Immunological tools for the characterization of the humoral immune response to *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*).

by

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Declaration

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Abstract

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is a chronic infectious disease known to occur in free-ranging mammals, captive wildlife, and livestock. *M. bovis* forms part of the *Mycobacterium tuberculosis* complex (MTC), a genetically related group of bacteria that cause tuberculosis in humans or other species. bTB eradication efforts are complicated by the occurrence of wildlife reservoirs of the disease such as the African buffalo (*Syncerus caffer*). Buffalo in the Kruger National Park and Hluhluwe-iMfolozi Game Reserve are affected by endemic bTB and there is the potential for *M. bovis* to spill-over into other domestic or wild animals, including zoonotic transmission to humans. Current standard diagnostic tests lack the required sensitivity to successfully detect all infected animals and are known to be ineffective in animals who have become anergic in the late stages of disease. Antibodies have been shown to correlate with disease pathology due to a high antigen burden and are able to be detected in anergic animals. The aim of this study was to characterize the humoral (antibody mediated) immune response in bTB-positive buffalo using different molecular techniques. Towards this aim, an in-house PPD ELISA was developed to detect antibodies against *M. bovis* and was compared to the Chembio® DPP VetTB, a commercial antibody assay. Both tests were able to detect *M. bovis*-specific antibodies in bTB-positive buffalo and there was good correlation between the two. We aimed to further assess the humoral response by investigating the diagnostic performance of monocyte chemotactic protein 1 (MCP-1), a cytokine involved in the activation of B-cell proliferation and class switching. There was a significant difference in antigen-specific MCP-1 release between bTB-positive and bTB-negative buffalos, suggesting there is promise for the use of MCP-1 as a biomarker, although sensitivity was low. Furthermore we evaluated the plasma stability of the MCP-1 protein during storage on protein saver cards (PSC) as well as after heat-treatment at 65°C. After 11 days at room temperature

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on the PSC, MCP-1 was still detected at similar levels. MCP-1 concentrations were higher after heat-treatment and correlated with the dilution factor. Lastly, we aimed to determine the stability of 8 reference genes to be used in a whole-blood qPCR gene expression assay in buffaloes. Selected gene transcripts of the African buffalo were sequenced using primers from the domestic cow and it was shown that *PPIA* is the most stable reference genes whereas *GAPDH* is the most unsuitable for use under our experimental conditions. In conclusion, the use of serological tests for the detection of *M. bovis* infected buffalo are disadvantaged by their low sensitivity, although using them in combination with conventional tests may provide useful information on the immune status of the animal. MCP-1 shows promise as a biomarker of disease in buffalo and its stability means that transporting of plasma samples can be made safe and efficient by heat-treating samples and storing them on PSC. Further research is needed to fully optimize a diagnostic GEA for bTB in buffaloes, however, we have shown that *PPIA* is a suitable reference gene for whole-blood stimulation in African buffaloes.

Opsomming

Beestuberkulose (bTB) word veroorsaak deur die bakterie *Mycobacterium bovis* en is 'n kroniese siekte in beeste en wild. *M. bovis* is deel van die *M. tuberculosis* kompleks, 'n groep geneties verwante bakterie wat tuberkulose veroorsaak in die mens en verskeie ander spesies. Die bekeuring van bTB word bemoeilik deur die voorkoms van die siekte in wild spesies soos die Kaapse buffel (*Syncerus caffer*). Buffels in die Kruger Nasionale Park en Hluhluwe-iMfolozi Park is endemies geïnfekteer en die moontlikheid van oordrag na vee en ander wild spesies, asook na die mens, bestaan. Onlangse navorsing het bevind dat die huidige beskikbare diagnostiese toetse nie die nodige sensitiwiteit beskik om alle geïnfekteerde diere te identifiseer nie, asook oneffektief is in diere met n gevorderde siekte toestand.

Vorige navorsing het bevind dat die mate van teenliggaam teenwoordigheid in geinfekteerde diere sterk korreleer met sy siektestoestand as gevolg van die groot hoeveelhede antigeen teenwoordig in letsels. Die doel van die studie was om die humorale immuun reaksie in bTB-positiewe buffels te karakteriseer deur gebruik te maak van verskeie molekulêre tegnieke. In lig van hierdie doelwit is 'n PPD ELISA ontwikkel om die teenwoordigheid van teemliggaampies teen *M. bovis* te meet en die resultate te vergelyk met die van 'n kommersiële beskikbare toets, die Chembio DPP toets. Albei toetse kon die *M. bovis*-spesifieke teenliggaampies in die bTB-positiewe groep optel en albei toetse se resultate het goed gekorreleer.

Die humorale reaksie was verder gekarakteriseer deur die vrystelling van MCP-1 in *M. bovis* antigeen gestimuleerde bloed te meet. Hierdie sitokien is verantwoordelik vir B-sel proliferasie asook klas omskakeling gedurende gevorde infeksies. Daar was 'n betekenisvolle verskil gevind in antigeen-spesifieke MCP-1 vrystelling tussen bTB-positiewe en bTB-negatiewe buffels. Hierdie resultate ondersteun die potensiele gebruik van MCP-1 as 'n biologiese merker van infeksie, al was die sensitiwiteit laag. Verder was die stabiliteit van die MCP-1 sitokien

geevalueer deur die meting daarvan na storing van bloed plasma op Whatman filtreer papier, asook na die hitte-behandeling daarvan by 65 ℃. Na 11 dae op filtreer papier was die konsentrasie van MCP-1 dieselfde as in plasma gestoor by -80 ℃. MCP-1 plasma konsentrasies was hoër na hitte behandeling as by -80 ℃ storing en het goed gekorreleer met die verdunnings faktor.

Laastens het ons die stabiliteit van 8 verskillende verwysings gene ge-evalueer om die mees stabiel gene te identifiseer vir die verdere gebruik daarvan in n kwantitatiewe ware tyd polimerase kettingreaksie toets vir die diagnose van bTB in buffels. Verder was die mees stabiele gene se boodskapper RNA nukleotied volgorde bepaal deur "primers" te ontwerp vanaf gepubliseerde beskikbare informasie beskikbaar oor elk een vir beeste. Die mees stabiel verwysings geen was *PPIA*, met *GAPDH* as die minder toepaslike kandidaat gedurende hierdie eksperimentele omstandighede.

Ten slotte, serologiese toetse se gebruik vir die diagnose van *M. bovis* infeksie in buffels word benadeel deur hulle lae sensitiwiteit wel die gebruik van hierdie toetse in kombinasie met konvensionele toetse mag dalk nuttige informasie bied oor die immuun status van n dier. MCP-1 toon potensiaal as n biologiese merker van infeksie in buffels en die stabiliteit onder verskeie kondisies ondersteun die moontlikheid om die vervoer van bloed monsters meer doeltreffend en veiliger te maak deur die geprosesseerde plasma by hoë temperature te skok en te stoor op filtreer papier. Alhoewel ons bewys het dat die *PPIA* die mees toepaslike verwysings geen is vir die gebruik in n diagnostiese geen uitdrukkings-toets vir bTB diagnose in Kaapse buffels, word verdere optimaliserings navorsing wel voorgestel.

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Abbreviations

°C	Degrees Celsius
μg	Microgram
μl	Microliter
μM	Micro Molar
3D	Three dimensional
AA	Amino acid
Ab	Antibody
ACTB	Beta actin
B2M	Beta-2-Microglobulin
BB	Blocking buffer
BCG	Bacillus Calmette–Guérin
bp	Base pair
BSA	Bovine serum albumin
bTB	Bovine tuberculosis
cDNA	Complimentary DNA
CFP-10	Culture filtrate protein
CFT	Caudal fold test
CMI	Cell-mediated immunity
Cq	Quantification cycle
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPP	Dual path platform
DTH	Delayed type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secretory antigenic target
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEA	Gene expression analysis
H_2O_2	Hydrogen peroxide
H3F3A	H3 histone, family 3A
HPRT1	Hypoxanthine phosphoribosyltransferase 1

IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IGRA	Interferon gamma release assay
IL	Interleukin
IP-10	Interferon gamma-induced protein 10
IU	International Units
kb	Kilo bases
kDa	Kilo Daltons
KLH	Keyhole Limpet Hemocyanin
M	Molar
mAb	Monoclonal antibody
MAPIA	Multi-antigen print immunoassay
MCP-1	Monocyte chemoattractant protein-1
ME	Mercaptoethanol
min	Minutes
ml	Millilitre
mm	Millimetre
MPB	Proteins produced by M. bovis
MPT	Proteins produced by M. tuberculosis
mQFT	Modified QFT
mRNA	Messenger Ribonucleic acid
MTC	Mycobacterium tuberculosis complex
NCBI	National Center for Biotechnology Information
ng	Nano gram
NK	Natural killer
nm	Nano meter
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pg	Pico gram
pI	Isoelectric points
PPD-A	Avium purified protein derivative

PPD-B	Bovine purified protein derivative
PPIA	Peptidylprolyl isomerase A
PSC	Protein saver cards
QFT	QuantiFeron TB test
qPCR	Quantitative real-time Polymerase Chain Reaction
RNA	Ribonucleic acid
RT	Room temperature
RU	Reflectance units
s	Second
SCITT	Single intradermal comparative tuberculin test
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SFT	Skin fold thickness
SNP	Single-nucleotide polymorphism
TB	Tuberculosis
TBP	TATA-binding protein
Th0	Naïve T-cell
Th1	T-helper type-1
Th2	T-helper type-2
Tm	Melting temperature
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour necrosis factor
TST	Tuberculin skin test
USA	United States of America
UTR	Untranslated region
WB	Whole-blood
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta
ΔOD	Delta-OD

Chapter 1 - Introduction:

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis,* is a chronic infectious disease known to occur in free-ranging mammals, captive wildlife, and livestock (Fitzgerald et al. 2013). *M. bovis* forms part of the *Mycobacterium tuberculosis* complex (MTC), a genetically related group of bacteria that can cause tuberculosis in humans or other species (Michel 2008). bTB has become an issue of public health and economic importance due to increasing wildlife-livestock-human interfaces, with worldwide annual losses to agriculture of $3 billion (Garnier et al. 2003). The outcome of infection with *M. bovis* may vary, depending on the host species and other factors, such as age, animal behaviour, route of infection and environmental conditions (Neill et al. 2001). Common routes of exposure in cattle include direct inhalation of aerosols containing *M. bovis* from infected animals, or indirectly from contaminated water and/or pastures (Hassan et al. 2014). Infections may remain subclinical for years and can be reactivated during stress, old age or in immunocompromised individuals (De Vos et al. 2001). As the disease progresses, animals develop lesions in the infected tissues. Lesions in bovids are manifested as granulomas or tubercles which are characterized by a caseous or mineralized necrotic core of tissue surrounded by a zone of inflammation. The size can vary from microscopic to macroscopic lesions measuring several centimetres, while their distribution, appearance and severity differs between as well as within host species (Michel 2008). Tuberculous lesions in African buffalo (*Syncerus caffer*) occur most frequently in lymphatic tissue of the thoracic cavity, namely the bronchial and mediastinal lymph nodes (Laisse et al. 2011). The second most frequent site for lesions are lymph nodes of the head region such as the retropharyngeal and submaxillary nodes, and also tonsillar tissues from field cases of bovine tuberculosis (Neill et al. 2001, Laisse et al. 2011). Often tuberculous lesions in the lungs are recorded less frequently than that of the thoracic lymph nodes; however, these lower numbers may be due to the difficulty of detecting very small, singular lesions in the lungs due

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to their large volume. The almost exclusive distribution of lesions in the lung and upper respiratory tract suggests that the primary route of infection in African buffalo is aerosol inhalation (Neill et al. 2001, Laisse et al. 2011). African buffalo may remain infected for many years before death; however, those that reach an advanced stage of disease may show clinical signs including coughing, debilitation, emaciation, and lagging behind when chased (De Vos et al. 2001, Renwick et al. 2007).

M. bovis is known to have one of the widest host ranges of all the MTC bacteria and can spill-over into a variety of domestic and wild animals, including zoonotic transmission to humans (Musoke et al. 2015). Worldwide, more than 60 wild mammal species have been shown to be infected with *M. bovis* (de Lisle et al. 2002). The control of bTB is complicated by the occurrence of wildlife reservoirs of the disease such as the African buffalo, European badger (*Meles meles*), white-tailed deer (*Odocoileus virginianus*), and brush-tail possum (*Trichosurus vulpecula*) which pose a threat for reinfection of livestock (De Garine-Wichatitsky et al. 2013, Fitzgerald et al. 2013). African buffalo in the Kruger National Park and Hluhluwe-iMfolozi Game Reserve are affected by endemic bTB (Fitzgerald et al. 2013). Transmission to other wildlife species in these parks occurs through spill-over from buffalo through predation, scavenging or contaminated habitat, although direct transmission is also a possibility (Michel et al. 2006).

In South Africa there is a need to develop novel diagnostic strategies to effectively manage and prevent the spread of *M. bovis*. One of the main approaches to controlling bTB in livestock herds is test and slaughter. This method of disease control has been effective in eradicating TB from certain locations, and relies on the slaughter of any animals testing positive on the TST. Test and slaughter has been widely used with great success in countries such as New Zealand, Australia and the USA (Tweddle and Livingstone 1994, Olmstead and Rhode 2004). However, the effectiveness of this strategy is limited where free-ranging wildlife reservoirs of the disease

are present. Logistical, economic, and safety constraints of wildlife capture and handling, lack of available diagnostic tools, and lack of information about infected populations complicates surveillance and management efforts (Kaneene et al. 2010). Poorly established control programs in countries such as South Africa are due to a lack of economic and socio-political driving forces (Michel et al. 2006). In order to effectively monitor and manage bovine tuberculosis in wildlife, advances in ante-mortem diagnostics tests are required for earlier detection of infected individuals to prevent intra- and inter-species transmission and develop control programs (Kaneene et al. 2010, Michel et al. 2006).

Currently used diagnostic tests for bTB in cattle rely on the detection of cell-mediated immunity (CMI) to *M. bovis* antigens. The primary ante-mortem test, the single intradermal comparative tuberculin test (SICTT), measures a delayed type hypersensitivity (DTH) response to mycobacterial antigens, specifically *M. bovis* purified protein derivative (PPD-B) and *M. avium*-PPD (PPD-A). The test relies on the measurement of dermal inflammation three days after intradermal injections in the skin of the caudal fold (CFT) or neck (SICTT). A difference in skin thickness of 4mm or more at the PPD-B injection site compared to the PPD-A site is considered definitively positive while a measurement of between 2mm and 4mm is a suspected positive result (Schiller et al. 2010). The advantages of the skin test are its low cost and high availability. However, this test has been shown to lack both sensitivity and specificity (Schiller et al. 2010, Rothel et al 1990). It also requires that the animals are handled twice and the test cannot be repeated for approximately 90 days because of immunological sensitization to the PPD antigens. Movement of animals require that all individuals test negative on successive skin tests, therefore a long waiting period between re-testing can delay the whole procedure (Rothel et al 1990).

An alternative to the SICTT is the interferon gamma release assay (IGRA), an *in vitro* diagnostic test that quantifies the secretion of interferon gamma (IFN-γ) by lymphocytes in

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response to pathogen-specific antigen stimulation. IFN-γ in the plasma supernatant is quantified in a sandwich enzyme-linked immunosorbent assay (ELISA) using detection antibodies specific to the species of interest (Schiller et al. 2010). This assay is significantly more sensitive than the SCITT and results can be produced within 24 hours (Wood et al., 1990). Commercially available IFN-γ ELISA based tests such as the BOVIGAM® (Prionics, Switzerland) have come onto the market as ancillary tests for bTB detection in cattle (Bass et al. 2013). Limitations of the IFN-γ assay are reduced specificity, high cost and high logistical demands (Vordermeier et al. 2006, de la Rua-Domenech et al. 2006). IGRAs also require that species-specific antibodies are used for the detection of IFN-γ, thus they have only been developed for a few select species including: European badger (Cousins and Florisson, 2006), African buffalo (Grobler et al. 2002), domestic cattle (Wood et al. 1991), red deer (Slobbe et al. 2000), and pigs (Cousins and Florisson, 2006). More recently the specificity of IGRAs has been improved by using antigens specific to *M. bovis*, such as 6 kDa early secretory antigenic target (ESAT-6) and the 10 kDa culture filtrate protein (CFP-10), which are both highly expressed by *M. bovis* (Vordermeier et al. 2001). This has led to the development of the modified QuantiFERON TB-Gold (mQFT) assay, an adapted version of a human TB test, which has been described for the testing of buffaloes (Parsons et al. 2011).

Detection of additional biomarkers involved in the antigen–specific immune response to *M. bovis* has the potential to significantly increase the sensitivity and specificity of bTB diagnostic tests. The discovery of novel biomarkers for use in animal studies is aided by the identification of candidate targets studied in human tuberculosis patients (Ruhwald et al. 2009). Recently, a candidate biomarker, interferon gamma-induced protein 10 (IP-10) was identified as an alternative to measuring IFN-γ release in a cohort of *M. bovis* exposed African buffaloes, with the potential for increased sensitivity over conventional IGRAs (Goosen et al. 2015). Another candidate biomarker identified previously, monocyte chemoattractant protein-1 (MCP-1), was

shown to have promise as a diagnostic marker for *M. bovis* infection in African buffaloes. This chemokine was also found in far greater abundance in antigen-stimulated plasma of infected buffaloes compared to human patients with TB (Goosen et al. 2014). MCP-1, a member of the C-C chemokine family, is involved in recruiting monocytes, memory T lymphocytes, and natural killer (NK) cells to sites of infection. It is mainly produced by monocytes and macrophages in response to oxidative stress, cytokines or growth factors released during an inflammatory response (Deshmane et al. 2009). MCP-1 has also been shown to be essential in the formation of granulomas and provides protection against tuberculosis in the mouse model via recruitment of antigen specific T-lymphocytes to the infected tissue (Kipnis et al. 2003). In a study of human tuberculosis patients, MCP-1 had a higher sensitivity and specificity for diagnosing active tuberculosis compared to IFN-γ and was useful in differentiating between active and latent tuberculosis (Wang et al. 2015).

bTB is characterized by a predominantly cell-mediated response during the early and intermediate stages of infection with a humoral (antibody-mediated) immune response appearing later in the course of infection (Ritacco et al. 1991). CMI or T-helper type-1 (Th1) responses involve macrophages, dendritic cells and an adaptive T-cell response, and are controlled by cytokines released from antigen-specific T-cells. The result of the CMI response is the formation of the granuloma in which bacteria are walled off to prevent their spread. A significant biological challenge to the host immune response is a balance between the antimicrobial activity needed to kill the pathogen while at the same time limiting its own tissue damage. T-helper type-2 (Th2) responses are required to inhibit Th1 responses in order to limit physiological damage but they can also contribute to pathology. Th1 cells enhance cell mediated immunity and are the primary source for the inflammatory cytokines IFN-γ, interleukin (IL)-2, tumour necrosis factor (TNF)-α and TNF-β, which are involved in the activation of macrophages and lymphocytes for the destruction of bacterial pathogens

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(Romagnani et al. 2000). Th2 cells activate and maintain the humoral immune response by producing various cytokines such as IL-4, IL-5, IL-10 and IL-13. Th2 cytokines are responsible for inducing strong antibody responses, eosinophil activation and inhibition of several macrophage functions (Romagnani et al. 2000, Gu et al. 2010). Studies have shown that during the course of infection, cattle may convert from a Th1 response to a Th2 response and this conversion is associated with increased pathology (Hernández-Pando et al. 1996, Thacker et al. 2007). It has been reported that antibody responses to *M. bovis* infection correlate with disease pathology, likely due to increased antigen burden (Harboe et al. 1990, Lyashchenko et al. 2004). In cases of advanced tuberculosis, cattle may become immunologically anergic; these animals lack a measureable CMI response but can have high circulating antibody levels (Neill et al. 2001). Therefore, diagnostic tests such as IGRAs that rely on the detection of Th1 cytokines may be more useful for identifying animals in the early stages of infection, whereas antibody based diagnostics or tests detecting cytokines involved in the Th2 response could be used to identify animals at a more advanced stage of disease (Ritacco et al. 1991). Other advantages of serological diagnostic tests include the rapidity in which responses can be detected as well as the stability of antibodies during transport and storage (Green et al. 2009, Lyashchenko et al. 2013).

