MYCOBACTERIUM TUBERCULOSIS COMPLEX-SPECIFIC ANTIGENS FOR USE IN SERODIAGNOSIS OF BOVINE TUBERCULOSIS

BY

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Submitted in partial fulfillment of the requirements for the degree of

Masters of Science (Veterinary Science)

In the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science,

University of Pretoria

October, 2012

DECLARATION

I, Boitumelo Modise, declare that this dissertation which I hereby submit to the University of Pretoria for the degree of Master of Science (Veterinary Science) is my own work and has not previously been submitted by me for a degree at any other university.

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Boitumelo M. Modise

DEDICATION

To “the love of my life” Kebadire Tlotleng, our daughter Tlotlang and son Kevin

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to the following people and organizations/institutions who were involved in this research project to make it a success:

Dr Jeanni Fehrsen, my supervisor, who at all cost gave useful advice, ideas, support and for her patient guidance.

Prof. Anita Michel, my co-supervisor, for organizing funds and allowing me the opportunity to work on this project and providing serum samples. Her valuable input is really appreciated.

Dr Dion Du Plessis, Manager of the New Generation Vaccine Unit, for his insightful criticisms as well as the Immunology staff at ARC-OVI for providing use of their facilities and creating a good working environment. **Ms Susan Grant**, my office mate, for her support and assistance with Coral Draw.

Acknowledgement to my former and new HOD of DVTD, **Prof Koos Coetzer** and **Prof Darrell Abernethy** respectively, for their moral support.

Dr E. Kekgonne Baipoledi, Former Head of Botswana National Veterinary Laboratory for always believing in my abilities and his encouragement.

Recognition also to **Ms Tiny Hlokwe** and **Ms Noma Gcebe** for providing the *M. bovis* strain; **Dr Andy Potts** and **Mr Bryan Peba** from ARC-OVI & **Dr Akin Jenkins** from DVTD for providing serum samples.

Ms Maria Mtswini the librarian, **Dr Nlingisisi Babayani**, colleagues at ARC-OVI and DVTD for their support and contributions. **Dr Mmamohale Chaisi**, **Ms Codellia Mashau**, **Ms Petunia Malatji**, **Ms Elizabeth Debeila**, **Ms Ayesha Hassim**, **Ms Betty Ledwaba, Ms Mokete Moetela**, **Mrs Enala Lubinga and Mrs Abubakar Adamu** for your friendship, hospitality and entertainment during my stay in South Africa. I will miss you.

Deutsche Forschungsgemeinschaft (DFG) for their financial support of the project.

The department of Botswana Veterinary Services for giving me permission to further my studies.

My dear husband **Dr Kebadire Tlotleng**, for encouraging me to undertake MSc research in the line of bovine tuberculosis. You are a "*star".* I left you with our two year old son and four year old daughter whom you dealt with in my absence. Your unwavering love, motivation, great patience and good-natured support inspired me to never give up during the tough and challenging times of the research. You have always stood by me and I really appreciate that.

To my daughter **Tlotlang** and son **Kevin** whom I left when still very young, I am deeply sorry for the time that we spent apart.

My parents**, Mr and Mrs Onalenna Kenanao Modise** and my father in-law, **Mr Makhutle Malakane**, for their endless love and moral support from the time I started working on this research project to the end. Thanks for the words of wisdom Mum, Dad and father in-law. To my sisters and brothers, I thank you for all the encouragement and unequivocal support.

Last, but by no means least, I thank **God** for his grace, love and protection, for without him this project will not be successful.

Joshua 1:9; Be strong and courageous, do not be frightened or dismayed, for the lord your God is with you wherever you go.

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LIST OF ABBREVIATIONS

ARC-OVI		Agricultural Research Council-Onderstepoort Veterinary Institute
AUC	-	Area under the curve
BCG	-	Bacille Calmette Guerin
bp	-	Base pair
BSA	-	Bovine serum albumin
BTB	-	Bovine tuberculosis
°C	-	Degree Celsius
CITT		Comparative intradermal tuberculin test
cm	-	Centimeter
CMI	-	Cell mediated immunity
CPF-10	-	Culture filtrate protein-10
DNA	-	Deoxyribonucleic acid
dNTP -	-	Deoxyribonucleotide triphosphate
DTH	-	Delayed type hypersensitivity
EB	-	Elution buffer
E. coli	-	*Escherichia coli*
ELISA	-	Enzyme-Linked Immunosorbent assay
ESAT-6	-	Early secretory antigenic target-6
FITC	-	Fluorescein isothiocyanate
FN	-	False negative
FP	-	False positive
FPA	-	Fluorescence polarization assay
Frag	-	Fragment
g	-	Relative centrifugal force
g	-	Grams
h	-	Hour(s)
His-tag	-	Histidine tag
HRP	-	Horseradish peroxidase

IFN-γ	-	Interferon gamma
Ig		Immunoglobulin
IgG	-	Immunoglobulin class G
IgY	-	Immunoglobulin classY
IMAC		Immobilized metal affinity chromatography
IPTG	-	Isopropyl ß-D-thiogalactopyranoside
kDa	-	kilo Dalton
l	-	Litre
LB	-	Luria-Bertani
LIDS	-	Lithium dodecyl sulfate
M	-	Molarity
mA	-	Milliamp(s)
M. bovis	-	*Mycobacterium bovis*
mg	-	Milligram(s)
MGFP	-	Monster green fluorescent protein
min	-	Minute(s)
ml	-	Millilitre(s)
mm	-	Millimetre(s)
mM	-	Millimolar
mP	-	Millipolarization
MP	-	Milk powder
MPB70		Mycobacterial protein bovis 70
MPB83		Mycobacterial protein bovis 83
MPB64		Mycobacterial protein bovis 64
MTBC	-	*Mycobacterium tuberculosis* complex
M. tuberculosis		*Mycobacterium tuberculosis*
MWCO(s)	-	Molecular weight cutt-off(s)
N	-	Normality
ng	-	Nanogram(s)
nm	-	Nanometre(s)
NPV	-	Negative predictive value
OD	-	Optical density
OPD	-	*O*-phenylenediamine

PAGE -	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
PEG	-	Polyethylene glycol
pmol	-	Picomole
PPD	-	Purified protein derivative
PPV	-	Positive predictive value
PVDF	-	Poly vinylidene fluoride
r	-	Recombinant
ROC	-	Receiver operator characteristic
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
RT	-	Room temperature
s	-	Second(s)
SDS	-	Sodium dodecyl sulphate
SOE	-	Splicing by overlap extension
spp	-	Species
TB	-	Tuberculosis
TE	-	Tris-EDTA
Th1	-	T helper 1
Th2	-	T helper 2
Tm	-	Melting Temperature
TN	-	Test negative
TP	-	Test positive
TST	-	Tuberculin skin test
UV	-	Ultraviolet
V	-	Voltage
WHO	-	World Health Organization
µg	-	Microgram(s)
µl	-	Microlitre(s)
µM	-	Micromolar
%	-	Percent

SUMMARY

MYCOBACTERIUM TUBERCULOSIS COMPLEX-SPECIFIC ANTIGENS FOR USE IN SERODIAGNOSIS OF BOVINE TUBERCULOSIS

by

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Bovine tuberculosis (BTB) is a zoonotic disease that affects domestic and wild animals, and humans. It is caused by *Mycobacterium bovis* (*M. bovis*) and has a wide host range. The effective control of BTB is of paramount importance and this can be achieved through the use of accurate and comprehensive diagnostic tests. The most widely used methods to detect BTB are the skin test and *in vitro* gamma interferon assay which do not detect anergic animals, but serological tests such as ELISA and fluorescence polarization assay (FPA) have been found promising in ancilliary tuberculosis diagnosis. The overall aim was to study *M. tuberculosis* complex (MTBC) protein, mycobacterial protein bovis 70 (MPB70) as a target for serological assays in the detection of antibodies to bovine tuberculosis.

The MPB70 protein was expressed, purified and labeled with fluorescein (FITC). The *mpb70* gene was fragmented into three regions without disrupting predicted epitopes. The resulting protein Fragments were expressed as fusion proteins with the monster green fluorescent protein (MGFP). The recombinant MPB70 (rMPB70) and the expressed gene fragments 2 & 3 were tested in immunoblots and ELISAs. The rMPB70 and fragment 2-MGFP reacted with chicken antibodies raised against rMPB70 and immune sera from BTB infected buffaloes. MPB70 peptides were synthesized as an approach to identify even smaller antigenic regions. The peptides BT1G (residues 31-45) and BT51L (residues 81-95) were recognised by anti-MPB70 chicken antibodies in the ELISA and fall within fragment 1 and 2, respectively. The tracers (rMPB70-FITC, fragment 2-MGFP fusion and peptides BT1G & BT51L) were tested in the FPA, but the results failed to distinguish between immune sera from chickens immunized with rMPB70 and negative control sera.

Even though the FPA was not successful, the MPB70 fragment 2-MGFP fusion protein, which was recognized by sera from BTB infected buffaloes, was tested in an ELISA using panels of sera from uninfected and naturally *M. bovis* infected buffaloes and cattle. The diagnostic performance of the ELISA was, however, overall unsatisfactory and hence of very limited use as a serological test to detect antibody responses to BTB as a stand-alone assay. Sera from some of the animals gave false positive reactions indicating that MPB70 was not sufficiently specific for serodiagnosis of *M. tuberculosis* complex infections.

CHAPTER 1

1. LITERATURE REVIEW

1.1 Introduction

Tuberculosis (TB) is a contagious, usually fatal disease that affects one third of the world's population, approximately 1.8 billion people per year (Todar, 2009). The disease is caused by a closely related group of bacteria known as the *Mycobacterium tuberculosis* complex (MTBC). The members of the MTBC are closely related genetically, sharing 16S rRNA sequences (Boddinghaus *et al*., 1990; Abass *et al*., 2010). Genome sequencing has revealed that the MTBC are over 99.9% identical at the nucleotide level (Streevatsan *et al*., 1997). The MTBC species include *Mycobacterium tuberculosis*, *M. bovis, M. africanum, M. microti and M. canetti, M. bovis BCG, M. pinnipedii, M. caprae, oryx bacillus, dassie bacillus* and *M. mungi* (Smith *et al*., 2006; Abass *et al*., 2010; Alexander *et al*., 2010). These pathogens have preferred hosts, zoonotic potential and reservoirs, except for *M. mungi,* whose host spectrum and transmission dynamics still remains unclear (Ayele *et al*., 2004; Abass *et al*., 2010; Alexander *et al*., 2010).

Mycobacterium tuberculosis is the main causative agent of human tuberculosis whereas *M. bovis* mainly causes bovine tuberculosis (BTB) in cattle. *Mycobacterium bovis* has a broad host range that includes farm animals, wildlife and humans (Van Embden *et al*., 1995; Harrington *et al.*, 2007, 2008). BTB is a zoonotic disease which is emerging as a wildlife disease in southern Africa. Its impact is noticed worldwide, causing major economic losses as it affects animal health, productivity and international trade (Porphyre *et al*., 2007; Ngandolo *et al*., 2009). Studies have shown that wildlife and domestic animals share common *M. bovis* genotypes; suggesting some form of transmission between these animal species (Aranaz *et al*., 1996; Naranjo *et al*., 2008).

In some instances, wildlife may act as reservoirs of *M. bovis* infection and therefore pose a threat to other wildlife, especially valuable and endangered species, and domestic animals (Lisle *et al*., 2002; Michel, 2002; Renwick *et al*., 2007). Currently, *M. bovis* infection is reported in more than 40 free-ranging wildlife species (Michel *et al*., 2010). According to Morris and Pfeiffer (1995), an infected wild animal can be classified as either a maintenance or a spillover host, depending on the dynamics of the infection. The Infection in a maintenance host can persist within the species in the absence of cross-transmission from other species (Cousins & Florisson, 2005; Renwick *et al*., 2007). African buffalo (*Syncerus caffer*) in South Africa (Michel *et al*., 2006), badgers (*Meles meles*) in Ireland and the United Kingdom (Phillips *et al*., 2003; Griffin *et al*., 2005) and brush-tailed possums (*Trichosurus vulpecula*) in New Zealand (Coleman *et al*., 2006; Porphyre *et al*., 2007) have all been recognized as potential maintenance hosts of *M. bovis*. Spillover hosts or dead–end hosts have only a limited capacity to transmit the infection within the population in the absence of a persistent alternative source of infection (Renwick *et al*., 2007). Michel and co-workers (2009) have shown through genetic typing that the spillover of *M. bovis* from buffaloes to lions does exist in the Kruger National Park. The infection has also spilled over from buffaloes into additional species including leopards (*Panthera pardus*) and cheetahs (*Acinonyx jubatus*) (De Vos *et al*., 2001). The continued transmission of *M. bovis* from free ranging wildlife reservoirs to domestic livestock hampers BTB eradication and control programmes in several countries (Cousins, 2001; Michel *et al*., 2006).

1.2 Aetiology

The members of the *Mycobacterium tuberculosis* complex are Gram positive, aerobic, non-motile, acid-fast, slow growing bacteria (Wayne & Kubica, 1986; Kaneene *et al*., 2004; Corner *et al*., 2011). They have a cell wall with high lipid content, which accounts for their slow growth, resistance to acids, desiccation and most disinfectants. *M. bovis* grows poorly or not at all on glycerol-based media which is usually used for isolation of *M. tuberculosis*. However, its growth is enhanced by the addition of sodium pyruvate instead of glycerol (WHO, 1996; Corner *et al*., 2011).

The organism is microaerophilic, negative for niacin accumulation and nitrate reduction (Ayele *et al*., 2004; Kubica *et al*., 2006). In contrast, *M. tuberculosis* is aerobic, positive for niacin accumulation and nitrate reduction. *M. bovis* is a robust pathogen but cannot thrive under hot, dry or sunny conditions, although it can remain viable for long periods in moist and warm soil. In drinking water it can survive for up to 18 days (Ayele *et al*., 2004; Good & Duignan, 2011; http://www.vetsweb.com).

1.3 Routes of *M. bovis* transmission

Mycobacterium bovis can be transmitted in a number of ways depending on the species involved. Infection can occur within and between domestic and wild animals; from animals to humans and vice versa; and between humans (O'Reilly & Dabon, 1995; Collins, 2000; http://www.hpa.org.uk). The possible routes of infection include respiratory, alimentary, congenital and cutaneous. However, these routes of infection are influenced by animal age, the type of species, species behaviour, environment, climate and existing farming practices (Neill *et al*., 1994; Ayele *et al*., 2004). Young cows and growing heifers are mostly at risk of infection with *M. bovis*, as are poorly nourished or stressed animals.

The respiratory (aerosol/droplet) and alimentary (oral) routes are the main transmission pathways (Neill *et al*., 1994; Kaneene *et al*., 2004; Renwick *et al*., 2007). Close, prolonged contact between infected and healthy animals facilitates the aerosol mode of transmission. This route of transmission is the most common in cattle but highest when domestic animals and ungulate wildlife share pasture or territory such as water points, wells, ponds or streams, salt supplementary points, feeders or shelter at night for protection against predators (Ayele *et al*., 2004; Renwick *et al*., 2007; Naranjo *et al*., 2008). The aerosol transmission is also common where farming is practiced intensively, especially in industrialised countries and during the movement of cattle through markets and between farms (Neill *et al*., 1994; Ayele *et al*., 2004). Wildlife species that are kept in confinement in zoos are also at risk of being infected by *M. bovis* through the respiratory route. Similarly, *M.*

tuberculosis may be transmitted by workers, veterinarians and the general public who visit the zoo (Kaneene *et al*., 2004).

The transmission of *M. bovis* infection from cattle to humans can be spread via aerosol droplets during direct contact with infected animals or mucous membranes and skin abrasions (Grange & Yates, 1994; Ashford *et al*., 2001). Farmers, veterinary staff, rural and abattoir workers, TB laboratory personnel, hunters and game and zoo keepers are at a high risk of contracting BTB as they regularly handle infected carcasses or animal reservoirs of *M. bovis* (O'Reilly & Dabon, 1995; Moda *et al*., 1996; Ashford *et al*., 2001). People who acquire infection by inhalation from infected cattle usually develop classic pulmonary TB similar to *M. tuberculosis* infection. Such patients can shed the organism from their airways back to cattle (Cosivi *et al*., 1998; http://www.vetmed.wisc.edu). Even though human-to-cattle transmission of *M. bovis* does occur, reports of such cases are scarce (O'Reilly & Daborn, 1995; Ayele *et al.*, 2004). The infection is usually via pasture or bedding contaminated with urine from patients with genito-urinary TB (O'Reilly & Daborn, 1995; Grange, 2001; Ayele *et al.*, 2004). Human-to-human aerosol spread of *M. bovis* is uncommon in immunocompetent individuals (O'Reilly & Daborn, 1995; Grange, 2001; Ayele *et al*, 2004). However, in most developing countries humans are vulnerable due to HIV/AIDS, reduced access to health services and poverty (Ayele *et al.*, 2004). TB cases due to *M. bovis* in HIV patients resemble disease caused by *M. tuberculosis* (Cosivi *et al*., 1998). As with *M. tuberculosis, M. bovis,* too, has the capacity to acquire drug resistance (Rivero *et al*., 2001; Ayele *et al.*, 2004) and this is a major concern for HIV patients in developing countries.

The alimentary route of infection is possible when an infected animal excretes *M. bovis* in sputum, milk, draining sinuses, pus, urine or faeces and other animals subsequently consume the contaminated material (Renwick *et al*., 2007). This mode of transmission has been detected in calves that consume milk from the infected dam and in cattle that graze in contaminated pastures (Kaneene *et al*., 2004). Similarly, humans are able to contract the infection from cattle via consumption of

raw, unpasteurized milk (Grange & Yates, 1994; Moda *et al*., 1996; Michel *et al.*, 2010). The infection acquired through ingestion of *M. bovis* in milk is more likely to result in non-pulmonary forms of the disease and most of the time the organism is located in the gastrointestinal (GI) tract and related lymph nodes (Ayele *et al.*, 2004). Inter-specific encounters account for a second means of alimentary transmission, for example when predators become infected by consuming infected prey (Morris *et al.*, 1994). This route of transmission is of great concern in Africa's conservation areas as it affects high profile wild carnivores which share territory with infected prey species (Renwick *et al*., 2007). Feral swine in Australia (Corner *et al*., 2002) and Hawaii (Essey *et al*., 1981) and wild boar in Spain (Gortazar *et al.*, 2008, 2011; Mentaberre *et al*., 2012) that scavenge on contaminated carcasses are also at great risk.

Percutaneous infection is a less known mode of transmission. It has been observed in kudus where contaminated thorns either scratch their ears or cause micro-lacerations of the oral and pharyngeal mucosa (Thorburn & Thomas, 1940; Renwick *et al*., 2007; Bengis *et al*., 2012). It has also been recorded in badgers in England as a result of infection by bite wounds (Mahmod *et. al.*, 1987; Corner *et al*., 2011). Specific behavior such as social interaction or intra-species aggression between lions facilitates percutaneous transmission through bites and claw wounds (Michel *et al*., 2006; Bengis *et al*., 2012). Congenital and genital infections are rarely found (Ayele *et al*., 2004).

1.4 Immune response to *M. bovis*

A 'spectrum' of immune responses exists within *M. bovis* infection (Ritacco *et al*., 1991; Neill *et al*., 1994; Pollock *et al*., 2005) with cell-mediated immune (CMI) responses dominating in the early stages of infections. CMI responses involves the activity of T helper 1 (Th1) cells which releases proinflammatory cytokines including interferon-γ (IFN-γ, Pollock *et al*., 2001, 2002; Welsh *et al*., 2005). It is generally accepted that CMI responses play an important role in protective immunity (Neill *et al*., 1994; Boom, 1996) and may benefit the host by initiating processes which may

destroy or inhibit mycobacteria. Alternatively, a delayed type hypersensitivity (DTH) reaction may result (Neill *et al*., 1994; Thom *et al*., 2004). As the disease progresses, dominance shifts from a Th1 to T helper 2 (Th2) immune response with an associated anergy of cellular responses. Antibodies produced by B-cells develop (Ritacco *et al*., 1991; Welsh *et al.*, 2005) with the onset of clinical signs as shown in Figure 1.1.

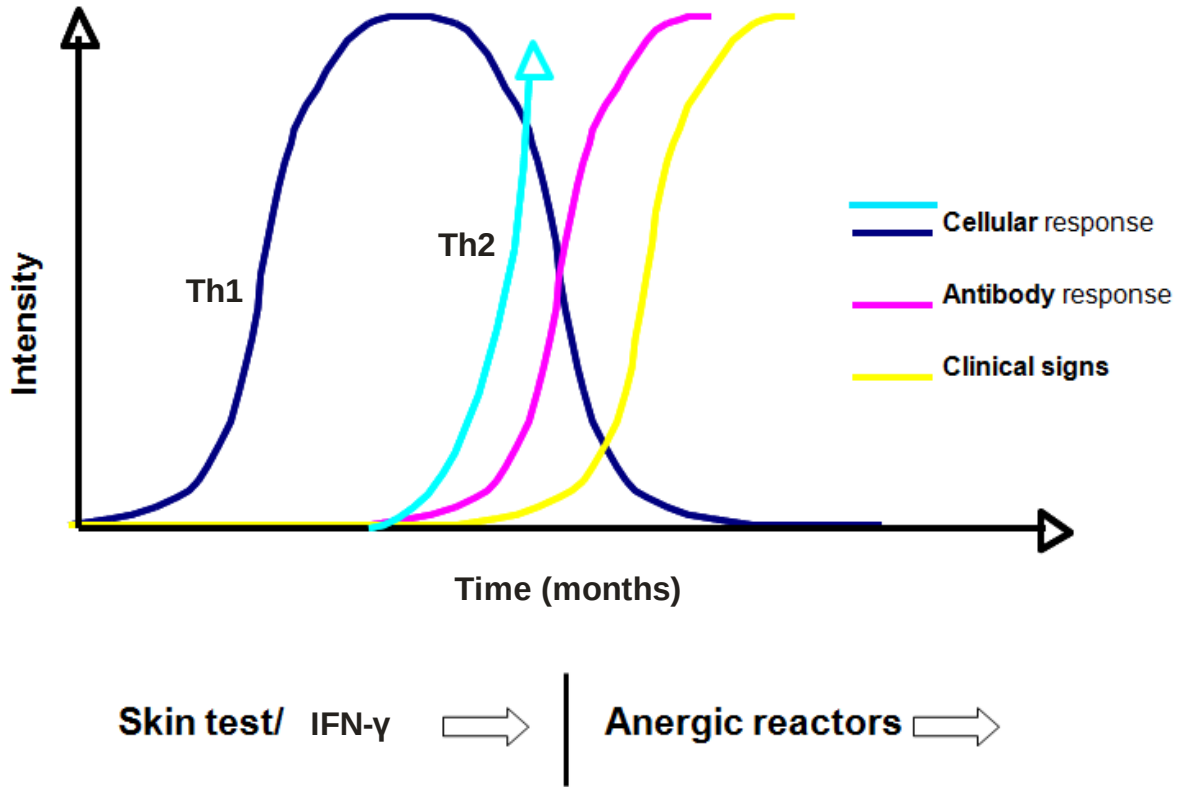


Figure 1.1: Immune response to *M. bovis* infection.

