimulated blood for some animals, all assays were not performed on all animals.

4.3.2 Effect of the SICCT on absolute IFN-ɣ concentrations in response to PPD and PC-HP

The change in IFN-ɣ release in response to bPPD, aPPD and PC-HP from pre- to post-SICCT was measured by the Mabtech IFN-ɣ ELISA in 55 animals from group 1 and 21 buffaloes from group 2. The median IFN-ɣ concentrations (pg/ml) in response to these antigens for animals from group 1 are presented in Table 9, and from group 2 in Table 10.

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Table 9: Median concentrations (pg/ml) and interquartile range (IQR) of IFN-ɣ release in response to Bovigam® antigens pre- and post-SICCT for buffaloes from group 1, i.e. Bovigam®-positive on any assay pre SICCT.

	Pre-SICCT		**Post-SICCT**	
Condition	**Median**	**IQR**	**Median**	**IQR**
bPPD – Nil	903.7	191.2 to 2191	395.7	148.9 to 996.9
aPPD – Nil	79.54	37.58 to 204	71.6	29.4 to 158
bPPD – aPPD	785.6	181.6 to 2050	287.9	79.8 to 796.6
HP – Nil	559.2	96.9 to 1628	171.4	38.67 to 370.9

Table 10: Median concentrations (pg/ml) and interquartile range (IQR) of IFN-ɣ release in response to Bovigam® antigens pre- and post-SICCT for buffaloes from group 2, i.e. pre-SICCT negative on all Bovigam® assays and SICCT.

	Pre-SICCT		**Post-SICCT**	
Antigen	**Median**	**IQR**	**Median**	**IQR**
bPPD – Nil	44.02	0 to 107.2	43.14	2.6 to 71
aPPD – Nil	57.5	0 to 80.5	45.5	22.8 to 75.9
bPPD – aPPD	0	-22.4 to 33.5	-10.8	-26.7 to 8.6
HP – Nil	0	0 to 12.52	0.4	0 to 18.3

Using the Wilcoxon signed rank test, a significant decrease ($p < 0.0001$) in IFN-ɣ release in response to bPPD (Fig 9a) and in the differential response to bPPD and aPPD (Fig 9c) was observed from pre- to post-SICCT for animals from group 1. However, there were no significant differences ($p = 0.09$) in the median IFN-ɣ concentration between these time points in response to aPPD (Fig 9b). Furthermore, a significant decrease ($p < 0.0001$) in response to PC-HP was observed from pre- to post-SICCT (Fig 9d). For group 2, no significant differences ($p > 0.05$) in IFN-ɣ release between pre- and post-SICCT were observed.

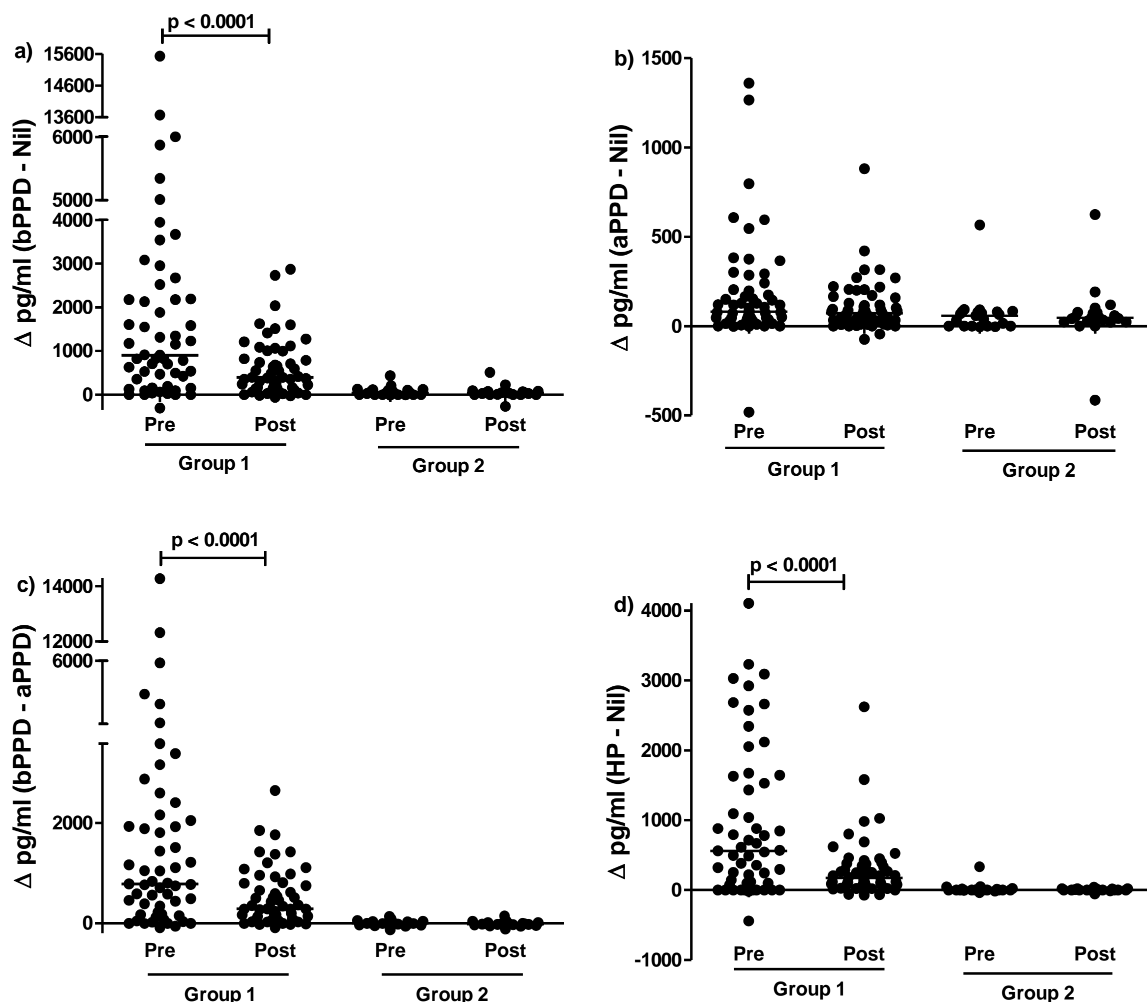

Figure 9: IFN-ɣ release (pg/ml) in response to a) bPPD, b) aPPD, c) bPPD – aPPD and d) PC-HP as measured by Mabtech IFN-ɣ ELISA pre- and post-SICCT in groups 1 and 2. Differences between pre- and post-SICCT time point concentrations were compared using the Wilcoxon signed rank test. Horizontal lines represent medians.

4.3.3 Effect of the SICCT on the Bovigam® test outcome

Forty-nine buffaloes from group 1, 21 buffaloes from group 2 and 5 buffaloes from group 3 were tested by the Bovigam® assay to measure the change in IFN-ɣ release from pre- to post-SICCT in response to bPPD, aPPD and PC-HP and PC-EC peptides, respectively. Median OD values of IFN-ɣ levels in response to these antigens for group 1 are shown in Table 11 and for group 2 in Table 12. For group 3, median OD values of IFN-ɣ in response to bPPD at pre- and post-SICCT were 0.09 (IQR: 0.06 to 0.2) and 0.4 (IQR: 0.16 to 1.4), respectively, and that of the differential

response to bPPD and aPPD at pre- and post-SICCT, were 0.08 (IQR: 0.04 to 0.09) and 0.3 (IQR: 0.1 to 1.1), respectively.