MCP-1 plays a critical role in the polarization of Th0 cells towards a Th2 phenotype by stimulating the production of IL-4. This also inhibits the production of pro-inflammatory (Th1) IFN-γ-secreting cells (Gu et al. 2000). Th2 activation induces the proliferation of antibody producing B-cells and is required for immunoglobulin subclass switching, therefore mice deficient in MCP-1 are unable to mount effective Th2 responses (Gu et al. 2000). Due to its ability to attract monocytes, MCP-1 is thought to have a pathobiological role in inflammatory disorders and high levels of MCP-1 in infected animals may therefore represent a sign of pathology or advanced stages of disease (Kipnis et al. 2003). The role of this cytokine in Th2

activation and its association with pathology make MCP-1 a promising biomarker in the investigation of the immune response to *M. bovis* infection in African buffalo.

The ELISA technique is used to measure the binding of antibodies to antigen fixed on a solid-phase adsorbent, such as a polystyrene microtitre plate. An ELISA which detects antibodies against *M. bovis* was reported in 2008 for wild boar with a sensitivity and specificity of 73% and 96%, respectively (Aurtenetxe et al. 2008). Another technique used for the detection of antibodies to mycobacterial antigens is the multi-antigen print immunoassay (MAPIA). The MAPIA consists of a cocktail of antigens immobilized on a nitrocellulose membrane in narrow strips. The membrane is incubated with test sera and antibody-antigen complexes are visually detected (Lyashchenko et al. 2000). Using this method, antibodies against multiple antigens can be screened in a single serum sample. Advantages of the MAPIA over the ELISA technique include an efficient and cost effective method for screening antibodies to many antigens, high versatility and the potential to evolve into a rapid point-of-care diagnostic (Lyashchenko et al. 2000). The poor sensitivity of serological tests has slowed the widespread use of these assays for early detection of bTB in cattle. Reports have shown that not all cattle with tuberculous lesions have a detectable antibody response, however this seems to vary between species as serological tests for bTB in elephants and wild boar have reported greater sensitivities, i.e. 100% and 89.6%, respectively (Boadella et al. 2011, Lyashchenko et al. 2006).

Several new serological tests with more promising accuracy have recently emerged (Green et al. 2009, Waters et al. 2011). The Chembio DPP VetTB® assay is an example of a commercially available test designed to measure antibody responses to *M. bovis* specific antigens. The DPP VetTB® assay utilizes a two-step process aimed at rapid detection of specific antibodies and has been validated for use in animal species including red deer (Buddle et al. 2010), white-tailed deer (Lyashchenko et al. 2013), African and Asian elephants (Greenwald et al. 2009). The membrane strip contains two test antigen bands, T1 (MPB83

protein) and T2 (ESAT-6/CFP-10 fusion protein), which allows differential immunoglobulin G (IgG) antibody detection. Species-nonspecific detection occurs by binding of colloidal gold particles coupled to recombinant protein A/G with the antigen-antibody complexes fixed to the membrane (Lyashchenko et al. 2013). Another rapid serological diagnostic test, the Chembio STAT-PAK, first used in 2003 for the detection of bTB in Eurasian badgers, has become a popular tool for the serodiagnosis of bTB in a variety of wildlife species (Greenwald et al., 2003). The STAT-PAK detects antibodies to a cocktail of *M. bovis* antigens and results can be provided in 20 minutes. The test is validated for use in elephants (Lyashchenko et al. 2006) and cervids (Gowtage-Sequeira et al. 2009), although it is not currently available.

Quantitative real-time Polymerase Chain Reaction (qPCR) is an additional technique which can be used to investigate the gene expression of cytokines involved in the Th2 immune response to *M. bovis* infection. Therefore, helping us to better understand the complex interactions which occur between host and pathogen (McGuire et al. 2005). Genes involved in the immune response may be either up- or down-regulated during microbial infection, therefore studying gene expression in combination with protein secretion could help better characterize the immune status of the animal. Targeted immuno-specific bovine cDNA microarray analysis has been used to study the gene expression profile of *M. bovis* infected cattle (Meade et al. 2006, Meade et al. 2007, MacHugh et al. 2009, Lim et al. 2012). In a study evaluating the expression of 1336 genes, 244 were differentially expressed between antigen-stimulated and non-stimulated peripheral blood mononuclear cells (PBMC) with the majority (137) of those genes showing significant down-regulation (Meade et al. 2006). Meade et al. (2007) investigated differences in gene expression of *ex vivo* PBMC between *M. bovis* infected cattle and healthy cattle, without *in vitro* antigen stimulation. Their results confirmed earlier findings and showed that out of 378 genes identified as differentially expressed between the two groups, 244 (65%) of those genes were down-regulated. These variations can provide a gene expression

profile which may be useful to the characterization of the immune response to *M. bovis* infection and lead to the development of improved diagnostics and/or vaccines (Thacker et al. 2007). Parsons et al. (2011) developed a diagnostic gene expression assay for the detection of *M. bovis* infected African buffalo using cDNA from antigen-stimulated whole-blood samples. The IFN-γ gene was significantly upregulated in antigen-stimulated samples from bTB-positive buffalo compared to bTB-negative buffalo. Furthermore, they were able to distinguish between the two groups with a sensitivity and specificity of 80% and 95%, respectively (Parsons et al. 2011).

qPCR is a highly sensitive and accurate technique for the quantification of mRNA transcripts and has become the method of choice for measuring changes in gene expression levels. The relative quantification method for qPCR gene expression analysis is preferred over absolute quantification when the absolute copy number of mRNA is not required (Bustin et al. 2000, Pfaffl et al. 2001). In relative quantification, the expression of your gene/s of interest is calculated in relation to a reference gene subjected to the same experimental conditions. The assumption is that the reference gene(s) is expressed constitutively and therefore the levels of expression remain the same between sample types and under different experimental treatments (Bustin et al. 2000, Hugget et al. 2005). Reference genes such as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta actin (*ACTB*) have been commonly used in early gene expression studies, however, a number of studies have shown that expression of reference genes is influenced by sample type and experimental treatments (Ronbinson et al. 2007, Varshney et al. 2012, Lisowski et al. 2008). It is therefore a necessary prerequisite to appropriately validate reference genes under specific experimental conditions before they can be used for accurate gene quantification. Previous studies evaluating gene expression in cattle were referred to for a selection of commonly used reference genes (Vorachek et al. 2013, Lisowski et al. 2008, Varshney et al. 2012). The 8 reference genes selected were: *GAPDH*,

ACTB, Beta-2-Microglobulin (*B2M*), Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (*YWHAZ*), Peptidylprolyl isomerase A (*PPIA*), H3 histone, family 3A (*H3F3A*), TATA-binding protein (*TBP*), and Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). Software programs such as geNorm (Vandesompele et al. 2002) and NormFinder (Andersen et al. 2004) have been developed which use different algorithms to assess the stability of candidate reference genes. Using multiple reference genes in the analysis can improve the accuracy of quantification (Vandesompele et al. 2002). Further optimization of gene expression assays could provide useful tools for characterizing the gene expression profile in *M. bovis* infected animals and lead to the development of more sensitive diagnostic tests for bTB.

In summary, bTB is a chronic disease that is maintained by free-ranging wildlife reservoirs such as the African buffalo with the potential to spill-over to livestock, other wildlife species, and even humans. Diagnostic tools offering accurate and rapid detection of infected animals are required to effectively monitor and develop management strategies for bTB in wild populations. Currently available diagnostic tests have been shown to lack the sensitivity required when testing for bTB in certain species. False negative animals may result in introduction of disease into new areas when translocated, or perpetuate infection in current locations. Serological diagnostic tests show promise for detecting infected animals which may be missed by conventional CMI based tests, especially in advanced disease. Although humoral responses appear to correlate with disease pathology, they are known to lack sensitivity in certain species. Therefore, characterization of the immune response to bTB can help identify potential targets that when incorporated into novel diagnostics, will improve the detection of *M. bovis* infected animals.

The present study aimed to develop an in-house ELISA for the detection of antibodies against *M. bovis* antigens in order to evaluate the humoral immune response in bTB-positive African buffalo. Secondly, MCP-1, which plays a role in stimulating the antibody-mediated immune response to infection, was evaluated as a diagnostic biomarker of disease in buffalo. In addition, expression stability of 8 reference genes was determined for potential use in a gene expression assay using antigen-stimulated whole blood to detect *M. bovis* infection in buffalo.

Chapter 2 - Literature review:

The dominant immunogenic proteins of *Mycobacterium bovis*: MPB70 and MPB83.

2.1   Introduction

Mycobacterium bovis is part of the *Mycobacterium tuberculosis* complex (MTC), a genetically related group of bacteria that can infect and cause tuberculosis in a wide range of mammal species, including humans (Michel 2008). During the course of microbial infection, various molecules (proteins) associated with the causative organism are recognized by cells of the immune system (Fifis et al. 1994). This leads to a complex cascade of immune responses which can be measured using several techniques. Some proteins are more capable than others of inducing an immune response in the host, due to certain biological traits such as the amount of protein secreted or its chemical complexity, collectively referred to as the immunogenicity (Hewinson et al. 1996). Proteins with high immunogenicity are therefore important potential targets for use in diagnostic tests for infectious diseases such as tuberculosis. A family of highly immunogenic proteins derived from mycobacteria are the so-called MPB and MPT proteins.

The term 'MPB' is used to refer to proteins produced by *M. bovis*, while 'MPT' refers to proteins from the closely related species *M. tuberculosis*. The numbers 70 and 83 relate to the relative mobility of 0.70 and 0.83 by native polyacrylamide gel electrophoresis at pH 9.4. MPB70 was first purified from culture filtrates of *M. bovis* BCG and it was subsequently found to elicit a delayed type hypersensitivity (DTH) response in guinea pigs sensitized with certain BCG strains but not by other BCG strains or members of the *Mycobacterium tuberculosis* complex (MTC) (Nagai et al. 1981, Miura et al. 1983). Antibody responses to MPB70 were found to follow a very similar pattern among these strains (Hasløv et al. 1987). Further analyses

using radioimmunoassay inhibition test showed the presence of MPB70 in the BCG strains that induced DTH however, it was found to be in very low amounts in strains unable to induce this response (Harboe and Nagai 1984, Hasløv et al. 1987). These studies formed the basis for dividing BCG strains and other mycobacteria into the two main groups now known as MPB70 high producers and MPB70 low producers. It was discovered that low levels of MPB70 were found in sonicated extracts from whole washed mycobacterial cells, indicating this protein was actively secreted from the cells. These findings were supported by the observation that large amounts of heat killed BCG Tokyo cells induced very low level of DTH compared to small doses of live BCG Tokyo (Miura et al. 1983).

Early knowledge of MPB83 was limited by the lack of genomic data and because the protein had not yet been purified. Bands in western blotting thought to be glycosylated MPB70 were actually MPB83, and the relationship between these products was shown using a monoclonal antibody which strongly recognized both proteins (Fifis et al. 1992, Wiker et al. 1996). MPB83 was later isolated and purified to near homogeneity from *M. bovis* BCG Tokyo culture filtrate by Harboe et al. (1995). In the same study they were able to show that peptides of MPB83 isolated after cyanogen bromide cleavage had widespread homology as well as distinct variations in the amino acid sequence compared to that of MPB70 (Harboe et al. 1995, Terasaka et al. 1989). Guinea pigs sensitized with live BCG Tokyo also developed a DTH response to intradermally injected MPB83 (Harboe et al. 1995). Further characterization of these proteins was required in order to determine their potential value for the diagnosis of bovine tuberculosis.

2.2   Properties of MPB70 and MPB83

There are two genes encoding the proteins MPB70 and MPB83 that lie within an operon of six genes, with the gene of *mpb83* (Mb2898) located approximately 2.3kb upstream of *mpb70*

(Mb2900) (Matsuo et al. 1996, Radford et al. 1990). The orthologs of *mpb83* and *mpb70* found in *M. tuberculosis*, *mpt83* and *mpt70*, are 100% identical at the sequence and amino acid level (Wiker et al. 1998, Hewinson et al. 1996). Both genes encode pre-proteins containing typical hydrophobic signal peptides which are cleaved following translocation. Cloning and sequencing of *mpb70* revealed a mature protein of 163 amino acids in length and a signal peptide at the N-terminus consisting of 30 amino acids (Terasaka et al. 1989, Radford et al. 1990). In later studies when *mpb83* was cloned and sequenced, it was discovered to code for a 200 amino acid mature peptide with an N-terminal signal peptide of 20 amino acids (Matsuo et al. 1996). Although the sequence and size of signal peptides between the two proteins are quite different, there is high homology, around 73%, within regions of the mature protein (Matsuo et al. 1996, Wiker et al. 1998). Both the nucleotide and amino acid sequences for MPB70 and MPB83 were later confirmed when the complete genome of *M. bovis* was published in 2003 (Garnier et al. 2003).

The molecular mass (kDa) of both proteins are very similar as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); however, the isoelectric points (pI) of MPB70 (pI = 4.8) and MPB83 (pI = 4.7) are slightly different (Harboe et al. 1995, Wiker et al. 1998). MPB70 migrates with an apparent molecular mass of 22 kDa under reduced conditions with mercaptoethanol (ME) and 15 kDa when unreduced (Wiker et al. 1998). The native MPB83 protein has a molecular mass of 23 kDa when reduced with ME and 22 kDa in unreduced conditions (Hewinson et al. 1996, Wiker et al. 1998). Both proteins contain two cysteine residues which are widely separated and the formation of internal disulphide bridges between these cysteines produces rings structures in both proteins. These disulphide bridges alter the three-dimensional (3D) structure of the molecules and can therefore influence the speed at which the peptides migrate in SDS-PAGE (Wiker et al. 1998). The internal disulphide bridges that were predicted by SDS-PAGE have been confirmed for MPB70 after its 3D

structure was determined by NMR spectroscopy (Carr et al. 2003). The 3D structure of MPB83 has not yet been determined.

Before the identification of MPB83 as a distinct protein to MPB70, a 25 kDa band in SDS-PAGE was observed to be recognized by monoclonal antibodies against MPB70. It was discovered that this protein was also glycosylated and it was therefore reported as being a glycosylated form of MPB70 (Fifis et al. 1991). The 25 kDa antigen was subsequently purified and the amino acid sequence revealed this was a protein encoded on a separate gene to *mpb70* (Harboe et al. 1995), thus proving that the 25 kDa antigen is a glycosylated form of the 23 kDa MPB83 protein. A unique difference of mature MPB83 compared to MPB70 is the presence of a classic lipoprotein motif as well as a unique amino acid insert of 25 peptides in its N-terminus containing a glycosylation site (Hewinson et al. 1996, Vosloo et al. 1997). Biochemical and structural analysis of MPB83 demonstrated that this protein is *O*-glycosylated with mannose via threonine residues. Three mannose units were found attached to two threonine residues approximately 25 residues from the N-terminal region of the mature peptide (Michell et al. 2003). Mannose residues were joined by $1 \rightarrow 3$ linkages, in contrast to the $1 \rightarrow 2$ linkage previously described for the antigen MPB32 (Dobos et al. 1996). The 23 kDa unglycosylated form is not observed when *mpb83* is cloned into *M. smegmatis*. This may suggest that *M. bovis* has peptidases involved in post-translational modification which are not found in other non-pathogenic mycobacteria (Michell et al. 2003). The exact role of these glycosylations is unclear although it has been proposed that they protect lipoproteins from cleavage in the N-terminal region which is vulnerable to proteolytic cleavage (Dobos et al. 1996).

MPB70 has been shown to be highly soluble and present in large amounts in the culture filtrate of *M. bovis* or BCG high producing strains. However, when protein extracts are prepared from bacterial cells prewashed with an aqueous buffer, MPB70 is barely detected (Wiker et al. 1991). This demonstrates that MPB70 is actively secreted into the culture medium when cells are

grown in liquid media and are therefore not associated with the cell surface. In contrast, using flow cytometry and monoclonal antibodies against MPB83, it was shown that this protein was found on the surface of *M. tuberculosis* complex cells and anchored to the cell wall via the N-terminal lipid group (Harboe et al. 1998). The purified native 23-kDa MPB83 molecule is also a soluble secreted (nonlipoprotein) variant of the 26-kDa lipoprotein.

2.3   Differential expression of MPB70 and MPB83

A key phenotypic difference between *M. bovis* and *M. tuberculosis* is in the production of the proteins MPB70 and MPB83. *M. bovis* has a high level of expression for these proteins *in vitro* whereas their production in *M. tuberculosis* is very low (Harboe and Nagai 1984, Hewinson et al. 1996). Later findings showed that all MTC members tested (*M. tuberculosis, M. africanum, M. microti*), were low producers of MPB70 compared to *M. bovis* (Liebana et al. 1996). Also, levels of MPB70 and MPB83 expression varied between different strains of BCG (Table 1) (Wiker et al. 1996). BCG strains obtained from the Pasteur Institute before 1927 have high levels of MPB70 and MPB83 production, while strains obtained after 1931 have much lower production, most likely due to *in vitro* mutations during this period (Behr and Small. 1999, Charlet et al. 2005). The pattern of MPB70 high- and low-producing substrains of BCG is only partially applicable for MPB83. Strains that are MPB70 low producers are also MPB83 low producers, however, the expression of MPB83 varies much more than the expression of MPB70 in the MPB70 high-producing strains (Wiker et al. 1996).

Table 1 – The production of MPB70 in various strains of *M. bovis* and other MTC members is grouped into high and low producers.

MPB70 high producers	**MPB70 low producers**
M. bovis AF2122/97	*M. tuberculosis* H37Rv
M. bovis AN5	*M. africanum*
M. bovis Ravenel	*M. microti*
M. bovis 04-303	*M. bovis* BCG Pasteur
M. bovis BCG Tokyo	*M. bovis* BCG Copenhagen
M. bovis BCG Moreau	*M. bovis* BCG Glaxo
M. bovis BCG Russia	*M. bovis* BCG Beijing
M. bovis BCG Sweden	*M. bovis* BCG Tice

Interestingly, another secreted protein specific to the MTC named MPB64 was found to exhibit a similar expression pattern to MPB70 and MPB83 in BCG strains (Harboe et al. 1986, Wiker et al. 1996). MPB64 is only secreted by *M. tuberculosis* and *M. bovis*, and therefore appears to be highly specific for these two species (Tamada et al. 2012). High levels of MPB64 in the BCG high-producing strains correlates with levels of MPB70, suggesting that these genes are partly controlled by the same regulatory mechanism (Wiker et al. 1996). However, in the MPB70 low-producers, MPB64 was not detected at all and it was subsequently shown that the *mpb64* gene was deleted from all MPB70 and MPB83 low-producing BCG strains (Li et al. 1991). This was the first gene deletion discovered in BCG and the results seemed to indicate that the amount of MPB70 was related to the presence of the MPB64 gene or other factors (Matsuo et al. 1995, Li et al. 1991). In contrast, MPB64 is expressed in high levels by *M. tuberculosis* even though MPB70 and MPB83 are expressed in low levels when compared to *M. bovis* and BCG high-producers (Wiker et al.

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1996). These observations suggest that there is a different mechanism to explain the variable expression of these two proteins between mycobacterial species.

Observed differences in MPB70 and MPB83 secretion between the substrains may be due to variance in the coding or non-coding regions of the specific genes. Cloning and sequencing of *mpb70* and *mpb83* showed that there was no difference in nucleotide sequence between the high- and low-producing substrains. Furthermore, the upstream and downstream regulatory regions also had identical nucleotide sequences (Matsuo et al. 1995, Hewinson et al. 1996). In an attempt to explore the underlying differences in protein production, Matsumoto et al. (1995) cloned the *mpb70* gene from BCG Tokyo (high-producer) into BCG Pasteur (low-producer) but was unable to restore levels of MPB70 to those recorded in wild-type BCG Tokyo. Additionally, by targeted gene expression analysis using qPCR, differences were seen in the amount of MPB70 mRNA produced by each substrain. In BCG Tokyo the mRNA was readily detected, while in BCG Pasteur it was found at a very low level (Matsuo et al. 1995). Both these results suggest that the different levels of secreted MPB70 observed were due to the efficiency of gene transcription inherent to the parent BCG strain.