1.5 Clinical signs

Bovine tuberculosis infection usually progresses very slowly, and therefore it may take several months or even years for clinical signs to develop (Henning, 1956; Keet, 2000). Early infections are often asymptomatic and can progress to active disease when the animal is stressed or suffering from old age (Vos *et al*., 2001; Renwick *et al*., 2007). The most common clinical signs of BTB include progressive emaciation, weakness, a low-grade fever, breathing difficulties, and lameness especially in

carnivores (Kaneene & Thoen, 2004; McGeary, 2008). A cough may be detected that worsens especially in cold weather or when the animal exercises. Lymph nodes may enlarge, rupture and drain (Kaneene & Thoen, 2004; OIE Terrestrial manual, 2009). Enlarged lymph nodes may also obstruct blood vessels, airways or the digestive tract. If the digestive tract is involved, intermittent diarrhea and constipation may be seen.

1.6 Pathogenesis

Pathogenesis of BTB varies within and between species, resulting from different routes of infection, excretion and transmission patterns (Drewe *et al*., 2009). Generally infection with *M. bovis* starts with the inhalation of a single bacillus in an aerosol droplet that enters the respiratory tract and lodges within the alveolar surface of the lung (Neill *et al*., 1991; Ayele *et al*., 2004). Inhaled bacilli are ingested by alveolar macrophages that may either clear the infection or allow the mycobacteria to replicate intracellularly. In the latter case, the macrophages loaded with mycobacteria migrate through lymphatic vessels to the lymph nodes where a cell mediated immune response develops (Spitznagel & Jacobs, 1993; Thoen & Chiodini, 1993). The lymphokines released by lymphocytes attract, immobilize and activate monocytes, lymphocytes and neutrophils at the site of infection (Thoen & Chiodini, 1993; Smith, 2003), but none of these mononuclear cells kill the bacteria very efficiently. Primary lesions or foci (granulomas) begin to form. At this stage the immune system manages to contain the spread of the organisms (Spitznagel & Jacobs, 1993; Smith, 2003). As a delayed hypersensitivity reaction develops, infected macrophages are killed and caseous necrosis (cell destruction) forms at the center of the granulomas with a boundary of epithelioid cells, granulocytes, lymphocytes and giant cells (Thoen & Chiodini, 1993; Neill *et al*., 1994). The caseous necrotic center may calcify and a classic ‘tubercle’ forms as the lesion becomes surrounded by granulation tissue and a fibrous capsule. This is a characteristic of a lesion caused by *M. bovis* in cattle and other bovids (Neill *et al*., 1994; Lisle *et al*., 2002; Ayele *et al*., 2004).

The tubercle is usually a round firm white or yellowish nodule which measures roughly 1-3 cm in diameter. Cut sections of tubercles prepared for histology show dry, yellowish, caseous, necrotic cellular debris at the center. Tuberculosis lesions in cattle are most frequently found in the lungs and associated lymph nodes. Lesions can also be found in mesenteric lymph nodes, liver and other organs (Ayele *et al*., 2004; Medeiros *et al*., 2010). The appearance, nature and distribution of lesions in wildlife sometimes differ substantially from those found in cattle. The primary lesions can heal completely. However, if the infection is not completely contained by the immune system, the bacilli will escape from the lesion by natural ducts and spread hematogenously to lymph nodes and other organs of the body (Ayele *et al*., 2004; Merckvet manual, 2011) and cause smaller tubercles known as 'miliary tuberculosis".

1.7 Mycobacterial proteins

During mycobacterial infection, the host is exposed to several antigenic proteins produced by the Mycobacterium. Many of these proteins have been studied, cloned, purified and characterized (Terasaka *et al*., 1989; Yamaguchi *et al*., 1989; Matsuo *et al*., 1996). Of importance are MPB70, mycobacterial protein bovis 83 (MPB83), mycobacterial protein bovis 64 (MPB64), early secretory antigenic target 6 kilodaltons (kDa) (ESAT-6) and culture filtrate protein 10 kDa (CFP-10), most of which are restricted to the *Mycobacterium tuberculosis* complex with some exceptions (Harboe *et al*., 1986; Wiker *et al*., 1998, Skjøt *et al*., 2000). Their use in serological and cellular immune response studies has formed the basis for improved diagnostic tests for tuberculosis and vaccine development for both bovine and human tuberculosis.

The MPB70 protein is secreted by *M. bovis* and other members of the *M. tuberculosis* complex and has been widely used in diagnosis of BTB. It is an active component of tuberculin (Harboe *et al*., 1990) and forms a major component of the *M. bovis* culture filtrate (Fifis *et al*., 1989; 1991; Wood *et al*., 1992; Lin *et al*., 1996). It can stimulate both cellular and humoral immune responses and it is able to elicit a

delayed-type hypersensitivity response in *M. bovis* infected cattle (Nagai *et al*., 1981, Harboe *et al*, 1986, Fifis *et al*., 1991, 1994). The gene encoding MPB70 has been cloned, sequenced and the protein expressed in *E. coli.* The mature protein is 163 amino acid residues in size and is secreted from mycobacterial cells following cleavage of a 30 amino acid residue N-terminal secretory signal sequence (Terasaka *et al*., 1989; Radford *et al*., 1990; Hewinson *et al*., 1993). The signal sequence is not involved in the antibody response (Radford *et al*., 1990). MPB70 has an estimated molecular mass of between 16 and 23 kDa depending on the method of estimation (Nagai *et al*., 1991; Surujballi *et al*., 2002).

The MPB70 protein has been found to contain at least three distinct *M. bovis*-specific epitopes (Wood *et al*., 1988) using mouse monoclonal antibodies, although some cross reactivity of at least one epitope with *Nocardia asteroids* was observed (Harboe & Nagai., 1984; Harboe *et al*., 1986). Omission of these cross reactive epitopes can improve the specificity of diagnostic tests. Radford *et al*. (1990) scanned and mapped linear B-cell epitopes within mature MPB70 using octapeptides (8-mers) with one amino acid residue overlap. Monoclonal antibodies SB9 and SB10 reacted with residues 45-49 and 53-57, while *M. bovis* infected cows reacted strongly with residues 51-62, 62-69, 103-107 and 141-147 (Figure 1.2). Wiker *et al*. (1998) covered the signal sequence and mature MPB70 by using 20-mer peptides with 10 amino acid residue overlap and found epitope mapping with monoclonal antibodies to be in agreement with the findings of Radford *et al*. (1990). Bovine and rabbit sera (Wiker *et al*., 1998) showed a response within the same region as the bovine antibodies used in the study done by Radford *et al*. (1990). However, the bovine antibodies used by Wiker *et al*. (1998) recognized a wider spectrum of amino acid residues. By screening a panel of overlapping peptides (Lightbody *et al*., 2000), using sera from cattle immunized with recombinant (r) MPB70 and cattle infected with *M. bovis,* two regions of residues 31-70 and 101-120 were found and confirmed the positions of epitopes in the regions 51-70 and 103-107 which have already been identified by Radford *et al*. (1990) and Wiker *et al*. (1998). Studies have shown that antibodies to MPB70 are detected at a late stage of *M. bovis* infection (Harboe *et al*.,

1990; Wiker *et al*., 1998; Harrington *et al*., 2008). Therefore, this study focused on MPB70 protein as a marker for detection of late BTB infections.

As T-cell mediated immune responses predominate in the early stages of *M. bovis* infection, T-cell epitopes have been identified in order to understand these responses and to design improved diagnostic tests for BTB (Pollock *et al*., 1994). Bovine T-cell epitopes have been mapped for MPB70 using *in vitro* lymphocyte proliferative responses (Pollock *et al*., 1994), *in vitro* IFN-γ responses to overlapping peptides (Lightbody *et al*., 1998) and truncated recombinant products (Billman-Jacobe *et al*., 1991). All were shown to be important in cell-mediated immunity.

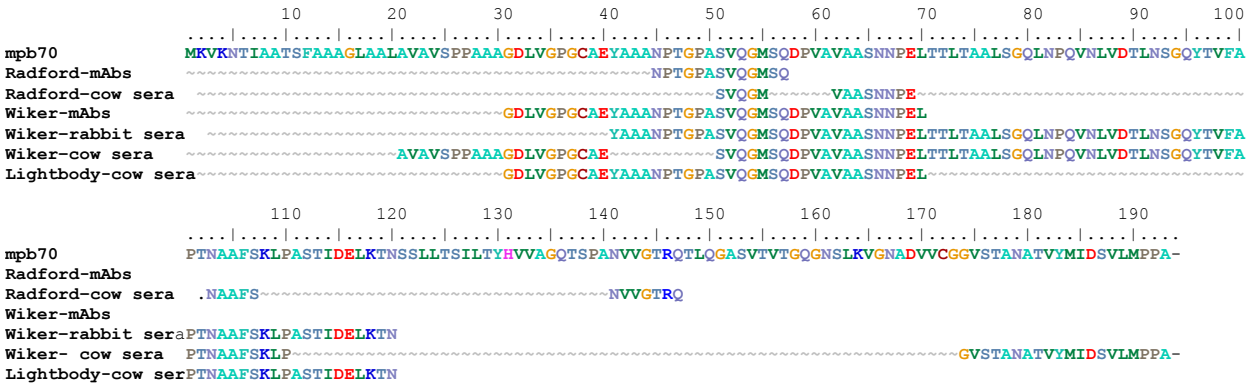


Figure 1.2: The epitopes of MPB70 shown by different researchers: Radford *et al.* (1990) using monoclonal antibodies and cow sera; Wiker *et al*. (1998) using monoclonal antibodies, polyclonal rabbit sera and cow sera; Lightbody *et al*. (2000) using cow sera.

Another major secreted protein found in the culture filtrate is MPB83 (Harboe *et al*., 1998; Wiker *et al*., 1998), usually a glycosylated lipoprotein located at the cell surface (Vosloo *et al*., 1997; Harboe *et al*., 1998). It has been cloned, sequenced and the protein expressed in *E. coli*. The mature protein is 196 amino acid residues long (Carr *et al*., 2003) with a pro-lipoprotein signal peptide of 20 amino acid residues (Matsuo *et al*., 1996; Vosloo *et al*., 1997). Residues 33-195 of mature MPB83 are over 80% identical to full length mature MPB70 (74% identity over 163 residues) (Matsuo *et al*., 1996; Wiker *et al*., 1998., Carr *et al*., 2003). MPB83 induces

strong T-cell responses in *M. bovis* infected cattle (Fifis *et al*., 1994; Vordermeier *et al*., 1999). It is also a major B-cell target (O'Loan *et al*., 1994; McNair *et al*., 2001) and antibody responses to MPB83 have been found to appear early in the *M. bovis* infection (O'Loan *et al*., 1994; Waters *et al*., 2006).

According to Harboe *et al*. (1986), MPB64 is a secreted protein which was first isolated from the culture filtrates of *M. bovis* BCG Tokyo. It is also found in *M. tuberculosis* (Nagai *et al*., 1991; Harboe *et al*., 1998). MPB64 contains both T- and B-cell antigenic targets in *M. bovis* infected cattle (Fifis *et al*., 1989; 1991; Wood *et al*., 1992). Furthermore, it can elicit a DTH response (Harboe *et al*., 1986). Like other proteins mentioned above, it has been cloned, sequenced and the protein expressed *E. coli*. The mature protein consists of 205 amino acid residues and a putative signal peptide of 22 amino acid residues (Yamaguchi *et al*., 1989).

ESAT-6 and CFP-10 proteins are strongly immunogenic, IFN-γ inducing antigens of tuberculous mycobacteria (Waters *et al*., 2004; Palmer & Waters, 2006). The two proteins form a 1:1 heterodimeric complex with each other (Renshaw *et al*., 2002). The sequence of the *cfp10* gene is approximately 40 % identical to ESAT-6 (Berthet *et al*., 1998; Skjøt *et al*., 2000). Genes for the two proteins have been found present in *M. tuberculosis, M. africanum* and virulent *M. bovis*, although they are absent in *M. bovis* BCG and many environmental as well as non-tuberculous mycobacteria (van Pinxteren *et al*., 2000; Waters *et al*., 2004) with the exception of *M. kansasii, M. marinum, M. leprae* and *M. smegmatis* (Sorensen *et al*., 1995; Harboe *et al*., 1996; Gey van Pittius *et al*., 2001). It has been proven that tuberculin skin test can distinguish between *M. bovis* infected and BCG vaccinated cattle when ESAT-6 and CFP-10 are used as test reagents (Vordermeier *et al*., 2000, 2001; Hu *et al*., 2011). In addition, when ESAT-6 is used, it can differentiate between cattle infected with *M. bovis* and cattle sensitized by environmental strains (Pollock & Anderson, 1997a & b). ESAT-6 and CFP-10 are able to elicit T-cell responses. When these proteins are used as stimulating antigen, the specificity of IFN-γ based assay is improved compared to bovine PPDs (van Pinxteren *et al*., 2000; Vordermier *et al*., 2001;

Buddle *et al*., 2003). Recently Kwok *et al*. (2010) reported that ESAT-6 and CFP-10 elicited a humoral immune response two weeks post challenge, with heat-inactivated *M. bovis* wild-type strain, indicating an early humoral status. However, this was shown in a rabbit model and remains to be proven in cattle and wildlife.

1.8 Diagnosis

The effective control of BTB in cattle and wildlife is of paramount importance and can be achieved through the use of accurate and comprehensive diagnostic tests. There are various tests available. However, no single test can diagnose BTB at all stages of infection. Broadly, BTB diagnostic tests can be divided into four groups: those based on the detection of cellular immune response to infection; those which rely on the observation of pathological changes (histopathology); those which determine the presence of *M. bovis* organisms (culture and polymerase chain reaction) and assays which detect antibody response to infection.

Diagnostic tests based on a cellular immune response include the tuberculin skin test and the *in vitro* IFN-γ assay. Both of these assays detect the early stages of the disease, but do not detect anergic animals. Anergic animals are those animals which can no longer mount CMI responses and are thought to be heavily diseased and highly infective (Ngandolo *et al*., 2009). The tuberculin skin test measures CMI dependent delayed–type hypersensitivity reaction in response to tuberculin. In contrast, the IFN-γ test is a generic assay that measures IFN-γ that is released from antigen–sensitised lymphocytes following overnight incubation with tuberculin. IFN-γ assay detects infection in animals before the onset of the DTH skin response (Pollock & Neill, 2002; de la Rua-Domenech *et al*., 2006). The tuberculin skin test is impractical for routine use in free-ranging wildlife as the animals have to be contained for 72 hours (h) and are required to be handled twice over this period, thus increasing the risk of capture-associated injuries and deaths (Harrington *et al*., 2008; Keet *et al*., 2010). In addition, the immobilization costs are high. Other limitations of this test are the difficulties in defining proper test sites for different species like

rhinoceros and elephants (Morar *et al*., 2007), lack of information regarding concentrations, dosages and preparations of tuberculin to use (Keet *et al*., 2010). Samples for IFN-γ assays require processing within 24 h, which is impractical for samples from remote areas in the field. Both the IFN-γ assays and the tuberculin skin test have been reported to lack sensitivity and specificity (Jolley *et al*., 2007; Kwok *et al*., 2010).

Histopathology detects pathological changes through the examination of histological sections of suspect tuberculous lesions (Lisle *et al*., 2002). Diagnostic sensitivity of the test can be increased by using it alongside with culture of the mycobacteria (Liebana *et al*., 2008). The disadvantage of histopathology is that it is conducted postmortem. In addition, variation in the appearance of lesions among different animal species infected with BTB makes diagnosis difficult. Granulomatous lesions for tuberculosis on histopathology can be mistaken for those caused by bacteria such as *Staphylococci*, *Actinomyces*, or *Actinobacillus* and fungi including *Aspergillus* and *Crypyococcus* (Lisle *et al*., 2002). Hence, false negative and false positive diagnoses can be made. Irrespective of the outcome of results, tissues are best tested for confirmation by culture and PCR.

Culturing of the organism from affected tissues is still considered to be the 'gold standard' method for detection of *M. bovis,* followed by confirmation using polymerase chain reaction (PCR) (Jolley *et al*., 2007; Kwok *et al*., 2010). Due to the slow growth rate of MTBC bacilli, culture takes a long time to produce a result, and this is impractical for field testing (Kaneene & Thoen, 2004). Advances in molecular biology have led to the development of rapid, sensitive and specific tests to detect mycobacteria based on amplification of unique mycobacterial DNA or RNA target fragments by PCR (Mikota *et al*., 2001; Medeiros *et al*., 2010). Other PCR-based techniques include sploligotyping which is based on polymorphisms of the chromosomal direct repeat loci containing variable numbers of short direct repeats interspersed with non-repetitive spacers (Kamerbeek *et al*., 1997), restriction fragment length polymorphism (RFLP) and variable number tandem repeat typing.

These nucleic acid assays have been widely used in the epidemiological studies of BTB and TB control (Medeiros *et al*., 2010). However, in spite of all the advantages, PCR tests must be performed under carefully controlled conditions to avoid cross contamination and false positive tests (Lisle *et al*., 2002; Kaneene & Thoen, 2004; OIE Terrestrial Manual, 2009).

Serological tests are used to detect the host antibody response to mycobacterial antigens. The antibody-based assays exist in different formats, including immunochromatographic lateral flow like tests such as the STAT-PAK assay; blotting methods like the multi-antigen print immunoassay (MAPIA); the enzyme linked immunosorbent assay (ELISA) and the fluorescence polarization assay (FPA). STAT-PAK is a novel and rapid test that uses a cocktail of selected *M. bovis* antigens including MPB83, ESAT-6 and CFP-10. It is easy to perform and can be run in the field, however, it is costly and requires species-specific reagents. The test has shown good potential for detecting BTB in a number of species (Lyashchenko *et al*., 2007, 2008) including badgers (Greenwald *et al*., 2003; Chambers *et al*., 2008), elephants (Greenwald *et al*., 2009; Lyashchenko *et al*., 2006) and wild deer species (Gowtage-Sequeira *et al*., 2009).

MAPIA is based on the immobilization of antigens onto nitrocellulose membranes by semi-automated micro spraying, followed by standard chromogenic immunodevelopment (Lyashchenko *et al*., 2000). It is an efficient and cost-effective method for large scale antigen screening. The test however cannot be run in the field and requires species-specific reagents. The antigens that have been used in MAPIA include MPB83, ESAT-6, CFP10, and MPB70. However, MPB83 is the most recognized antigen by cattle and most wildlife species including badger, deer, possums and wild boar (Waters *et al*., 2004; Lyashchenko *et al*., 2004, 2008; Lesellier *et al*., 2008; Buddle *et al*., 2010).

ELISA has been widely used over the years for diagnosis of BTB. It has been suggested as a complement to tests based on cellular immunity (OIE Manual of Terrestrial Animals, 2009). In addition to being a simple, rapid and low cost test (Harrington *et al*., 2008; Hu *et al*., 2011), it can be used for high throughput testing and the cut-off point can be adjusted to suit the purpose of the test (Chambers *et al*., 2012). Despite all the advantages, it requires species-specific reagents and has a low sensitivity and moderate specificity (Ritacco *et al*., 1990; McNair *et al*., 2001). Many antigens have been employed in ELISA. They include complex antigens such as purified protein derivative (PPD) and single or closely associated purified antigens from *M. bovis* (Ritacco *et al*., 1987; Lilenbaum *et al*., 1999; Waters *et al*., 2006). ELISA using *M. bovis* PPD antigen has shown to detect antibodies to mycobacteria successfully but lack specificity (Ritacco *et al*., 1987; Hammam *et al*., 1989). The use of proteins like MPB70 and MPB83 as capture antigens in ELISA has demonstrated good specificity but lack sensitivity (Wood *et al*., 1992; Yearsley *et al*., 1998; McNair *et al*., 2001). On the other hand, a cocktail of different purified antigens in ELISA has shown improved sensitivity and specificity. Liu *et al*. (2007) reported that a combination of MPB70, MPB83 and ESAT-6 showed a sensitivity of 69.4%, which is higher than 18% and 37.5% reported by Wood *et al*. (1992) and McNair *et al*. (2001) respectively, when a single protein was used.