Table 11: Median OD values and interquartile range (IQR) of IFN-ɣ release in response to Bovigam® antigens pre- and post-SICCT for buffaloes from group 1, i.e. Bovigam®-positive at pre-SICCT time point.

	Pre-SICCT		**Post-SICCT**	
Condition	**Median**	**IQR**	**Median**	**IQR**
OD^{bPPD} - OD^{Nil}	0.9	0.22 to 1.9	0.5	0.15 to 0.96
OD^{bPPD} - OD^{aPPD}	0.8	0.2 to 1.6	0.38	0.04 to 0.84
OD^{aPPD} – OD^{Nil}	0.08	0.03 to 0.2	0.06	0.02 to 0.19
OD^{HP} – OD^{Nil}	0.3	0.09 to 1.0	0.18	0.002 to 0.3
OD^{EC} – OD^{Nil}	0.36	0.1 to 0.96	0.1	0.01 to 0.3

Table 12: Median OD values and interquartile range (IQR) of IFN-ɣ in response to Bovigam® antigens pre- and post-SICCT for buffaloes from group 2, i.e. Bovigam® and SICCT-negative.

	Pre-SICCT		**Post-SICCT**	
Antigen	**Median**	**IQR**	**Median**	**IQR**
OD^{bPPD} - OD^{Nil}	0.01	-0.002 to 0.05	0.04	0.02 to 0.1
OD^{bPPD} - OD^{aPPD}	0.002	-0.01 to 0.02	-0.02	-0.04 to 0.03
OD^{aPPD} – OD^{Nil}	0.009	0 to 0.03	0.05	0.02 to 0.18
OD^{HP} – OD^{Nil}	-0.004	-0.01 to 0.01	0.004	-0.01 to 0.02
OD^{EC} – OD^{Nil}	-0.002	-0.01 to 0.006	0.005	-0.03 to 0.02

Using the Wilcoxon signed rank test for group 1, significant decreases in IFN-ɣ levels in response to bPPD (OD^{bPPD} – OD^{Nil}) ($p = 0.0008$; Fig 10a) and in the differential response to bPPD and aPPD (OD^{bPPD} – OD^{aPPD}) ($p = 0.0006$; Fig 10c) were observed from pre- to post-SICCT. However, there was no significant difference ($p = 0.5$; Fig 10b) in IFN-ɣ release in response to aPPD (OD^{aPPD} - OD^{Nil}) between these time points (Fig 9b). Furthermore, IFN-ɣ levels produced in response to PC-HP (OD^{HP} – OD^{Nil}; Fig 11a) and PC-EC (OD^{EC} – OD^{Nil}; Fig 11b) decreased significantly ($p < 0.0001$) from pre- to post SICCT. In groups 2 and 3, no significant differences ($p > 0.05$; Wilcoxon

signed rank test) in IFN-ɣ levels in response to any Bovigam® antigen were observed between these time points.

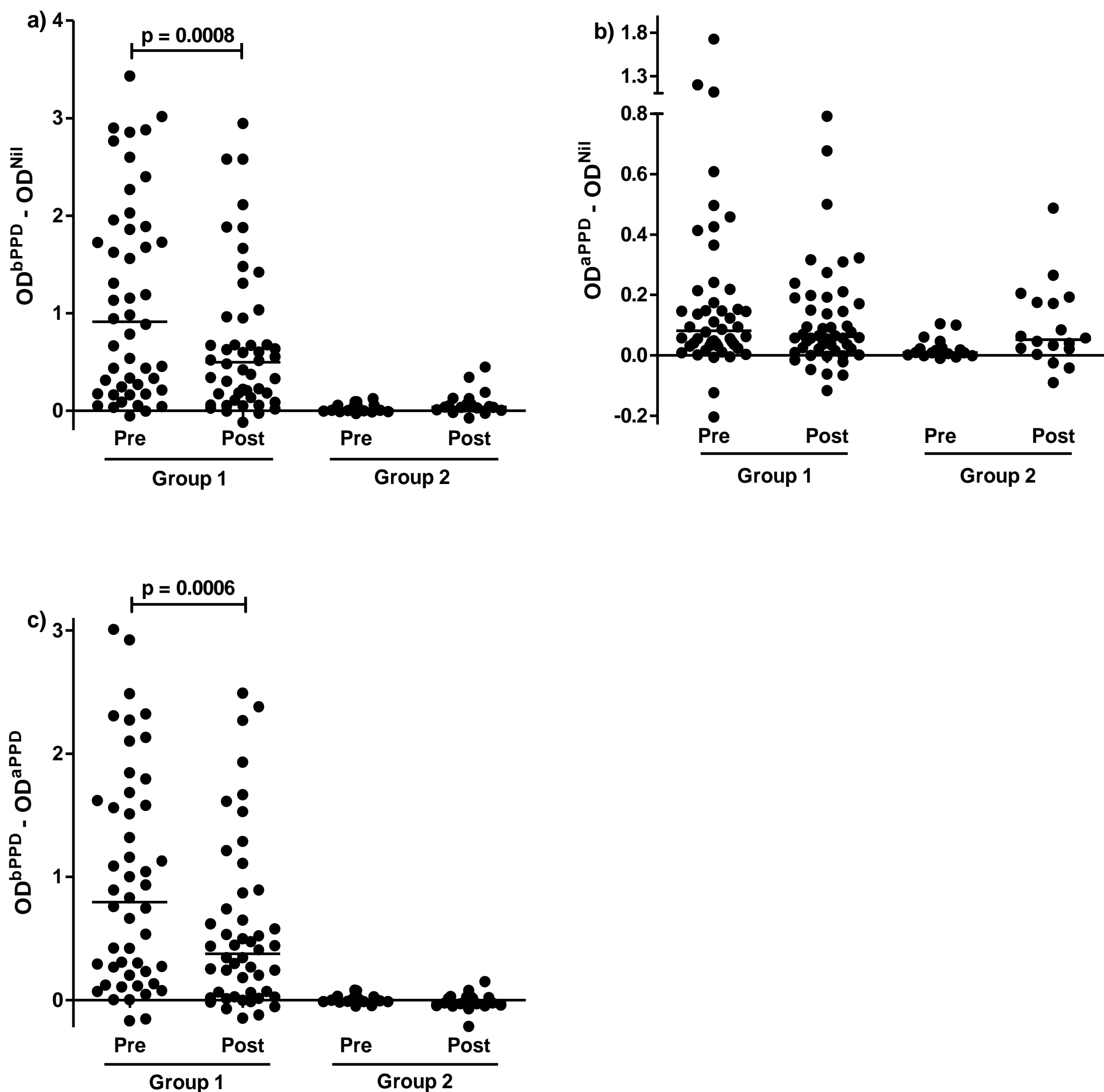

Figure 10: IFN-ɣ release in response to a) bPPD ($OD^{bPPD} – OD^{Nil}$), b) aPPD ($OD^{aPPD} – OD^{Nil}$) and c) the differential response to these antigens ($OD^{bPPD} – OD^{aPPD}$) pre-SICCT and post-SICCT as measured by the Bovigam® assay in animals from group 1, i.e. Bovigam®-positive at the pre-SICCT time point, and group 2, i.e. Bovigam® and SICCT-negative. Differences in median OD were compared using Wilcoxon matched pair test. Horizontal bars represent medians.