BCG strains have accumulated a number of mutations such as single-nucleotide polymorphisms (SNPs) and deletions during their half a century of *in vitro* passage (Behr et al. 1999). Since some of these mutations affect known regulatory genes, it was therefore hypothesized that a mutation in a regulatory gene was causing the variance in production of MPB70 and MPB83 (Behr et al. 1999, Charlet et al. 2005). Using microarray analysis, a genetic region showing consistent down regulation in low-producing strains contained the gene for sigma factor K (*sigK*), a transcriptional regulator (Charlet et al. 2005). Subsequently, it was shown that all eight low producing BCG strains had the same point mutation in the start codon of *sigK*. This G → A mutation at the third position resulted in a AUA start codon which significantly affected levels of transcription. When wild-type *sigK* from a BCG high-producer was complemented into a low-producing strain the

results showed a marked increase in MPB70 and MPB83 protein production, at levels consistent with high-producing strains. These results show that sigma factor K positively regulates the expression of MPB70 and MPB83 (Charlet et al. 2005).

The *sigK* start codon in *M. tuberculosis* and *M. bovis* contains the wild-type AUG sequence, even though MPB70 and MPB83 are differentially expressed *in vitro* by these two species. These differences were thought to occur due to unregulated activity of *sigK* in *M. bovis*. The repressor protein, Anti-SigK is coded by the gene *Rv0444c* which is co-transcribed and lies immediately downstream of *sigK*. Sequencing analysis revealed that *Rv0444c* in *M. bovis* contains two non-synonymous SNPs, C320T and C551T, resulting in a non-functional protein product (Saïd-Salim et al. 2006). The sequence was then compared to members of the MTC with low MPB70/MPB83 production, such as *M. tuberculosis* H37Rv and *M. africanum*. All low-producing strains had the wild-type sequence for Anti-SigK, indicating that the increased expression of MPB70 and MPB83 in *M. bovis* is due to a dysfunctional anti-SigK (Saïd-Salim et al. 2006). The increased expression of MPB83 and MPB70 in *M. bovis* compared to other MTC members means that more specific bTB diagnostic tests can be developed, thus eliminating the potential cross-reactivity between immunogenic proteins of various pathogenic mycobacteria.

2.4   Immunologic characteristics of MPB70 and MPB83

2.4.1   Monoclonal antibodies

Monoclonal antibodies (mAbs) that bind to MPB70 and MPB83 have been produced by various independent groups (Wiker et al. 1998, Wood et al. 1988, Lyashchenko et al. 1993, Lyashchenko et al. 2001). In many cases, antibodies have shown strong binding to both proteins, providing further evidence of the structural homology between these two proteins (Wiker et al. 1998). The antibodies 12/6/1 and SB10 react to shared epitopes of MPB70 and

MPB83 and were found to bind to both proteins during western blot analysis (Wiker et al. 1996). Importantly, antibodies that bind specifically to only one of the proteins are useful for allowing researchers to distinguish between MPB70 and MPB83 (Wiker et al. 1996). Wood et al. (1988) produced several mAbs, named the SB series (SB1 - SB10), which were specific to antigens in the culture filtrate of *M. bovis*. These antibodies all bound to culture filtrate proteins of *M. bovis* and *BCG* while showing minimal cross-reactivity to *M. tuberculosis* H37Rv proteins. The anti-MPB70 antibodies of the SB series were found to be reactive to three different molecular species of 20 kDa, 22 kDa and 25 kDa, suggesting possible cross reactivity with other antigens (Wood et al. 1988). In a later study, MPB70 was further purified to homogeneity and used to produce mouse monoclonal antibodies with high affinity against MPB70. The monoclonal antibodies Bov-1, -2 and -3 were found to recognize different epitopes of MPB70, therefore indicating the presence of at least 3 binding sites on the protein. However, Bov-1 was the most potent mAb and reacted with only a single molecular species in the culture filtrate of *M. bovis* (Haga et al. 1992). More recently, mAbs have been produced and described as entirely specific to their corresponding protein, for example MBS43 binds specifically to MPB83 whereas 1-5C binds specifically to MPB70 (Wiker et al. 1996, Wiker et al. 1998). In a study investigating new antibodies to MPB70 and MPB83, Lyashchenko et al. (2001) identified that most mAbs produced against these proteins were of IgG1, IgG2a or IgG2b isotypes. They found several antibodies reacted with both proteins, however two new mAbs, 1-5A and 3-5C bound exclusively to MPB70. They further showed that these new antibodies had identical reactivity to the previously discovered anti-MPB70 antibody 1-5C (Lyashchenko et al. 2001). The discovery of novel mAbs is an important step for the development of serological assays for bTB.

2.4.2   Serological diagnostics

The high expression of MPB70 and MPB83 in *M. bovis* has led researchers to investigate whether antibody responses against MPB70 or MPB83 could be used as an indicator of *M. bovis* infection. One of the first studies was performed using sera from a herd of 44 cattle naturally infected with *M. bovis* using MPB70 as a capture antigen on an ELISA plate. This assay reported a sensitivity of 73% and 88% specificity, using histopathological diagnosis as the gold standard (Harboe et al. 1990). A number of studies investigating the diagnosis of bTB using MPB70 in serological assays reported variable sensitivities (18%-73%), although high specificities have been observed (88-96%) (Wood et al. 1992, Sugden et al. 1997, Lyashchenko et al. 2000, Cho et al. 2007). Humoral responses are generally low or absent in the first stages of the infection period but are known to significantly increase in later stages of disease (Fifis et al. 1994). Antibodies against MPB70 have been shown to develop between 18-22 months after experimental infection of cattle with *M. bovis*; in contrast antibodies to MPB83 can be detected as early as 4 weeks post-infection (O'Loan et al. 1994, Waters et al. 2006). Low sensitivities (37%-37.5%) have also been recorded for serological assays detecting antibodies against MPB83, however the specificity (89%-98%) remains high (Goodger et al. 1994, McNair et al. 2001). Due to the high specificity achieved using these antigens in serological tests, there is still potential for the development of cost effective and specific diagnostic assays for the detection of bTB. There is a renewed interest in antibody based diagnostics with recent studies showing promising results for detecting infected animals missed by conventional TB diagnostics such as the tuberculin skin test (TST) and Interferon Gamma (IFN-γ) Release Assays (IGRA) (Koo et al. 2005, Green et al. 2009, Casal et al. 2014). Serological assays using MPB83 for the detection of *M. bovis* infection have now been developed and used in a number of different species including cattle, badgers, wild boar, non-human primates and white-tailed deer (Liu et al. 2007, Greenwald et al. 2003, Lyashchenko et al. 2007, Lyashchenko et al.

2008). Waters et al. (2011) described the performance of the IDEXX *M. bovis* ELISA (IDEXX Laboratories, Inc., Maine, USA) assay which detects antibodies against both MPB70 and MPB83 for the diagnosis of bTB in cattle. In this study they reported a sensitivity of 63% and a specificity of 98% with samples from naturally infected cattle (Waters et al. 2011). Additionally the IDEXX *M. bovis* ELISA was able to detect antibodies against both proteins in milk samples from bulk tanks of *M. bovis* infected dairy herds (Waters et al. 2011). In a separate study, using MPB70 and MPB83 separately as capture antigens in an ELISA, a significant difference in antibody response was observed for cattle infected with *M. bovis* compared to those infected with *M. avium* subsp. *paratuberculosis*, therefore showing promise as a tool to distinguish between these two infections (Marassi et al. 2009). A commercially available test produced by Chembio® called the Dual Path Platform VetTB (DPP) (Chembio Diagnostic Systems, Inc., NY, USA) uses lateral-flow technology to detect antibodies against MPB83 and the fusion protein ESAT-6/CFP-10. The VetTB DPP was found to have a sensitivity and specificity of 72% and 98%, respectively, in white-tailed deer naturally infected with *M. bovis* (Lyashchenko et al. 2013). MPB70 and MPB83 both show promising results for use as capture antigens, although further research is required to optimize test performance and determine the specific value of serological bTB diagnostic tests in different species. The availability of rapid serodiagnostic tests for use in a wide range of animal species is important for the early detection of *M .bovis* infection, and because of their relatively high specificity they can be used as a screening tool for disease surveillance in wildlife where handling of animals presents risks to both the animal and staff.

2.4.3   Cellular immune response

T-cell responses were characterized during the initial experiments on purification of MPB70 when it was established that this protein induced a strong skin test reaction in guinea pigs sensitized with BCG Tokyo, but not *M. tuberculosis* (Nagai et al. 1986). The peak of the skin

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test response to MPB70 was found to be 15 weeks after sensitization of guinea pigs with BCG Tokyo (Haslov et al. 1987). The cytokines IFN-γ and tumour necrosis factor-α (TNF-α), both involved in the immune response to mycobacterial infections, are induced *in vitro* in response to stimulation of peripheral blood mononuclear cells (PBMC) with MPB70 (Al-Attiyah et al. 2006). A study by Olsen et al. (2005) showed that natural killer (NK cells) isolated from clinically healthy calves produced IFN-γ in response to bovine PPD and ESAT-6 but not to MPB70. The IFN-γ response to the MPB70 antigen has been extensively studied for the diagnosis of tuberculosis, however, the ESAT-6/CFP-10 mixture has been observed to induce a much greater response (Pollock et al. 2000, Al Attiyah et al. 2006, Aangaard et al. 2006). Aangard et al. (2006) used MPB70 as a stimulation antigen in a CMI-based whole blood assay to detect IFN-γ in TST-positive cattle and cattle from a TB-free herd. Animals from herds of different prevalence were tested at three separate sites and the test sensitivity and specificity was determined to be 5%, 56%, 36% and 90%, 100%, 83%, respectively (Aangard et al. 2006). Although the T-cell response to MPB83 is less well characterized than that of MPB70, early studies showed that intradermally injected MPB83 induced a DTH response in guinea pigs sensitized with live BCG Tokyo (Harboe et al. 1995). MPB83 was shown to induce a stronger IFN-γ response in whole-blood cultures compared to MPB70 in 10 cattle that were TST-positive. In the same study MPB83 was able to differentiate between infected cattle and cattle vaccinated with BCG Pasteur (Vordermeier et al. 2000). MPB70 and MPB83 induced a low and moderate proliferative response, respectively, in stimulated PBMC from skin test positive cattle, however the response induced by ESAT-6 was much greater (Vordermeier et al. 1999). These results were confirmed by another group who evaluated the T-cell response in experimentally infected cattle to a panel of mycobacterial antigens including PPD, ESAT-6, MPB70 and MPB83. Although cell proliferation and release of IFN-γ was induced in PBMCs stimulated with MPB70 and MPB83, the responses to ESAT-6 and PPD were higher (Rhodes

et al. 2000). Therefore, due to low sensitivity, neither MPB70 nor MPB83 has the potential for use in a single-antigen-based diagnostic assay, but they have shown promise when included in protein cocktails. Using a protein cocktail composed of ESAT-6, MPB64, and MPB83 to stimulate the proliferation of PBMC, researchers were able to differentiate between animals that were TST-positive and those vaccinated with BCG (Vordermeier et al. 1999). In another study, a cocktail consisting of ESAT-6, CFP-10, MPB70 and MPB83 administered via intradermal injection elicited a DTH response in 78% of TST-positive cattle naturally infected with *M. bovis*. Additionally, this protein cocktail was highly specific and no DTH response was induced in naïve or BCG-vaccinated calves (Whelan et al. 2010). Both MPB70 and MPB83 appear to have poor IFN-γ inducing capabilities compared to ESAT-6 and CFP-10, however, when they were included in the skin test cocktail, skin test responder frequency increased from 3/12 using only ESAT-6 and CFP-10, to 6/12 (Whelan et al. 2010). These results suggest that the use of multi-antigen cocktails containing different immunogenic proteins can increase detection of bTB in cattle.

2.4.4 T-cell epitopes

In order to identify new candidate peptides for antigen stimulations or sub-unit vaccines it is crucial to identify the epitopes recognized by T-cells. Linear T-cell epitopes to MPB70 were mapped using thirteen 25-mer synthetic peptides with a 10 amino acid overlap, covering the entire protein sequence. The peptides showing the strongest responses in the PBMC proliferation assay were peptides 8, 12 and 13, which correspond to amino acids (AA) 106-130, 166-189 and 180-193, respectively (Al Attiyah et al. 2003). The same peptides were found to induce the strongest IFN-γ response after stimulation of PBMC from BCG-vaccinated healthy subjects (Al Attiyah et al. 2003). Proliferation assays of PBMC from *M. bovis* experimentally infected cattle demonstrated the immunogenicity of the MPB70 peptides at AA 118-135 and 174-193 which correspond to peptides 8 and peptide 13 of the previous study.

These peptides showed reactivity to naturally infected cattle that were TST-positive, however they were not recognized by non-infected controls (Pollock et al. 1994). These results were further confirmed by two separate studies. Lightbody et al. (1998) found strong T-cell epitopes in AA 81-100 and 171-193 in both MPB70 immunized cattle and cattle experimentally infected with *M. bovis*, whereas Tollefsen et al. (2002) discovered the middle (AA 75-140) and C-terminal (AA 160-193) regions contained epitopes which induced a cellular response in immunized mice. T-cell epitopes to the MPB83 antigen are less well characterized however they have been described. Vordermeier et al. (2000) assessed T-cell proliferation in response to peptide stimulation in cattle experimentally infected with *M. bovis* and field TST-positive cattle, and found that more than half of the animals reacted to peptides 20, 21 and 22 which correspond to the amino acids 184-205, 195-214, 204-225, respectively. More recently it was shown that MPB83 has Th1 cell epitopes scattered throughout the protein sequence, however, the three peptides that induced the strongest responses were amino acids 151-175, 166-190 and 196-220 (Mustafa et al. 2011). The first vaccine study in cattle using a DNA vaccination expressing either MPB70 or MPB83 showed that all calves, vaccinated with the MPB83 expressing plasmid, demonstrated a strong Th1 response characterized by IFN-γ release, whereas the response to vaccination with MPB70 was less effective. Importantly, the specificity of the skin test was not compromised (Vordermeier et al. 2000). The protective effect of MPB70 and MPB83 DNA vaccinations prior to intratracheal challenge with *M. bovis* has been investigated, however it was determined that the cattle were not protected from developing bTB by the vaccines (Wedlock et al. 2003).

2.5   Conclusion

The extensive studies performed on these two proteins have identified them as highly immunogenic and specific protein targets for the detection of *M. bovis* infection in a variety of animals. Although their use in CMI based diagnostic assays has shown a lack of sensitivity when compared to ESAT-6 or CFP-10, serological assays using either one or a combination of MPB70 and MPB83 have shown promise as a highly specific diagnostic tool for the rapid detection of *M. bovis* infection. Further studies are required to evaluate the immunogenicity and diagnostic potential of these proteins in other wildlife species as tools for the diagnosis of bTB.

Chapter 3 - Materials and Methods:

3.1 Animals

African buffaloes were captured and tested between 2011 and 2015 in the Hluhluwe-iMfolozi Park, Kwazulu-Natal, South Africa, as part of an ongoing bTB control program. Animals were chemically immobilized as previously described (Parsons et al. 2011) and subjected to the Single Comparative Intradermal Tuberculin Test (SCITT) as described below. During immobilization, whole-blood (WB) was drawn into heparinised blood collection tubes and serum tubes (both BD Biosciences, New Jersey, USA) via venipuncture of the jugular vein. Serum tubes were centrifuged at 3000 x g for 10 min after which the serum fraction was aliquoted into cryovials and stored at -20°C. Buffalo were defined as bTB-positive if they tested positive based on the SCITT and one ancillary interferon gamma (IFN-γ) release assay (IGRA) as described below (3.2), i.e. either the modified QuantiFERON® TB-Gold (mQFT) (Qiagen, Venlo, Limburg, Netherlands) or the Bovigam® assay (Prionics, Schlieren-Zurich, Switzerland). Animals that tested negative for both the SCITT and IGRA were defined as bTB-negative. Ethical approval for this study was granted by the Stellenbosch University Animal Care and Use committee (Protocol #: SU-ACUM13-00016).

3.2 Ante-mortem bTB diagnostic tests and sample processing

3.2.1 SCITT

Skin fold thickness (SFT) was measured at injection sites using Hauptner calipers (Kyron Laboratories, Benrose, South Africa). Thereafter, 0.1 ml (50 000 IU) *M. bovis* purified protein derivative (PPD-B) and 0.1 ml (25 000 IU) *M. avium* PPD (PPD-A) (both WDT, Hoyerhagen, Germany) were injected intradermally on either side of the neck. The SFT measurements were repeated after 72 hours in all animals with palpable inflammation at injection sites. Animals with an increase in SFT of 2 mm or greater at the *M. bovis* PPD injection sites compared to the *M. avium* PPD injection sites were defined as SCITT-positive.

3.2.2 mQFT

One ml of heparinized whole blood (WB) from each animal was transferred into a QFT-TB Antigen tube (containing ESAT-6, CFP-10 and TB7.7 peptides) and a QFT-Nil tube (containing saline) (Qiagen, Venlo, Limburg, Netherlands). The tubes were shaken before incubation at 37°C for 20 hours after which they were centrifuged at 3000 x g for 6 minutes and the plasma fraction transferred into a second tube and stored at -80°C. The remaining cell pellet was resuspended in 1.3 ml RNAlater® (Ambion, California, USA). Plasma harvested from the QFT tubes was used in an in-house IGRA (mQFT assay) utilizing a commercial bovine IFN-γ ELISA kit (Mabtech AB, Nacka Strand, Sweden) to quantify the concentration of IFN-γ. An animal was defined as mQFT-positive if the IFN-γ concentration in the plasma of the TB tube was 66pg/ml or more greater than in plasma from the Nil tube (Parsons et al. 2011).

3.2.3 Bovigam®

Two-hundred and fifty (250) µl of WB was added to each of four 2 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 25 µl solutions of PPD-B (Prionics, Schlieren-Zurich, Switzerland) (at a final concentration of 24 µg/ml), PPD-A (Prionics, Schlieren-Zurich, Switzerland) (at a final concentration of 24 µg/ml), filter-sterilized phosphate buffered saline (PBS) and Pokeweed mitogen (PWM) (Sigma-Aldrich, St. Louis, MO,USA) (at a final concentration of 5 µg/ml), respectively. Following incubation at 37°C for 20 hours, tubes were centrifuged at 2000 x g for 10 min and the plasma fraction transferred to a separate tube and stored at -80°C. All further analyses were done according to the manufacturer's instructions.

3.2.4 Peptide stimulation for MCP-1

One ml WB was added to six 2 ml microcentrifuge tubes (Eppendorf, Hamburg, Germany) which contained 20 µl of either saline (Nil), a 1 µg/µl solution of Keyhole Limpet Hemocyanin (KLH) peptides (Amino acid sequence: WQALQEKRGLPSDRAD (BIOPEP, Stellenbosch, South Africa) diluted in PBS and another diluted in 0.1% Dimethyl sulfoxide (DMSO), a 1 µg/µl solution of Bovine serum albumin (BSA) peptides (Amino acid sequence: KYLYEIARRHPYFYAPELL) (BIOPEP, Stellenbosch, South Africa) diluted in PBS and another diluted in 0.1% DMSO, and lastly a 0.1% solution of DMSO in PBS. The tubes were gently agitated and incubated at 37°C for 20 hours. Hereafter, tubes were centrifuged at 2000 x g for 10 min and the plasma fraction transferred to a separate tube. Plasma samples were stored at -80°C until tested. DMSO was used as a solvent to dissolve the peptides, a

control condition using DMSO alone was included to evaluate its effect on MCP-1 induction.

3.3 Serological assays

3.3.1   PPD ELISA

Flat-bottomed 96-well microtitre plates (Nunc, New York, USA) were coated with 100 µl of a 10 µg/ml PPD-B solution (Prionics, Schlieren-Zurich, Switzerland) in 0.05M carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. For each well coated with antigen a corresponding control well was coated with blocking buffer (BB) consisting of 5% milk powder (Clover, Roodepoort, South Africa) in PBS + 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA). After incubation, plates were decanted and washed five times with PBS containing 0.05% Tween-20, then blocked with 200 µl/well of BB for 1 hour at room temperature (RT). The plates were washed as above and either serum or plasma samples diluted 1:200 in BB were added to duplicate wells (100 µl/well). Plates were incubated at RT for 1 hour and then washed five times as above. Plates were then incubated with 100 µl/well of peroxidase-conjugated recombinant protein A/G (Thermo Scientific, MA, USA) diluted 1:100 000 in PBS, for 1 hour at RT. Plates were washed as above before addition of 100 µl/well of the colorimetric substrate 3,3',5,5'-tetramethylbenzidine (TMB) (BD Biosciences, New Jersey, USA) and subsequently incubated in the dark for 15 minutes. The reaction was stopped using 50 µl/well of 2M H_2SO_4 and the optical density (OD) of each well was measured at 450nm using a LT-4000 Microplate Reader (BMG Labtech, Vienna, Austria). For each animal, an assay result was calculated as the mean OD value of the PPD-coated wells minus that of the BB-coated wells. The PPD ELISA was used as a

measure of antibody levels only and not as a diagnostic, therefore no positive threshold was calculated.