Fluorescence polarization assay is a simple and rapid test which detects and measures the binding of small fluorescent–labeled molecules (tracers) to large molecules (binding partners) like antibodies and receptors (Plackett *et al*., 1989). The principle behind the test is that polarized light is applied to a free tracer in a solution, causing molecules to rotate very fast and resulting in the emission that is depolarized by the rapid rotational diffusion that occurs during the lifetime of the excited state. Conversely, if polarized light is applied to the tracer bound to its binding partner, the molecules rotate very slowly, resulting in the subsequent emission that remains polarized (Jolley & Nasir, 2003; Kimple *et al*., 2008; Figure 1.3). The depolarization is quantified as fluorescence polarization (FP) by measuring the intensity of the emission perpendicular and parallel to the plane of excitation (Kimple *et al*., 2008). FP is expressed as milliPolarization (mP). Tracers used in FPA

include protein antigens, hormones and peptide epitopes (Nasir & Jolley, 1999; Jolley & Nasir, 2003).

The FPA does not require species-specific reagents and can be performed in a portable instrument in the field or in the laboratory using equipment for high throughput testing. FPA using MPB70 protein labeled with fluorescein (Lin *et al*., 1996, Surujballi *et al*., 2002) has been shown to detect antibodies to *M. bovis*. It demonstrated a high diagnostic sensitivity of 92.9% for a small panel of culture positive bovine sera from Canadian cattle (n=28) and specificity of 98.3% for a large panel of presumed negative sera (n=5666, Surujballi *et al*., 2002). The sensitivity decreased to 33% when samples from animals at various stages of *M. bovis* infection were tested (Harrington *et al*., 2008). Jolley *et al*. (2007) compared the sensitivity and specificity of FPA, using fluorescein labeled peptide 733 derived from MPB70, to PCR and it corresponded to 61.5% and 98.5% respectively. The FPA has also been employed to detect antibodies to *Brucella* spp (Nielson *et al*., 1996, 2001; Lin & Nielson, 1997) and *Salmonella* spp using *O*-polysaccharide as the target (Nasir *et al*., 2000; Jolley *et al*., 2001, 2002). Tencza *et al*. (2000) used a fluorescein labeled peptide derived from gp45 transmembrane protein as a tracer to detect equine infectious anemia virus.

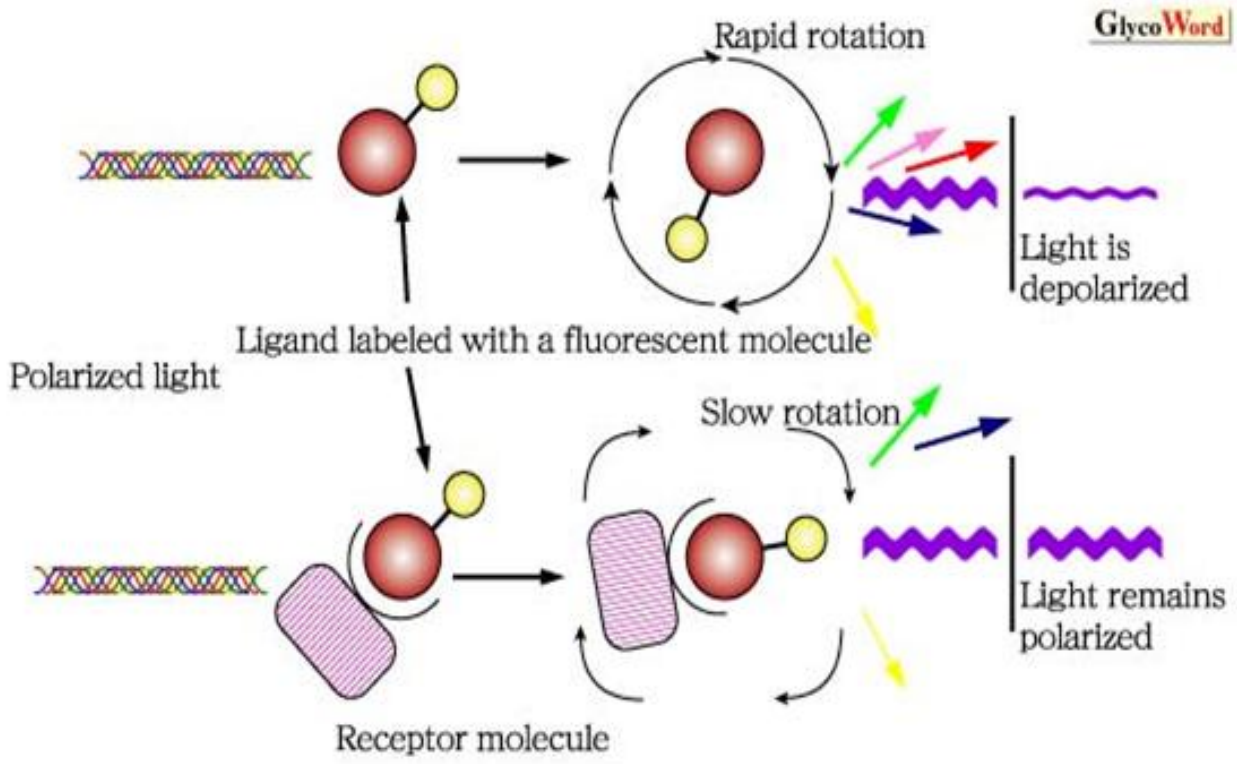


Figure 1.3: Diagrammatic illustration of fluorescence polarization assay (Taken from http://glycoforum.gr.jp)

1.9 Problem and hypothesis

MPB70 is a highly specific antigen and marker for the detection of late *M. bovis* infections, hence a good diagnostic target. It has been widely used as a component of tuberculin in the skin test measuring CMI response and also in serodiagnosis. The most widely used methods to detect BTB are the skin test and *in vitro* IFN-γ which do not detect anergic animals. However, serological tests such as FPA and ELISA have been found promising in ancillary tuberculosis diagnosis although the specificity and sensitivity of these tests need to be improved. FPA appears to be a good option because there is no species-specific reagent needed, making it a suitable test for BTB in wildlife. A number of MPB70-specific epitopes have already been determined and have been incorporated into diagnostic tests. This project focuses on identifying additional MPB70-specific epitopes to use in the FPA and ELISA. The identification of new epitopes has the potential to improve the sensitivity and specificity of each of these tests and may contribute to the current knowledge of MPB70 epitopes.

1.10 General Objectives

- To study *Mycobacterium tuberculosis* complex protein MPB70 as a target for serological assays in the detection of antibodies to bovine tuberculosis.

1.11 Specific Objectives

- To use MPB70 protein as a marker for detection of late BTB infections
- To clone the *mpb70* gene and express the protein in *E. coli*
- To identify the epitope-containing regions by using different prediction programmes and expressing gene fragments
- To synthesize peptides derived from immune-reactive protein products
- To incorporate identified tracers in an FPA and ELISA
- To evaluate the FPA and ELISA with sera from naturally infected cattle and buffaloes

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Mycobacterial strains

A field strain of *M. bovis* (TB 3894B) from the Kruger National Park isolated from a buffalo was used in this study. Genomic DNA which had been extracted with the PUREGENE DNA extraction kit (Gentra Systems) was provided by Tiny Hlokwe and Nomakorinte Gcebe from Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) TB laboratory.

2.2 Control chicken IgY

Antibodies which were raised against recombinant (r) MPB70 protein in chickens were provided by Dr Fehrsen from the ARC-OVI Immunology laboratory. The chicken antibody immunoglobulin class Y (IgY) were isolated from eggs (as a pre-immunization control sample and as antibodies collected at days 30, 80 and 100 after inoculating rMPB70) using a method adapted from Polson *et al*. (1985). The egg yolk and white was separated, and the yolk volume (*X* ml) was measured. The phosphate buffered saline (PBS, 4X ml) and pulverized PEG 6000 [(5 × 5*X*)/100] g were added. After dissolving the PEG, the mixture was centrifuged at 5,000 x g for 20 minutes (min) and the supernatant was poured through cotton wool into a measuring cylinder (*Y* ml). The PEG [(*Y* × 8.5)/100] g was added again, dissolved and left to stand for 10 minutes. The mixture was pelleted by centrifugation (Sorvall RC 5B plus, USA) with Sorval GSA rotor at 5,000 x g for 25 min and the pellet was dissolved in 2.5*X* ml PBS. The PEG [(12 × 2.5*X*)/100] g was added once more, dissolved and left to stand for 10 min. The mixture was centrifuged at 5,000 x g for 20 min, the supernatant was discarded and the pellet dried by spinning. The final pellet was dissolved in 0.25*X* ml PBS and the concentration of the IgY was

determined spectrophotometrically (Shimadzu Corporation, Japan) at 280 nm (A_{280} of 1= 1.4 mg/ml).

2.3 Serum samples

Different panels of sera were used in the study which were provided by Prof. Anita Michel and Dr Akin Jenkins from University of Pretoria and Dr Andy Potts and Mr Bryan Peba from ARC-OVI. The panels comprised the following sera:

2.3.1 Group 1: Characterized buffalo sera used to initially characterise proteins

The BTB status of the buffaloes from which the serum samples were collected:

i) Sample no 1 (KPN Buff 98/42): culture and Stat-Pak positive
ii) Sample no 2 (KPN Buff 9806): culture and histopathology positive
iii) Sample no 3 (LM19): culture, IFN-γ and histopathology positive
iv) Sample no 4: IFN-γ negative
v) Sample no 5: IFN-γ negative

2.3.2 Group 2: Panel of buffalo sera used for ELISA

Information and origin of the samples (Appendix 2)

a. The BTB status of the buffaloes from which the serum samples were collected:

i) 48 Bovigam negative
ii) 18 buffaloes with tuberculous lesions
iii) 35 Mycobacterium exposed (non-tuberculous) buffaloes

b. The BTB status of the cattle from which the serum samples were collected:

i) 50 BTB free cattle from different commercial dairy farms with negative BTB history
ii) 32 sera from tuberculin skin test positive cattle
iii) 10 Mycobacterium exposed (non-tuberculous) cattle

2.4 Expresso™ T7 Cloning and Expression System

In order to produce recombinant proteins (MPB70, monster green fluorescent protein and MPB70 fragments), the genes of interest were cloned into an expression vector using the Expresso™ T7 cloning and Expression System (Lucigen corporation, US).

The Expresso™ T7 cloning and Expression System is a simple, rapid enzyme-free cloning system that allows expression of 6xHis tagged proteins. It contains pre-processed pETite™ N-His and pETite™ C-His vector DNA, HI-Control™ 10G Chemically Competent cells for cloning and HI-Control BL21 (DE3) Chemically Competent cells for protein expression. The pETite vectors are provided in a pre-processed and linearized format that enables precise, directional cloning of inserts. The vectors encode either an N-terminal or C-terminal 6xHis tag for easy and rapid affinity purification. In addition, they include signals for expression such as T7-*lac* promoter, ribosome binding site, and translational start and stop codons (Figure 2.1). The small size (2.2 kb) of the vectors facilitates cloning of larger inserts. No enzymatic treatment or purification of the PCR product is required and no restriction enzymes are used hence no limitations on sequence junctions. After amplification of the target gene, the PCR product is mixed with the pETite vector and transformed directly into chemically competent HI-Control 10G cells. The HI-Control 10G cells are an *E. coli* strain ideal for cloning and propagation of plasmid clones. They have a recA-end-A genotype which allows the recovery of high quality plasmid DNA. They have been optimized for high efficiency transformation. After cloning, the recombinant plasmids are transferred to HI-Control BL21 (DE3) cells to express the cloned genes from the T7 promoter.

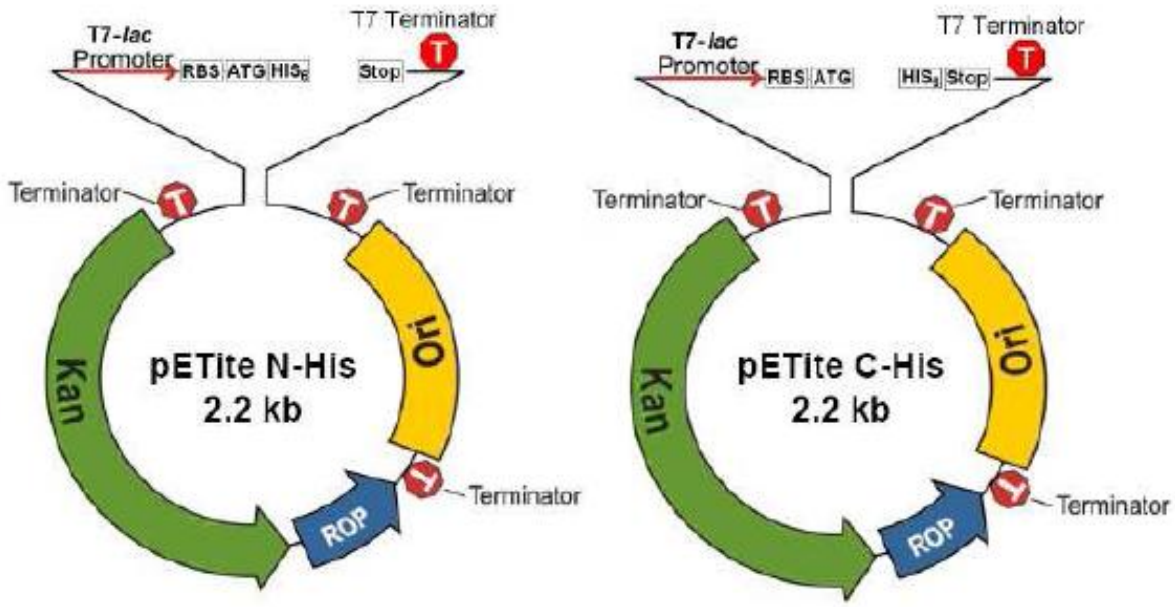


Figure 2.1: pETite Vectors (Taken from Expresso™ T7 cloning and Expression System manual).

2.4.1 Primer design

In order to facilitate enzyme-free cloning (Section 2.5) with the pETite vectors, the DNA to be inserted must be amplified with primers that append appropriate flanking sequences to the gene of interest. The primers were designed as described in the manual for Expresso™ T7 cloning and Expression System, to include 15-18 nucleotides of overlap with ends of the vector (Figure 2.2 & Table 2.1). The flanking sequences were used to fuse the target protein to either an amino-terminal 6xHis tag (pETite N-His kanamycin vector) or carboxyl-terminal 6xHis tag (pETite C-His kanamycin vector). The melting temperature (Tm) of each primer was determined at http://eu.idtdna.com/analser/Application and they were synthesized by Inqaba Biotechnical Industries (Pretoria, South Africa).

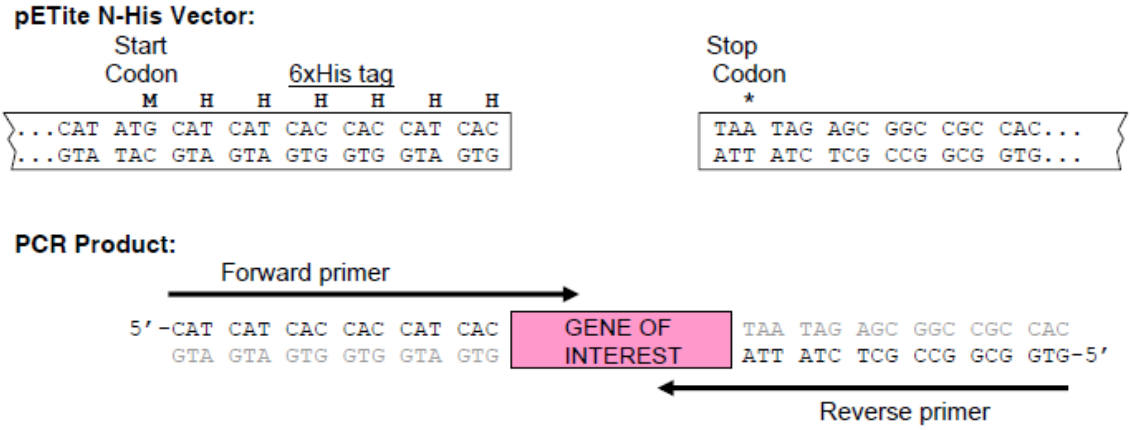


Figure 2.2: Insertion of a gene into pETite N-His vector (Taken from Expresso™ T7 cloning and Expression System manual).

2.4.2 MPB70 gene

The primers (Table 2.1) were designed to amplify the mature MPB70 protein from the gene sequence accessed from the GenBank (D38230), with flanking sequences to fuse the MPB70 to pETite N-His kanamycin vector. The genomic *M. bovis* field isolate DNA (described in section 2.1) was used as a template. One and quarter units of TaKaRa Ex Taq™ enzyme (TaKaRa, Japan) was used for the PCR comprising of 5 µl of 10X TaKaRa Ex Taq reaction buffer (TaKaRa, Japan), 4 µl of deoxynucleotide triphosphate mix (dNTPs, 2.5 mM each, TaKaRa, Japan), 1 µl of 10 µM each of forward and reverse primers, and template DNA (<500 ng). Deionized water was added to obtain a total volume of 50 µl. The PCR amplification was performed in a 5341 Epigradient S Thermal Cycler (Eppendorf, Germany) under the following reaction conditions: initial incubation at 94ºC for 1 min for one cycle, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing temperature at 55ºC for 30 s and extension at 72ºC for 1 min. A final extension was carried out at 72ºC for 5 min. For agarose gel electrophoresis, samples were prepared by adding 5 µl amplicon to 1 µl of 6 x bromo phenol blue loading dye and loaded on a 2% gel (Bioline, UK) containing 0.5-1 µg/ml ethidium bromide. Either the molecular ruler Hyperladder I or II (Bioline, UK) or both were included to estimate the size of the DNA fragments. The gel was run at 100V and 400 mA for 45 min. The DNA

fragments were visualized under UV light and then extracted with the QIAquick Gel Extraction Kit (QIAGEN, Germany) and quantified using ND-1000 UV/VIS NanoDrop spectrophotometer (NanoDrop Technologies, USA). Low yield DNA was precipitated with 1/10 volume of sodium acetate and 2.5 volume of cold absolute ethanol overnight at -20ºC. The DNA was pelleted by centrifugation (Eppendorf, 5415 R) at 13000 rpm for 15 min at 4ºC and dissolved in 500 µl of 70% ethanol to remove the salts. Following centrifugation at 13000 rpm for 10 min, the resulting pellet was left to air dry for 15-20 min, dissolved in a small volume of elution buffer (EB) buffer (10 mM Tris•Cl, pH 8.5) and stored at -20ºC.

Table 2.1: Primers used to amplify the *mpb70* gene, *mpb70* gene fragments and monster green fluorescent gene.

Region amplified	Primers' sequence	Expected PCR product size (bp)
mpb70 gene	Forward: 5'-CATCATCACCACCATCACGGCGATCTGGTGGGCCCGG-3' Reverse; 5'-GTGGCGGCCGCTCTATTACGCCGGAGGCATTAGC-3'	560-570
mgfp gene	Forward: 5'-GAAGGAGATATACATATGGGCGTGATCAAGCCCGAC-3' Reverse; 5'-GTGATGGTGGTGATGATGGCCGGCCTGGCGGG-3'	683
mpb70 gene fragment 1	Pair 1 (F1a) Forward: 5'-GAAGGAGATATACATATGGGCGATCTGGTGGGCCC-3' Reverse: 5'-GTCGGGCTTGATCACGCCGAGCTGGCCCGACAGTGC-3'	185
	Pair 2 (F1b) Forward: 5'-GCACTGTCGGGCCAGCTCGGCGTGATCAAGCCCGAC-3' Reverse: 5'–GTGATGGTGGTGATGATGGCCGGCCTGGCGGG–3').	683
mpb70 gene fragment 2	Pair 1 (F2a) Forward: 5'-GAAGGAGATATACATATGGCACTGTCGGGCCAGCTC-3' Reverse: 5'-GTCGGGCTTGATCACGCCGCCGGCCACTACGTGG-3'	214
	Pair 2 (F2b) Forward: 5'-ACCACGTAGTGGCCGGCGGCGTGATCAAGCCCGAC-3' Reverse: 5'–GTGATGGTGGTGATGATGGCCGGCCTGGCGGG–3'	683
mpb70 gene fragment 3	Pair 1 (F3a) Forward:5'-GAAGGAGATATACATATGAGCATCCTGACCTACCACG-3' Reverse: 5'-GTCGGGCTTGATCACGCCCGCCGGAGGCATTAGCAC-3'	239
	Pair 2 (F3b) Forward: 5'-GTGCTAATGCCTCCGGCGGGCGTGATCAAGCCCGAC-3 Reverse primer: 5'–GTGATGGTGGTGATGATGGCCGGCCTGGCGGG–3'	683

2.4.3 Monster green fluorescent protein (MGFP) gene

The monster green fluorescent protein (MGFP) was included as a control therefore primers (Table 2.1) were designed with flanking sequences to fuse the *mgfp* to pETite C-His kanamycin vector. The *mgfp* gene was amplified similarly to the *mpb70* but with a Tm of 57ºC.

2.4.4 MPB70 gene fragments

The online computer prediction programmes COBEpro (Sweredoski & Baldi, 2008), BCPRED (EL-Manzalawy *et al*., 2008), BepiPred (Larsen *et al*., 2006), ABCpred (Saha & Raghava, 2006) and AAPPred (Davydov & Tonevitskiĭ, 2009) were used to predict epitopes on the *mpb70* gene. The prediction of epitopes was based on parameters like hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains. The *mpb70* gene was fragmented into three regions to include the predicted epitopes and the primers for each fragment were designed as for the *mgfp* gene (Table 2.1). In addition, the primers included sequence overlaps to enable splicing by overlap extension (SOE) to the MGFP encoding gene prior to insertion into the pETite C-His vector (Figure 2.3).