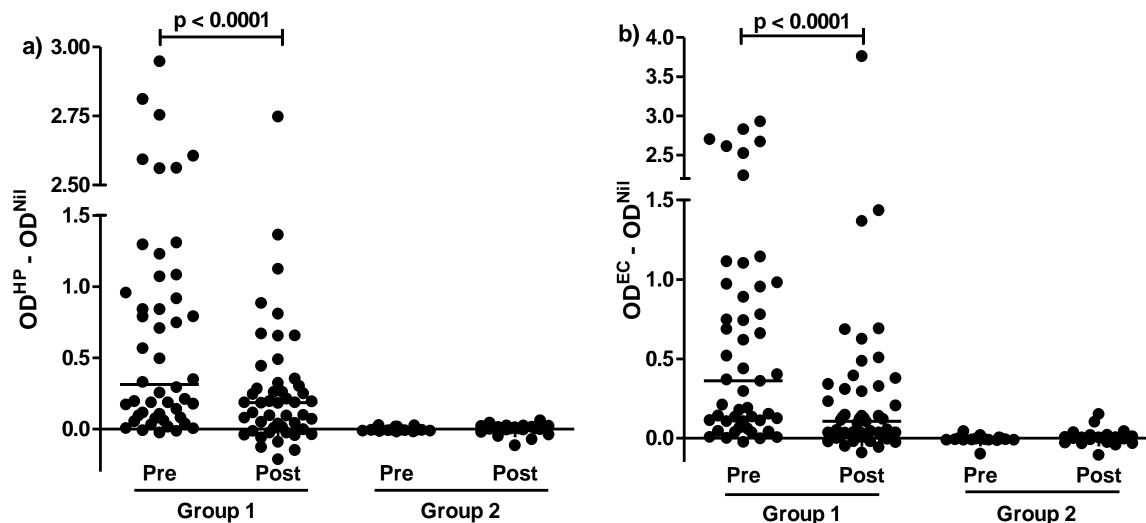

Figure 11: IFN-ɣ release in response to a) PC-HP ($OD^{HP} - OD^{Nil}$) and b) PC-EC ($OD^{EC} - OD^{Nil}$) pre-SICCT and post-SICCT as measured by Bovigam® in animals from group 1, i.e. pre-SICCT Bovigam®-positive, and group 2, i.e. Bovigam® and SICCT-negative. Differences in median OD were compared using Wilcoxon matched pair test. Horizontal bars represent medians. Diagnostic cut-off values, i.e. 0.1, are indicated by the dotted line.

The proportions of test-positive and –negative animals from group 1 pre- and post-SICCT are summarised in Table 13. Significantly greater proportions of animals tested positive pre-SICCT compared to post-SICCT on the Bovigam® PPD ($p = 0.046$) and PC-EC assays ($p = 0.0036$), but not with the Bovigam® PC-HP assay ($p = 0.08$), as determined by McNemar's test. Post-SICCT test outcomes of group 2 showed that 1 of 21 (4.8%) buffaloes were Bovigam® PPD positive, none were PC-HP positive and 2 of 21 (9.5%) buffaloes were PC-EC positive. However, there were no significant changes ($p > 0.05$; McNemar's test) in test outcome (Bovigam® PPD, PC-EC and PC-HP assays) between pre- and post-SICCT for group 2. In group 3, 4 of 5 buffaloes (80%) were Bovigam® PPD-positive post-SICCT and 3 of 5 (60%) animals were positive on the PC-HP and PC-EC assays, respectively. These 3 animals were the same individuals on both assays. The proportion of test-positive animals was, however, not significantly different ($p > 0.05$; McNemar's test) between the 2 time points.

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Table 13: Proportions of test-positive and –negative buffaloes pre- and post-SICCT from group 1 as determined by Bovigam® PPD, PC-EC and PC-HP assays.

	Test-positive		**Test-negative**	
Bovigam® assay	**Pre-SICCT**	**Post-SICCT**	**Pre-SICCT**	**Post-SICCT**
PPD	40/48 (83%)	33/48 (69%)	8/48 (17%)	15/48 (31%)
PC-EC	38/49 (78%)	25/49 (51%)	11/49 (22%)	24/49 (49%)
PC-HP	35/48 (73%)	27/48 (56%)	13/48 (27%)	21/48 (44%)

Relative sensitivities were calculated as the proportion of Bovigam®-positive animals from those that were *M. bovis*-positive and/ or had BTB-like lesions with a score of 2 (several small foci or a single large lesion) or 3 (multifocal or confluent lesions) (W. Goosen pers. comm.).

For the Bovigam® PPD assay, there was no significant change (p = 0.2; McNemar's test) in relative sensitivity from pre-SICCT (96.7%; CI: 82.8 – 99.9%) to post-SICCT (86.7%; CI: 69.3 – 96.2%). Similarly, there were no significant differences (p = 0.4; McNemar's test) in relative sensitivity between pre-SICCT (83.3%; CI: 65.3 – 94%) and post-SICCT (73.3; CI: 54.1 – 87.7%) for the Bovigam® PC-HP assay or between pre-SICCT (83.9%; CI: 66.3 – 94.6%) and post-SICCT (64.5%; 45.4 – 80.8%) for the Bovigam PC-EC assay (p = 0.08; McNemar's test).

4.3.4 Change in IP-10 production and test outcome from pre-SICCT to post-SICCT

IP-10 concentrations in response to PC-EC peptides were measured pre- and post-SICCT in 49 buffaloes from group 1 and 17 animals from group 2. The median IP-10 concentrations for animals from group 1 pre- and post-SICCT were 8171 pg/ml (IQR: 2063 to 10044) and 3135 pg/ml (1105 to 7964), respectively. The median IP-10 concentrations for animals from group 2 pre- and post-SICCT were 261 pg/ml (-266.4 to 656.2) and 84.55 pg/ml (-92.3 to 385), respectively. In group 1, IP-10 concentration decreased significantly (p = 0.0005) from pre- to post-SICCT (Fig 12), but no significant difference (p = 0.7) was observed between these time points for group 2, as determined by the Wilcoxon signed rank test.