3.3.2 Dual Path Platform (DPP) VetTb® assay

The assay was performed according to the manufacturer's instructions as follows: 5 µl of serum or plasma was added to the sample well followed by 2 drops of buffer. After 5 minutes, 4 drops of buffer were added to the conjugate well. After 15 minutes the test results were read, animals were classified as antibody-positive if there was a visible line present on either band, while those with no visible line were classified as antibody negative. A DPP optical reader device (Chembio Diagnostic Systems Inc., USA) was used to measure the reflectance of test strips and a result was quantified as a numerical score, represented as reflectance units (RU).

3.3.3 Statistical analysis

Spearman's rank correlation was performed using GraphPad Prism (version 5.00) to evaluate the correlation between the PPD ELISA and DPP VetTB assay for the 20 bTB-positive buffalo. A t-test was performed to compare the means between the bTB-positive and bTB-negative cohorts on the PPD ELISA.

3.4 MCP-1 diagnostic performance

3.4.1 MCP-1 ELISA

Anti-bovine MCP-1 capture antibody (catalog no. PB0092B-100; Kingfisher Biotech Inc., St Paul, MN, USA) diluted 1:400 in PBS was added to each well (100 µl/well) of

a 96-well flat-bottom polystyrene plate (Nunc, New York, USA) and incubated overnight at 4°C. Plates were decanted and then washed five times with a wash buffer made up of PBS and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). A blocking buffer (BB) consisting of 0.1% bovine serum albumin (BSA) (Roche, Basel, Switzerland) and 0.05% Tween 20 in 1X PBS was added at a volume of 200 µl to each well before being incubated for 1 hour at RT. The plates were washed as above before addition, to each well, of 100 µl of plasma, diluted 1:100 in BB. In addition, a dilution series of recombinant bovine MCP-1 protein (catalogue no. RP0027B-005; Kingfisher Biotech Inc., St Paul, MN, USA) in BB, ranging from 0 to 2500 pg/ml was added to separate wells. Samples were incubated for 2 hours at RT. Following another wash step, the plates were incubated at RT for 1 hour after addition of 100 μl/well of biotinylated anti-bovine MCP-1 detection antibody (catalogue no. PB0091b-050; Kingfisher Biotech Inc., St Paul, MN, USA) diluted 1:4000 in BB. The plates were washed as above and 100 µl/well of streptavidin-horseradish peroxidase (R&D Systems, USA) diluted 1:200 in BB was added to each well and incubated for 30 min at RT. After a final wash step, 100 μl/well of TMB was added to each well and incubated for 15 min in the dark. The reaction was stopped with 50 μl/well of 2M H2SO4, and the optical density (OD) was measured at 450nm using a LT-4000 Microplate Reader (Labtech, Vienna, Austria). The MCP-1 concentration in each sample was calculated in GraphPad Prism (version 5.00) using the standard curve generated from the serial dilution of recombinant bovine MCP-1. The antigen-specific release of MCP-1 was determined by subtracting the MCP-1 concentration in the Nil sample from the MCP-1 concentration in the corresponding TB-antigen stimulated sample.

3.4.2   Protein stability

3.4.2.1   Plasma storage stability

The stability of plasma MCP-1 on Whatman® protein saver cards (PSC) (Sigma-Aldrich, St. Louis, MO, USA) was assessed in samples from two animals; one with a high concentration of MCP-1 (59164.79 pg/ml) and one with a lower concentration (5707.53 pg/ml). Plasma was spotted onto PSC in duplicate volumes of 1 µl and 5 µl and left to dry for 30 min. The card was then sealed in a plastic sleeve and stored in the dark at RT for 11 days. In addition, aliquots of plasma samples were stored in microcentrifuge tubes at -80°C for this period. Hereafter, a paper punch was used to cut out a 5 mm circle of card containing the dried plasma spot. The concentration of MCP-1 in all samples was measured by ELISA, as described above, with the following modification: for plasma stored on PSCs, a single punched-out circle was added to a well containing 100 µl of BB.

3.4.2.2   Heat-treatment stability

Plasma from two buffaloes was diluted in BB in individual tubes to a final volume of 200 µl at dilutions of 1:5, 1:20 and 1:100. The tubes were then placed in a heat-rack, preheated to 65°C, and heat-shocked for 20 min. MCP-1 protein levels were quantified via ELISA using the heat-shocked plasma as test sample, the 1:100 dilution was added directly to the wells of the ELISA plate whereas the 1:5 and 1:20 were diluted appropriately in blocking buffer to 1:100 before being added to the wells. A plasma sample that was diluted to 1:100 and stored at 4°C during the 20 min heat-shock was used as a control to assess the effect of heat on the protein.

3.4.3   Statistical analysis

A t-test was performed in GraphPad Prism (version 5.00) to calculate if there was a significant difference between the means of bTB-positive and bTB-negative animals when evaluating the diagnostic performance of MCP-1. When comparing three or more conditions for the peptide stimulation, a Kruskal-Wallis test was performed; if the medians were significantly different ($p<0.05$) a Mann Whitney test was performed between individual groups.

3.5 Validation of reference gene stability

3.5.1   Sample selection

The samples used were obtained from a QFT-TB and QFT-Nil tube from 8 bTB-positive animals in order to assess the variation of gene expression between the two treatments. RNAlater®-stabilized whole-blood samples were used for RNA extraction as described below.

3.5.2   RNA extraction and reverse transcription

Total RNA was isolated from samples using the RiboPure™-Blood Kit (Ambion, California, USA) according to the manufacturer's protocol. The quantity and quality of extracted RNA was estimated using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and samples stored at −80°C. RNA samples with A260/A280 > 1.8, A260/A230 between 2.0-2.2 and a concentration of >30 ng/ml were chosen for cDNA synthesis. First-strand cDNA was synthesized from 200 ng of RNA from each sample using the QuantiTect Reverse Transcription Kit (Qiagen, Netherlands). The

cDNA sample was then diluted in RNAse-free H$_2$0 to a final volume of 40 µl and stored at −80°C.

3.5.3 Sequencing of buffalo reference genes

For selected reference genes (Table 1), the mRNA sequence of the domestic cow *(Bos Taurus)* was obtained from the nucleotide database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) and Ensemble genome browser (http://www.ensembl.org/). Regions in the 5' untranslated region (5′ UTR) and 3'UTR of these sequences were selected for primer binding, thereby ensuring complete coverage of the gene transcript. Using the online software Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), primers were designed to prime in the 5'UTR and 3'UTR regions. The cDNA from two animals was used for PCR as follows. Each PCR reaction consisted of 2.5 µl 10x PCR buffer, 0.125 µl HotStarTaq DNA polymerase (both Qiagen), 1 µl template cDNA, 0.5 µl (0.2 mM each) dNTPs (Promega Corporation, USA), 1 µl (0.4 µM) of each primer (Inqaba biotec, Pretoria, RSA) and 18.875 µl of DNAse-free H$_2$O (Applied Biosystems, California, USA). An initiation step of 95°C for 15 min was followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Thereafter, a final extension step at 72°C for 10 min. Gel electrophoresis was performed on the resulting PCR products; bands of the correct corresponding sizes confirmed that amplification of the transcripts was successful. PCR products were sequenced using a 3130xl Genetic Analyzer (Applied Biosystems, California, USA) according to the manufacturer's recommendations.

3.5.4   qPCR assay development

Primers for qPCR were designed using Primer3Plus and the African buffalo gene sequences obtained as above. Primer selection criteria included an amplicon size between 80 – 150bp, a GC content of 40-60% and an optimal Tm of 60°C. All primers were designed either to anneal across exon-exon boundaries, or anneal to exons separated by a large intron, thus minimizing genomic DNA amplification.

qPCRs were performed in 96-well MicroWell™ plates (Sigma-Aldrich, St. Louis, MO, USA) using the CFX96 real-time PCR detection system (Biorad, California, United States). Reactions were performed in triplicate with each well containing 5 µl iTaq™ Universal SYBR® Green supermix (Biorad, California, United States), 1 µl cDNA, 0.5 µl of each primer (a final concentration of 250nM each) and 3 µl of DNAse-free H_2O. A negative control (all reagents except template cDNA) was included for each assay to confirm the absence of any DNA contamination. The cycling conditions consisted of an initial denaturation at 95°C for 30 seconds (s), followed by 40 cycles of 5 s denaturation at 95°C and 30 s annealing-elongation at 60°C. A melt curve analysis from 70°C-90°C with increments of 0.5°C was used to ensure a single product was being amplified in each reaction.

3.5.5   Optimization

Annealing temperature for each primer set was optimized using a temperature gradient with temperatures of 57°C, 60°C and 63°C. PCR efficiency for each primer set was calculated using a standard curve made up of serial dilutions of pooled cDNA samples from 2 selected animals with the highest mRNA concentrations (118.9 ng/µl and 112.9

ng/µl). Beginning at approximately 60 ng/µl, the cDNA sample was serially diluted 4-fold covering 7 concentration points with 3 replications at each point.

3.5.6   Data analysis

Data from the qPCR reactions was acquired using Bio-Rad CFX Manager software (version 3.1) and recorded as Quantification cycle (Cq) values, i.e. the PCR cycle at which a fluorescence signal is first detected above an automatically determined threshold value. The mean Cq value for each reference gene was calculated from the 3 replicates of each sample and used for further analyses. The PCR efficiency of each primer set was calculated from the Cq values of the standard curve using GraphPad Prism software (version 5.00) and the equation $E = 10^{[-1/slope]}$. Relative gene expression data were analysed using the *geNorm* (Vandesompele et al. 2002) and *NormFinder* (Andersen et al. 2004) Microsoft Excel applets. A *NormFinder* package which runs in the 'R' software environment (version 2.10.0) was also used in order to compare results between the two methods. Cq values were converted into relative quantities, and the data were inputted into both software programs according to the specified format.

Chapter 4 - Results:

4.1 Animal cohorts

Archived plasma samples (collected between 2011 and 2013) and prospectively collected plasma and serum samples (collected in 2015) were utilized in separate sub-studies as follows:

1)   *Cohort 1 - Pilot study: PPD ELISA vs DPP VetTB (3.3)*

       Archived plasma samples of 20 bTB-positive buffaloes sampled in 2012.

2)   *Cohort 2 - Prospective study: PPD ELISA (3.3)*

       Serum samples, collected in 2015, from 30 bTB-positive buffaloes and 32 bTB-negative buffaloes, sampled pre-TST.

3)   *Cohort 3 - Pilot study: MCP-1 (3.4)*

       Archived plasma, collected in 2013, derived from mQFT-processed blood from 17 bTB-positive buffalo and 20 bTB-negative buffalo, sampled pre-TST.

4)   *Cohort 4 - Prospective study: MCP-1 (3.4)*

       The plasma samples used for this study were from 10 bTB-positive buffalo and 9 bTB-negative buffalo sampled in 2015 and stimulated in mQFT. Samples were collected post-TST.

5)   *Cohort – 5 Reference gene selection (3.5)*

       Samples from 8 bTB-positive buffalo sampled in 2011 and stimulated in mQFT were used. Animals were selected based on RNA concentration and quality.

4.2 Serology

Antibody responses in 20 bTB positive African buffalo (QFT and TST positive reactors) were evaluated using an in-house PPD ELISA and results compared with the Chembio® DPP VetTB assay. Figure 1 shows the antibody response analysed using the PPD ELISA (ΔOD) and Chembio® DPP assay (reflectance units, RU). Results displayed for the DPP assay correspond to the T1 antigen test line (MPB83) only. Based on the presence of a visible line on either antigen band, 9/20 buffalo were seropositive. Values for each assay are displayed in Table 1 in Appendix I.
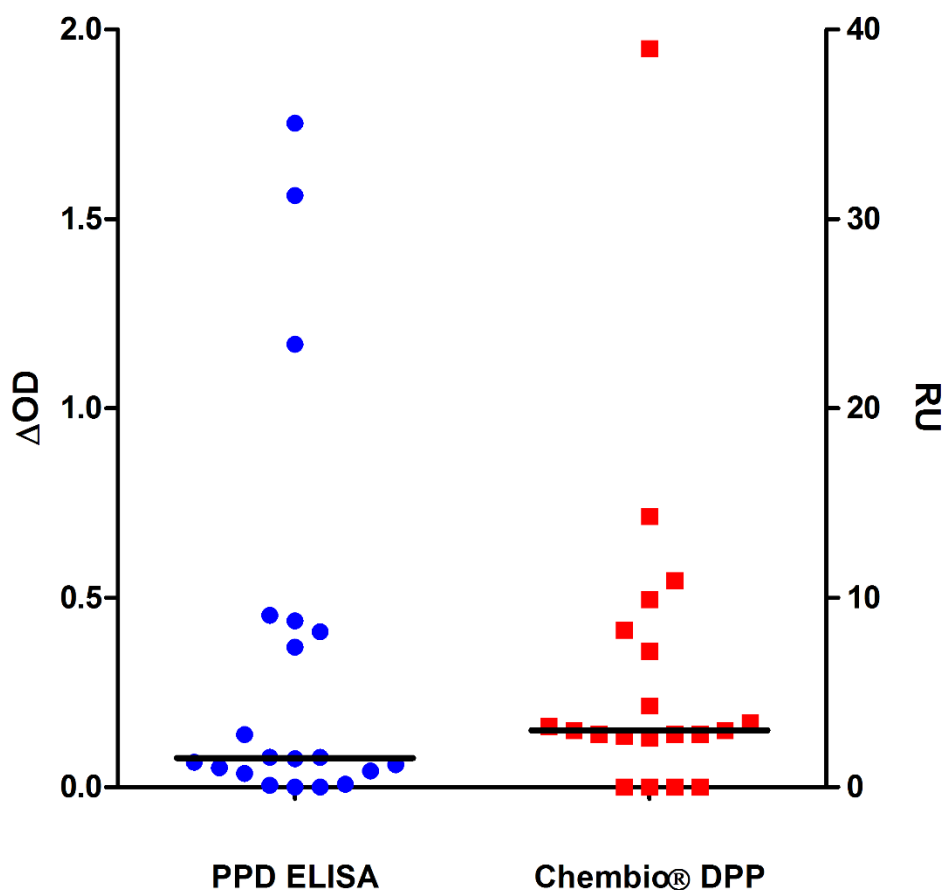
Figure 1. IgG antibody titres against PPD as measured by ELISA (ΔOD) and against MPB83 measured using a commercial rapid test (RU) in a cohort of 20 bTB-positive buffalo. Solid lines represent medians.

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There was a significant correlation (p = 0.0008) observed between the antibody response measured by PPD ELISA and the Chembio® DPP assay as determined by Spearman's rank correlation coefficient (r = 0.6868) (Figure 2).
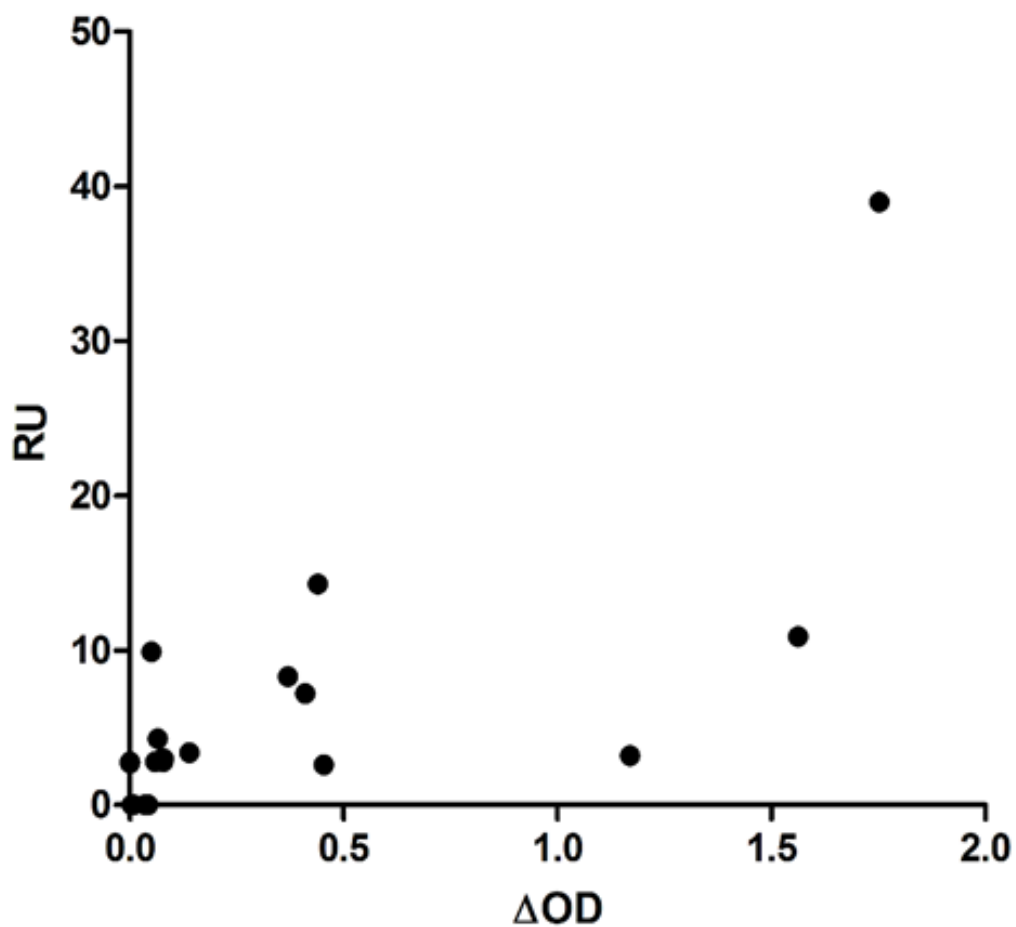
Figure 2. Scatterplot showing significant correlation in IgG antibody titers between the Chembio DPP assay (RU) and PPD ELISA (ΔOD) in bTB-positive buffalo (p = 0.0008, Spearman's r = 0.6868).