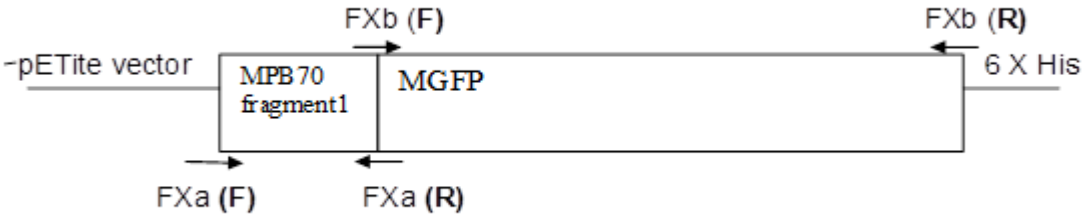


Figure 2.3: Illustration of position of the primers for SOE and cloning of fragments into the pETite vector. FX = Fragment 1, 2 or 3.

For each *mpb70* gene fragment, two sets of primers (Table 2.1, Figure 2.3) were used: The F1a forward/ F1a reverse (amplicon 1) and F1b forward/ F1b reverse (amplicon 2) primers were used in the fragment 1. For the fragment 2, the F2a forward/ F2a reverse (amplicon 1) and F2b forward/ F2b reverse (amplicon 2) primers were used while the F3a forward/ F3a reverse (amplicon 1) and F3b forward/ F3b reverse (amplicon 2) primers were used in the fragment 3. The PCR reaction mixture consisted of 5 µl 10X TaKaRa Ex Taq reaction buffer, 4 µl dNTPs mix (2.5 mM of each) and 1.25 Units of TaKaRa Ex Taq™ enzyme, 1 µl of 10 µM of each primer, 1 µl MPB70 plasmid DNA (for amplicons 1) and 1 µl MGFP plasmid DNA (for amplicons 2) and deionized water to obtain a total volume of 50 µl. The PCR amplification was performed the same as that of the *mgfp* gene.

Prior to splicing, the amplicons were precipitated with an equal volume of isopropanol, pelleted by centrifugation (Eppendorf, 5415 R) at 13000 rpm & 4ºC for 15 min and resuspended in 20 µl deionized water. The amplicons were then electrophoretically separated on a 2% blue agarose gel stained with crystal violet as described by Rand (1996) at a concentration of 10 mg/ml, then extracted and purified from the gel using QIAquick gel extraction kit (QIAGEN, Germany) and quantified using a NanoDrop spectrophotometer.

The splicing was performed to join two amplicons of each gene fragment in a reaction comprising of equal molar amounts of the DNAs, 5 µl 10X TaKaRa Ex Taq reaction buffer, 4 µl dNTPs mix (2.5 mM of each) and 2.5 U of TaKaRa Ex Taq™. Three units of *Pfu* DNA polymerase was added for proofreading and deionized water to make a final volume of 50 µl. The reaction conditions were; initial incubation at 94ºC for 1 min, 15 cycles of denaturation at 94ºC for 30 s, annealing temperature at 60ºC for 30 s and extension at 72ºC for 2 min. A final extension was carried out at 72ºC for 5 min. A portion (2 µl) of the resulting product was amplified further in a "pull-through" reaction by adding 2 µl of 20 pmol/reaction of 5' (FXa forward) and 3' (FXb reverse) primers with 10 µl of 10X reaction buffer, 8 µl dNTPs mix (2.5 mM of each), 2.5 U of TaKaRa Ex Taq™ and deionized water to make a final volume of 100 µl. The reaction conditions were the same as above but for 25 cycles. The DNA products were visualized by 2% agarose gel stained with ethidium bromide.

2.5 Cloning and sequencing

The *mpb70* and *mgfp* genes were cloned individually and the *mpb70* gene fragments were cloned as fusions with the *mgfp* encoding gene using a rapid enzyme-free Expresso™ T7 cloning and Expression System following the manufacturer's instructions. Between 25 and 100 ng of each of the resultant PCR product (*mpb70* gene, *mgfp* gene and MPB70 fragment MGFP fusion proteins) was mixed with 2 µl pETite vector and transformed into the HI-Control 10G competent *E. coli* cells by incubation on ice for 30 min. The cells were given a heat shock treatment at 42ºC for

45 s in pre chilled 15 ml disposable polypropylene culture tubes (17 x 100 mm) followed by incubation on ice again for 2 min. Recovery medium (960 µl) was added to the cells in the culture tube and incubated for 1 h at 37ºC with shaking at 250 rpm. Transformants were selected by plating 100 µl of the transformed cells on Luria-Bertani (LB) agar plates (Appendix 1) containing 30 µg/ml Kanamycin. The remaining transformed cells were concentrated by centrifugation (Eppendorf, 5415 R) at 12,000 x g for 2 min at 4ºC. Ninety percent of the supernatant was discarded and the remainder was used to resuspend the pellet which was also plated. All the plates were incubated overnight at 37ºC.

Colonies were picked at random, placed in 50 µl deionized water and mixed to screen for presence of the insert DNA using colony PCR as described in the Expresso^{TM} T7 cloning and Expression System instructions. A “master plate” was prepared by plating 0.5 µl of each suspension on LB agar plates containing 30 µg/ml of Kanamycin and incubated at 37ºC overnight. The remaining suspensions were placed in a heat block (100ºC) for 5 min followed by incubation on ice for 2 min and centrifugation (Eppendorf, 5415 R) at 12,000 x g & 4ºC for 2 min. The supernatant was collected and used as template for colony PCR. The reaction mixture used in the PCR comprised of 0.5 µl of 10 pmol/reaction pETite T7 forward and reverse primers, 12.5 µl 2X GO Taq PLUS GREEN master mix (Invitrogen) and 11.5 µl template DNA. The following reaction conditions were used: 94ºC for 1min, 30 cycles of (94ºC for 30s, 55ºC for 30s, and 72ºC for 1 min) and 72ºC for 5 min.

To prepare plasmid of PCR positive clones, colonies were picked from the master plate and grown in LB medium containing 30 µg/ml of Kanamycin and incubated at 37ºC overnight. This was followed by isolation of the recombinant plasmid DNA using the QIAprep® spin miniprep kit (QIAGEN, Germany). The pETite T7 forward and reverse primers (3.2 pmol each) were used to prepare the sequencing reactions and sent to the ARC-OVI Sequencing Laboratory (Pretoria, SA) to confirm the junction of the insert with the vector as well as to check if the correct coding

sequence was cloned. The sequences were analyzed and edited using the Bioedit programme, version 7.0.5.3 (10/28/05).

2.6 Protein expression

The *mpb70* and *mgfp* genes were expressed as polyhistidine protein fusions while the *mpb70* gene fragments as green fluorescent and polyhistidine fusion proteins. Small scale protein expression was performed to determine if the recombinant proteins were expressed and to evaluate their solubility. The plasmids containing the verified clones were transformed into the competent HI-Control BL21 (DE3) *E. coli* cells following the manufacturer's instructions. The pETite plasmid DNA (0.1-10 ng) was added to 40 µl of the competent HI-Control BL21 (DE3) *E. coli* cells in pre-chilled 15 ml disposable polypropylene culture tubes and incubated on ice for 30 min. The cells were given a heat shock treatment at 42ºC for 45 s followed by incubation on ice again for 2 min. Following inoculation in 960 µl of the recovery medium, the cells were incubated for 1 h at 37ºC with shaking at 250 rpm. The transformants were plated as before (Section 2.4).

A colony of BL21 (DE3) *E. coli* cells containing the transformed plasmid was inoculated into 5 ml LB broth containing 30 µg/ml kanamycin and incubated at 37ºC with shaking at 250 rpm. Glucose (5%) was added if the culture was grown overnight before the isopropyl β-D-thiogalactopyranoside (IPTG) induction to maintain repression of the *lacUV5* and *T7-lac* promoters. The following morning the culture was diluted 1:100 into the LB medium containing 30 µg/ml kanamycin. The cultures were grown and monitored with an interval of one hour until an optical density at 600 nm (OD_{600}) reached 0.5-1.0. An aliquot of uninduced cells (1 ml) was collected by pelleting in a microcentrifuge (Eppendorf, 5415 R) at 12,000 x g for 1 min at 4ºC. The cell pellet was resuspended in 50-65 µl of Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer (Appendix 1) and stored at -20ºC until required. To induce protein expression, 1 mM IPTG was added to the remaining log phase cultures of *E. coli* and propagated for a further 3 h. Prior to harvesting, an

aliquot (1 ml) of the induced cells was collected, centrifuged (Eppendorf, 5415 R) at 12,000 x g & 4ºC for 1 min to collect bacterial pellet and stored at -20ºC for small scale protein extraction. The remaining induced cells were harvested by centrifugation (KOBUTA 8100, Japan) at 3,100 x g for 5 min and the bacterial pellet stored at -20ºC for later use.

The BugBuster Master Mix method is similar to BugBuster® protein extraction reagent (Novagen®, Merck KGaA, Darmstadt, Germany) except that lysonase is already in the mix. Total protein was extracted by either using the BugBuster® protein extraction reagent or the BugBuster Master Mix. For the BugBuster® protein extraction reagent method, the cell pellet (Section 2.6, from 1 ml induced cells) was resuspended in 200 µl 1x BugBuster® protein extraction reagent followed by addition of 1 µl lysonase (10 µl per gram wet pellet, Novagen®, Merck KGaA, Darmstadt, Germany) and incubated at room temperature on a rotating mixer for 20 min. The extract was centrifuged at 16,000 x g for 20 min at 4ºC. The supernatant, containing the soluble protein fraction was collected. The pellet, containing insoluble protein was resuspended with 200 µl 1x BugBuster® protein extraction reagent. The protein fractions (soluble and insoluble) were mixed with 200 µl of SDS-PAGE loading buffer and boiled at 95-100ºC for 5 min. The proteins were separated by SDS-PAGE, stained with Coomassie brilliant blue or transferred to polyvinylidene difluoride (PVDF) membrane for immunoblotting to evaluate the protein expression and protein solubility. The remaining pellet of induced cells (≈ 5 ml culture) was extracted similarly in 1 ml 1x BugBuster® protein extraction reagent and 5 µl lysonase and stored at -20ºC for inclusion body purification.

For the large scale protein expression, two 50 ml cultures were used and the cell pellet resuspended in 2.5 ml 1x BugBuster® and 25 µl lysonase. The extract was centrifuged (Sorvall RC 5B plus, USA) with Sorval SS-34 rotor at 16,000 x g for 20 min at 4ºC and the pellet kept for the inclusion body purification.

2.7 Protein purification

From this point onwards both the BugBuster Master Mix and the BugBuster® protein extraction reagents will be referred to as BugBuster. The inclusion bodies (from 5 ml bacterial culture) were purified on a small scale using 1/5 culture volume of 1x BugBuster (1 ml) and on a large scale (from 50 ml bacterial culture) using 2.5 ml 1x BugBuster. Addition of 6 volumes of 1x BugBuster with a further vortexing for 1 min and centrifugation (Eppendorf, 5415 R) at 5,000 x g for 15 min at 4ºC followed. Three further washes with half the original culture volume of 1x BugBuster with vortexing and centrifugation steps as before were performed. A final wash step was followed by resuspending the pellet with the same amount of 1x BugBuster and the same vortexing step but centrifugation (Eppendorf, 5415 R) at 16,000 x g for 15 min at 4ºC. The supernatant was removed and the final pellet of the purified inclusion bodies were resuspended in 1ml of 1x binding buffer (or 5 ml for large scale) containing 6 M urea (denaturant) followed by incubation on ice for 1 h to completely solubilize the protein. The insoluble material was removed by centrifugation (Eppendorf, 5415 R) at 16,000 x g for 30 min at 4ºC and the supernatant was filtered through a 0.45-µm membrane for immobilized metal affinity chromatography (IMAC).

The polyhistidine-tagged recombinant MPB70, MGFP and MPB70 fragment (Frag) MGFP fusion proteins were purified on a small scale by IMAC under denaturing conditions using His•Bind resin (Novagen). The buffers were prepared as described in the manual. Novagen His•Bind chromatography columns were prepared by adding 1 ml sterile deionized water to the dry column. The column top was pushed gently using a gloved finger to make the column wet and to start the flow. The His•Bind Resin (400 µl) was transferred to the column and allowed to pack under gravity. Three washes were performed to charge and equilibrate the column (1 volume equivalent to settled volume of 200 µl). The first wash was done with 3 vol sterile deionized water, followed by 5 vol 1 x charge buffer and lastly with 3 vol 1 x binding buffer. The cleared lysate from the inclusion bodies was loaded onto the column and allowed to flow through. The column was washed with 10 vol 1x binding buffer followed by 6 vol 1x wash buffer. The bound protein was eluted with 6 vol 1 x elution

buffer. When elution was complete, the His•Bind resin was regenerated for re-use by washing the column with 3 vol 1x strip buffer. Samples were collected at different stages from the flow through, washes and eluates. Aliquots (30 µl) of the collected fractions were mixed with 30 µl of SDS-PAGE loading buffer, boiled at 95-100ºC for 5 min and analyzed with SDS-PAGE (stained with Coomassie brilliant blue stain) and western blotting to determine the purity and integrity of the purified protein samples. The remaining eluted protein was kept at -20ºC for dialysis. The large scale purification was performed as above, but with 4 ml of His•Bind Resin in the column (1 volume equivalent to settled volume of 2 ml).

2.8 Dialysis and protein concentration

The eluted proteins were dialyzed using Slide-A-Lyzer® Dialysis Cassettes (Thermo Scientific, USA) of molecular weight cut off (MWCO) of 7,000 to remove the urea and imidazole, and exchange the elution buffer with phosphate buffered saline (PBS). The eluted proteins (Section 2.7) were introduced by penetrating the gasket with a syringe needle. The membrane was placed in a 1 L beaker with PBS and left for a few hours or overnight stirred at 4ºC. The PBS was changed 3 times at regular intervals. The sample was withdrawn from the cassette into a syringe and transferred to a tube and the protein concentration was determined spectrophotometrically (Shimadzu Corporation, Japan) at 280 nm. The theoretical extinction coefficient was worked out for the sequences with *mpb70 gene* A_{280} of 1 = 0.319 mg / ml, *mgfp* gene A_{280} of 1 = 1.176 mg / ml, Frag 2-MGFP A_{280} of 1 = 1.013 mg / ml and Frag 3-MGFP A_{280} of 1 = 1.002 mg / ml (http://web.expasy.org/cgi-bin/protparam/protparam). The protein solutions were further concentrated by centrifugal Vivaspin filters (Vivascience, UK) of 10, 000 MWCO. A volume of up to 6 ml protein solution was loaded in to the concentrator (upper chamber) and centrifuged at 3000 rpm until the volume was reduced to between 100 and 500 µl. The protein concentration was determined again.

2.9 Peptide synthesis

Peptides were chosen based on predicted epitopes by various computer programmes (Section 2.4.4) and from previous studies that defined antigenic regions of MPB70 protein with sera from *M. bovis* infected bovine (Radford *et al*., 1990; Wiker *et al*., 1998; Lightbody *et al*., 2000). Fifteen peptides of 15 amino acid residues overlapping by 5 residues were synthesized by GenScript, USA. The peptides had a fluorescein (FITC-Ahx) attached on the N-terminus so that they could be used as tracers in FPA. Lyophilised peptides were resuspended at a final concentration of 2 mg/ml following manufacturer's instructions and stored at -20°C.

2.10 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Reducing SDS-PAGE was carried out with 4% stacking gel and 12.5% separating gel (Appendix 1) according to standard procedures (Laemmli, 1970) using a Scie plas mini gel apparatus (BioExpress, Kaysville, USA). Twenty microliters of protein samples (uninduced protein samples, soluble and insoluble protein fractions or purified protein fractions) and 10 µl of Precision Plus Protein Kaleidoscope Standard protein marker (BioRad) were loaded and the SDS-PAGE conducted at 150V and 400 mA for 1 h to 1.5 h. The separated rMPB70 / rMGFP / Frag-MGFP fusion proteins were either stained with Coomassie brilliant blue for 1h followed by destaining for 1 h with 0.04% acetic acid or stained with Acqua stain (Vacutec, South Africa) for 15 min without destaining.

2.11 Immunoblot

For immune-blot analysis, 20 µl of the collected samples were separated as described in section 2.10 and transferred electrophoretically onto PVDF membranes (Invitrogen) using a Scie plas Trans-blot semi transfer cell and conducted at 100V and 400 mA for 1 h. The *E. coli* cells without an expression construct were included as a negative control. The PVDF membrane was cut into strips and blocked either with 1% (w/v) bovine serum albumin (BSA) in 1X Tris Buffered Saline with 10%

Tween 20 (TBST, KPL-USA), pH 7.6 wash solution or 2% (w/v) fat-free milk powder (MP; Elite)/PBS at RT with shaking for 1 h. This was followed by incubation in the primary antibodies at room temperature with shaking for 1 h. The primary antibodies used included polyclonal rabbit anti-*M. bovis* (DakoCytomation, Denmark) diluted 1:500 with 1% (w/v) BSA block solution or sera diluted 1:50 in either 1% (w/v) BSA block solution or 2% (w/v) MP/PBS/0.05% Tween 20 (T). After washing the membrane strips three times with either 1X TBST or PBS/0.05T, they were immersed in secondary antibodies and incubated at room temperature with shaking for 1 h. The secondary antibodies used included polyclonal swine anti-rabbit IgG/HRP (DakoCytomation, Denmark) diluted 1:1000 with 1% (w/v) BSA block solution, polyclonal rabbit anti-bovine IgG/IgM/IgA/Peroxidase (Thermo Scientific, USA), polyclonal rabbit anti-bovine Igs/HRP (DakoCytomation, Denmark), sheep anti-bovine Igs/HRP (The Binding Site, UK) all diluted 1:1000 with either 1% (w/v) BSA block solution or 2% (w/v) MP/PBS/0.05T. HisDetector (KPL, USA) western blot was also performed to detect His-tagged protein fusions and was carried out as above, but eliminating primary and secondary antibodies steps replacing it by the addition of Nickel-HRP (KPL, USA) diluted with 1% (w/v) BSA block solution. The membrane strips were washed again three times with either 1X TBST or PBS/0.05T prior to addition of the substrate. TMB was used as a substrate for 5-15 min and the enzyme reaction was stopped by soaking the membrane in water.

2.12 Enzyme-linked immunosorbent assay (ELISA)

The purified rMPB70 protein and Frag-MGFP fusion proteins were tested against the respective chicken anti-rMPB70 IgY in an ELISA. The following concentrations: 10, 20, 40 & 80 µg/ml in PBS were prepared and 50 µl per well was used to coat 96-well Nunc maxisorp microtiter plates (Thermo Fisher Scientific, Denmark) overnight at 4ºC. The plates were blocked with 2% (w/v) MP/PBS for 1 h at 37ºC in a moist chamber and then washed with PBS/0.05T three times. The chicken anti-rMPB70 IgY was diluted (20, 40, 60 & 80 µg/ml in 2% (w/v) MP/PBS) and 50 µl was added in duplicate wells. Duplicate wells without anti-rMPB70 IgY were included as a control in which the chicken anti-rMPB70 IgY was replaced with 1 X PBS. The control was

used to check background reaction of secondary antibodies in the next step. The plates were incubated for 1 h 30 min to 2 h at 37ºC and washed as before. The secondary antibodies, goat anti–chicken IgG/HRP (Serotec, USA) diluted 1:500 with 2% (w/v) MP/PBS, were added. The plate was incubated for 1 h at 37ºC and washed as before. After a final wash, 50 µl of substrate solution (1 OPD tablet (Sigma) in 5 ml 0.1 M citrate buffer, pH 4.5 and 2.5 µl 30% hydrogen peroxide) was added to each well. The plate was incubated at RT for 45 min. The reaction was stopped by the addition of 50 µl 2 N sulphuric acid in each well. Optical density (OD) readings were measured at 492 nm (OD_{492}) with a microplate reader (Multiskan Ex, Thermo Electron Corporation). For the peptides, the plates were coated with 50 µl of 10 µg/ml anti-FITC (Millipore Corp/ CHEMICON International Incorporation, California) per well overnight at 4ºC. The blocking and washing steps were performed as above followed by addition of 50 µl of peptides per well diluted 1/1000 (1.4 µM). The plates were incubated for 1.5 h to 2 h at 37ºC and washed. Addition of the chicken anti-rMPB70 IgY followed by 1 h incubation at 37ºC and the remaining steps were followed as for rMPB70 protein and Frag-MGFP fusion protein samples described above.

When the rMPB70 protein and the Frag-MGFP fusion proteins were tested with immune buffalo sera, the wells were blocked and washed as for the chicken IgY described in the preceding paragraph. The buffalo sera were diluted 1:25 with 2% (w/v) MP/PBS and 50 µl was added in duplicate wells. Duplicate wells containing all reagents except serum were included as a control in which the serum was replaced with 1 X PBS. The plates were incubated for 1.5 h to 2 h at 37ºC and washed as above. Two anti–bovine-HRP preparations were compared: 1:500 polyclonal rabbit anti-bovine IgG/IgM/IgA/Peroxidase (Thermo Scientific, USA) and 1:300 monoclonal mouse anti–bovine IgG/HRP (Serotec, USA) and all were diluted in 2% (w/v) MP/PBS. After the addition of the conjugate, the remainders of steps were conducted as described for the chicken IgY.

2.12.1 MPB70 Frag 2-MGFP fusion protein ELISA

The Frag 2-MGFP protein fusion was solubilized in 2 M urea, diluted in PBS to 20 µg/ml and 50 µl was used to coat the ELISA plate overnight at 4 °C. The plates were blocked with 2% (w/v) MP/PBS for 1 h at 37ºC in a moist chamber and then washed with PBS/0.05T three times. The buffalo and cattle sera were diluted 1:25 with 2% (w/v) MP/PBS and 50 µl was added in duplicate wells. Positive (culture, IFN-γ and histopathology positive serum sample no 19) and negative (IFN-γ negative serum sample no 4 or 5) controls were included. The plates were incubated for 1.5 h to 2 h at 37ºC and washed as above. The secondary antibodies, monoclonal mouse anti–bovine IgG/HRP (Serotec, USA) diluted 1:300 with 2% (w/v) MP/PBS, were added. After the addition of the conjugate, the remainders of steps were conducted as described for the chicken IgY. To minimise variation between two plates, the optical density of the negative control was subtracted from the average duplicate OD_{492} of cattle and buffalo test sera. Receiver Operator Characteristic (ROC) curve analysis (http://analyse-it.com) was used to determine the cut-off points from the OD readings. Sensitivity and specificity were calculated by ROC analysis at the different cut-off points. The area under the ROC curve using a 95% confidence interval was calculated to assess the diagnostic performance of the ELISA. The cut-off chosen from the ROC curve was used in two by two tables to determine the test positive (TP), that is the number of diseased animals that tested positive by ELISA; false negative (FN), the diseased animals that tested negative by ELISA; test negative (TN), the disease free animals that tested negative by ELISA and false positive (FP), the disease free animals that tested positive by ELISA. The sensitivity, specificity, positive predictive value (PPV) and negative predictive values (NPV) were calculated.