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Using a diagnostic cut-off value of 2258 pg/ml (W. Goosen, pers. comm.), 37 of 49 (75.5%) buffaloes from group 1 were IP-10 positive pre-SICCT, of which 30 buffaloes plus no additional animals (61.2%) tested positive post-SICCT. The proportion of IP-10 positive animals was significantly greater pre-SICCT than post-SICCT (p = 0.02; McNemar's test). No animals from group 2 were IP-10 positive pre- or post-SICCT. The relative sensitivity of the IP-10 assay decreased significantly (p = 0.04; McNemar's test) from pre-SICCT (96.9%; CI: 83.8 – 99.9%) to post-SICCT (78.1%; CI: 60 – 90.7%). This was calculated as the proportion of IP-10 positive animals that were culture positive and/or had visible lesions with a score of 2 (several small foci or a single large lesion) or 3 (multifocal or confluent lesions, as previously described (Palmer et al., 2007)

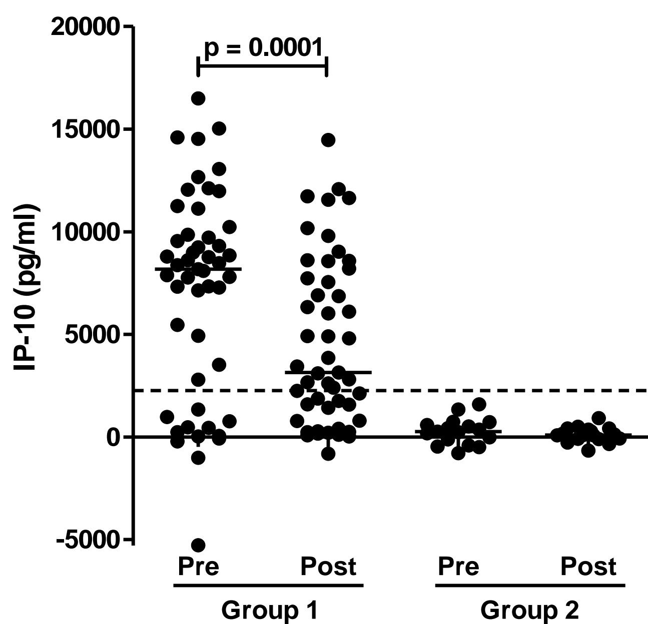

Figure 12: IP-10 release (pg/ml) in response to PC-EC pre- and post-SICCT in groups 1 and 2. A significant difference (p < 0.0001; paired t test) between pre- and post-SICCT was observed in group 1. The diagnostic cut-off value, i.e. 2258 pg/ml, is indicated by the dotted line. Horizontal lines represent medians.

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4.3.5 Change in cortisol release from pre- to post-SICCT

Cortisol levels in serum samples were measured by a commercial competitive ELISA at the pre-SICCT and post-SICCT time points in 14 buffaloes that showed the greatest increase (Group A) and 18 buffaloes that showed the greatest decrease (Group B) in IFN-ɣ production from pre- to post-SICCT on the Bovigam® assay. The mean cortisol concentrations (ng/ml) pre- and post-SICCT of group A were 540.1 (standard deviation, SD: 439.2) and 595.8 (SD: 470.9), respectively, whereas the mean cortisol concentrations of group B pre- and post-SICCT were 758.6 (SD: 692.6) and 599.4 (SD: 563.2), respectively. No significant differences ($p > 0.05$; paired t test) in cortisol concentrations were observed between the pre- and post-SICCT time points in group A and group B (Fig 13). Also, there were no significant differences ($p > 0.05$; unpaired t test) in cortisol concentrations between group A and group B at either time points (Fig 13).

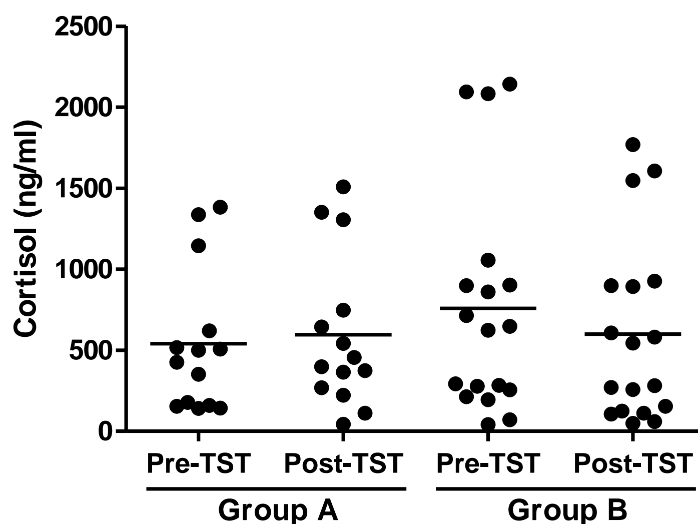

Figure 13: Cortisol concentration (ng/ml) as measured by a commercial competitive ELISA pre- and post-SICCT for buffaloes with an increase (Group A; n = 18) and a decrease (Group B; n = 18) in IFN-ɣ production on the Bovigam® assay. Horizontal lines represent means.

Chapter 5: Discussion

5.1 Progression of immunological responses in naturally *M. bovis*-infected African buffaloes

Animals that displayed chronic Bovigam®-positivity (group 1) showed good agreement between test outcomes for the Bovigam®, QFT IFN-ɣ and QFT IP-10 assays. In contrast, animals which showed recent conversion to Bovigam®-positivity (group 2) and those classified as Bovigam®-negative (group 3), showed poor agreement between Bovigam® and QFT test outcomes.

Interestingly, all the chronically Bovigam®-positive buffaloes were consistently QFT IP-10 and QFT IFN-ɣ positive at all six captures for the one year period, whereas the majority of newly Bovigam®-positive animals were consistently QFT IP-10 and QFT IFN-ɣ negative. Also, the magnitude of IFN-ɣ and IP-10 release did not change significantly over time in either group. Other studies reported a significant increase in IFN-ɣ release between two to eight weeks after experimental infection of cattle, which remained elevated until the end of the 20 – 22 week study duration, at which point the cattle had developed BTB-like lesions (Buddle et al., 1995; Pollock and Andersen, 1997b; Rhodes et al., 2000b, 2000c). Also, IFN-ɣ release in response to bPPD and ESAT-6 was similar throughout the infection period (Rhodes et al., 2000a, 2000b, 2000c; Vordermeier et al., 2002; Waters et al., 2010). Since the buffaloes from group 1 were already Bovigam®-positive before inclusion in the study, we do not know when an increase in IFN-ɣ release in response to PPD and ESAT-6 occurred. However, similar to the cattle studies, this group remained IFN-ɣ and IP-10 positive throughout the study. Group 1 buffaloes probably did not have advanced BTB, since it has been shown that IFN-ɣ release decrease at advanced stages of disease in chronically *M. bovis*-infected cattle; in some cases this may result in a negative test result (Welsh et al., 2005). The results from group 2 did not agree with the cattle studies. These buffaloes were Bovigam® PPD positive for approximately four months prior to inclusion in the study and for another six months during the study, but remained QFT-negative. Since the cattle studies showed that there was no significant difference in IFN-ɣ release in response to ESAT-6 or bPPD, we speculate that the difference in response between the two antigens may be due to exposure or infection with environmental mycobacteria. Environmental mycobacteria have been shown to cause a decrease in specificity of

the Bovigam® PPD assay (Buddle et al., 2009; Thom et al., 2004; Wood and Jones, 2001). However, ESAT-6 and CFP-10 have been used to increase specificity of CRAs, since the RD1 genes encoding these proteins are deleted from most environmental mycobacteria, with a few exceptions (Pollock and Andersen, 1997a; Thom et al., 2004; Waters et al., 2004). Therefore, with a lower specificity, the Bovigam® assay could be identifying greater numbers of false-positive animals, whereas the QFT result may reflect the true result for this group, i.e. *M. bovis*-uninfected. Another possible explanation for the group 2 results is that the buffaloes had a low level infection and the response to the ESAT-6 and CFP-10 antigens were present, but not at sufficient levels to exceed the threshold of detection in the assay.