The antibody response to bovine PPD was evaluated by ELISA for a cohort of bTB negative (n = 32) and bTB positive (n = 30) African buffaloes characterized using the Bovigam® and TST (Cohort 2). There was no significant difference (p = 0.8380) observed between the means for the bTB-negative (mean = 0.7521) and bTB-positive (mean = 0.7301) groups (Figure 3).
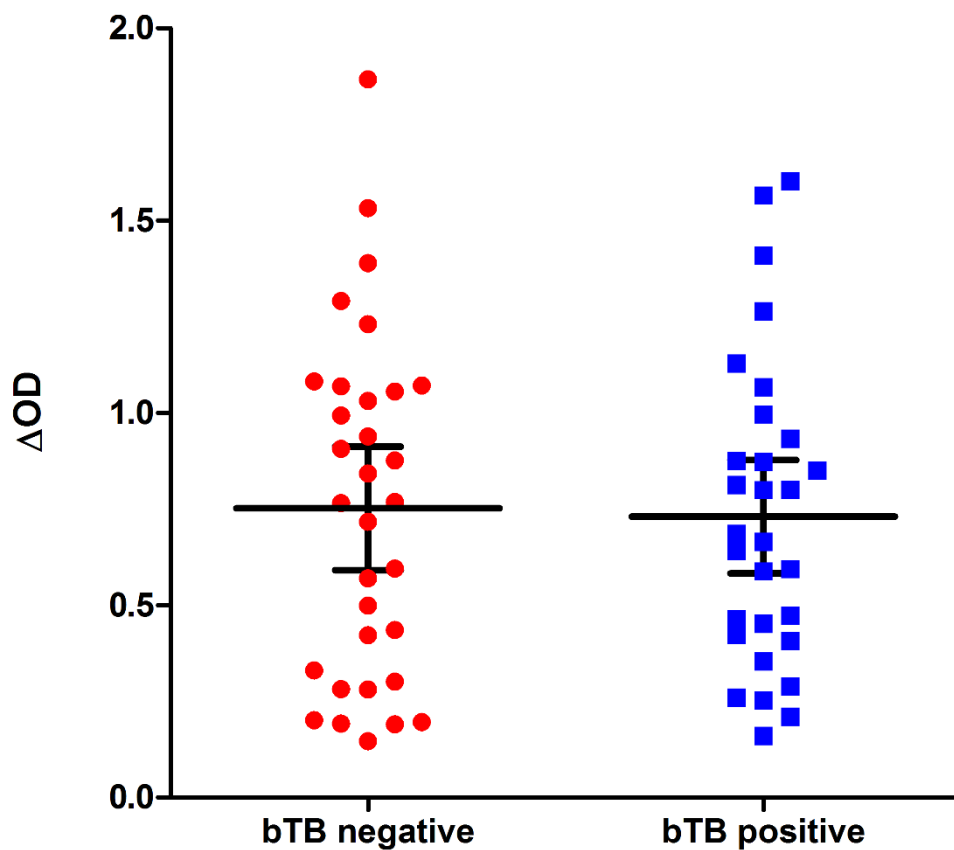
Figure 3. PPD specific antibody response measured by ELISA. Lines represent the mean and 95% confidence interval (CI). bTB-negative 95% CI = 0.5913 to 0.9128; bTB-positive 95% CI = 0.5828 to 0.8773

4.3 MCP-1 diagnostic performance

4.3.1   MCP-1 ELISA

The ESAT-6/CFP-10-specific release of MCP-1 was compared in bTB positive (n = 17) and bTB negative buffaloes (n= 20) (Cohort 3). There was a significant difference between the means of these groups (p = < 0.0001) (Figure 4). The mean ΔMCP-1 value determined for the bTB positive group was 16210 pg/ml while the mean for the bTB negative group was -10793 pg/ml.
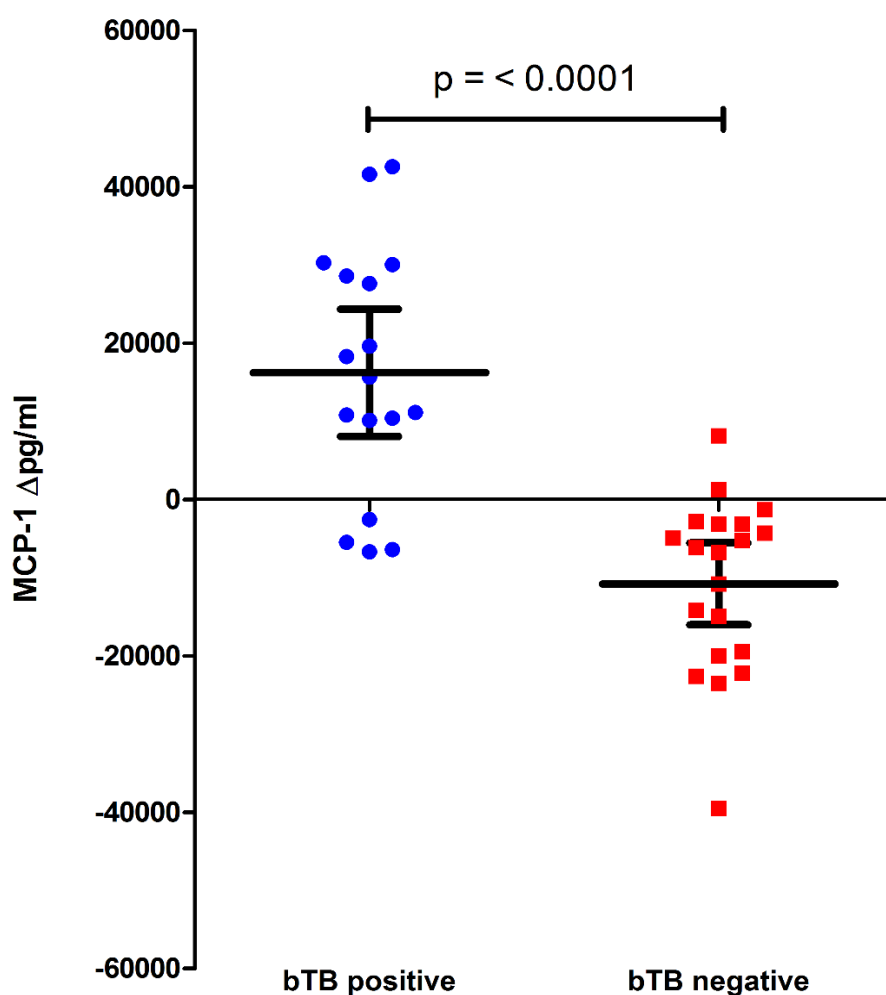
Figure 4. ESAT-6/CFP-10 antigen specific release of MCP-1 calculated as delta-pg/ml between an antigen stimulated sample and a control (Nil) sample. Mean and 95% CI shown.

For bTB-positive (n = 10) and bTB-negative (n = 9) buffaloes (Cohort 4), the antigen-specific release of MCP-1 was calculated as the difference in the MCP-1 concentration of the antigen (ESAT-6/CFP-10) stimulated sample and the control (Nil) sample (ΔMCP-1). The mean ΔMCP-1 concentration for the bTB-positive animals was 47 869 pg/ml and 23 061 pg/ml for the bTB-negative animals (Figure 5). The ΔMCP-1 results for these groups was significantly different (p = 0.0139).
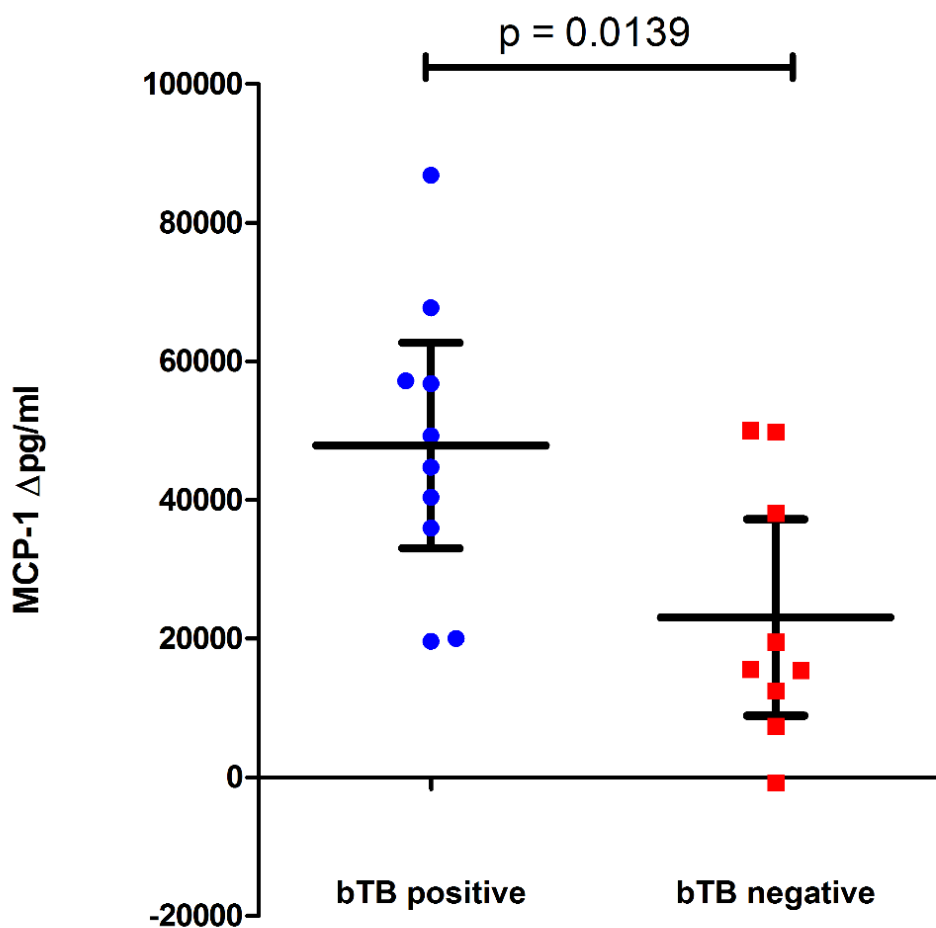
Figure 5. ESAT-6/CFP-10 antigen specific release of MCP-1 calculated as delta-pg/ml between an antigen stimulated sample and a control (Nil) sample. Lines represent the mean and 95% CI. bTB positive 95% CI = 33047 to 62690; bTB negative 95% CI = 8873 to 37250.

4.3.2 Plasma storage stability

The protein stability of MCP-1 after storage for 11 days on protein saver cards and frozen at -80°C was evaluated by ELISA. For buffalo B10, the MCP-1 concentration was 59 164 pg/ml in plasma stored at -80°C; 44 445 pg/ml in the 1 µl aliquot stored on a PSC; and 42 360 pg/ml in the 5 µl aliquot stored on a PSC. For buffalo B8, the MCP-1 concentration was 5707 pg/ml in plasma stored at -80°C; 819 pg/ml in the 1 µl aliquot stored on a PSC; and 3608 pg/ml in the 5 µl aliquot stored on a PSC (Figure 6).
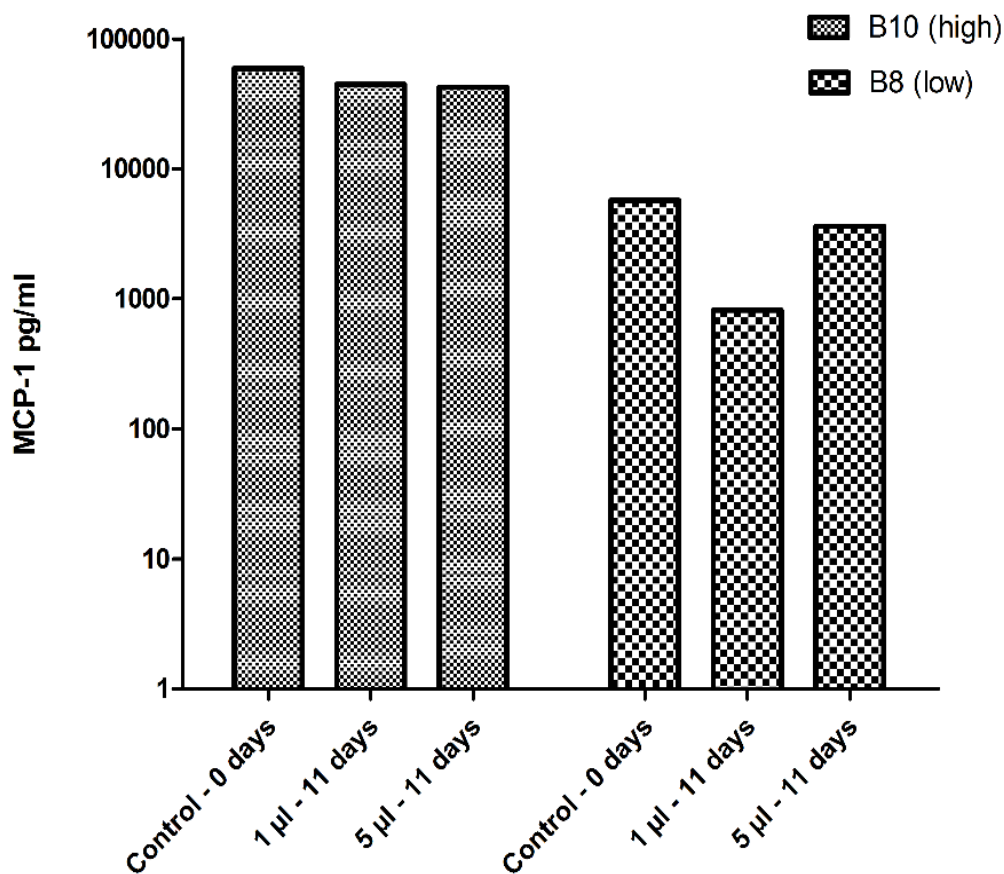
Figure 6. MCP-1 concentration of two bTB-positive buffaloes after storage of plasma samples on protein saver cards in aliquots of 1 µl and 5 µl for a period of 11 days.

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4.3.3   Heat-treatment stability

The ability of plasma MCP-1 to withstand heat-treatment was assessed. For buffalo B41, the MCP-1 concentration in the untreated plasma sample was 52 118 pg/ml. In plasma diluted 1:5, 1:20 and 1:100 in BB and heated for 20 min at 65°C, the MCP-1 concentrations were 48 935 pg/ml, 56 907 pg/ml, and 61 436 pg/ml, respectively (Figure 7). For buffalo B32, the MCP-1 concentration in the untreated sample was 22 977 pg/ml. In plasma diluted 1:5, 1:20 and 1:100 in BB and heated for 20 min at 65°C, the MCP-1 concentrations were 24 326 pg/ml, 30 126 pg/ml and 36 238 pg/ml, respectively (Figure 7).
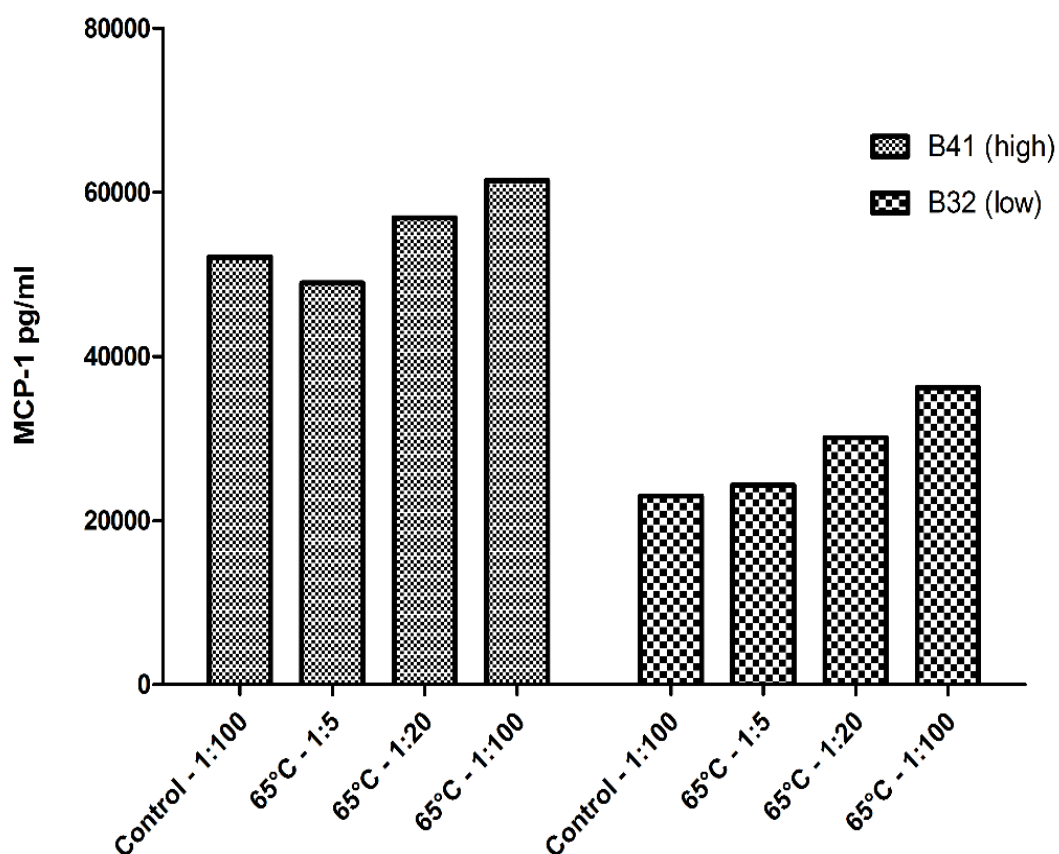
Figure 7. Graph representing the plasma concentration of MCP-1 from two bTB-positive buffaloes after 20 minutes of heat-treatment at 65°C for various dilutions.

4.3.4 MCP-1 induction in response to peptides

Stimulation with irrelevant peptides was investigated in an attempt to decrease the non-specific induction of MCP-1 observed previously (Figure 4). There was a large induction of MCP-1 in the sample co-incubated with 0.1% DMSO solution compared to the Nil (Saline) sample. When the induction of MCP-1 was measured between the 0.1% DMSO solution and the KLH and BSA peptide solutions dissolved in 0.1% DMSO, there was a decrease in MCP-1 concentration in both peptide samples compared to DMSO alone (Figure 8).
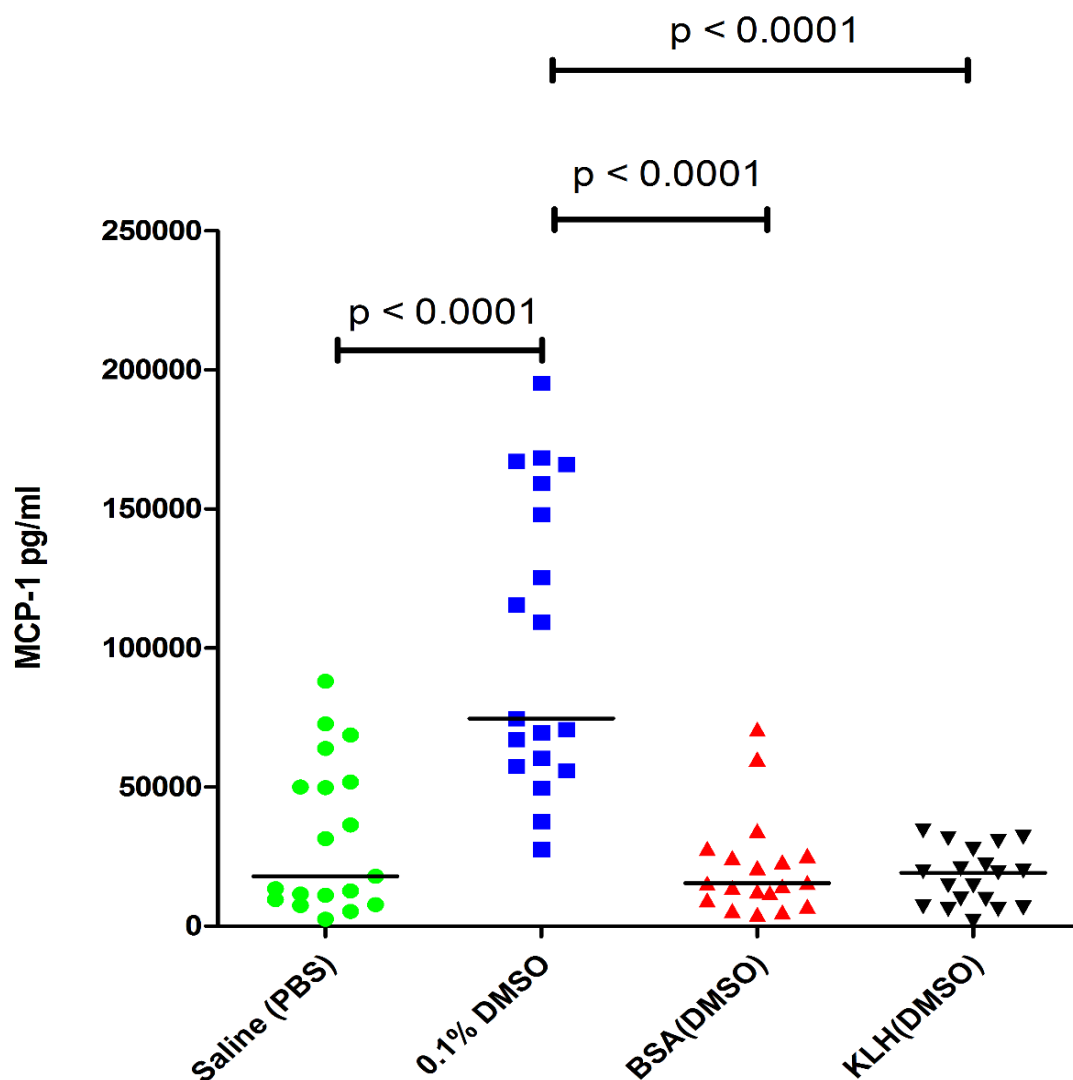
Figure 8. Induction of MCP-1 in response to stimulation with a 0.1% DMSO solution compared to saline. Furthermore an observed reduction in MCP-1 when stimulated with BSA or KLH peptides dissolved in 0.1% DMSO. Lines represent medians.

A Kruskal-Wallis test showed a significant difference ($p = < 0.0001$) between the median MCP-1 concentrations of the conditions. A Wilcoxon matched pairs test showed a significant difference ($p < 0.05$) between the medians of saline (17 942 pg/ml) compared to 0.1% DMSO (74 712 pg/ml), 0.1% DMSO (74 712 pg/ml) compared to BSA(DMSO) (15 470 pg/ml) and 0.1% DMSO compared to KLH(DMSO) (19 228 pg/ml). There was no significant difference observed between the medians of BSA(DMSO) and KLH(DMSO) samples themselves (Figure 8).