	Dis +	Dis -
test +	TP	FP
test-	FN	TN
total	(TP + FN)	(FP+TN)

Sensitivity = TP / (TP + FN) Specificity = TN / (TN + FP)

PPV = TP / (TP + FP) PPV = TN / (TN + FN)

2.13 Fluorescence polarization assay antigen

The purified rMPB70 protein was labeled with the Pierce NHS-Fluorescein Antibody Labeling kit (Thermo Scientific, USA) according to the manufacturer's instructions. Briefly, 40 µl of Borate Buffer (0.67 M) was added to 0.5 ml of 2 mg/ml of MPB70 in PBS. The prepared MPB70 solution was added to the vial of NHS-Fluorescein reagent, mixed by pipetting up and down and vortexing until the reagent was dissolved. The vial was centrifuged briefly to collect the sample at the bottom of the tube. Following centrifugation, the reaction mixture was incubated at RT for 1 h in the dark. The Purification Resin was mixed thoroughly and 400 µl of the suspension was added to each of the two spin columns which had already been placed in microcentrifuge collection tubes. The spin columns were centrifuged at 1,000 x g for 30-45 s to remove the storage solution. The used collection tubes were discarded and columns placed in new collection tubes. The labeling reaction (250 µl x 2) was added to each spin column, vortexed briefly and centrifuged at 1,000 x g for 30-45 s to collect the purified proteins. The samples were pooled from both columns and stored in aliquots at -20ºC. The concentration of the labeled purified rMPB70 was determined spectrophotometrically at 280 nm and 495 nm. The rMPB70 protein concentration (M) and degree of labeling (Moles fluor per mole protein) were calculated as follows:

$$M = [(A_{280} - (A_{max} \times CF)] / \varepsilon_{protein} \times \text{dilution factor (DF)}$$

Moles fluor per mole protein = (A_{max} of the labeled MPB70) / (ε_{fluor} x M) x DF

$\varepsilon_{protein}$ = protein molar extinction coefficient

CF = Correction factor = $A_{280} \div A_{max}$

ε_{fluor} = 70,000 (NHS-Fluorescein molar extinction coefficient)

2.14 Fluorescence polarization assay

Polarization measurements were conducted using a PHERAstar microplate reader (BMG Labtech, Germany) with the fluorescence polarization module. The FPA reader measures fluorescence using polarized excitation filter of 485 nm and

emission filter of 520 nm. Two measurements are taken on every well, which is from fluorescence intensities parallel (A) and perpendicular (B) to the excitation plane. The number of flashes was set to 200. The FPA was performed as previously described by Jolley *et al*, 2007 with some modifications. A range of tracer concentrations from 0.66 µM to 3.3 µM was tested. Different lithium dodecyl sulfate (LiDS) concentrations were added to the sample buffer (0.4%, 0.2% or 0.1% LiDS to PBS, and PBS without LiDS) to prevent possible nonspecific interactions between the tracer and other serum components.

To set up the FPA reader, the gain of the parallel and perpendicular channel was calibrated so that the rMPB70-FITC and peptides-FITC had a polarization value of ~35 mP and 400 mP for the Frag-MGFP fusions. The gain adjustment needed to be reoptimized whenever a different tracer is used or the tracer concentration is changed. Two hundred microliters of the sample buffer was transferred into two wells of a 96–well flat bottomed black microtiter plate (Greiner). Ten microliters of fluorescein labeled tracer (rMPB70/ peptide) or Frag-MGFP fusions with a starting concentration of 1.32 µM were added in duplicate wells containing sample buffer and mixed by shaking on a microplate shaker for 5 min. The plate was incubated at RT for 10 min and one of the wells containing tracer was used to determine gain adjustment and height measurement.

In the assay, the control chicken antibodies were used in the place of serum samples because they contained a known amount of antibodies. The chicken anti-rMPB70 IgY antibodies D80 (positive control) and D0 (negative control) were diluted to 1 mg/ml in PBS with a starting concentration of 0.1% (w/v) LiDS. Two hundred microliters of these solutions were transferred into duplicate/ triplicate wells and also 200 µl of sample buffer was transferred into duplicate/ triplicate wells. The plate was incubated at RT for 30 min to equilibrate and a blank reading taken (parallel and perpendicular blank). Ten microliters (1.32 µM) of fluorescein labeled tracer (rMPB70/ peptide) or Frag-MGFP fusions was added to the wells containing either sample buffer, positive or negative chicken IgY controls. The plate was mixed and incubated at RT for 10 min. The plate was read again. The background correction

was done by subtracting blank parallel (A blank) and perpendicular (B blank) from the intensity readings in each well. Results were expressed as mP values calculated as follows:

$$mP = \frac{(\text{Channel A blank subtracted} - \text{Channel B blank subtracted})}{(\text{Channel A blank subtracted} + \text{Channel B blank subtracted})} \times 1000$$

A = Parallel emission intensity measurement

B = Perpendicular emission intensity measurement

A blank and B blank = Measurement for background

Channel A blank subtracted = (A - A blank)

Channel B blank subtracted = (B - B blank)

CHAPTER 3

3. RESULTS

3.1. Recombinant MPB70 protein

3.1.1 Protein expression and purification

The gene encoding MPB70 was amplified by PCR. In the first attempt the PCR products obtained were 2000 bp which was bigger than the expected 560-570 bp (results not shown). A possible explanation is that the primers designed included a signal sequence thereby causing mispriming because most signal sequences of BTB proteins are similar. A new forward primer spanning the mature MPB70 protein which excludes the signal sequence was designed and a product with the expected size was obtained (Figure 3.1 A).

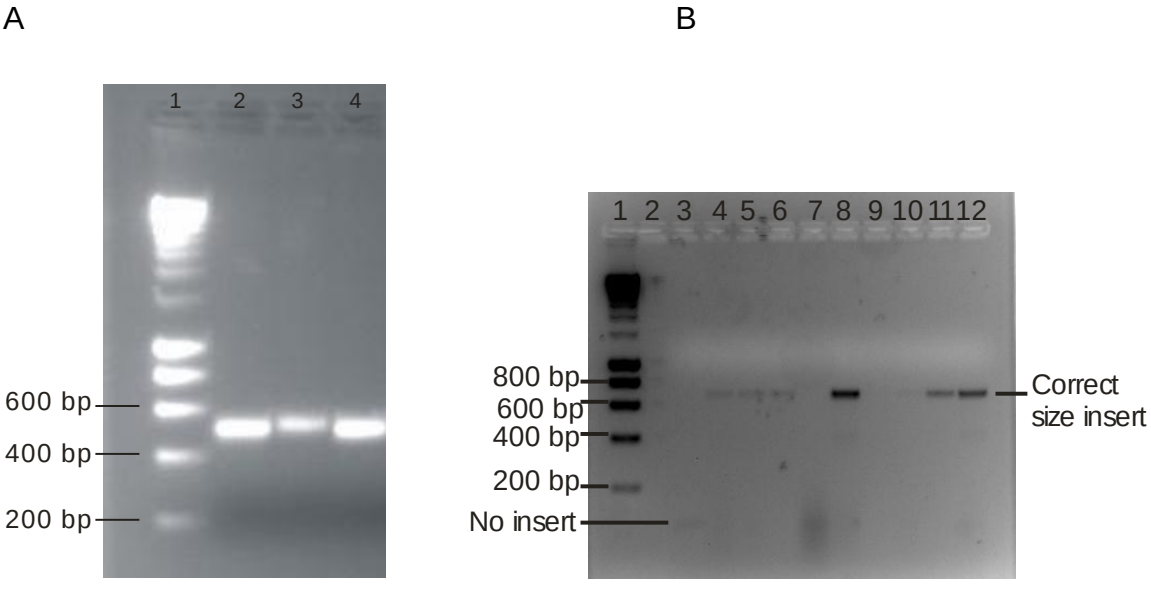


Figure 3.1: A: Agarose gel electrophoresis of the *mpb70* gene amplified with PCR; Lane 1, Hyperladder I marker (Bioline); Lanes 2 to 4, amplicons. B: Agarose gel electrophoresis analysis of the colony PCR products of the MPB70 transformants; Lane 1, Hyperladder I marker (Bioline); Lane 2, empty well; lanes 3 & 7, no insert (clones 1 & 5, 180 bp); lanes 4, 5, 6, 8, 9, 10, 11 & 12, correct size insert (clones 2, 3, 4, 6, 7, 8, 9 & 10, 740-750bp).

The amplified *mpb70* gene was mixed with the pETite vector and transformed into the HI-Control 10G cells (Section 2.5). Transformants were subjected to colony PCR to verify the recombinant clones. Agarose gel electrophoresis of PCR products showed that insert sizes of 740-750 bp were as expected (180 bp vector + 560-570 bp DNA insert, Figure 3.1 B). The insert DNA was sequenced and aligned with the *mpb7*0 gene sequence from GenBank (D38230). Clone 4 had the correct coding sequence which was in the correct reading frame with the pETite vector (Figure 1 & 2 of Appendix 3).

The verified plasmid clone 4 was transformed into *E. coli* BL21 (DE3) cells and protein expression was induced with IPTG. As a negative control, *E. coli* cells without an expression construct were also induced with IPTG. From the small scale protein expression, SDS-PAGE showed that the rMPB70 was expressed as a 22 kDa protein at high levels and was present in the insoluble fraction (Figure 3.2 A). HisDetector western blot analysis (Figure 3.2 B) confirmed that the rMPB70 contained a histidine tag by a band of the same molecular mass, even though there was a lot of background reaction. HisDetector showed that the 22 kDa band reacted strongly with Ni-HRP which was absent in the negative control. The blot showed the presence of a small amount of the soluble rMPB70. The polyclonal rabbit anti-*M. bovis* was also used to detect the rMPB70 (Figure 3.3). Although there was a significant degree of cross-reactivity of the anti *M. bovis* antibodies with *E.coli* proteins, these antibodies target the expressed rMPB70 (lane 2) but no band of similar size in the negative control lane.

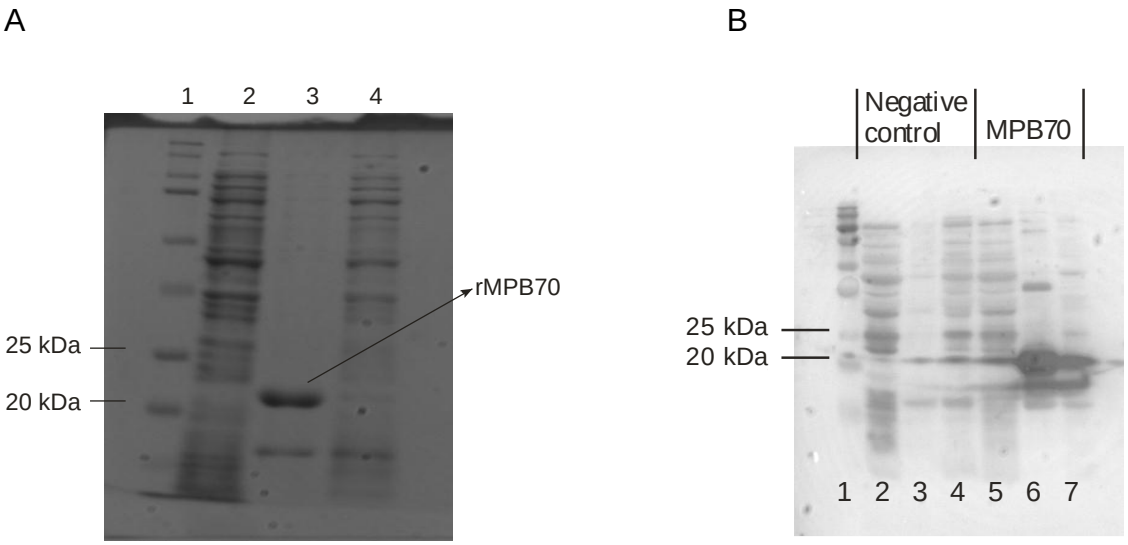


Figure 3.2: A: Coomassie Brilliant Blue stained SDS-PAGE of the expressed rMPB70 protein in *E. coli*; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lane 2, *E. coli* before IPTG induction; lane 3, insoluble protein after IPTG induction (pellet); lane 4, soluble protein after IPTG induction (supernatant). B: HisDetector immunoblot analysis of the expressed rMPB70 protein in *E. coli* using Ni-HRP; Lane 1; SDS-PAGE Broad range marker (BIO-RAD); Lanes 2 & 5, *E. coli* before IPTG induction; Lanes 3 & 6, insoluble protein after IPTG induction (pellet); Lanes 4 & 7, soluble protein after IPTG induction (supernatant). Negative control is the *E. coli* cells without an expression construct.

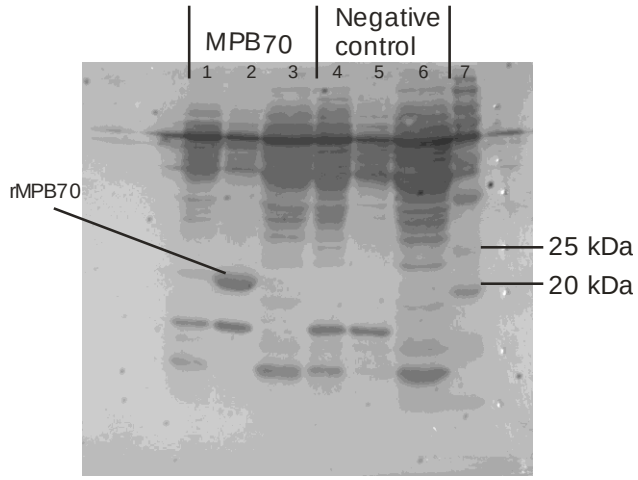


Figure 3.3: Immunoblot analysis of the rMPB70 protein expressed in *E. coli* using polyclonal rabbit anti-*M. bovis*; Lane 7; SDS-PAGE Broad range marker (BIO-RAD); Lanes 3 & 6, *E. coli* before IPTG induction; Lanes 2 & 5, insoluble protein after IPTG induction (pellet); Lanes 1 & 4, soluble protein after IPTG induction (supernatant). Negative control is the *E. coli* cells without mycobacterial DNA insert.

As the rMPB70 was expressed as an insoluble protein, it was purified from the inclusion bodies followed by IMAC using urea as a denaturant. The rMPB70 preparation was highly concentrated in the inclusion body preparation (Figure 3.4, lane 3) and contained some additional proteins, but after purification the protein was >90% pure (Figure 3.4, lane 7). The sacrifice was some loss of protein during the purification (Figure 3.4, lane 4).The eluted rMPB70 contained urea and imidazole which were removed by dialysis with PBS.

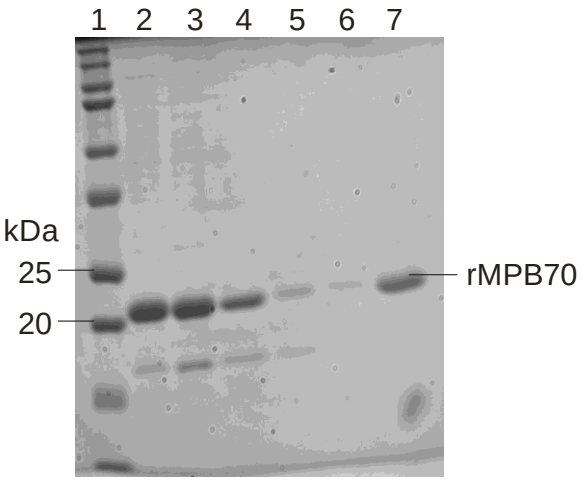


Figure 3.4: Coomassie Brilliant Blue stained SDS-PAGE of the rMPB70 samples after each purification step; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lane 2, crude insoluble protein extract; lane 3, purified inclusion bodies; lane 4, column flow through; lane 5, wash 1; lane 6, wash 2; lane 7, eluted purified rMPB70.

3.1.2 Testing with immune sera

Before the purified rMPB70 protein was labeled with fluorescein and tested in the FPA, it was characterized in an immunoblot and ELISA using the immune sera from BTB infected buffaloes. The rabbit antibodies raised against bovine Igs used in the assay reacted with the rMPB70 in the immunoblot (Figure 3.5). Therefore, positive sera could not be distinguished from negative sera because of these reactions. Using BSA in the blocking buffer instead of milk powder, cross absorbing the reacting antibodies with the rMPB70 and including goat serum in the blocking buffer had no effect on the reactions. Four different conjugates; that is, three polyclonal anti-bovine IgG and one monoclonal anti–bovine IgG/HRP antibodies were tested.

Only the monoclonal antibody showed no cross reaction (results not shown). Initially polyclonal antibodies were tried since buffalo sera were also to be tested and it was thought that the monoclonal antibody would be too specific to react with the buffalo Igs. In the ELISA using the monoclonal antibody (Figure 3.6), two of the serum samples from BTB infected buffaloes reacted with the rMPB70 (samples 2 & 3) while serum sample 1 had optical densities (OD) of 0.122 and 0.114 both which were higher than the ODs of the serum samples from BTB negative buffaloes (samples 4 & 5). The serum samples from BTB negative buffaloes yielded signals < 0.1 OD while the serum samples from BTB infected buffaloes yielded signals > 0.1 OD. Urea in the ELISA was used to solubilize the insoluble proteins and expose epitopes when coating the ELISA plates. A signal increase of 17% was observed when urea was used in coating the ELISA plates. The ELISA showed that the rMPB70 was recognised by antibodies in the sera from tuberculous positive animals; therefore it was ready to be labeled with fluorescein and tested in the FPA.

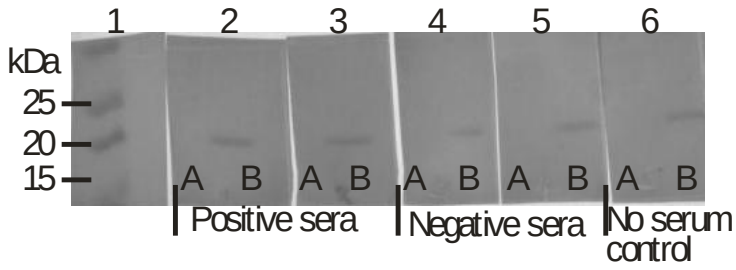


Figure 3.5: Immunoblot analysis of the rMPB70 with anti-bovine-HRP; Strip 1, SDS-PAGE Broad range marker (BIO-RAD); strip 2, positive sera diluted 1:50; strip 3, positive sera diluted 1:100; strip 4, negative sera diluted 1:50; strip 5, negative sera diluted 1:100; strip 6, no serum sample. A: *E coli* control and B: rMPB70.

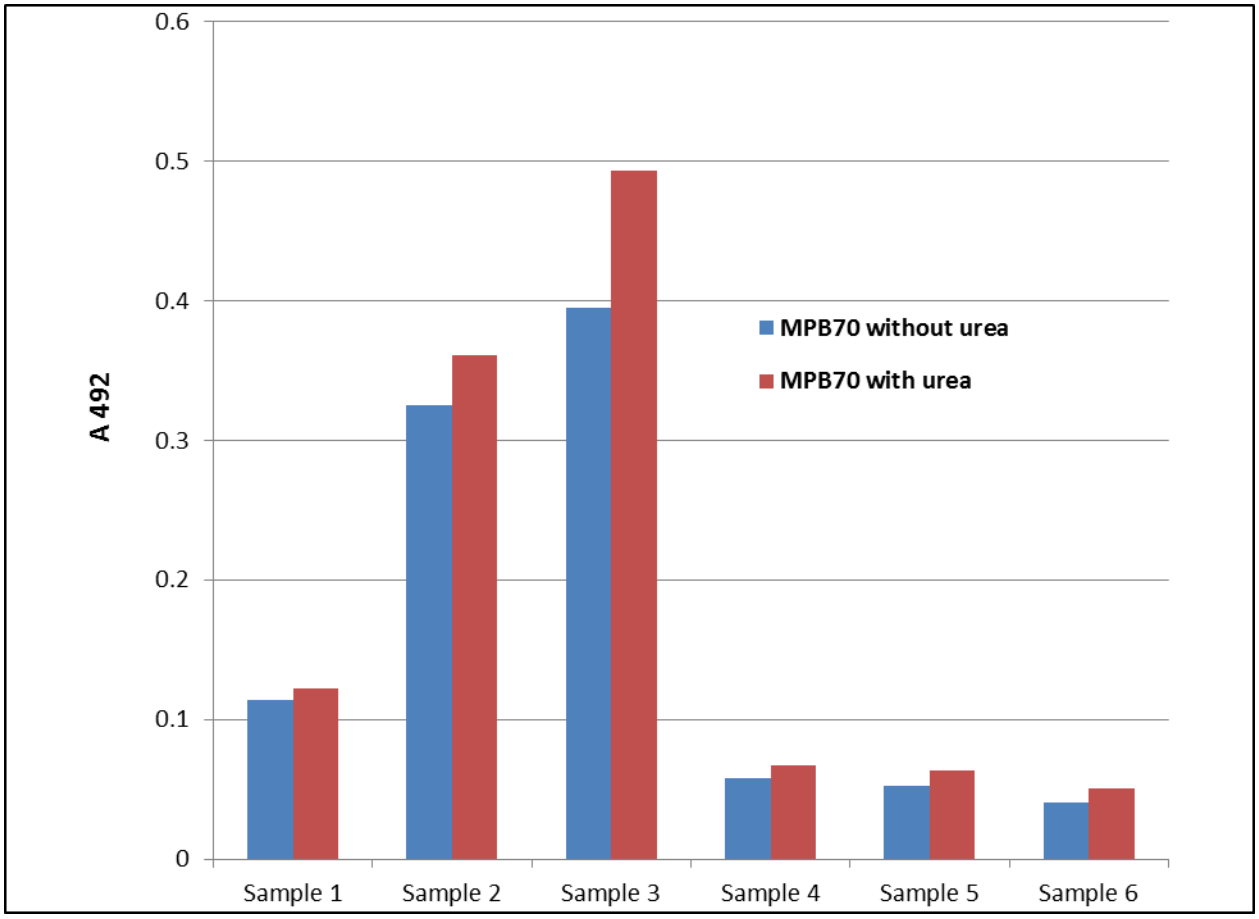


Figure 3.6: Results of the ELISA showing sera from BTB infected and uninfected buffaloes, reacting with the rMPB70. The BTB status of the buffaloes from which the serum samples were collected is shown: Sample 1, culture and Stat-Pak positive; sample 2, culture and histopathology positive; sample 3, IFN-γ and histopathology positive; samples 4 & 5, IFN-γ negative; sample 6, control containing all reagents except the serum. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm.