The majority of animals from the Bovigam®-negative group (group 3) were QFT IFN-ɣ negative, but QFT IP-10 positive. Therefore, both tests that measured IFN-ɣ resulted in a negative test result, whereas the assay that measured IP-10 resulted in a positive test result. IP-10 is typically upregulated by IFN-ɣ and occurs in significantly greater concentrations, about 100-fold greater, in stimulated blood compared to IFN-ɣ (Chakera et al., 2011; Chegou et al., 2014; Ruhwald et al., 2009). Therefore, if the buffaloes were in the early stage of *M. bovis*-infection, antigen-specific T-lymphocyte numbers could be low, which would result in low IFN-ɣ production. However, even at low levels, IFN-ɣ can result in the activation of IP-10 release that is high enough to be detected by ELISA (Goosen et al., 2014). Therefore, we speculate that this group may have been early *M. bovis*-infected animals, which could result in detection of IP-10 but low (undetectable) levels of IFN-ɣ. However, as described in cattle studies, significant increases in cytokine release were observed two to eight weeks post-infection (Buddle et al., 1995; Pollock and Andersen, 1997b; Rhodes et al., 2000b, 2000c). We would therefore expect to have seen a response during the 12 month period in which they were sampled. A possible explanation for this discrepancy is that cattle in the previously mentioned studies were experimentally infected, which may have resulted in exposure to greater numbers of *M. bovis* organisms than the buffaloes from our field study. This would lead to IFN-ɣ responses at an earlier time point post infection than in a natural system. This hypothesis is supported by findings of Rhodes et al. (2000c), in which cattle that were experimentally infected with very low doses of *M. bovis* showed no significant increase in IFN-ɣ

release by 20 weeks post-infection. It may therefore indicate that variation in time for antigen-specific cytokine responses to take place after infection may be related to mycobacterial load and disease development.

The immune response to *M. bovis* typically involves a dominating T helper type 1 (Th1) CMI response in early stages of infection, which is associated with high levels of IFN-ɣ release. As disease progresses, a more dominant T helper type 2 (Th2) response develops, associated with a decreased CMI response (decreased IFN-ɣ release) and an increase in the humoral response (Palmer et al., 2006; Pollock et al., 2005; Welsh et al., 2005; Wood and Jones, 2001). Welsh et al. (2005) further showed that antibody responses developed shortly (approximately 2 weeks) after the initial CMI response in the early stages of infection, followed by a decrease in IFN-ɣ release in response to bPPD, and increased antibody levels as disease progressed in cattle. However, our study showed that there was no significant change in cytokine release or antibody levels during the 1 year study period in any of the three groups of buffaloes. Also, in contrast to the cattle studies, there was no significant correlation between QFT IFN-ɣ and QFT IP-10 release and antibody levels for chronically infected buffaloes (group 1) over time. Furthermore, the IFN-ɣ and IP-10 responses fluctuated between animals and over time, as observed by others (Rhodes et al., 2000b; Waters et al., 2006). One buffalo from group 3 was euthanised due to respiratory compromise and lesions characteristic of BTB were found at necropsy. This buffalo was QFT-positive, but Bovigam®-negative. This may have been caused by a high response to aPPD due to exposure or infection with environmental mycobacteria, resulting in a false-negative Bovigam® PPD test result. Although this animal had gross evidence of disease, there was no significant change in IFN-ɣ and IP-10 release or in circulating antibody levels over time. QFT IFN-ɣ and QFT IP-10 assay results did, however, remain positive and antibody levels were similar to the median ODs of all three groups. These findings did not agree with Welsh et al. (2005) that showed that antibody levels correlated with the development of pathology in experimentally infected cattle, while IFN-ɣ was a poor indicator of pathology at advanced stages of disease (Welsh et al., 2005). In our study, the QFT assays correctly identified the buffalo with BTB pathology, whereas antibody levels remained unchanged. Other studies also found that circulating antibody levels to bPPD remained low and

unchanged for the duration of disease development, even though the cattle had BTB lesions on post-mortem examination (Buddle et al., 1995). From our study, we believe that a one year period may not be long enough to see significant changes in antibody and cytokine release in a naturally *M. bovis* exposed group of Bovigam®-positive buffaloes. However, since the Bovigam®-positive buffaloes had detectable CMI responses throughout the study period, but not strong antibody responses, and QFT assays correctly identified the buffalo with BTB, we recommend that diagnostic assays based on the CMI response, rather than the humoral response, should be used for the early diagnosis of BTB in buffaloes, which is supported by findings of other studies (de la Rua-Domenech et al., 2006; Palmer et al., 2006; Welsh et al., 2005). Furthermore, using both PPD and ESAT-6 antigens in diagnostic assays for disease surveillance would improve the sensitivity and specificity to correctly identify test-positive animals, even when environmental mycobacteria are present or infection levels are low (Parsons et al., 2011; Wood and Jones, 2001). Also, including assays that measure IP-10 release would allow detection of buffaloes at earlier stages of disease than with IFN-ɣ assays.

There were some limitations in this study. Most importantly was the small sample size of all three groups used in this study, which may have influenced our results in investigating the temporal changes in immune responses in these animals. Naturally *M. bovis*-infected or exposed buffaloes were used for the study. Therefore, we did not know how long these animals had been infected. Also, since there were no necropsy or culture results for these animals, we were unable to determine the true infection status of the animals. However, the presence of BTB lesions in one study buffalo confirmed the presence of disease in the herd.

In summary, there was no significant change in IFN-ɣ or IP-10 release or in circulating antibody levels for the duration of the study, even in the case of confirmed BTB disease. A one-year study period appeared to be an insufficient duration to investigate changes in immune responses in Bovigam®-positive buffaloes. Results from chronically Bovigam®-positive animals (i.e. animals suspected to have chronic infection) showed good agreement between the QFT IFN-ɣ, QFT IP-10 and Bovigam® assays. However, results from the group of animals that recently converted to Bovigam®-positivity showed poor agreement between the Bovigam® and QFT assays, which may

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have been associated with exposure to environmental mycobacteria. Finally, results from group 3 buffaloes (Bovigam®-negative) showed poor agreement between the IP-10 and IFN-ɣ assays, which could be attributed to greater IP-10 sensitivity at early stages of infection. Based on our limited findings, serial testing with consistent positive responses may increase confidence in determining true infection status in buffaloes, although changes in cytokine levels may vary over time. Further studies are needed to understand trends in cytokine release over time in naturally infected buffaloes.