As calculated by a Kruskal-Wallis test, there was no significant difference observed between the medians of samples co-incubated with saline (PBS) and samples stimulated with BSA and KLH peptides dissolved in PBS (Figure 9).
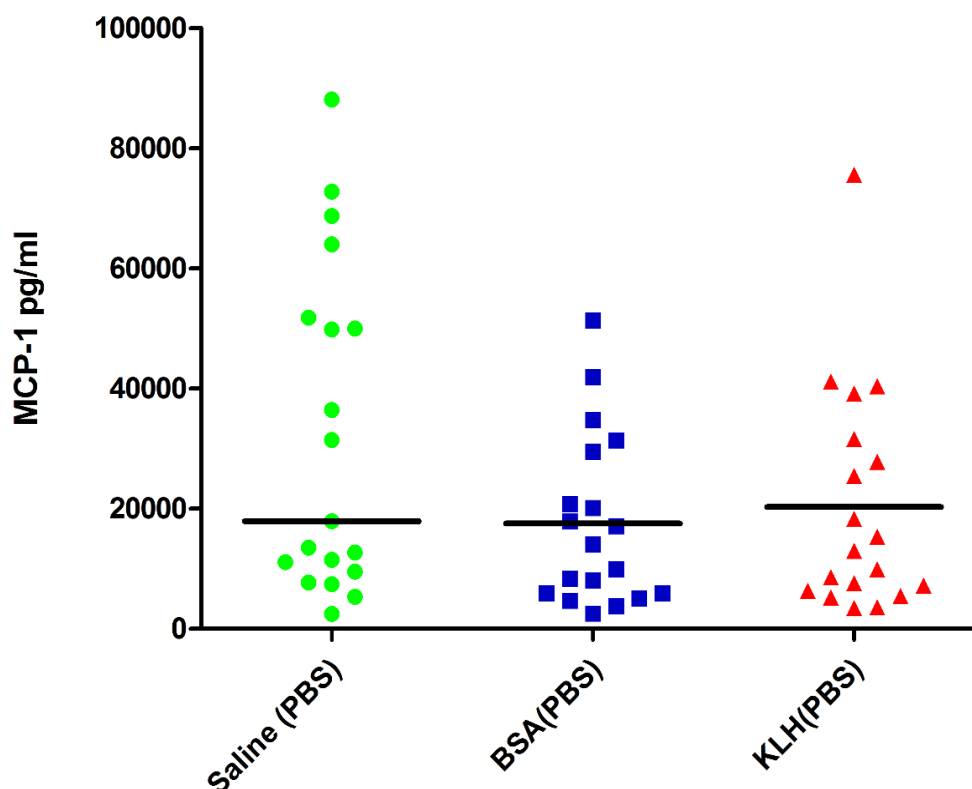
Figure 9. Co-incubation of blood samples with saline compared to stimulation with BSA and KLH peptides dissolved in PBS revealed no significant difference ($p = 0.2112$) between the median MCP-1 concentrations.

4.4 Reference gene selection

4.4.1 Reference gene sequencing

Table 2 lists primers used for sequencing of selected reference gene mRNA transcripts of the African buffalo. PCR products were analysed via gel electrophoresis to confirm they were the correct length. Transcript sequences, aligned to those of the domestic cow, are presented in Appendix II. The mRNA transcripts of the buffalo reference genes shared homology of between 98% and 100% with the transcripts of the cow.

Table 2. Primers used for the sequencing of selected African buffalo reference genes, indicating gene name, primer sequence, primer binding region and PCR product size.

Gene name	Primer sequence (5'-3')	Region	Product size (bp)
GAPDH	F: TAACTTCTGTGCTGTGCCAG R: AGATTCTCAGTGTGGTGGAG	5'UTR 3'UTR	1059
ACTB	F: TCGACACCGCAACCAGTTCG R: GGGTGTAACGCAGCTAACAG	5'UTR 3'UTR	1087
B2M	F: CCACCGCGCAGCTGCTGCAA R: GAATGTTCAAATCTCGATGGTGC	5'UTR 3'UTR	407
YWHAZ	F: TCCGGACACAGAACATCCAGT R: GCAGACAAAAGTTGGAAGGCA	5'UTR 3'UTR	788
PPIA	F: CCTTGCAGACGCCGCCACCT R: GGTGCTTAAGTAAAACACAAGTC	5'UTR 3'UTR	570
H3F3A	F: AAGTAAGGAGGTCTCTATACC R: GTTTCCCATCATAGTGGATTC	5'UTR 3'UTR	455
TBP	F: TTGCCAAGAAGGTGAACGTC R: GAGGCAGGAGAACACAGCAG	5'UTR 3'UTR	980
HPRT1	F: CTCCCCAGACTGCCTTCAG R: CCAAACTCAACTCGGACTCTC	5'UTR 3'UTR	715

4.4.2   qPCR development

The primers used for qPCR and subsequent gene expressions stability analysis are listed in Table 3. The qPCR amplicon sizes for each gene were confirmed by gel electrophoresis, furthermore the amplicon of each primer set produced a characteristic melt curve (data not shown). Annealing temperature for all primer sets of 60°C was chosen for the analysis. The efficiency of all qPCRs ranged from 100% – 119%, and all r^2 values were >0.980 (Table 3).

Table 3. Reference genes included in the gene expression stability analysis, indicating gene name, primer sequence, amplicon size, PCR efficiency and r^2 value.

Gene name	Primer sequence (5'-3')	Amplicon size (bp)	PCR Efficiency	r^2
GAPDH	F: ATGGTGAAGGTCGGAGTGAC R: ACGATGTCCACTTTGCCAGA	86	108%	0.998
ACTB	F: GGCATCCTGACCCTCAAGTA R: CACACGGAGCTCGTTGTAGA	102	109%	0.999
B2M	F: CGTGGCCTTGGTCCTTCT R: TTTGGCTTTCCATCTTCTGG	111	100%	0.999
YWHAZ	F: AGACGGAAGGTGCTGAGAAA R: CGTTGGGGATCAAGAACTTT	123	119%	0.995
PPIA	F: GGCCGCGTCTCTTTTGAG R: TCCTTTCTCTCCAGTGCTCAG	84	104%	0.996
H3F3A	F: GGGTGAAGAAACCTCATCGT R: AGACGCTGGAAGGGAAGTTT	109	103%	0.999
TBP	F: ACAACAGCCTCCCACCGTAT R: GGCTGTGGAGTCAGTCCTGT	115	103%	0.999
HPRT1	F: TGGATTACATCAAAGCACTGAA R: TATGTCGCCTGTTGACTGGT	107	109%	0.999

4.4.3   Reference gene expression stability analysis

The average stability values for each reference gene were calculated to determine the stability in expression of genes in the antigen-stimulated and unstimulated samples. The expression stability of the 8 reference genes as determined by geNorm are shown in Figure 10 with a lower M value representing a more stable gene. The expression stability of the tested genes were observed to be different, with the M values ranging from 0.22 to 0.47. The average M value of the pair *H3F3A* and *PPIA* was lowest at 0.22 and this pair was identified as the most suitable reference genes. The M value of *GAPDH* was the highest at 0.47 and therefore represented the most unstable reference gene.
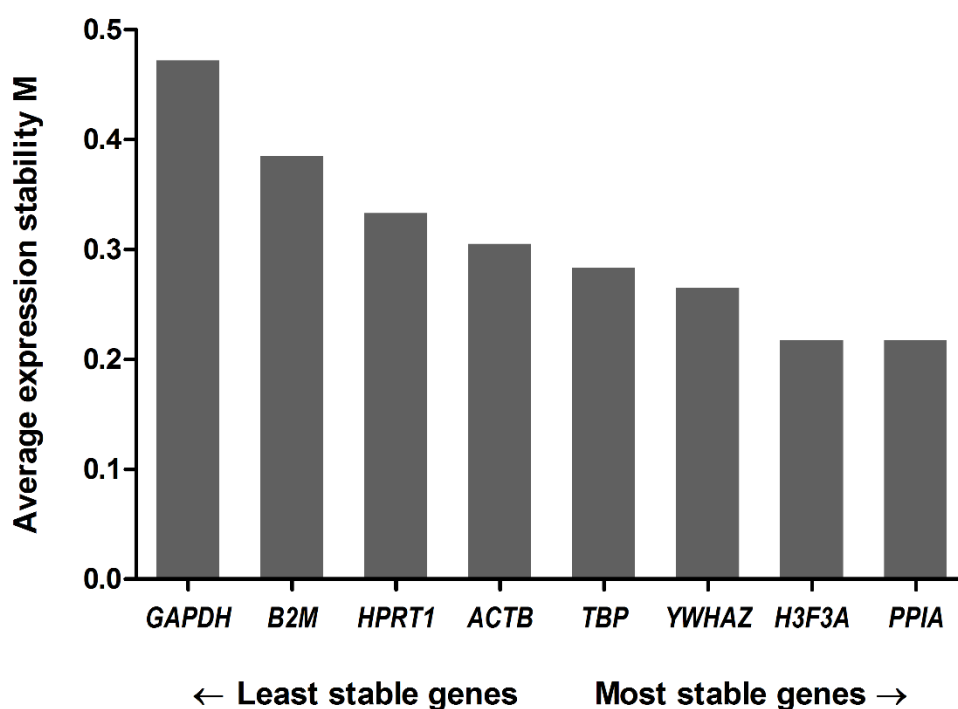
Figure 10. The average expression stability values (M) of each reference gene as calculated using the geNorm Excel applet. The lower the M value, the more stable the gene is under the tested conditions. In this case the most stable genes would be *H3F3A* and *PPIA*.

The NormFinder algorithm was also used to calculate the expression stability of the 8 reference genes. Higher expression stability is indicated by a lower stability value, referred to as the NormFinder stability index. Figure 11 shows the expression stability of each gene as calculated by a NormFinder Excel applet as well as a NormFinder algorithm run using the 'R' software package.
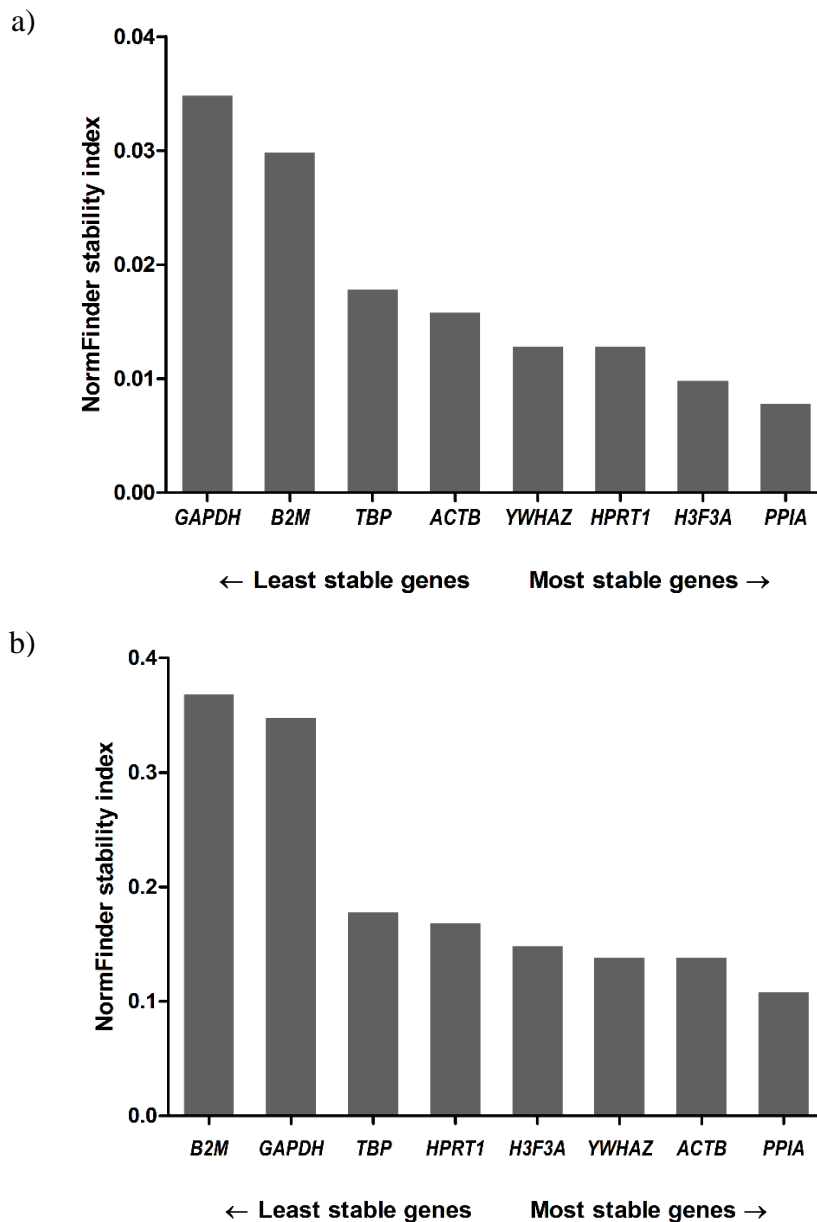
Figure 11. NormFinder gene expression stability results. a) Gene expression stability calculated using a NormFinder Excel applet. b) Gene expression stability results determined via an algorithm run using the 'R' software package.

The stability values calculated using the NormFinder Excel applet ranged from 0.008 to 0.035, the two best performing reference genes were *PPIA* (0.008) and *H3F3A* (0.010). The two most unstable genes according to the Excel applet were *GAPDH* (0.035) and *B2M* (0.030) (Figure 11a). The NormFinder 'R' algorithm was in agreement with the Excel applet in selecting the most stable reference gene as *PPIA* with a stability value of 0.11, closely followed by the two next most stable genes *ACTB* and *YWHAZ* which had a stability value of 0.14. The two worst performing reference genes according to the 'R' algorithm were *GAPDH* (0.35) and *B2M* (0.37) (Figure 11b).

Chapter 5 - Discussion:

Our results show that the use of bovine PPD as a capture antigen in an indirect ELISA is capable of detecting *M. bovis*-specific antibodies in bTB-positive buffalo. This was confirmed by using a commercial rapid test capable of detecting antibodies against the antigens MPB83 and ESAT-6/CFP-10 of *M. bovis.* To our knowledge, this is the first study conducted in South Africa to evaluate the use of a PPD ELISA for the detection of humoral responses in bTB-positive African buffalo. In an initial study, the animals tested revealed a broad range of PPD-specific antibody titres. There were a few high (n = 3/20) and intermediate (n = 4/20) reactors to PPD while the majority of animals showed a negligible PPD-specific antibody response. The DPP VetTB assay was chosen because of its reported ability to detect antibodies against *M. bovis* in other species, and to evaluate its use in buffalo compared to the in-house PPD ELISA (Greenwald et al. 2009, Lyashchenko et al. 2013). For the DPP VetTB, a similar pattern of antibody responses was observed, with 9/20 (45%) of the bTB-positive buffalo identified as being sero-positive to the MPB83 antigen. In contrast, few animals (2/20) were sero-reactive to ESAT-6/CFP-10, a finding similar to Green et al. (2009) we showed that antibody reactivity to MPB83 is a more consistent indicator of *M. bovis* infection than reactivity to ESAT-6/CFP-10. Further analysis showed that there was a significant correlation between the results of the PPD ELISA and the DPP VetTB, thus indicating that both assays are able to detect humoral responses to *M. bovis* infection in African buffalo. MPB83 expression is known to be high in *M. bovis*, therefore the correlation between the PPD ELISA and the DPP VetTB may be due to antibodies binding MPB83 in the PPD (McNair et al. 2001).

In order to further characterize the antibody response to infection, the in-house ELISA was evaluated in a larger cohort of animals (Cohort 2). PPD-specific-Antibodies were detected in both positive and negative buffalo with a widely overlapping range of titres with no significant

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difference in titre between the groups (Figure 3). A similar overlap of antibody responses was observed by other researchers using a PPD ELISA to measure antibody levels in a cohort of 283 cattle from bTB-infected herds and 277 cattle from a bTB-free area (Amadori et al. 1997). Our results demonstrate that the use of this PPD ELISA for detection of *M. bovis*-specific antibodies may not be a sufficiently specific tool for the diagnosis of bTB in African buffalo. The overlapping antibody response seen between the bTB-positive and negative groups may be due to shared antigens between *M. bovis* and related bacterial species which can include other mycobacteria (such as environmental non-tuberculous mycobacteria), including *M. avium*, or gram-positive bacteria A potential control for this could be the inclusion of an *M. avium* PPD-coated well in the assay, allowing for measurement of the differential antibody response to *M. bovis* and *M. avium*. The bTB-negative buffalo in the present study were all from an area endemic for bTB, it is possible they have been exposed to *M. bovis* and could be at an early stage of infection, however they may have also become anergic and therefore lack a CMI response. In agreement with our findings, other studies evaluating the performance of a PPD ELISA for the detection of antibodies against *M. bovis* in cattle have shown varying sensitivity and specificity. Ritacco et al. (1986) recorded a sensitivity of 90% in *M. bovis* culture-positive cattle and a specificity of 89.8% in a herd of healthy cattle from a bTB-free area. However, when the study was later repeated using new cohorts the sensitivity dropped to 73% while the specificity was higher at 94% (Ritacco et al. 1990). Another study which showed promising results was conducted by Lilenbaum et al. (1999) who determined the sensitivity and specificity of a PPD ELISA in cattle to be 86.7% and 90.6%, respectively. In contrast, there have been reports of low sensitivity, 63% and 48%, as well as low specificity, 52% and 55%, for the detection of antibodies in cattle using a PPD ELISA (Auer et al. 1987, Sayin et al. 2013). It is therefore evident that test performance of the PPD ELISA in cattle can vary, this may depend on the location where the samples were collected, since it has been shown that

prevalence and stage of disease affects diagnostic sensitivity and specificity (Bentley et al. 2012).

Although serological tests have limitations for the diagnosis of bTB, they have several advantages over the TST; animals are only handled once for the collection of blood samples, and sampling can be repeated without affecting the immune sensitization of the animal. Antibodies tend to appear in more advanced stages of disease when there is a high antigenic load and have been shown to correlate with disease pathology. Therefore serological tests can be useful in identifying animals that are anergic, and which fail to respond to CMI based diagnostics such as the TST (Neill et al. 2001, Plackett et al. 1989).

Two highly immunogenic proteins of *M. bovis*, MPB70 and MPB83 have been used as coating antigens in ELISAs in an attempt to increase the detection of antibodies against *M. bovis* in infected animals. Wood et al. (1992) developed an ELISA using MPB70 and reported a sensitivity and specificity of 18.1% and 96.4%, respectively, in cattle infected with *M. bovis* and another group from a TB-free area. In another study which used MPB83 as a capture antigen, sensitivity was estimated at 37.5% while specificity was 89%. Although the sensitivities for assays using these proteins were very low, the high specificities indicate potential for their use in serological assays for screening large herds for *M. bovis* (McNair et al. 2001). Other serological assays making use of the immunogenicity and specificity of these proteins include the commercially available IDEXX *M. bovis* Ab test which uses an ELISA for the detection of antibodies against both MPB70 and MPB83 (Waters et al. 2011). This test, in combination with conventional CMI tests, may increase detection of infected animals that may be missed by the TST alone. Validation of the IDEXX in a cohort of naturally infected cattle reported an apparent test sensitivity of 63% and specificity of 98%; however sensitivity was shown to increase with disease severity (Waters et al. 2011).