Control antibodies (provided by Dr Jeanni Fehrsen, ARC-OVI) were made by injecting chickens with the rMPB70. Two chickens were each injected with 100 µg of purified rMPB70 in 250 µl PBS mixed with 250 µl adjuvant (ISA206). Three boosts were given to the chickens on intervals of 21 days. Only one chicken laid eggs from which IgY were isolated. The chicken anti-rMPB70 IgY were isolated pre-immunization at day 0, at days 30, 80 & 100 after inoculation with the rMPB70 and were tested for reaction with the rMPB70 in the ELISA. From day 80, the antibodies yielded a signal of absorbance of ≤ 0.35 (Figure 3.7), which was considered low. However, the signal was greatly improved by increasing the concentration of the chicken antibodies from 20 to 80 µg/ml (Figure 3.8). Three concentrations (40, 60 &

80 µg/ml) of chicken anti-rMPB70 IgY antibodies from D0 & D80 were tested for reaction with the rMPB70 in the ELISA. The chicken anti-rMPB70 IgY antibodies isolated at D0 gave an OD value of 0.6 in all the different concentrations while the chicken anti-rMPB70 IgY antibodies isolated at D80 had a highest OD value at a concentration of 60 µg/ml (OD 1.39), followed by 80 µg/ml (OD 01.1) and concentration 40 µg/ml being the lowest (OD 0.78). Therefore the antibodies could be used in the FPA as controls.

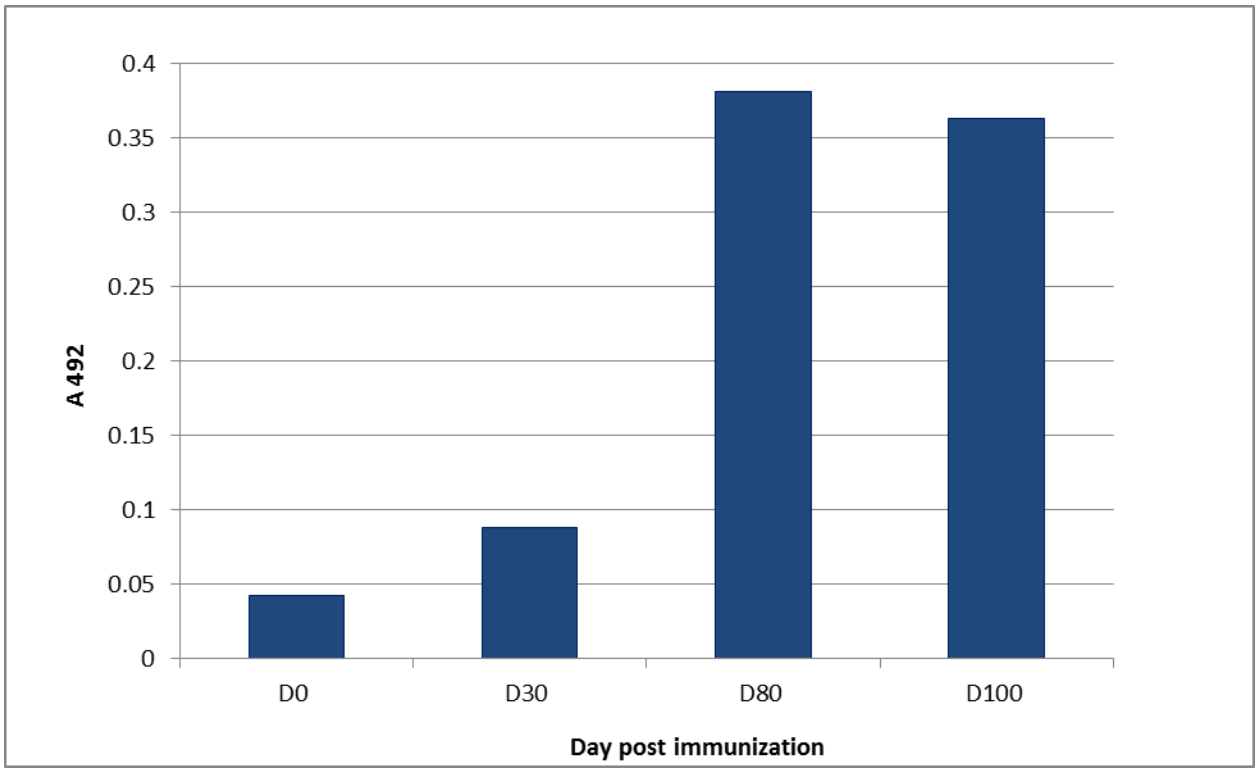


Figure 3.7: Results of the ELISA showing chicken anti-rMPB70 IgY antibodies (IgY = 20 µg/ml) reacting with the rMPB70. D0, anti-rMPB70 IgY antibodies isolated from the eggs before immunization with the rMPB70; D30, D80 & D100, anti-rMPB70 IgY antibodies isolated from the eggs on days 30, 80 & 100 respectively after immunization with the rMPB70. The plotted OD-values are the average of duplicate readings of chicken anti-rMPB70 IgY antibodies at absorbance 492 nm.

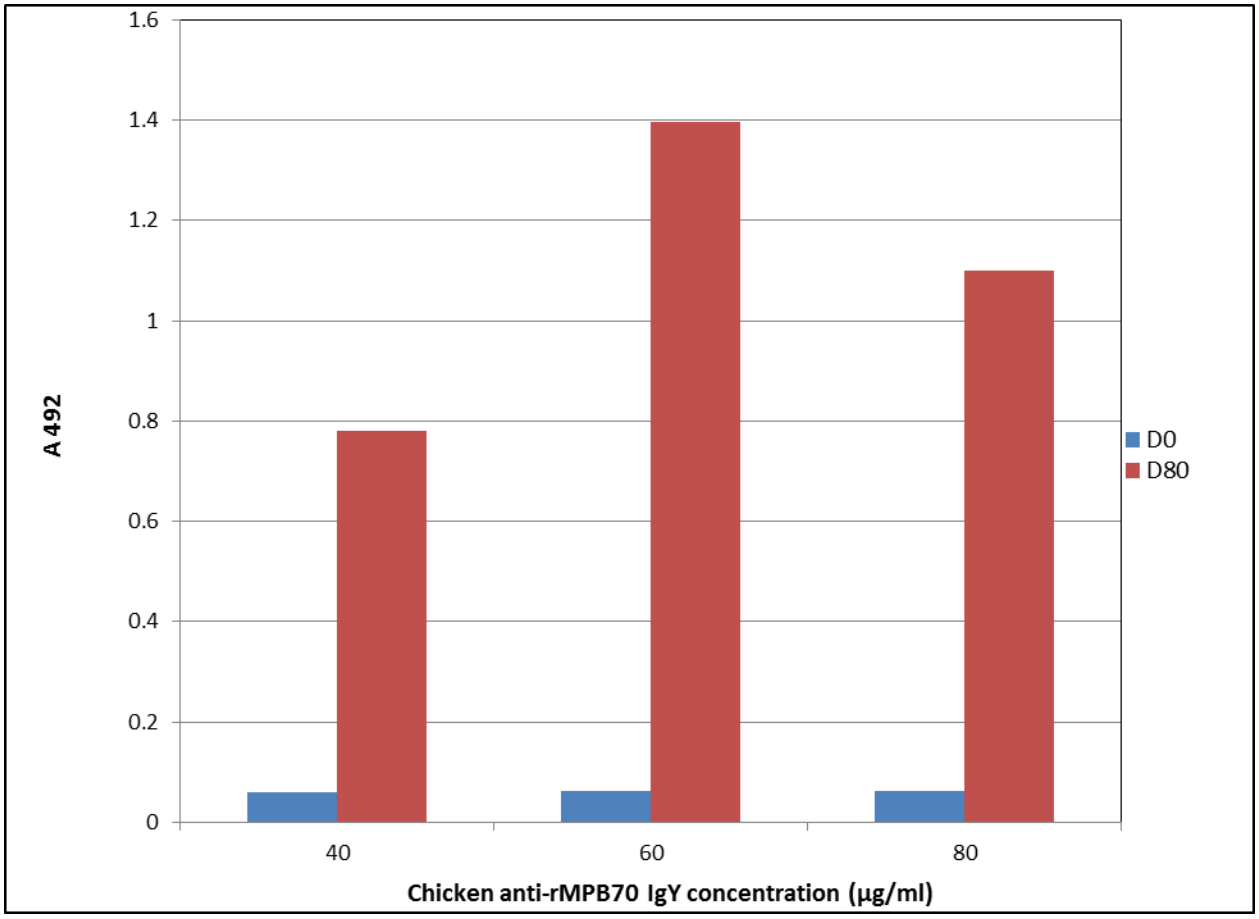


Figure 3.8: Results of the ELISA showing chicken anti-rMPB70 IgY antibodies at different concentrations (40, 60 & 80 µg/ml) reacting with the rMPB70. D0, anti-rMPB70 IgY antibodies isolated from the eggs before immunization with the rMPB70; D80, anti-rMPB70 IgY antibodies isolated from the eggs on days 80 after immunization with the rMPB70. The plotted OD-values are the average of duplicate readings of chicken anti-rMPB70 IgY antibodies at absorbance 492 nm.

3.2 MPB70 fragments

In order to identify antigenic regions on the MPB70, one approach was to fragment the protein and test each fragment for immuno-reactivity. For the FPA, the tracer must be able to fluoresce. Therefore we investigated whether fusing the gene fragments to an auto-fluorescent protein could yield molecules suitable to be used in the FPA.

3.2.1 Monster green fluorescent protein

3.2.1.1 Protein expression and purification

Green fluorescent protein (GFP) is widely used as a fusion tag (Chalfie *et al*., 1994; Ren *et al*., 1996; Muki *et al*., 2012). In this study, MGFP which is encoded by an improved synthetic version of the *gfp* was used. The MGFP was chosen as a fusion partner for the MPB70 fragments because it is auto-fluorescent and hence there is no need for labeling. To be able to use the MGFP as a control, it was cloned individually. Primers were designed to amplify the *mgfp* gene. Agarose gel electrophoresis showed a PCR product of the expected size of 683 bp (Figure 3.9 A).

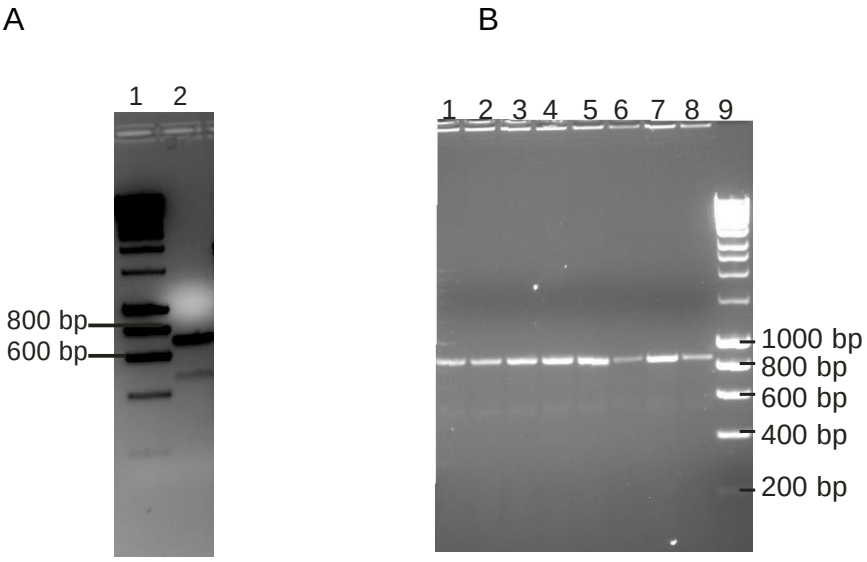


Figure 3.9: Agarose gel electrophoresis of the *mgfp* gene amplified with PCR; Lane 1, Hyperladder I marker (Bioline); Lane 2: Amplicon. B: Agarose gel electrophoresis analysis of the colony PCR products of the MGFP transformants; Lanes 1-8, positive clones 1-8 (863 bp); Lane 9, Hyperladder I marker (Bioline).

The cloning, sequencing and protein expression were performed as for the *mpb70* gene. The colony PCR verified that recombinant clones contained the correct size insert of 863 bp (Figure 3.9 B). The sequencing analysis showed that the MGFP clone 4 was in the correct reading frame with the pETite vector and had the right coding sequences (Figures 3 & 4 of Appendix 3). The *mgfp* gene was expressed in

E. coli as an insoluble 26 kDa protein as expected (Figure 3.10).The HisDetector immunoblot analysis confirmed that the protein contained a histidine tag (data not shown). The *E. coli* culture expressing MGFP was placed on a slide with a cover slip and viewed under a fluorescence microscope at 40 X magnification (Figure 3.11 A) and fluorescence was seen from the motile *E. coli*. The *E. coli* was further cultured on Luria broth (LB) agar (Appendix 1) plates containing IPTG and the following day viewed under UV transilluminator. Greenish colonies of the *E. coli* were seen fluorescing (Figure 3.11 B), therefore it could be used as a fluorescent control. The rMGFP was purified from the inclusion bodies using urea as a denaturant. The SDS-PAGE analysis (Figure 3.12) showed that the inclusion body preparation was highly concentrated. Further purification with immobilized metal affinity chromatography yielded a pure protein. The protein could therefore be used as a control in the FPA.

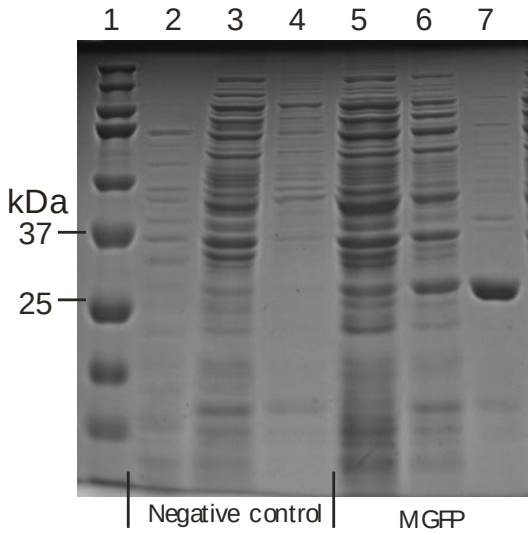
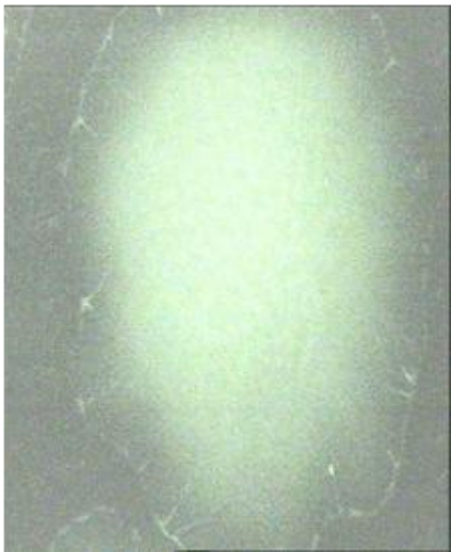


Figure 3.10: Coomassie Brilliant Blue stained SDS-PAGE of the expressed rMGFP protein in *E. coli*; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lanes 2 & 5, *E. coli* before IPTG induction; lanes 3 & 6, soluble protein after IPTG induction (supernatant); lanes 4 & 7, insoluble protein after IPTG induction (pellet). Negative control is the *E. coli* cells without an expression construct.

A



B

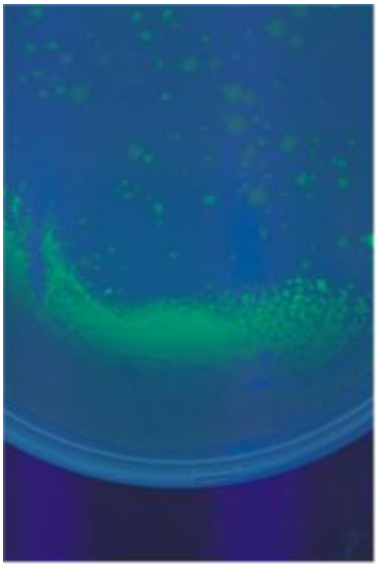


Figure 3.11: A: A culture *E. coli* expressing MGFP viewed under fluorescence microscope. B: Colonies of *E. coli* expressing MGFP viewed under UV transilluminator.

A

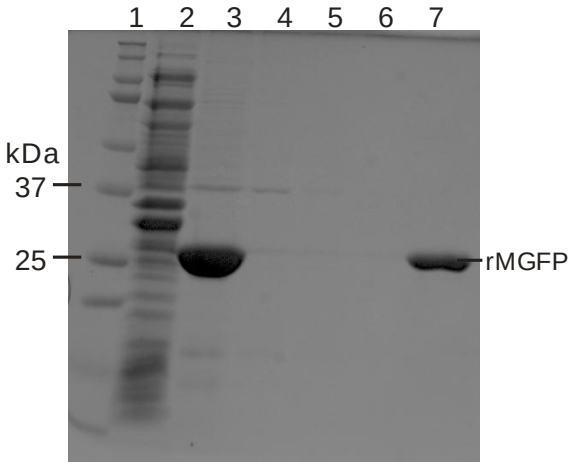


Figure 3.12: Coomassie Brilliant Blue stained SDS-PAGE of the rMGFP samples after each purification step; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lane 2, crude insoluble protein extract; lane 3, purified inclusion bodies; lane 4, column flow through; lane 5, wash 1; lane 6, wash 2; lane 7, eluted purified rMGFP.

3.2.2 MPB70 fragment MGFP fusion proteins

Epitopes on MPB70 were predicted using COBEpro (Sweredoski and Baldi, 2008), BCPRED (EL-Manzalawy *et al*., 2008), BepiPred (Larsen *et al*., 2006), ABCpred (Saha and Raghava, 2006) and AAPPred (Davydov and Tonevitskiĭ, 2009) epitope prediction programmes. All these prediction programmes were useful and were based on parameters like hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains. The prediction programmes automatically rated each epitope from 0 to 1. Epitopes chosen with the highest scores were considered in the analysis.

The *mpb70* gene was fragmented into three regions to include the predicted epitopes (Figure 3.13). The fragments were chosen based on where most of the epitopes on the *mpb70* gene were predicted to be located. In addition, the gene was divided in such a way that the fragments overlap to avoid disruption of the epitopes (Figure 3.13, Table 3.1 and Figure 11 of Appendix 4).

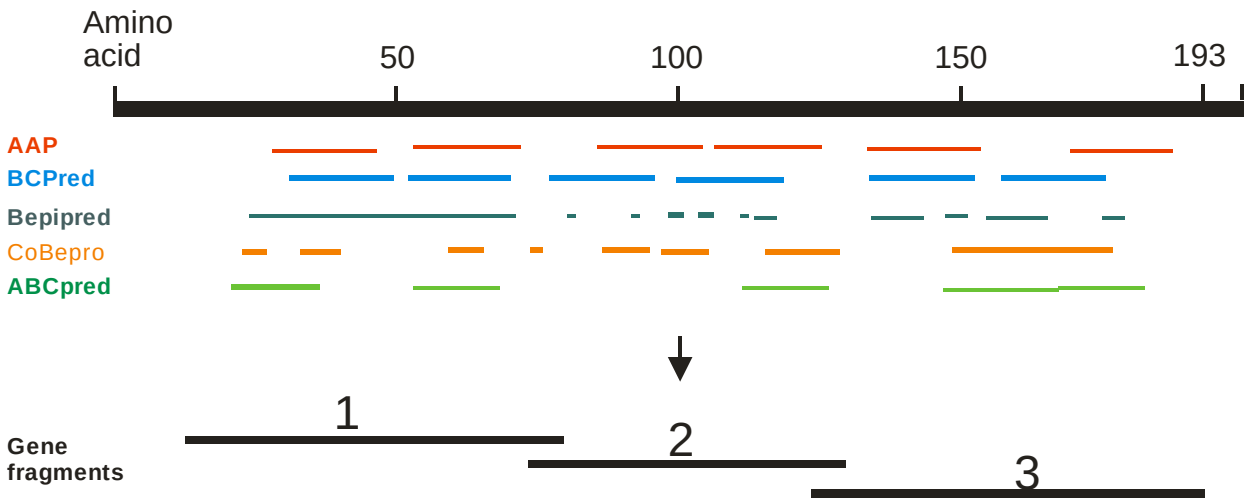


Figure 3.13: Diagrammatic illustration of the position of the predicted epitopes on the MPB70. Each line represents the epitopes predicted by the epitope prediction programmes and the gene fragments. Each epitope was rated and the epitopes chosen had the highest probability to be epitopes.