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5.2 Temporal changes in antigen-induced cytokine release in cattle

In SICT-positive cattle, high levels of PPD-specific (bPPD and aPPD) IFN-ɣ release occurred 1 – 3 weeks following the SICT and declined thereafter, suggesting immunological boosting by the skin test. Our results share similarities with Whipple et al. (2001) who reported a boost of IFN-ɣ in response to bPPD and aPPD between 3 and 28 days post-CFT, which declined thereafter. In contrast, Palmer et al. (2006) showed that the boosting effect lasted only between 3 and 7 days post-CFT, after which IFN-ɣ levels decreased. Furthermore, our results were similar to studies that reported a decline in IFN-ɣ release from 3 days post-SICCT (Whelan et al., 2004) and between 1 and 28 days post-SICCT (ÓNualláin et al., 1997). However, it seems more likely that the SICT caused a boost in IFN-ɣ release, followed by a decrease, since results from study cohort 2 showed an increase in IFN-ɣ release from 7 to 21 days post-SICT (not significant) followed by a significant decline in IFN-ɣ release by 78 days post-SICT. However, our result differed from studies that showed that the TST caused a boost in IFN-ɣ until 59 days post-CFT (Rothel et al., 1992; Wood and Rothel, 1994) or had no effect on IFN-ɣ production (Coad et al., 2010; Doherty et al., 1995; Gormley et al., 2004; Ryan et al., 2000). Moreover, Thom et al. (2006) reported a more pronounced IFN-ɣ boost in response to aPPD than in bPPD up to 7 days post-SICCT in *M. bovis*-positive cattle. In our study, the SICT-negative cattle were unaffected by the SICT, which agrees with other studies (Palmer et al., 2006; Thom et al., 2006).

Although we used animals from the same cattle herd, there were conflicting results between our two study cohorts when investigating the effect of the SICT on IFN-ɣ and IP-10 release in response to specific antigens, ESAT-6 and CFP-10. In the SICT-positive cattle from the first study cohort, high levels of IFN-ɣ were released 6 days after the SICT, which declined significantly by 41 days, again suggesting immunological boosting by the skin test. Our results were similar to other studies that reported a decline in IFN-ɣ release in response to ESAT-6 and CFP-10, from 7 days post-CFT (Palmer et al., 2006) and 3 days post-SICCT (Whelan et al., 2004). Our results differed from studies that found that the SICCT had no effect on IFN-ɣ production in response to ESAT-6 and CFP-10 (Coad et al., 2007; Thom et al., 2006). However, this observation was consistent with results from the second study cohort, which showed that there was no immunological boosting of

IFN-ɣ or IP-10, following the SICT, in the SICT-positive and negative groups. To our knowledge, this was the first study to investigate the effect of the SICT on IP-10 production in cattle.

The boosting effect of the SICT appears to affect cytokine production differently in response to specific antigens (ESAT-6, CFP-10) compared to PPDs. The number of QFT-positive, but not the Bovigam®-positive, animals decreased as the effect of the SICT diminished significantly by 41 days post-SICT. Also, by 41 days post-SICT, cattle which were culture-negative for *M. bovis* were all QFT IFN-ɣ negative, 6/29 were IP-10 positive, but 22/29 were Bovigam®-positive, indicating the high specificity of the QFT assays, especially the QFT IFN-ɣ assay, at this time point post-SICT. In fact, we found that the relative specificities of the QFT IFN-ɣ and QFT IP-10 assays, used alone or in combination, increased significantly by 41 days following SICT.

In the second study cohort, however, the SICT had no significant effect on test outcome and there was no significant difference in cytokine release between the SICT-positive and –negative groups. In addition, cytokine test-positive cattle were detected in both groups. Unfortunately, it is unknown whether there were any truly infected animals in the second study cohort, according to the gold standard, since they were not slaughtered. Possible explanations for the detection of CRA-positive animals in the SICT-negative group could be due to variation in the amount of time individuals may have been exposed or infected, or number of SICTs performed in these individuals. Test-positive cattle could also have been early-infected animals, since significant IFN-ɣ reactions can be detected by 15 days after *M. bovis*-infection, whereas a TST response is generally seen three to six weeks after infection (de la Rua-Domenech et al., 2006; Monaghan et al., 1994; Palmer et al., 2006).

The conflicting results between the two study cohorts and between the QFT and Bovigam® results may be due to exposure or infection with environmental mycobacteria, as indicated by the high aPPD response measured by Bovigam® in both study cohorts. Animals that are exposed or infected with environmental mycobacteria may also show a positive response to bPPD, since many of the antigens that are present in bPPD are shared between environmental mycobacteria and *M. bovis* (Pollock et al., 2005; Schiller et al., 2010). Pollock and Anderson (1997a) also demonstrated that cattle sensitised to environmental mycobacteria reacted to bPPD and aPPD, but not to ESAT-

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6 (Pollock and Andersen, 1997a). Therefore, the QFT assay, which incorporates ESAT-6 and CFP-10, should be able to distinguish between *M. bovis*-infections and environmental mycobacterial infections. We speculate that the immunological boosting effect that was seen in the Bovigam® assays following the SICT, may have been in response to sensitisation by environmental mycobacteria, rather than a response to *M. bovis* infection. This hypothesis is supported by the large number of false-positive cattle detected by Bovigam® compared to the significantly lower number of QFT-positive cattle at 41 days post-SICT. The SICT also identified large numbers of false-positive animals, especially in the first study cohort, where all 29 cattle were *M. bovis*-culture negative, but SICT-positive. Since the SICT only use bPPD, and not aPPD, it does not allow differentiation between responses to environmental mycobacteria compared to responses to *M. bovis,* which would reduce specificity (Coad et al., 2010; Palmer et al., 2006).

The large number of SICT-positive animals could also be due to repeated skin testing in this herd. Sensitisation to tuberculin following repeated skin tests has been reported in other studies (de la Rua-Domenech et al., 2006; Monaghan et al., 1994). The cattle herd used in our study was skin tested once per year, which is a much greater time interval than the recommended time of 60 days between TSTs (Coad et al., 2010; Ryan et al., 2000; Thom et al., 2006). Since the QFT assays are not affected by environmental mycobacteria, these results may be the more accurate representation of the test outcome of the assays. This explanation is supported by results from Palmer et al. (2006), which found that IFN-ɣ release was significantly lower in response to ESAT-6 and CFP-10 when compared to bPPD in *M. bovis*-uninfected cattle following the TST.

In order to minimise culling of false-positive cattle in herds with low BTB prevalence, it is important to have a diagnostic strategy that incorporates high specificity. The Bovigam® assay, which has reportedly high sensitivity (Wood and Jones, 2001), and the SICT, with its decreased specificity in herds exposed to environmental mycobacteria, were not suitable in this herd with low BTB incidence and suspected high environmental mycobacterial burden, since it identified large numbers of false-positive cattle. However, the QFT assays, especially the QFT IFN-ɣ assay, were more suitable for BTB control in this herd, since they had greater specificity and correctly identified all the animals from the first study cohort as test-negative on the QFT IFN-ɣ assay, with only a

small proportion of false-positive cattle on the IP-10 assay at day 41. Therefore, our results suggest that when performing serial testing, it is recommended to collect whole blood samples for the QFT IFN-ɣ assay at 41 days post-SICT, when the effect of the SICT has significantly decreased, to identify truly uninfected animals sooner than waiting to retest animals with the SICCT at 60 days (Coad et al., 2010; Palmer et al., 2006). Also, using the QFT IFN-ɣ assay at 41 days post-SICT, rather than earlier, may minimise culling of false-positive cattle, which would be economically advantageous and provide a more accurate assessment of the SICT reactors. However, the 41 day post-SICT testing does not support the recommended time frame of 3 to 30 days post-TST to collect blood for confirmatory *in vitro* assays (Palmer et al., 2006).