MCP-1 is essential for B-cell proliferation and antibody class switching, in addition it is involved in the formation of granulomas by recruiting immune cells to sites of infection (Deshmane et al. 2009). Therefore, we evaluated the use of MCP-1 as a diagnostic marker for *M. bovis* infection and found a significant difference in the mean MCP-1 concentration between bTB-positive and bTB-negative buffaloes (Cohort 3). However, the TB-antigen stimulated samples from bTB negative animals had lower plasma concentrations of MCP-1 compared to the Nil (saline) sample, which caused the differential concentration to be a negative value (Figure 4). The mechanisms resulting in this phenomenon are unclear, but incubation and handling of blood samples may cause spontaneous induction of MCP-1 by monocytes. Antigen processing by monocytes may alter the MCP-1 induction pathways, leading to less MCP-1 released in an antigen stimulated sampled compared to the Nil. This only occurs in bTB-negative animals that do not elicit a memory immune response to the antigens. In this study it was shown that MCP-1 concentrations in samples co-incubated with saline at 37°C for 24, 48 and 72 hours had a steady increase in MCP-1 concentration over time (data not shown), supporting the idea that MCP-1 is non-specifically released during incubation. To control for the non-specific response, we determined the effect on MCP-1 induction by co-incubating the blood with peptides that would not induce a memory immune response. DMSO was used as a solvent for the peptides because the rate of dissolution of peptides into these solvents is usually higher than in a water/solvent mixture (Narita et al. 1988). Therefore as a control we included 0.1% DMSO on its own as a co-incubation condition to evaluate its effect on MCP-1 induction. There was a significant increase in the concentration of MCP-1 in the sample co-incubated with 0.1% DMSO compared to the Nil (Saline) sample. This is in contrast to a study which showed that 1% DMSO decreased the induction of MCP-1 mRNA in a whole-blood stimulation assay (Xing et al. 2007b). However, a study by the same group determined that H_2O_2 in whole-blood induced significant increases in MCP-1, while MCP-1 production decreased at H_2O_2

concentrations higher than 1.0 mmol/L, suggesting a cytotoxic effect (Xing et al. 2007a). Knowing that MCP-1 induction due to H_2O_2 was concentration dependent, it is possible that lower concentrations of DMSO could induce MCP-1 while higher concentrations as seen by Xing et al. 2007b could have an inhibitory effect. However, this is still unknown and further research is required to understand the mechanisms of MCP-1 induction due to DMSO. Interestingly, in the samples stimulated with KLH and BSA peptides dissolved in 0.1% DMSO, there was a significant decrease in MCP-1 concentration compared to the 0.1% DMSO alone (Figure 8). This seems to suggest that in the presence of a peptide, MCP-1 induction is skewed or inhibited. There was no significant difference observed in MCP-1 levels between samples co-incubated with saline and samples stimulated with KLH and BSA peptides dissolved in saline (Figure 9). However, the range of MCP-1 concentrations is noticeably decreased when comparing the saline co-incubated samples to that of either peptide dissolved in saline, thus suggesting that the presence of the peptides affected the induction of MCP-1, albeit to a lesser effect then observed with DMSO.

A second cohort of bTB-positive and bTB-negative buffaloes (Cohort 4) also showed a significant difference in antigen-specific MCP-1 release between these groups (Figure 5). However, the slight overlap of MCP-1 concentrations between the groups suggests that it has a lower sensitivity than the detection of IFN-γ. In contrast to our previous observations, there was no reduction in MCP-1 concentration in the antigen-stimulated samples from bTB negative animals compared to saline samples. The mechanisms affecting MCP-1 induction are unclear, however, our results from a pilot study (data not shown) showing an increased non-specific induction of MCP-1 over time suggest that the length of time from when blood is first drawn to when the samples are processed could affect the results. The results from our initial observation on the diagnostic performance of MCP-1 were collected from buffalo before the TST was performed (Cohort 3), whereas the samples from our second observation were

collected from animals 3 days after the TST (Cohort 4), it is possible this could have an influence on the induction of MCP-1 due to immune sensitization.

The storage of stimulated plasma on protein saver cards (PSC) can facilitate the transport of plasma samples containing proteins of diagnostic importance (Duncombe et al. 2013). The PSC are small and light-weight and can be transported at ambient temperature without the need for cooling equipment. Results of MCP-1 storage stability experiments confirmed that the protein was still detected in equivalent amounts after 11 days of storage on protein saver cards (PSC) (Figure 6). Plasma from two animals were used, one with a high MCP-1 concentration and one with a low concentration to show that the initial concentration of plasma MCP-1 did not affect the stability of the protein. There was also no noticeable difference between the MCP-1 concentration of the 1 µl and 5 µl aliquots, although 5 µl is recommended because there is less chance for error. In a study investigating the stability of IFN-γ on PSC there was a progressive loss of IFN-γ signal over 10 days when stored at room temperature (Duncombe et al. 2013)

The heat stability of MCP-1 was assessed by heat-treatment of plasma at 65°C, a temperature used to inactivate harmful pathogens in samples. Results showed that MCP-1 can withstand a heat-treatment of 65°C for up to 20 minutes without any loss in signal. This property of MCP-1 could prove useful for applications which require inactivation of potential pathogens in plasma such as Brucella organisms (Weinhold et al. 2013) or Foot-and-Mouth Disease Virus (Kamolsiripichaiporn et al. 2007), while still maintaining the diagnostic use of the sample for cytokine determination. The heat-treated plasma could then be blotted onto a PSC and transported via post or courier in a safe and efficient manner. We observed that MCP-1 concentrations were higher in the heat-treated plasma samples compared to the control sample which was stored at 4°C. Plasma was diluted 1:5, 1:20 and 1:100 in PBS before heat treatment to increase volume to prevent the plasma coagulation. The control sample was diluted 1:100 in PBS and stored at 4°C during the heat shock step; this allowed the sample to be directly

transferred to the ELISA for MCP-1 quantification. Our results show that samples at the highest

dilution have greater levels of MCP-1 after heat treatment compared to lower dilutions and the

control (Figure 7). A possible explanation to describe these observations could include a

conformational change in the protein during heat-shock that allows extra epitopes of MCP-1 to

be exposed and therefore the binding of more antibodies to increase the signal (Zhang et al.

1999). Alternatively, heat-inactivation of an inhibitory molecule, such as an enzyme that breaks

down MCP-1, might increase detection of this molecule as enzymes are adversely affected by

high temperature (Bennett et al. 1969). For example, Trypsin is an enzyme involved in the

breakdown of many different proteins. Studies have shown that Trypsin inhibitors can be

inactivated after heat treatment at 70°C, thus it is possible that enzymes which break down

MCP-1 are inactivated by the heat treatment (Kozlowska et al. 1980).

In addition to evaluating antibody and cytokine production as a measure of humoral responses

to *M. bovis* infection in buffalo, investigation of changes in gene expression could better

characterize the complex immune response occurring in the host. For this purpose, we

investigated the expression stability of selected reference genes in antigen-stimulated blood

from bTB-positive buffaloes. To our knowledge this is the first study of the stability of selected

reference genes in antigen stimulated whole-blood samples from bTB-positive African buffalo.

The two software programs used for the stability analyses showed similar results, with *PPIA*

and *H3F3A* showing greatest stability (Figure 10 and 11). In contrast, *GAPDH* and *B2M*

showed much lower expression stability suggesting that they are unsuitable for use as reference

genes in African buffalo under the experimental conditions described.

Additionally, we sequenced the eight reference genes of the African buffalo using primers

designed from the corresponding genes in the domestic cow to assess homology between

species. All the sequenced genes shared high homology with their cow counterparts, ranging

from 98-100% (Appendix II). This could prove useful in designing qPCR primers for cows or buffalo for a non-species specific GEA.

Studies investigating the gene expression profile in response to bTB infection have been conducted in cattle using various cell and tissue types. The reference genes chosen for each study differed based on type of sample used. Two separate studies investigating the gene expression profiles of *M. bovis* antigen stimulated PBMC from infected cattle used *ACTB* and *GAPDH* as reference genes, respectively (Thacker et al. 2007, Blanco et al. 2011). *GAPDH* was used as a reference gene in qPCR GEA investigating the expression of genes in lymph nodes of *M. bovis* infected cattle (Widdison et al. 2006, Witchell et al. 2010), while another study investigating the expression of genes in the lungs of *M. bovis* infected cattle used the reference gene U1 small nuclear ribonucleoprotein (*U1*) (Shu et al. 2014). *ACTB* was used as a reference gene in the only other study of gene expression in African buffalo using *M. bovis* antigen stimulation of whole blood (Parsons et al. 2011). Vorachek et al. (2013) validated the stability of reference genes in bovine neutrophils in animals with and without selenium supplementation and discovered that the commonly used genes *ACTB* and *HPRT1* were unreliable, whereas *YWHAZ* and phosphoglycerate kinase (*PGK1*) ranked among the top most stable genes under their experimental conditions. Therefore genes commonly chosen as reference genes may not behave as expected depending on cell type and treatment conditions, hence why it is important to evaluate the expression stability of candidate reference genes for the specific experimental conditions. The role of a GEA for the diagnosis of bTB in Africa buffalo has been investigated with promising results, however, further research and optimization is required to develop more sensitive diagnostic tests for bTB (Parsons et al. 2011).

There were certain limitations to this study. Firstly, we did not include an avian PPD antigen coated well in the PPD ELISA; this would have allowed us to control for cross-reactivity

between antibodies against *M. bovis* and those against *M. avium*. Secondly, further experimentation is needed to determine the causing of the variations in MCP-1 concentrations of animals sampled on different occasions. This is most likely caused by a spontaneous MCP-1 release, which, if controlled for could improve the diagnostic performance of MCP-1. Thirdly, due to a lack of post-mortem pathological data, the stage of infection in the bTB-positive buffaloes was unknown. Without information on the stage of infection, correlations cannot be made between characteristics of the immune response and disease pathology.

Chapter 6 - Conclusion:

The in-house PPD ELISA was able to detect antibodies against *M. bovis* in bTB-positive buffalo. Although the diagnostic sensitivity of this assay was low, the results correlate with a commercial rapid test used to detect antibodies against MPB83. The high immunogenicity of MPB83, and its increased expression by *M .bovis* could suggest that the antibodies detected by the PPD ELISA are, in part, those against MPB83. Although MCP-1 shows promise as a diagnostic biomarker for bTB, it too has a poor sensitivity. Nonetheless, the stability properties of MCP-1 during storage and heat treatment, are characteristics which could prove particularly useful, especially for the detection of wildlife species co-infected with controlled diseases. However, further studies on this cytokine are needed to determine its true role as a diagnostic marker of bTB. The reference genes evaluated in this study can be used for the development of a diagnostic GEA with greater sensitivity, or to assess a gene expression profile of immune genes in bTB-positive buffalo. The use of diagnostic assays measuring both CMI and humoral immune response could provide useful information regarding the *M. bovis* infection status of the animal. Although serological tests have been shown to have low sensitivity, they may still be used as an adjunct test to help better characterize the immune response to *M. bovis* infection.

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Appendix I:

Table 1. Antibody titers as measured by the Chembio DPP VetTB assay represented as reflectance units (RU). Presence of a visible line on either band was used to define an animal as antibody-positive (highlighted in red). Antibody responses to PPD as determined via ELISA are represented as delta-OD between antigen and control wells.

| Buffalo | DPP (RU) | | PPD ELISA | |
	MPB83	ESAT-6/CFP-10	ΔOD	Visible line (Yes/No)
B2	0.0	0.0	0.005	N
B8	4.3	0.0	0.067	Y
B10	2.8	0.0	-0.010	N
B17	2.6	0.0	0.453	N
B23	7.2	0.0	0.410	Y
B24	3.4	0.0	0.138	Y
B25	14.3	0.0	0.439	Y
B26	0.0	0.0	0.036	N
B34	9.9	0.0	0.051	Y
B40	39.0	9.3	1.752	Y
B41	2.8	0.0	0.078	N
B44	2.7	0.0	-0.097	N
B48	0.0	0.0	0.008	N
B49	3.2	0.0	1.168	Y
B59	3.0	0.0	0.075	N
B61	10.9	82.3	1.562	Y
B63	0.0	0.0	0.043	N
B65	2.8	0.0	0.059	N
B67	3.0	0.0	0.078	N
B69	8.3	0.0	0.370	Y

Appendix II:

Sequences of selected reference genes of the African Buffalo

Note: Start and stop codons are underlined and annealing sites of the qPCR primers are highlighted in grey.

GAPDH: 99.20% nucleotide homology between cattle and buffalo sequences.

```
cow       ATGGTGAAGGTCGGAGTGAACGGATTCGGCCGCATCGGGCGCCTGGTCACCAGGGCTGCT
buffalo   ATGGTGAAGGTCGGAGTGAACGGATTCGGCCGCATTGGGCGCCTGGTCACCAGGGCTGCT
          **************************** **  ***********************

cow       TTTAATTCTGGCAAAGTGGACATCGTCGCCATCAATGACCCCTTCATTGACCTTCACTAC
buffalo   TTTAATTCTGGCAAAGTGGACATCGTCGCCATCAATGACCCCTTCATTGACCTTCACTAC
          ************************************************************

cow       ATGGTCTACATGTTCCAGTATGATTCCACCCACGGCAAGTTCAACGGCACAGTCAAGGCA
buffalo   ATGGTCTACATGTTCCAGTATGATTCCACCCACGGCAAGTTCCACGGCACAGTCAAGGCA
          ******************************************  *****************

cow       GAGAACGGGAAGCTCGTCATCAATGGAAAGGCCATCACCATCTTCCAGGAGCGAGATCCT
buffalo   GAGAACGGGAAGCTCGTCATCAATGGAAAGGCCATCACCATCTTCCAGGAGCGAGATCCT
          ************************************************************

cow       GCCAACATCAAGTGGGGTGATGCTGGTGCTGAGTATGTGGTGGAGTCCACTGGGGTCTTC
buffalo   GCCAACATCAAGTGGGGTGATGCTGGTGCTGAGTATGTGGTGGAGTCCACTGGGGTCTTC
          ************************************************************

cow       ACTACCATGGAGAAGGCTGGGGCTCACTTGAAGGGTGGCGCCAAGAGGGTCATCATCTCT
buffalo   ACTACCATGGAGAAGGCTGGGGCTCACTTGAAGGGTGGCGCCAAGAGGGTCATCATCTCG
          *********************************************************** 

cow       GCACCTTCTGCCGATGCCCCCATGTTTGTGATGGGCGTGAACCACGAGAAGTATAACAAC
buffalo   GCGCCTTCTGCCGATGCCCCCATGTTTGTGATGGGTGTGAACCACGAGAAGTATAACAAC
          ** ****************************************  ***************

cow       ACCCTCAAGATTGTCAGCAATGCCTCCTGCACCACCAACTGCTTGGCCCCCCTGGCCAAG
buffalo   ACCCTCAAGATTGTCAGCAATGCCTCCTGCACCACCAACTGCTTGGCCCCCCTGGCCAAG
          ************************************************************

cow       GTCATCCATGACCACTTTGGCATCGTGGAGGGACTTATGACCACTGTCCACGCCATCACT
buffalo   GTCATCCATGACCACTTTGGCATCGTGGAGGGACTTATGACCACCGTCCACGCCATCACT
          ********************************************  **************

cow       GCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAGCTGTGGCGTGACGGCCGAGGGGCT
buffalo   GCCACCCAGAAGACTGTGGATGGCCCCTCCGGGAAGCTGTGGCGTGACGGCCGAGGGGCT
          ************************************************************

cow       GCCCAGAATATCATCCCTGCTTCTACTGGCGCTGCCAAGGCCGTGGGCAAGGTCATCCCT
buffalo   GCCCAGAATATCATCCCTGCTTCTACTGGCGCTGCCAAGGCCGTGGGCAAAGTCATCCCT
          **************************************************  ********

cow       GAGCTCAACGGGAAGCTCACTGGCATGGCCTTCCGCGTCCCCACTCCCAACGTGTCTGTT
buffalo   GAGCTCAACGGGAAGCTCACTGGCATGGCCTTCCGCGTCCCCACTCCCAACGTGTCTGTT
          ************************************************************
```

80

```
cow      GTGGATCTGACCTGCCGCCTGGAGAAACCTGCCAAGTATGATGAGATCAAGAAGGTGGTG
buffalo  GTGGATCTGACCTGCCGCCTGGAGAAACCTGCCAAGTATGATGAGATCAAGAAGGTGGTG
         ************************************************************

cow      AAGCAGGCGTCAGAGGGCCCTCTCAAGGGCATTCTAGGCTACACTGAGGACCAGGTTGTC
buffalo  AAGCAGGCGTCAGAGGGCCCTCTCAAGGGCATTCTAGGCTACACTGAGGACCAGGTTGTC
         ************************************************************

cow      TCCTGCGACTTCAACAGCGACACTCACTCTTCTACCTTCGATGCTGGGGCTGGCATTGCC
buffalo  TCCTGCGACTTCAACAGCGACACTCACTCTTCTACCTTCGATGCTGGGGCTGGCATTGCC
         ************************************************************

cow      CTCAACGACCACTTTGTCAAGCTCATTTCCTGGTACGACAATGAATTTGGCTACAGCAAC
buffalo  CTCAACGACCACTTTGTCAAGCTCATTTCCTGGTACGACAATGAATTCGGCTACAGCAAC
         *********************************************** ************

cow      AGGGTGGTGGACCTCATGGTCCACATGGCCTCCAAGGAGTAA
buffalo  AGGGTGGTGGACCTCATGGTCCACATGGCCTCCAAGGAGTAA
         ******************************************
```

ACTB: 99.08% nucleotide homology between cattle and buffalo sequences.

```
cow      ATGTGCAAGGCCGGCTTCGCGGGCGACGATGCTCCCCGGGCCGTCTTCCCGTCCATCGTG
buffalo  ATGTGCAAGGCCGGCTTCGCGGGCGACGATGCTCCCCGGGCTGTCTTCCCCTCCATCGTG
         ****************************************** ******* ********

cow      GGGCGCCCCCGGCACCAGGGCGTAATGGTGGGCATGGGCCAGAAGGACTCGTACGTGGGG
buffalo  GGGCGCCCCCGGCACCAGGGCGTAATGGTGGGCATGGGCCAGAAGGACTCGTACGTGGGG
         ************************************************************

cow      GATGAGGCTCAGAGCAAGAGAGGCATCCTGACCCTCAAGTACCCCATTGAGCACGGCATC
buffalo  GATGAGGCTCAGAGCAAGAGAGGCATCCTGACCCTCAAGTACCCCATTGAGCACGGCATC
         ************************************************************

cow      GTCACCAACTGGGACGACATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTCCGT
buffalo  GTCACCAACTGGGACGACATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTCCGT
         ************************************************************

cow      GTGGCCCCTGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAAC
buffalo  GTGGCCCCTGAGGAGCACCCCGTGCTGCTGACCGAGGCCCCCCTGAACCCCAAGGCCAAC
         ************************************************************

cow      CGTGAGAAGATGACCCAGATCATGTTCGAGACCTTCAACACCCCTGCCATGTACGTGGCC
buffalo  CGTGAAAAGATGACCCAGATCATGTTCGAGACCTTCAACACCCCTGCCATGTACGTGGCC
         ***** ******************************************************

cow      ATCCAGGCTGTGCTGTCCCTGTATGCCTCTGGCCGCACCACCGGCATCGTGATGGACTCC
buffalo  ATCCAGGCCGTGCTGTCCCTGTATGCCTCTGGCCGCACCACTGGCATCGTGATGGACTCC
         ******** ******************************** ******************

cow      GGTGACGGGGTCACCCACACGGTGCCCATCTATGAGGGGTACGCCCTTCCCCATGCCATC
buffalo  GGTGACGGGGTCACCCACACGGTGCCCATCTACGAGGGGTACGCCCTTCCCCATGCCATC
         ******************************** ***************************

cow      CTGCGTCTGGACCTGGCTGGCCGGGACCTGACGGACTACCTCATGAAGATCCTCACGGAG
buffalo  CTGCGTCTGGACCTGGCTGGCCGGGACCTGACGGACTACCTCATGAAGATCCTCACGGAG
         ************************************************************

cow      CGTGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTCCGTGACATCAAGGAGAAG
buffalo  CGTGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTCCGTGACATCAAGGAGAAG
         ************************************************************

cow      CTCTGCTACGTGGCCCTGGACTTCGAGCAGGAGATGGCCACCGCGGCCTCCAGCTCCTCC
buffalo  CTCTGCTACGTGGCCCTGGACTTCGAGCAGGAGATGGCCACCGCGGCCTCCAGCTCCTCC
         ************************************************************
```

```
cow       CTGGAGAAGAGCTACGAGCTTCCTGACGGGCAGGTCATCACCATCGGCAATGAGCGGTTC
buffalo   CTGGAGAAGAGCTACGAGCTTCCTGACGGGCAGGTCATCACCATCGGCAATGAGCGGTTC
          ************************************************************

cow       CGCTGCCCTGAGGCTCTCTTCCAGCCTTCCTTCCTGGGCATGGAATCCTGCGGCATTCAC
buffalo   CGCTGCCCTGAGGCTCTCTTCCAGCCTTCCTTCCTGGGCATGGAATCCTGCGGTATTCAC
          ***************************************************** ******

cow       GAAACTACCTTCAATTCCATCATGAAGTGTGACGTCGACATCCGCAAGGACCTCTACGCC
buffalo   GAAACTACCTTCAATTCCATCATGAAGTGTGATGTCGACATCCGCAAGGACCTCTACGCC
          ******************************* ****************************

cow       AACACGGTGCTGTCCGGCGGGACCACCATGTACCCCGGCATCGCGGACAGGATGCAGAAA
buffalo   AACACGGTGCTGTCCGGCGGGACCACCATGTACCCCGGCATCGCAGACAGGATGCAGAAA
          ******************************************** ***************

cow       GAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATCGCGCCCCCTGAGCGC
buffalo   GAGATCACTGCCCTGGCACCCAGCACAATGAAGATCAAGATCATCGCGCCCCCTGAGCGC
          ************************************************************

cow       AAGTACTCCGTGTGGATTGGCGGCTCCATCCTGGCCTCGCTGTCCACCTTCCAGCAGATG
buffalo   AAGTACTCCGTGTGGATTGGCGGCTCCATCCTGGCCTCGCTGTCCACCTTCCAGCAGATG
          ************************************************************

cow       TGGATCAGCAAGCAGGAGTACGATGAGTCCGGCCCCTCCATCGTCCACCGCAAATGCTTC
buffalo   TGGATCAGCAAGCAGGAGTACGACGAGTCCGGCCCCTCCATCGTCCACCGCAAATGCTTC
          *********************** ************************************

cow       TAG
buffalo   TAG
          ***
```