Table 3.1: Amino acid sequence of MPB70 fragments.

Name	Amino-acid sequence	Site	Length
Fragment 1	GDLVGPGCAEYAAANPTGPASVQGMSQDPVAVAASNNPELTTLTAALSGQL	31-81	51
Fragment 2	ALSGQLNPQVNLVDTLNSGQYTVFAPTNAAFSKLPASTIDELKTNSSLLTSILTYHVVAG	76-135	60
Fragment 3	SILTYHVVAGQTSPANVVGTRQTLQGASVTVTGQGNSLKVGNADVVCGGVSTANATVYMIDSVLMPPA	126-193	68

The *mpb70* gene fragments and *mgfp* gene were fused by the spliced overlap extension (SOE) as described in section 2.4.4. The gene fragments were amplified and produced the products of the expected size with the exception of fragment 3 amplicon 1 (F3a) which was not amplified and had no product (Figure 3.14 A). A new forward primer was designed further upstream of the *mpb70* gene with a larger overlap with fragment 2 and resulted in the correct gene fragment being amplified (Figure 3.14 B). The products were joined to produce MPB70 fragment MGFP fusion genes before cloning (Figure 3.15).

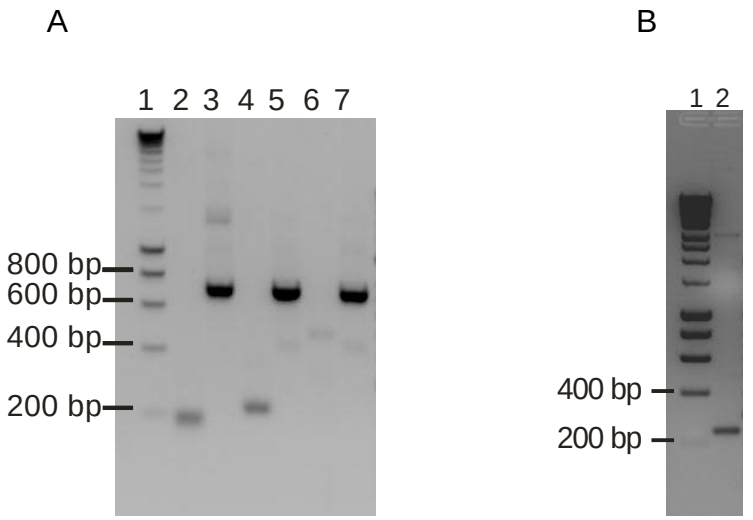


Figure 3.14: Agarose gel electrophoresis of the mpb70 gene fragments amplified with PCR. A: Lane 1, Hyperladder I marker (Bioline); Lane 2, F1a amplicon; lane 3, F1b amplicon; lane 4, F2a amplicon; lane 5, F2b amplicon; lane 6, F3a amplicon with old primers; lane 7, F3b amplicons. B: Lane 1, Hyperladder I marker (Bioline); lane 2, F3a amplicon with new primers.

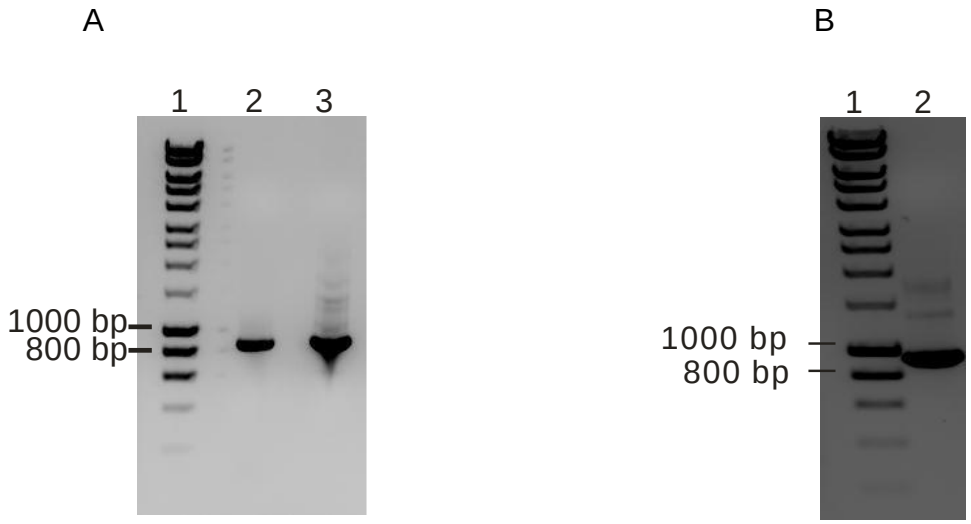


Figure 3.15: Agarose gel electrophoresis analysis of SOE-PCR of the MPB70 fragments. A: Lane 1, Hyperladder I marker (Bioline); lane 2, Fragment 1; lane 3, Fragment 2. B: Lane 1, Hyperladder I marker; lane 2, Fragment 3.

The SOE-PCR products were cloned, sequenced and the proteins expressed. The colony PCR verified that the recombinant clones contained the correct size insert (Figure 3.16). Sequencing the DNA inserts revealed that the Frag-MGFP fusions coding sequences were correct, in frame with the vector reading frame and could be translated into the correct amino acid sequences (clone 1 for Frag 1, clone 3 for Frag 2 and clone 2 for Frag 3, Figures 5-10 of Appendix 3).

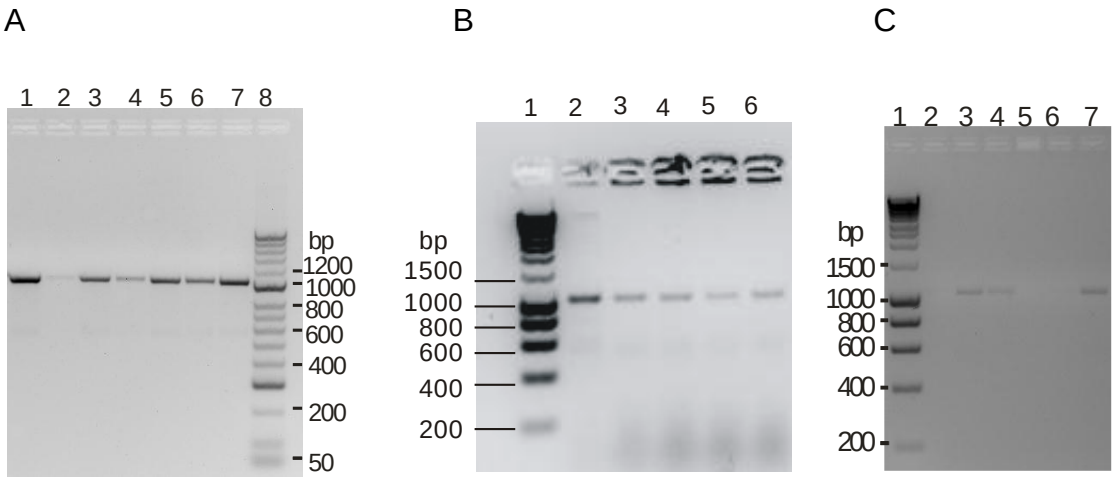


Figure 3.16: Agarose gel electrophoresis analysis of the colony PCR products of the fragment-MGFP fusion transformants. A: Frag 1-MGFP. Lanes 1-7, correct size insert (clones 1-7, 1048 bp); Lane 8, Hyperladder II marker (Bioline); B: Frag 2-MGFP. Lane 1, Hyperladder I marker (Bioline); Lanes 2-6, correct size insert (clones 1-5, 1077 bp); C: Frag 3-MGFP. Lane 1, Hyperladder I marker (Bioline); Lane 2, empty; Lanes 3-7, correct size insert (clones 1-5, 1102 bp).

The MPB70 fragments 2 and 3 were also found in the insoluble fraction and were expressed as 33 kDa and 34 kDa MGFP fusion proteins respectively (Figure 3.17 A). The yield of Frag 3-MGFP fusion protein was higher than that of Frag 2-MGFP fusion protein. Both the Frag-MGFP fusion proteins contained polyhistidine tags to aid in purification (Figure 3.17 B). There was no protein expressed from the Frag 1-MGFP fusion gene construct (Figures 3.17 A & B), even though the DNA sequence showed that the correct coding sequence was cloned in the correct reading frame with the vector sequence (Figure 5 & 6 of Appendix 3). The cause of the improper protein expression is unclear. Growing at 18°C and 30°C instead of 37°C and inducing the protein expression with 1 mM, 0.5 mM and 0.25 mM IPTG did not result in the Frag 1-MGFP fusion protein being expressed.

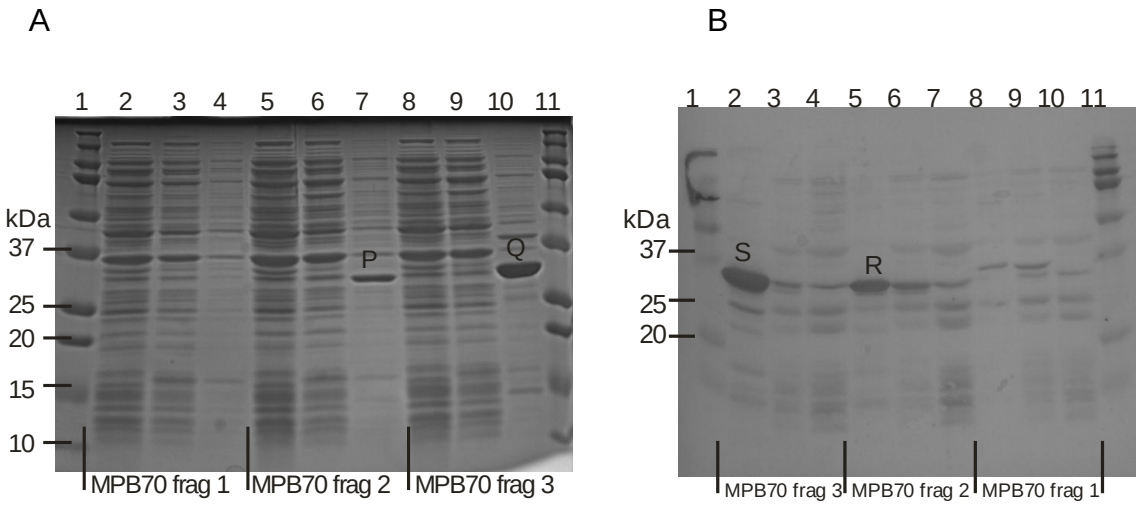


Figure 3.17: A: Coomassie Brilliant Blue stained SDS-PAGE of the expressed Frag-MGFP fusion proteins in *E. coli.* Lanes 1 & 11, SDS-PAGE Broad range marker (BIO-RAD); lanes 2, 5 & 8, *E. coli* before IPTG induction; lanes 3, 6 & 9, soluble protein after IPTG induction (supernatant); lanes 4, 7 & 10, insoluble protein after IPTG induction (pellet); P & Q indicate Frag 2^- and Frag 3^- MGFP fusion proteins. B: Immunoblot analysis of the expressed Frag-MGFP fusion proteins in *E. coli* using Ni-HRP; Lanes 1 & 11, SDS-PAGE Broad range marker (BIO-RAD); Lanes 2, 5 & 8, insoluble protein after IPTG induction (pellet); Lanes 3, 6 & 9, soluble protein after IPTG induction (supernatant); Lanes 4, 7 & 10, *E. coli* before IPTG induction; R & S indicate Frag 2^- and Frag 3^- his-tag fusion proteins.

As the Frag-MGFP fusion proteins were insoluble; the Frag 2-MGFP fusion protein was purified from the initial inclusion body preparation using BugBuster protein extraction reagent (Figure 3.18 A). The Frag 3-MGFP fusion protein needed further purification using His•Bind purification resin under denaturing conditions (Figure 3.18 B). The purified Frag-MGFP fusion proteins were placed in a Petri dish and viewed under UV light of a photo documentation system and fluorescence was seen (Figure 3.19), therefore the proteins were ready for the FPA.

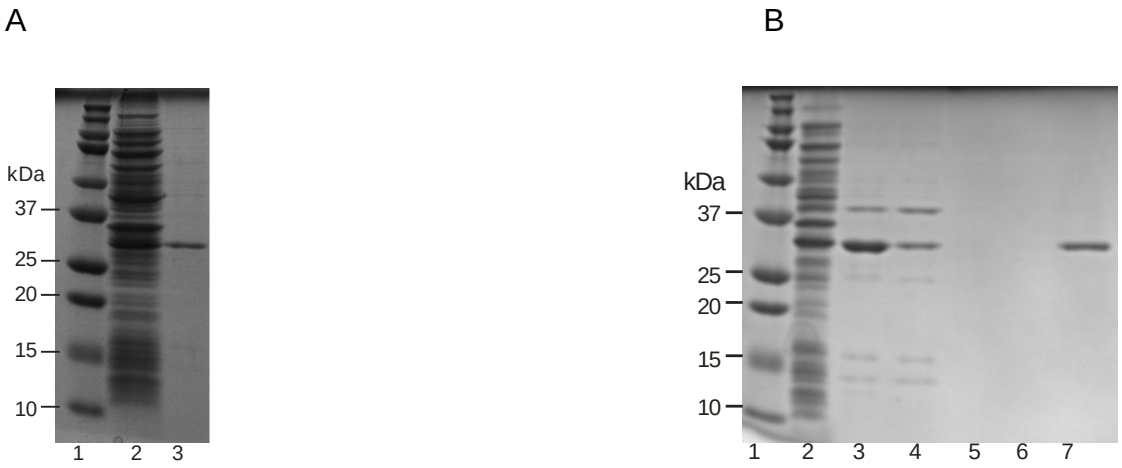


Figure 3.18: Coomassie Brilliant Blue stained SDS-PAGE of the Frag-MGFP fusion protein samples after each purification step; A: Frag 2-MGFP fusion protein; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lane 2, crude insoluble protein extract; lane 3, purified inclusion bodies. B: Frag 3-MGFP fusion protein; Lane 1, SDS-PAGE Broad range marker (BIO-RAD); lane 2, crude insoluble protein extract; lane 3, purified inclusion bodies; lane 4, column flow through; lane 5, wash 1; lane 6, wash 2; lane 7, eluted purified protein.

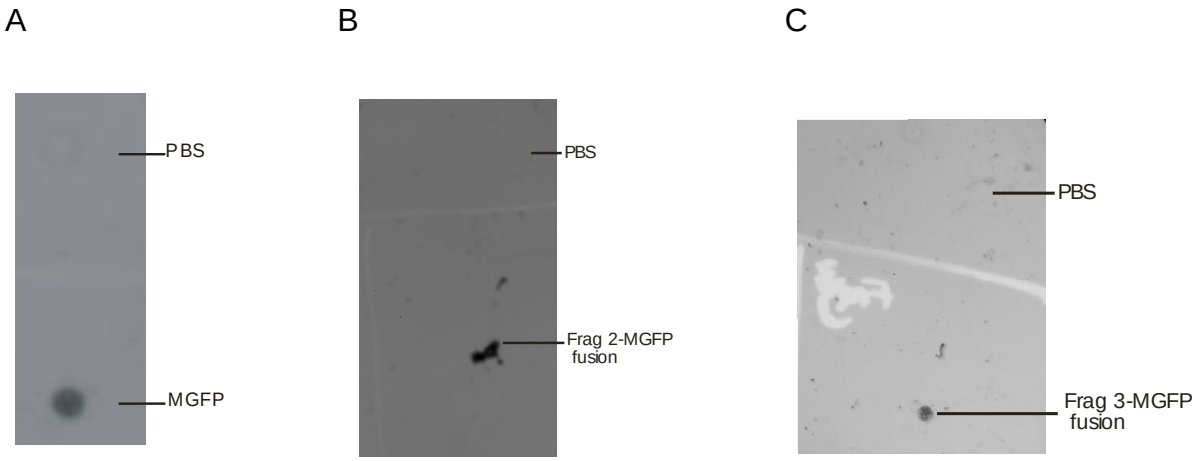


Figure 3.19: Photos of fluorescent proteins exposed to UV light. A: MGFP; B: Frag 2-MGFP fusion protein; C: Frag 3-MGFP fusion protein. A droplet of each of the proteins was placed in a Petri dish and placed in a photo documentation system and a photo taken. PBS, negative control.

3.2.3 Testing with immune sera

Like the rMPB70, the purified Frag-MGFP fusion proteins were characterized in the ELISA before tested in the FPA. The chicken anti-rMPB70 IgY antibodies reacted with the Frag 2-MGFP fusion protein but not the Frag 3-MGFP fusion protein (Figure 3.20). The positive control chicken anti-rMPB70 IgY antibodies (D 80) gave a signal

of 0.6 OD with Frag 2-MGFP fusion protein while Frag 3-MGFP fusion protein gave a signal similar to the negative control chicken antibodies (D0).

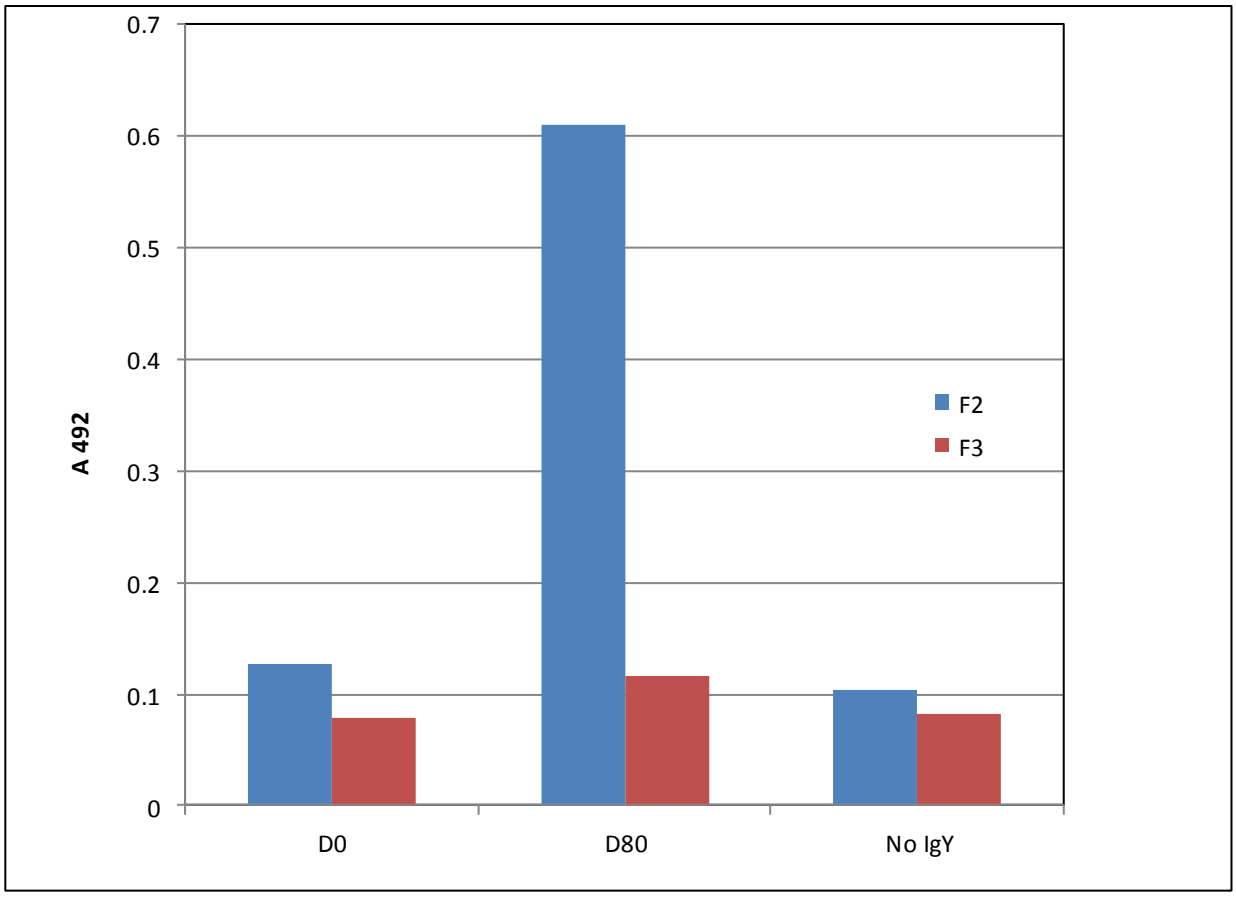


Figure 3.20: Results of the ELISA showing chicken anti-rMPB70 IgY antibodies (IgY = 60 µg/ml) reacting with the Frag-MGFP fusion proteins. D0, anti-rMPB70 IgY antibodies isolated from the eggs before immunization with the rMPB70; D80, anti-rMPB70 IgY antibodies isolated from the eggs on day 80 after immunization with the rMPB70; No IgY, a control containing all reagents except chicken anti-rMPB70 IgY antibodies; F2, Frag 2-MGFP fusion protein; F3, Frag 3-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of chicken anti-rMPB70 IgY antibodies at absorbance 492 nm. The plate was coated with 80 µg/ml Frag 2 & 3-MGFP fusion proteins.

When the Frag-MGFP fusion proteins were tested with the immune sera from BTB infected buffaloes (Figure 3.21), the serum sample no 3 reacted strongly with Frag 2-MGFP fusion protein while the Frag 3-MGFP fusion protein gave a signal of 0.33 OD, but the serum samples no 1 and 2 had OD-values lower than serum sample no 3. The history of the sera indicated that samples no 1 & 2 were from infected

buffaloes. The BTB status of the buffaloes from which serum samples no 1 and 2 were collected was culture and Stat-Pak (antibody) positive and culture and histopathology positive, respectively. The Frag 2-MGFP fusion protein showed potential to be tested as a target in the ELISA; therefore it was checked with panels of sera from naturally infected and uninfected cattle and buffaloes (Appendix 2).