Since the QFT assays appeared to have greater specificity, it is possible that the boosting effect of the SICT on these assays may be more useful in distinguishing truly infected from false-positive cattle. We investigated whether results from the 7 and 21 day time points of the QFT assays, which were combined as one time point, could be used to predict the outcome at 78 days post-SICT. We found that the results of 7/21 day time points from of the QFT IFN-ɣ and QFT IP-10 assays were poor indicators of positive test outcomes at 78 days post-SICT, since only 42% and 58% of the QFT IFN-ɣ and QFT IP-10, respectively, remained positive at 78 days post-SICT. However, the 7/21 day time point results could accurately be used to predict a negative test outcome at 78 days post-SICT, since 81% of the animals remained test-negative at both time points.

We would therefore recommend that the animals that were QFT-positive at 78 days post-SICT, especially the QFT-IFN-ɣ positive animals, should be regarded as suspect for BTB and be retested with the QFT assay at least 41 days following any skin testing. This is based on the QFT results from the first study cohort, which showed that the effect of the SICT diminished significantly by 41 days following the SICT, and the Bovigam® results of the second study cohort in which the test outcome was still affected by 21 days post-SICT. However, by 78 days post-SICT, the results did not appear to be significantly affected by the SICT. Therefore, the test results from this later time point should more accurately reflect the infection status of the individual.

One of the limitations of this study was that the initial categorisation of cattle in the study cohorts was based on the SICT, not the SICCT. Results from the subsequent SICCT in the second study

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cohort showed that the cattle were exposed to, or infected with environmental mycobacteria, since the response to aPPD was high. Only 4 cattle from the initial SICT-positive group of the second study cohort (n = 16) were SICCT-positive. SICCTs can distinguish between *M. bovis* infected animals and those that are exposed to, or infected with environmental mycobacteria, since the response to aPPD is considered in the test criteria (Gormley et al., 2006; Palmer et al., 2006; Thom et al., 2006). The change in SFT was significantly greater in the SICT-positive group compared to the negative group in response to both aPPD and bPPD; in addition, the SICCT-positive animals all came from the SICT-positive group. Therefore, the SICT did identify high responding (i.e. suspect) animals, which could be further tested using the SICCT. Applying the SICCT to only SICT-positive animals would decrease the cost and time of testing the entire herd with the SICCT, which requires two injections. However, the SICCT has been shown to be more specific (Buddle et al., 2009; Parsons et al., 2011), and therefore, it may decrease the number of cattle that may need further testing using CRAs.

Another limitation of this study was that cytokine release was only measured starting approximately 1 week following the SICT. Future research should include measurements of cytokine release pre-SICT and at scheduled time points after the SICT to determine the relative degree and duration of the boosting effect of skin tests on CRAs. Other limitations of the study include a lack of culture results for the second study cohort to determine true infection status, and lack of information on exposure to environmental mycobacteria.

In summary, the SICT appeared to cause a boost in IFN-ɣ release in response to bPPD and aPPD 1 – 3 weeks following the SICT, which decreased significantly thereafter. However, the IFN-ɣ response may not be *M. bovis*-specific, but could reflect a response to environmental mycobacteria. In this herd with low BTB prevalence, but suspected high levels of environmental mycobacteria, a test or test combination with high specificity would be recommended for future herd surveillance. The QFT assays, but not the SICT or the Bovigam® assay, appeared to provide more accurate results for cattle in this herd, due to high specificity and ability to differentiate between *M. bovis* infection and sensitisation to environmental mycobacteria. In addition, our results

support a recommendation to collect blood for CRAs around 41 days or later, to minimise the effect of the SICT on cytokine production.

5.3 Changes in antigen-induced cytokine release in African buffaloes following the SICCT

In Bovigam®-positive African buffaloes, there was a significant decrease in IFN-ɣ release in response to bPPD, and PC-HP and PC-EC peptides, but not to aPPD, 3 days following the SICCT. However, the SICCT did not affect IFN-ɣ release in Bovigam®/SICCT-negative buffaloes, which agrees with findings from a cattle study (Palmer et al., 2006). Moreover, our findings were similar to cattle studies that reported a significant decline in IFN-ɣ release in response to bPPD by 3 days post-SICCT (Whelan et al., 2004), between 3 and 7 days post-CFT (Rothel et al., 1992; Wood and Rothel, 1994) and 1 – 28 days post-SICCT (ÓNualláin et al., 1997). However, contrary to our findings, Thom et al. (2006) found that the SICCT only affected IFN-ɣ release in response to aPPD, but not to bPPD. Furthermore, PC-HP and PC-EC peptides contain *M. bovis*-specific antigens, ESAT-6 and CFP-10, and similarly to our study, Whelan et al. (2004) reported a decrease in IFN-ɣ release by 3 days post-CFT in response to these antigens in cattle. However, our study did not agree with studies that found the TST had no effect on IFN-ɣ release in response to ESAT-6 and CFP-10 (Coad et al., 2007; Thom et al., 2006). We also found significantly lower IP-10 release in response to PC-EC 3 days after the SICCT in Bovigam®-positive, but not in Bovigam®/ SICCT-negative animals. To our knowledge, this was the first study that investigated the effect of the SICCT on IP-10 release in buffaloes. Following the SICCT, changes in IP-10 release showed similar trends to those observed with IFN-ɣ. Furthermore, Bovigam®-positive animals and an increase in IFN-ɣ release in response to PPD, PC-HP or PC-EC were observed post-SICCT in a cohort of buffaloes (n = 5) that were SICCT-positive, but Bovigam®-negative pre-SICCT (group 3) However, this change was not significant. This has also been reported in other studies that found that the SICCT detected additional *M. bovis*-positive animals that were Bovigam®-negative (Gormley et al., 2006; Schiller et al., 2010; W. Goosen, pers. comm.). In group 1, the changes in IFN-ɣ and IP-10 release were large enough to result in a significant decrease in the proportion of Bovigam® PPD, Bovigam® PC-EC and IP-10 (PC-EC) positive buffaloes 3 days post-SICCT.

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However, the relative test sensitivities of the Bovigam® assays did not change significantly after the SICCT were performed, although the relative sensitivity of the IP-10 assay decreased significantly.

Since the TST was associated with a significant decrease in the number of IFN-ɣ and IP-10 positive animals from pre-SICCT to 3 days post-SICCT, it is possible that the number of false-negative buffaloes may increase after the SICCT using CRAs. This is especially apparent in the IP-10 assay, in which the relative test sensitivity decreased significantly at the second test point. Therefore, for BTB testing of buffaloes from this herd, we recommend that blood samples for the IFN-ɣ and IP-10 CRAs be collected at the time the SICCT is performed, rather than when the SICCT response is measured at 3 days. This would also allow identification and removal of greater numbers of test-positive animals, which would also improve BTB management in this location (HiP), especially since BTB prevalence is very high in certain areas, with reports of up to 54.7% in some herds (Jolles et al., 2005; le Roex et al., 2016).

Environmental mycobacteria have been shown to decrease the sensitivity of assays that use both aPPD and bPPD as antigens, since a large aPPD response to environmental mycobacteria may mask a bPPD response (Buddle et al., 2009; Parsons et al., 2011; Ryan et al., 2000). Also, a change in PPD-specific IFN-ɣ release following the TST may also be in response to environmental mycobacteria, rather than to *M. bovis,* as was seen in our cattle study. However, it is unlikely that our results were influenced by exposure to or infection with environmental mycobacteria, since the IFN-ɣ response to aPPD was low compared to bPPD.