_B2M:_ 98.6% nucleotide homology between cattle and buffalo sequences.

```
cow       ATGGCTCGCTTCGTGGCCTTGGTCCTTCTCGGGCTGCTGTCGCTGTCTGGACTGGACGCC
buffalo   ATGGCTCGCTTCGTGGCCTTGGTCCTTCTCGGGCTGCTGTCGCTGACTGGACTGGACGCC
          ********************************************* **************

cow       ATCCAGCGTCCTCCAAAGATTCAAGTGTACTCAAGACACCCACCAGAAGATGGAAAGCCA
buffalo   ATCCAGCGTGTTCCAAAGATTCAAGTGTACTCAAGACACCCACCAGAAGATGGAAAGCCA
          *********  *************************************************

cow       AATTACCTGAACTGCTATGTGTATGGGTTCCATCCACCCCAGATTGAAATTGATTTGCTG
buffalo   AATTACCTGAACTGCTATGTGTCTGGGTTCCATCCACCCCAGATTGAAATCGATTTGCTG
          ********************** ************************** *********

cow       AAGAATGGGGAGAAGATTAAATCGGAGCAGTCAGACCTGTCTTTCAGCAAGGACTGGTCT
buffalo   AAGAATGGGGAGAAGATTAAATCGGAGCAGTCAGACCTGTCTTTCAGCAAGGACTGGTCT
          ************************************************************

cow       TTCTACCTGCTGTCCCACGCTGAGTTCACTCCCAACAGCAAGGATCAGTACAGCTGCCGA
buffalo   TTCTACCTGCTGTCCCACGCTGAGTTCACTCCCAACAGCAAGGATCAGTACAGCTGCCGA
          ************************************************************

cow       GTGAAACACGTTACTTTGGAACAACCCCGGATAGTTAAGTGGGATCGAGACCTGTAA
buffalo   GTGAAACACGTTACTTTGGAACAACCCCGGATAGTTAAGTGGGATCGAGACCTGTAA
          ********************************************************
```

YWHAZ: 99.73% nucleotide homology between cattle and buffalo sequences.

```
cow        ATGGATAAAAACGAGCTGGTACAGAAGGCCAAACTGGCCGAGCAGGCTGAGCGATATGAT
buffalo    ATGGATAAAAACGAGCTGGTACAGAAGGCCAAACTGGCCGAGCAGGCTGAGCGATATGAT
           ************************************************************

cow        GACATGGCAGCCTGCATGAAGTCTGTAACTGAGCAAGGAGCTGAATTATCCAATGAGGAG
buffalo    GACATGGCAGCCTGCATGAAGTCTGTAACTGAGCAAGGAGCTGAATTATCCAATGAGGAG
           ************************************************************

cow        AGGAATCTTCTCTCAGTTGCTTATAAAAATGTTGTAGGAGCCCGTAGGTCATCTTGGAGG
buffalo    AGGAATCTTCTCTCAGTTGCTTATAAAAATGTTGTAGGAGCCCGTAGGTCATCTTGGAGG
           ************************************************************

cow        GTCGTCTCCAGTATTGAGCAAAAGACGGAAGGTGCTGAGAAAAAACAGCAGATGGCTCGA
buffalo    GTCGTCTCCAGTATTGAGCAAAAGACGGAAGGTGCTGAGAAAAAACAGCAGATGGCTCGA
           ************************************************************

cow        GAATACAGAGAGAAAATAGAGACCGAGCTAAGAGATATCTGCAATGATGTACTGTCTCTT
buffalo    GAATACAGAGAGAAAATAGAGACCGAGCTAAGAGATATCTGCAATGATGTACTGTCTCTT
           ************************************************************

cow        TTGGAAAAGTTCTTGATCCCCAACGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGAAA
buffalo    TTGGAAAAGTTCTTGATCCCCAACGCTTCACAAGCAGAGAGCAAAGTCTTCTATTTGAAA
           ************************************************************

cow        ATGAAAGGAGACTACTACCGCTACTTGGCTGAGGTTGCAGCTGGTGATGACAAGAAAGGG
buffalo    ATGAAAGGAGACTACTACCGCTACTTGGCTGAGGTTGCAGCTGGTGATGACAAGAAAGGG
           ************************************************************

cow        ATTGTGGACCAGTCACAGCAAGCATACCAAGAAGCTTTTGAAATCAGCAAAAAGGAAATG
buffalo    ATTGTGGATCAGTCGCAGCAAGCATACCAAGAAGCTTTTGAAATCAGCAAAAAGGAAATG
           ********  *****  ********************************************

cow        CAACCAACACATCCTATCAGACTGGGTCTGGCCCCTTAACTTCTCTGTGTTCTATTATGAG
buffalo    CAACCAACACATCCTATCAGACTGGGTCTGGCCCCTTAACTTCTCTGTGTTCTATTATGAG
           ************************************************************

cow        ATTCTGAACTCCCCTGAGAAAGCCTGCTCTCTTGCAAAGACAGCATTTGATGAAGCCATT
buffalo    ATTCTGAACTCCCCTGAGAAAGCCTGCTCTCTTGCAAAGACAGCATTTGATGAAGCCATT
           ************************************************************

cow        GCTGAACTTGATACATTAAGTGAAGAGTCATACAAAGACAGCACGCTAATAATGCAGTTA
buffalo    GCTGAACTTGATACATTAAGTGAAGAGTCATACAAAGACAGCACGCTAATAATGCAGTTA
           ************************************************************

cow        CTGAGAGATAACTTGACATTGTGGACATCGGATACCCAAGGAGACGAAGCTGAAGCAGGA
buffalo    CTGAGAGATAACTTGACATTGTGGACATCGGATACCCAAGGAGACGAAGCTGAAGCAGGA
           ************************************************************

cow        GAAGGAGGGGAAAATTAA
buffalo    GAAGGAGGGGAAAATTAA
           ******************
```

83

PPIA: 98.96% nucleotide homology between cattle and buffalo sequences.

```
cow       ATGGTCAACCCCACCGTGTTCTTCGACATCGCTGTCGACGGCGAGCCCTTGGGCCGCGTC
buffalo   ATGGTCAACCCCACCGTGTTCTTCGACATCGCCGTCGACGGCGAGCCCTTGGGCCGCGTC
          ******************************** ****************************

cow       TCTTTTGAGCTGTTTGCAGACAAAGTTCCAAAGACAGCAGAAAACTTTCGTGCTCTGAGC
buffalo   TCTTTTGAGCTGTTTGCAGACAAAGTTCCAAAGACAGCAGAAAACTTTCGTGCTCTGAGC
          ************************************************************

cow       ACTGGAGAGAAAGGATTTGGTTATAAAGGTTCCTGCTTTCACAGAATAATTCCGGGATTT
buffalo   ACTGGAGAGAAAGGATTTGGTTATAAAGGTTCCTGCTTTCACAGAATAATTCCGGGATTT
          ************************************************************

cow       ATGTGCCAGGGTGGTGACTTCACACGCCATAATGGTACTGGTGGCAAGTCCATCTATGGC
buffalo   ATGTGCCAGGGTGGTGACTTCACACGCCATAATGGTACTGGTGGCAAGTCCATCTATGGC
          ************************************************************

cow       GAGAAATTTGATGATGAGAATTTCATTTTGAAGCATACAGGTCCTGGCATCTTGTCCATG
buffalo   GAGAAATTTGATGATGAGAATTTCATTCTGAAGCATACAGGTCCTGGCATCTTGTCCATG
          *************************** ********************************

cow       GCAAATGCTGGCCCCAACACAAATGGTTCCCAGTTTTTCATTTGCACTGCCAAGACTGAG
buffalo   GCAAATGCTGGCCCCAACACAAATGGTTCCCAGTTTTTCATCTGCACTGCCAAGACTGAG
          ***************************************** ******************

cow       TGGTTGGATGGCAAGCACGTGGTACTTTGGCAAGGTGAAAGAGGGCATGAATATTGTGGA
buffalo   TGGTTGGATGGCAAGCATGTGGTC-TTTGGCAAGGTGAAAGAGGGCATGAATATTGTGGA
          ***************** *****   **********************************

cow       AGCCATGGAGCGCTTTGGGTCCAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGA
buffalo   AGCCATGGAGCGCTTTGGGTCCAGGAATGGCAAGACCAGCAAGAAGATCACCATTGCTGA
          ************************************************************
```

_H3F3A:_ 100% nucleotide homology between cattle and buffalo sequences.

```
cow       ATGGCTCGTACAAAGCAGACTGCCCGCAAATCGACCGGTGGTAAAGCACCGAGGAAGCAA
buffalo   ATGGCTCGTACAAAGCAGACTGCCCGCAAATCGACCGGTGGTAAAGCACCGAGGAAGCAA
          ************************************************************

cow       CTCGCTACAAAAGCCGCTCGCAAGAGTGCGCCCTCTACTGGAGGGGGTGAAGAAACCTCAT
buffalo   CTCGCTACAAAAGCCGCTCGCAAGAGTGCGCCCTCTACTGGAGGGGTGAAGAAACCTCAT
          ************************************************************

cow       CGTTACAGGCCTGGTACTGTGGCACTCCGTGAAATTAGACGTTATCAGAAGTCCACTGAA
buffalo   CGTTACAGGCCTGGTACTGTGGCACTCCGTGAAATTAGACGTTATCAGAAGTCCACTGAA
          ************************************************************

cow       CTTCTGATTCGCAAACTTCCCTTCCAGCGTCTGGTGCGGGAAATTGCTCAGGACTTCAAA
buffalo   CTTCTGATTCGCAAACTTCCCTTCCAGCGTCTGGTGCGGGAAATTGCTCAGGACTTCAAA
          ************************************************************

cow       ACAGATCTGCGCTTCCAGAGTGCAGCTATTGGTGCTTTGCAGGAGGCAAGTGAGGCCTAT
buffalo   ACAGATCTGCGCTTCCAGAGTGCAGCTATTGGTGCTTTGCAGGAGGCAAGTGAGGCCTAT
          ************************************************************

cow       CTGGTTGGCCTTTTTGAAGACACCAACCTGTGTGCTATCCATGCCAAACGTGTAACAATT
buffalo   CTGGTTGGCCTTTTTGAAGACACCAACCTGTGTGCTATCCATGCCAAACGTGTAACAATT
          ************************************************************

cow       ATGCCAAAAGACATCCAGCTAGCACGCCGCATACGTGGAGAACGTGCTTAA
buffalo   ATGCCAAAAGACATCCAGCTAGCACGCCGCATACGTGGAGAACGTGCTTAA
          **************************************************
```

TBP: 99.47% nucleotide homology between cattle and buffalo sequences.

```
cow        ATGGATCAGAACAACAGCCTCCCACCCTATGCCCAGGGCCTGGCCTCCCCTCAGGGTGCC
buffalo    ATGGATCAGAACAACAGCCTCCCACCGTATGCCCAGGGCCTGGCCTCCCCTCAGGGTGCC
           **************************** ********************************

cow        ATGACTCCTGGAATCCCTATCTTTAGTCCAATGATGCCTTACGGCACAGGACTGACTCCA
buffalo    ATGACTCCTGGAATCCCTATCTTTAGTCCAATGATGCCTTACGGCACAGGACTGACTCCA
           ************************************************************

cow        CAGCCTATTCAGAACACCAACAGTCTGTCTATTCTGGAGGAGCAGCAACGGCAGCAGCAG
buffalo    CAGCCTATCCAGAACACCAACAGTCTGTCTATTCTGGAGGAGCAGCAACGGCAGCAGCAG
           ******** ***************************************************

cow        CAGCAGCAACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGGCGGCAGTGGCA
buffalo    CAGCAGCAACAGCAG------------CAGCAGCAGCAGCAGCAGCAGGCAGCAGTGGCA
           ***************            ******************** *********

cow        GCCGTCCAGCAGTCGACGTCCCAGCAGGCAACCCAGGGGCCCTCGGGCCAGACACCACAG
buffalo    GCCGTCCAGCAGTCGACGTCCCAGCAGGCAACCCAGGGGCCCTCGGGCCAGACACCACAG
           ************************************************************

cow        CTCTTCCACTCACAGACTCTTACGACTGCACCCTTGCCGGGCACCACTCCCTTGTATCCC
buffalo    CTCTTCCACTCACAGACTCTTACGACTGCACCCTTGCCGGGCACCACTCCCTTGTATCCC
           ************************************************************

cow        TCCCCCATGACCCCCATGACCCCCATCACCCCTGCCACGCCAGCCTCAGAGAGCTCCGGG
buffalo    TCCCCCATGACCCCCATGACCCCCATCACCCCTGCCACGCCAGCCTCAGAGAGCTCCGGG
           ************************************************************

cow        ATCGTGCCGCAGCTGCAAAATATTGTATCCACAGTGAATCTTGGTTGTAAACTTGACCTA
buffalo    ATCGTGCCGCAGCTGCAAAATATTGTATCCACAGTGAATCTTGGTTGTAAACTTGACCTA
           ************************************************************

cow        AAGACCATTGCACTTCGTGCCCGAAATGCTGAATATAATCCCAAGCGTTTTGCTGCTGTA
buffalo    AAGACCATTGCACTTCGTGCCCGAAATGCTGAATATAATCCCAAGCGTTTTGCTGCTGTA
           ************************************************************

cow        ATCATGAGAATAAGAGAGCCCCGCACCACTGCACTGATATTCAGTTCTGGGAAGATGGTG
buffalo    ATCATGAGAATAAGAGAGCCCCGCACCACTGCACTGATATTCAGTTCTGGGAAGATGGTG
           ************************************************************

cow        TGCACAGGAGCCAAGAGTGAAGAACAGTCTAGACTAGCAGCAAGAAAATACGCCAGAGTT
buffalo    TGCACAGGAGCCAAGAGTGAAGAACAGTCTAGATTAGCAGCAAGAAAATACGCCAGAGTT
           ********************************* **************************

cow        GTACAGAAACTGGGTTTTCCAGCTAAGTTCTTGGACTTCAAGATTCAGAACATGGTGGGG
buffalo    GTACAGAAACTGGGTTTTCCAGCTAAGTTCTTGGACTTCAAGATTCAGAACATGGTGGGG
           ************************************************************

cow        AGCTGTGATGTGAAGTTCCCTATAAGGTTAGAAGGCCTTGTGCTTACCCACCAACAGTTC
buffalo    AGCTGTGATGTGAAGTTCCCTATAAGGTTAGAAGGCCTTGTGCTTACCCACCAACAGTTC
           ************************************************************

cow        AGTAGTTATGAGCCAGAGTTATTTCCTGGTTTAATCTACAGAATGATCAAACCGAGAATT
buffalo    AGTAGTTATGAGCCAGAGTTATTTCCTGGTTTAATCTACAGAATGATCAAACCGAGAATT
           ************************************************************

cow        GTTCTCCTTATTTTTGTTTCTGGAAAAGTTGTATTAACAGGTGCTAAAGTCAGAGCAGAA
buffalo    GTTCTCCTTATTTTTGTTTCTGGAAAAGTTGTATTAACAGGTGCTAAAGTCAGAGCAGAA
           ************************************************************

cow        ATTTATGAAGCCTTTGAGAACATCTACCCTATTCTGAAGGGTTTCAGGAAGACAACGTAA
buffalo    ATTTATGAAGCCTTTGAGAACATCTACCCTATTCTGAAGGGTTTCAGGAAGACTACGTAA
           ****************************************************** ******
```

HPRT1: 99.09% nucleotide homology between cattle and buffalo sequences.

```
cow        ATGGCGGCCCGCAGCCCCAGCGTGGTGATTAGCGATGATGAACCAGGTTATGACCTAAAT
buffalo    ATGGCGGCCCGCAGTCCCAGCGTGGTGATTAGCGATGATGAACCAGGTTATGACCTAAAT
           ************** *********************************************

cow        TTATTTTGTATACCCAATCATTATGCTGAGGATTTGGAGAAGGTGTTTATTCCTCATGGA
buffalo    TTATTTTGTATACCCAATCATTATGCTGAGGATTTGGAGAAAGTGTTTATTCCTCATGGA
           ***************************************** ******************

cow        CTAATTATGGACAGGACCGAACGGCTGGCTCGAGATGTGATGAAGGAGATGGGTGGCCAT
buffalo    CTAATTATGGACAGGACCGAACGACTGGCTCGAGATGTGATGAAGGAGATGGGTGGCCAT
           *********************** ************************************

cow        CACATTGTGGCCCTCTGTGTGCTCAAGGGGGGCTATAAGTTCTTTGCCGACCTGTTGGAT
buffalo    CACATTGTGGCCCTCTGTGTGCTCAAGGGGGGCTATAAGTTCTTTGCTGACCTGTTGGAT
           *********************************************** ************

cow        TACATCAAAGCACTGAACAGAAATAGTGACAAATCCATTCCTATGACTGTGGATTTTATC
buffalo    TACATCAAAGCACTGAACAGAAATAGTGACAAATCCATTCCTATGACTGTGGATTTTATC
           ************************************************************

cow        AGACTGAAGAGCTACTGTAACGACCAGTCAACAGGCGACATAAAAGTAATTGGTGGAGAT
buffalo    AGACTGAAGAGCTACTGTAACGACCAGTCAACAGGCGACATAAAAGTAATTGGTGGAGAT
           ************************************************************

cow        GATCTCTCAACTTTAACTGGAAAGAATGTCTTGATTGTCGAAGATATAATTGACACTGGG
buffalo    GATCTCTCAGCTTTAACTGGAAAGAATGTCTTGATTGTCGAAGATATAATTGACACTGGA
           ********* **************************************************

cow        AAGACAATGCAGACTTTGCTTGCCTTGGTCAAGAAGCATAAACCAAAGATGGTCAAGGTT
buffalo    AAGACAATGCAGACTTTGCTTGCCTTGGTCAAGAAGCATAAACCAAAGATGGTCAAGGTT
           ************************************************************

cow        GCGAGCTTGCTGATGAAAAGGACCCCTCGAAGTGTTGGATATAAACCAGACTTTGTTGGA
buffalo    GCGAGCTTGCTGATGAAAAGGACCCCTCGAAGTGTTGGATATAAACCAGACTTTGTTGGA
           ************************************************************

cow        TTTGAAATTCCAGACAAGTTTGTTGTGGGATATGCCCTTGACTATAATGAATACTTCAGG
buffalo    TTTGAAATTCCAGACAAGTTTGTTGTGGGATATGCCCTTGACTATAATGAATACTTCAGG
           ************************************************************

cow        GACTTGAATCACGTGTGTGTCATTAGCGAAACTGGAAAAGCAAAATACAAAGCCTAA
buffalo    GACTTGAATCACGTGTGTGTCATTAGCGAAACTGGAAAAGCAAAATACAAAGCCTAA
           ********************************************************
```

86