Since this study was performed on wild buffaloes, we hypothesised that stress factors affecting these animals, including capture by helicopter, captivity, decreased food and water intake, handling, fear and new surroundings, may have influenced our results. Studies have shown that stress causes a release of glucocorticoids, such as cortisol, which may lead to immune suppression and result in significantly reduced tuberculin reactions and IFN-ɣ release in cattle (Gormley et al., 2006). Likewise, a significant increase in glucocorticoids upon capture has also been reported in zebras (Franceschini et al., 2008) and suppression of T-cell function and increased serum cortisol levels in wild ferrets (Cross et al., 1999). However, our results showed

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that there were no significant changes in cortisol levels between pre- and post-SICCT in the group of animals that showed the greatest increase in IFN-ɣ release (group A) or in the group that showed the greatest decrease in IFN-ɣ release (group B). There was no significant difference between the two groups at either time point, although the mean cortisol concentration in group B was higher than that in group A at the pre-SICCT time point and the cortisol concentration decreased from pre- to post-SICCT in group B. Nevertheless, our results agreed with Brivio et al. (2015) that showed that capture and immobilisation of Alpine ibex did not significantly influence the faecal cortisol concentrations, although this was only tested in males. Moreover, it has been shown that immune suppression can continue for up to five weeks in deer (Griffin and Thomson, 1998) or 11 to 18 weeks after release, in zebras (Franceschini et al., 2008). Therefore, cortisol concentrations may have already increased by the time of the pre-SICCT measurement and remained elevated post-SICCT, which would explain why there was no significant change in cortisol concentrations between pre- and post-SICCT samples.

Chemical immobilisation was performed using etorphine hydrochloride, an opioid that is chemically related to morphine. Studies have shown that morphine may result in inhibition of immune responses (Garcia et al., 2012; Sacerdote, 2006), which may have contributed to a decline in IFN-ɣ release. However, the buffaloes were darted at both sampling sessions, i.e. pre- and post-SICCT, and therefore it is unlikely that the opioids caused the decrease in IFN-ɣ that was seen post-SICCT, since both time points would be influenced by drug administration.

There were some technical limitations that may have influenced the Bovigam® results. The researchers and location where the pre-SICCT Bovigam® assays were performed differed from conditions for the post-SICCT assays. The Mabtech IFN-ɣ ELISAs were performed to provide confirmation of the results from the Bovigam® assay, since these assays were performed at a single location by one person and both time points were performed on the same ELISA plate. The Mabtech IFN-ɣ ELISA measured the absolute IFN-ɣ release in response to bPPD, aPPD and PC-HP peptides and results were in agreement with the Bovigam® assays, suggesting that samples were not compromised. Another limitation of this study is that cytokine release was only measured at two time points, which limits our understanding of the effect of the SICCT on IFN-ɣ and IP-10

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release over time. However, due to the logistics and stress involved in capture and immobilisation of wild animals, sample collection for cytokine release assays was determined by the schedule for performing the SICCT (day 0 and day 3) to avoid additional handling.

In summary, to our knowledge this is the first study that has investigated the effect of the SICCT on cytokine assays in African buffaloes. Our results demonstrated IP-10 and IFN-ɣ release in response to specific (PC-HP and PC-EC) and sensitive antigens (PPD) was reduced post-SICCT in Bovigam®-positive animals, but remained unchanged in *M. bovis*-negative animals. The number of IFN-ɣ and IP-10 positive buffaloes and the relative sensitivity of the IP-10 assay decreased 3 days following the SICCT. In a herd with high *M. bovis* infection rates, effective BTB management efforts requires removal of as many infected buffaloes as possible. Based on our results, we recommend that blood samples be collected for the IP-10 and IFN-ɣ CRAs on the day the SICCT is performed, rather than 3 days later, since significantly greater numbers of animals are detected pre-SICCT.

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Chapter 6: Conclusion

The accurate detection of *M. bovis* infected buffaloes has been shown to be influenced by the stage of infection. Although no significant changes in CMI responses or circulating antibodies were observed in our first study over a 1 year period, it appeared that the accurate detection of *M. bovis*-infected buffaloes depended on the cytokine measured and the particular antigen used for whole blood stimulation. Buffaloes that may have been at early stages of infection, were identified by IP-10 assays but not by those measuring IFN-ɣ. Although both Bovigam® PPD and QFT assays were able to identify infected animals over time, QFT assays, using ESAT-6 and CFP-10, were more appropriate diagnostic assays than the Bovigam® PPD assay to use for animals exposed to environmental mycobacteria. However, in terms of changes in antigen-specific responses in individual infected buffaloes, we did not find obvious trends during the study period and further studies are needed to investigate changes in immunological responses that may affect diagnostic outcomes over time.

The SICT has been shown to affect IFN-ɣ and IP-10 production in cattle. PPD-specific cytokine production remained elevated 1-3 weeks following the SICT, which declined significantly by 41 days post-SICT. However, the effect was variable in response to ESAT-6/CFP-10, since cytokine release decreased between 6 and 41 days post-SICT in group 1, but remained unchanged between 7, 21 and 78 days in group 2. However, it appears as though immunological boosting of cytokine release may not be *M. bovis*-specific, but could reflect a cross-reactive response to environmental mycobacteria. This was supported by the large number of false-positive cattle detected by Bovigam® compared to the significantly lower number of QFT-positive cattle at 41 days post-SICT, at which point the effect of the SICT had significantly subsided. QFT assay results were not affected by environmental mycobacteria and correctly identified the animals from group 1 as test-negative at 41 days post-SICT. It is therefore recommended that cattle from areas with high environmental mycobacterial burden and low BTB incidence be tested with assays of high specificity, i.e. QFT assays, at 41 days or later after the application of the SICT.

In *M. bovis*-infected buffaloes, IFN-ɣ and IP-10 release and the number of test positive animals decreased significantly 3 days following the SICCT. However, since HiP has a high incidence of

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BTB in buffaloes, the aim is to remove all possible test-positive buffaloes from the herd. Therefore, we recommend taking blood samples on the day the SICCT is performed, rather than 3 days later. No change in cytokine release in *M. bovis*-uninfected buffaloes following the SICCT was observed.

Accuracy of ante-mortem diagnostic assays may be influenced by many factors, including the stage of *M. bovis* infection, exposure to environmental mycobacteria, differing high *M. bovis* prevalence, and performance of TSTs prior to *in vitro* immunological assays. Our study has shown that assays using specific antigens, ESAT-6 and CFP-10, improves the accuracy of detection in cattle herds with low BTB incidence and high environmental mycobacteria exposure, especially when the effect of the SICT has subsided by 41 days post-SICT. However, in buffalo herds with high BTB incidence, highly sensitive assays are required to identify all *M. bovis*-positive animals and CRAs have been shown to be significantly more sensitive before the SICCT is performed.

This was the first study that investigated the temporal changes in immune responses and the effect of the TST in African buffaloes. Also, to our knowledge, this was the first study to describe the effect of the TST on IP-10 production. This study contributed to improving our understanding of changes of immunological responses over time in buffaloes, the effects of environmental mycobacteria on the interpretation of ante-mortem diagnostic assays and the effect of the TST on CRAs accuracy.

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