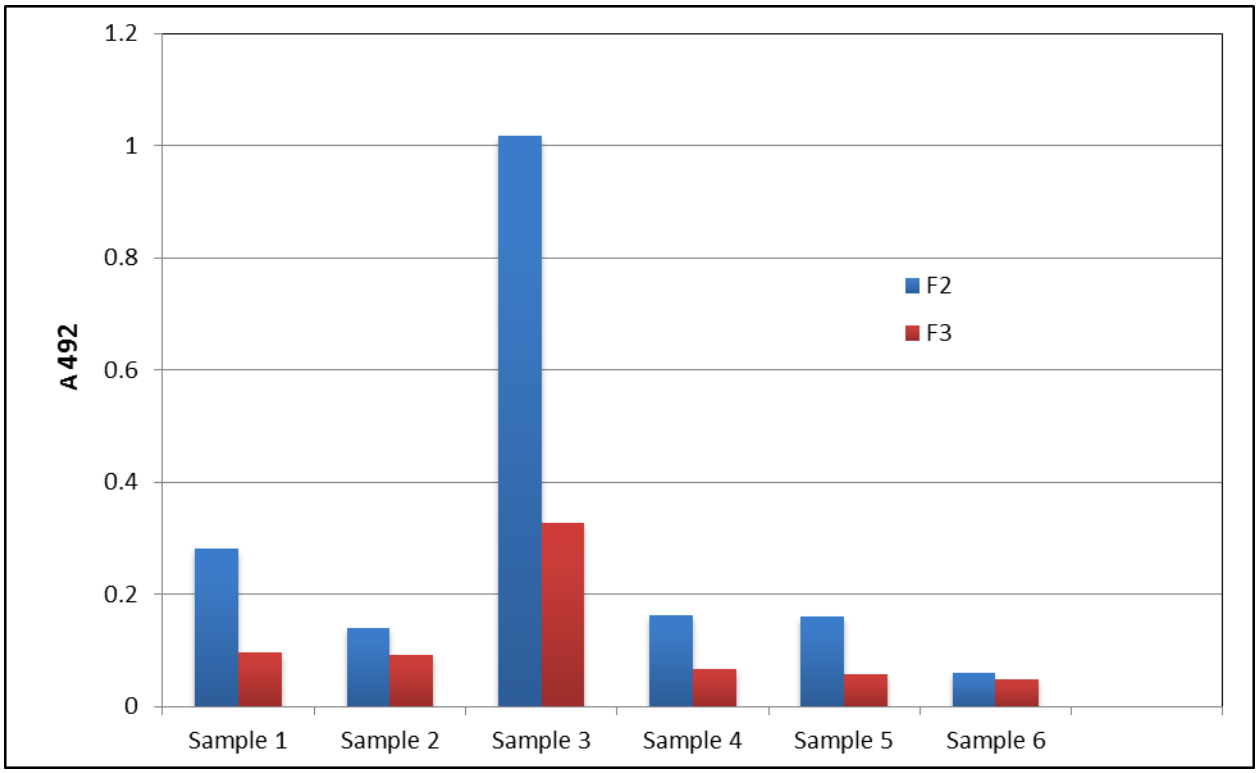


Figure 3.21: Results of the ELISA showing sera from BTB infected and uninfected buffaloes reacting with the Frag-MGFP fusion proteinss. The BTB status of the buffaloes from which the serum samples were collected is shown: Sample 1, culture and Stat-Pak positive; sample 2, culture and histopathology positive; sample 3, IFN-γ and histopathology positive; samples 4 & 5, IFN-γ negative; sample 6, control containing all reagents except serum; F2, Frag 2-MGFP fusion protein; F3, Frag 3-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm.

3.2.4 Testing panels of characterized sera using Frag 2-MGFP fusion protein

Ninety-two serum samples from cattle and 101 from buffaloes were tested in the ELISA using the Frag 2-MGFP fusion protein. The samples were split into BTB infected, non-tuberculous Mycobacterium exposed and negative groups each. The infected cattle and buffaloes were identified by tuberculin skin test and

histopathological (presence of tuberculous lesions) analysis, while uninfected and non-tuberculous Mycobacterium exposed cattle and buffaloes from which the sera were collected were identified using the Bovigam test. Fifty sera were from BTB free cattle from different commercial dairy farms with negative BTB history, 32 were from tuberculin skin test positive cattle and ten were from non-tuberculous Mycobacterium exposed cattle. The buffalo serum samples were comprised of 48 Bovigam negative sera, 18 sera from buffaloes with tuberculous lesions and 35 sera from non-tuberculous mycobacterium exposed buffaloes. It should be noted that the results of the tuberculin skin test or Bovigam test measure a cell mediated immune response which indicates BTB infection while lesions indicate the disease which can either be in an early or advanced stage and the antibodies increase with progressing disease. These criteria do not guarantee the prescence of antibodies in the sera.

For the results analysis, the OD_{492} of the negative control was subtracted from the OD_{492} of samples in order to minimise variation between plates (Figures 3.22 to 3.27). Fifty percent (16/32) of the sera from the tuberculin skin test positive cattle gave a signal between 0.2 OD and 0.5 OD while 21.9% (7/32) had low OD ≤ 0.2 and 28.1% (9/32) strongly reacted with the Frag 2-MGFP fusion protein with OD's ≥ 0.5 (Figure 3.22 & Tables 3.2 & 3.3). The sera from the tuberculin skin test positive cattle with low ELISA signals affected the sensitivity of the ELISA.

Twelve percent (6/50) of the sera from BTB free cattle reacted strongly with Frag 2-MGFP fusion protein with OD ≥ 0.5 (Figure 3.23 & Tables 3.2 & 3.3) and this in turn will affect the specificity of the ELISA. Forty-two percent (21/50) of sera from the BTB free cattle had OD between 0.2 and 0.5 and 46% (23/50) had OD ≤ 0.2.

Twenty-two percent (4/18) of sera from buffaloes with tuberculous lesions had OD ≥ 0.2 and 78% (14/18) sera had OD < 0.2 (Figure 3.25 & Tables 3.2 & 3.3) while 10.4% (5/48) of Bovigam negative buffalo sera had OD ≥ 0.2 (Figure 3.28 & Tables 3.2 & 3.3) and 89.6% (43/48) had OD<0.2.

The ELISA was applied to the sera from the cattle exposed to non-tuberculous Mycobacteria and 80% (8/10) of the sera reacted strongly with the Frag 2-MGFP fusion protein with OD ≥ 0.5 when compared to the BTB free cattle sera (Figure 3.24 & Tables 3.2 & 3.3). It is noteworthy that serum 3-11 which gave the strongest reaction in ELISA was negative in the Bovigam test. As it was seen with sera from cattle exposed to non-tuberculous mycobacteria, there was a high percentage (40%, 14/35) of sera from Mycobacterium exposed buffaloes that reacted with the Frag 2-MGFP fusion protein with OD ≥ 0.2 when compared to the Bovigam negative sera (Figure 3.27 & Tables 3.2 & 3.3).

For the cattle sera, the ELISA had a sensitivity of 28% and specificity of 88% at a cut-off point OD_{492} = 0.5. The PPV and NPV predictive values were 60% and 66% respectively (Table 3.3). For surveillance of BTB in large populations with unknown BTB status, a test with high specificity is needed. The cut-off point was adjusted in order to maximise specificity. By increasing the cut-off point from OD_{492} of 0.5 to 0.65, increased the specificity to 92% at the cost of sensitivity (22%) (Table 3.3). An area under the ROC curve of 0.6967 was observed (Figure 3.28 A) and this shows that the ELISA results were not by chance, but the value was not high enough to indicate real value for use as a serological test to detect antibody responses to BTB as a stand-alone assay.

For the buffalo sera, the sensitivity, specificity, PPV and NPV of the ELISA were 22%, 90%, 44% and 75% respectively at a cut-off point of OD_{492} = 0.2, (Table 3.3). The aim for the test was the same as for the cattle sera. The specificity was maximised to 94% by raising the cut-off point from OD_{492} of 0.2 to 0.3. However, the sensitivity decreased to 11% (Table 3.3). The area under the ROC curve was 0.7161 (Figure 3.28 B) and similarly as it was with the cattle sera, the ELISA was of no real value to measure antibody responses to BTB as a stand-alone assay.

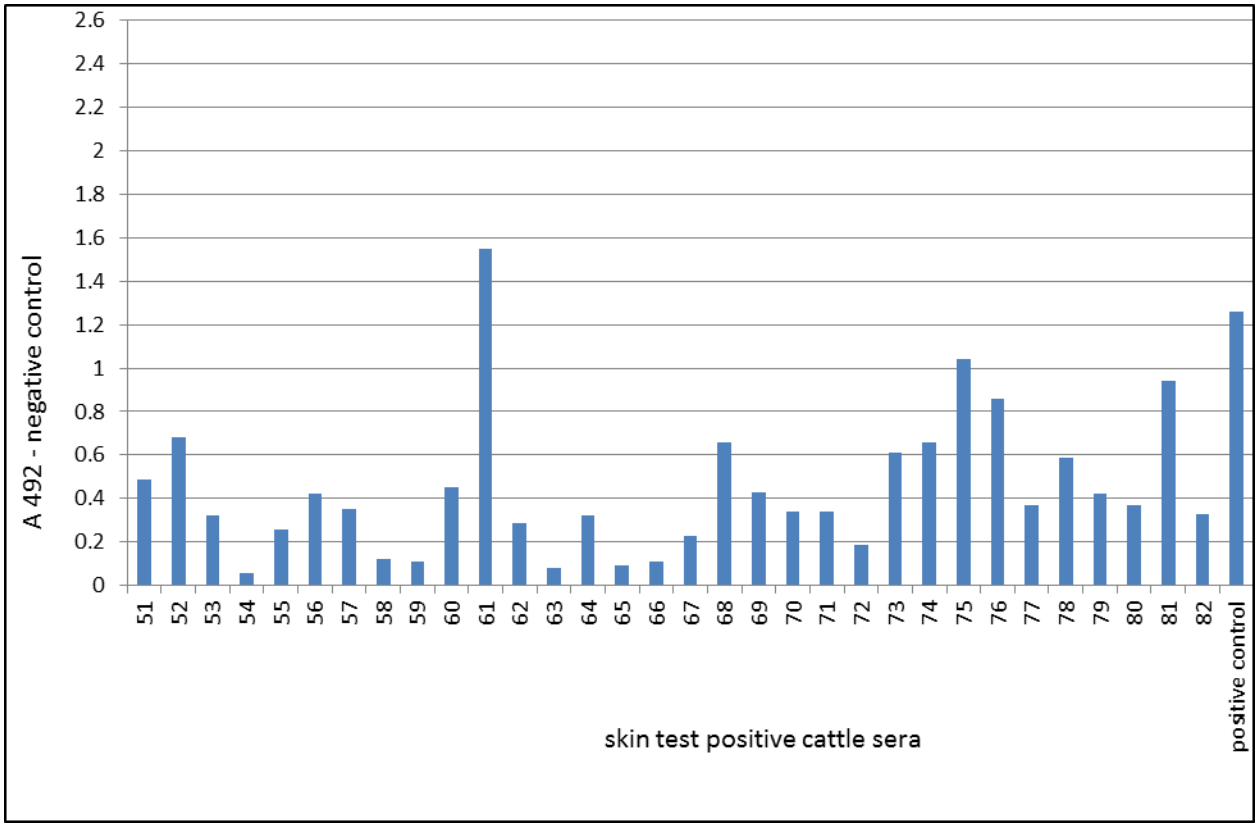


Figure 3.22: Results of the ELISA showing sera from the tuberculin skin test positive cattle reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

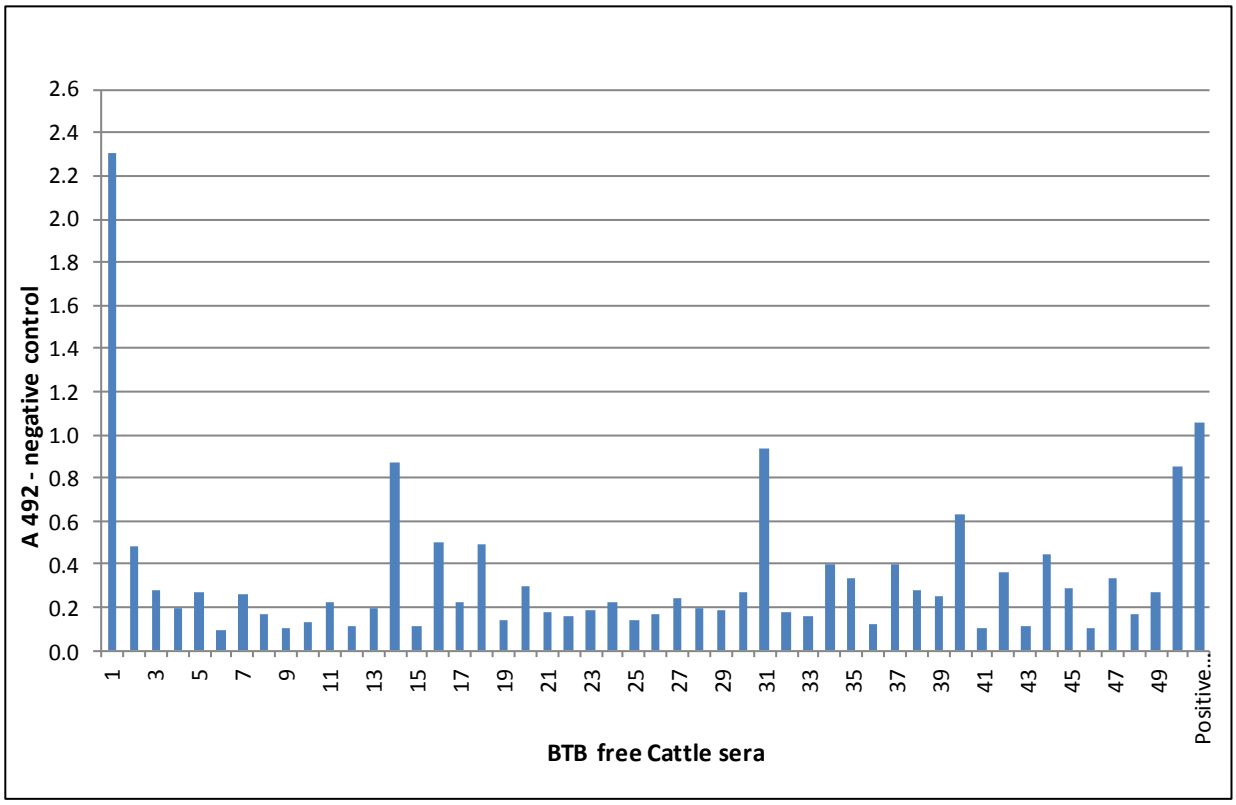


Figure 3.23: Results of the ELISA showing sera from the BTB free cattle reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

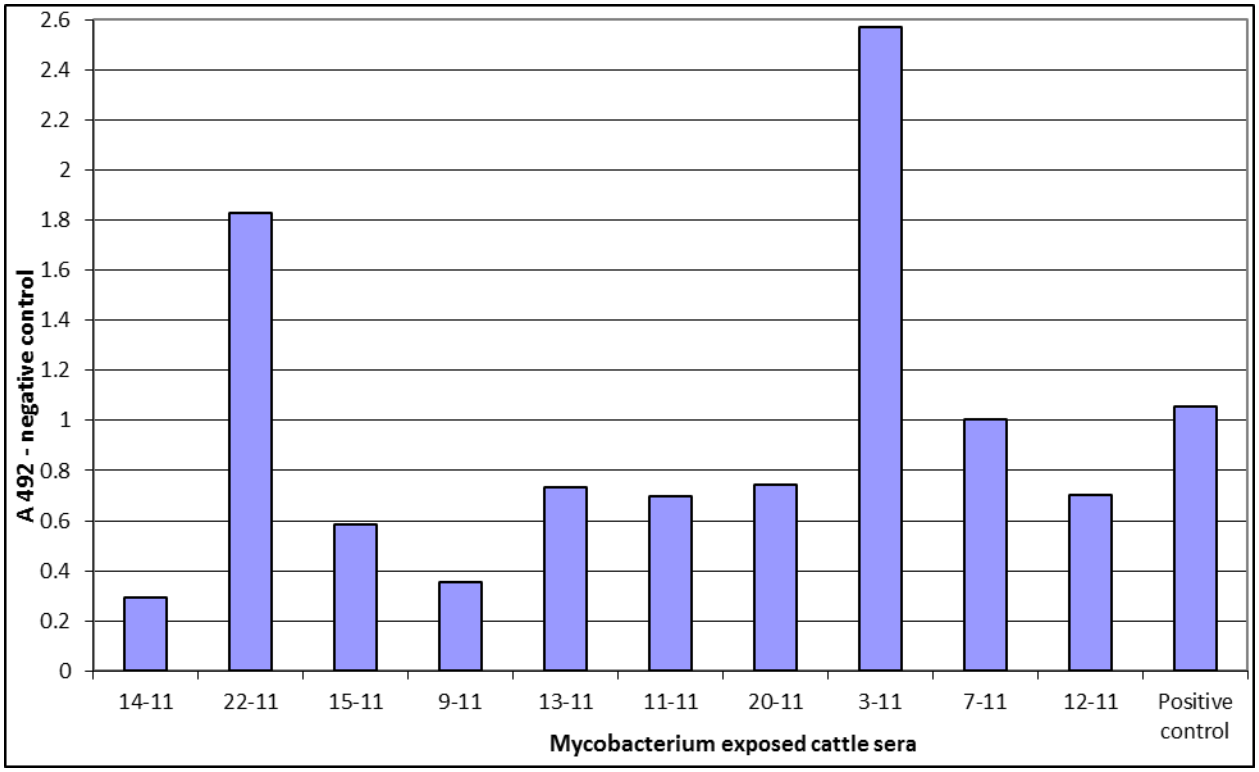


Figure 3.24: Results of the ELISA showing sera from the Mycobacterium exposed cattle reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

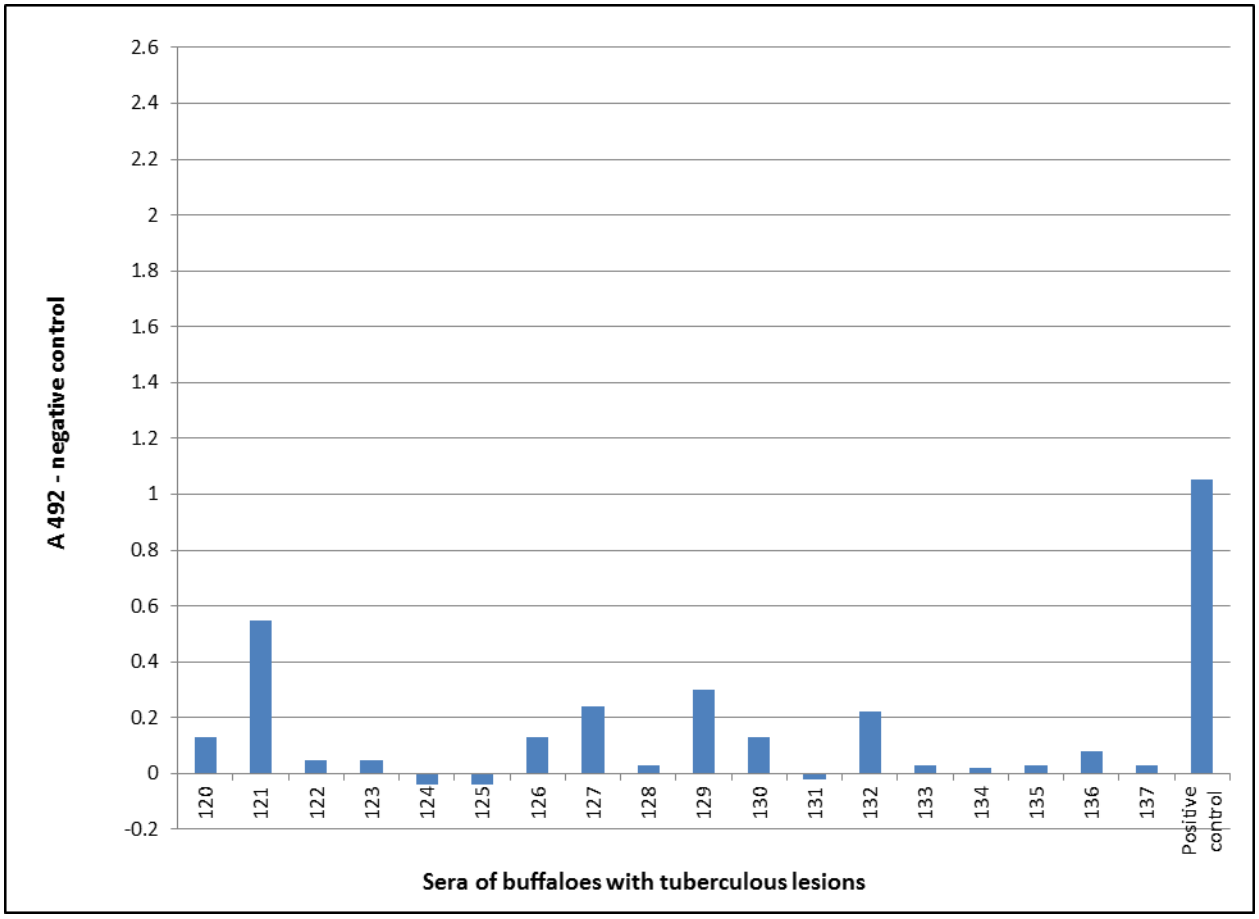


Figure 3.25: Results of the ELISA showing sera from the buffaloes with tuberculous lesions reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

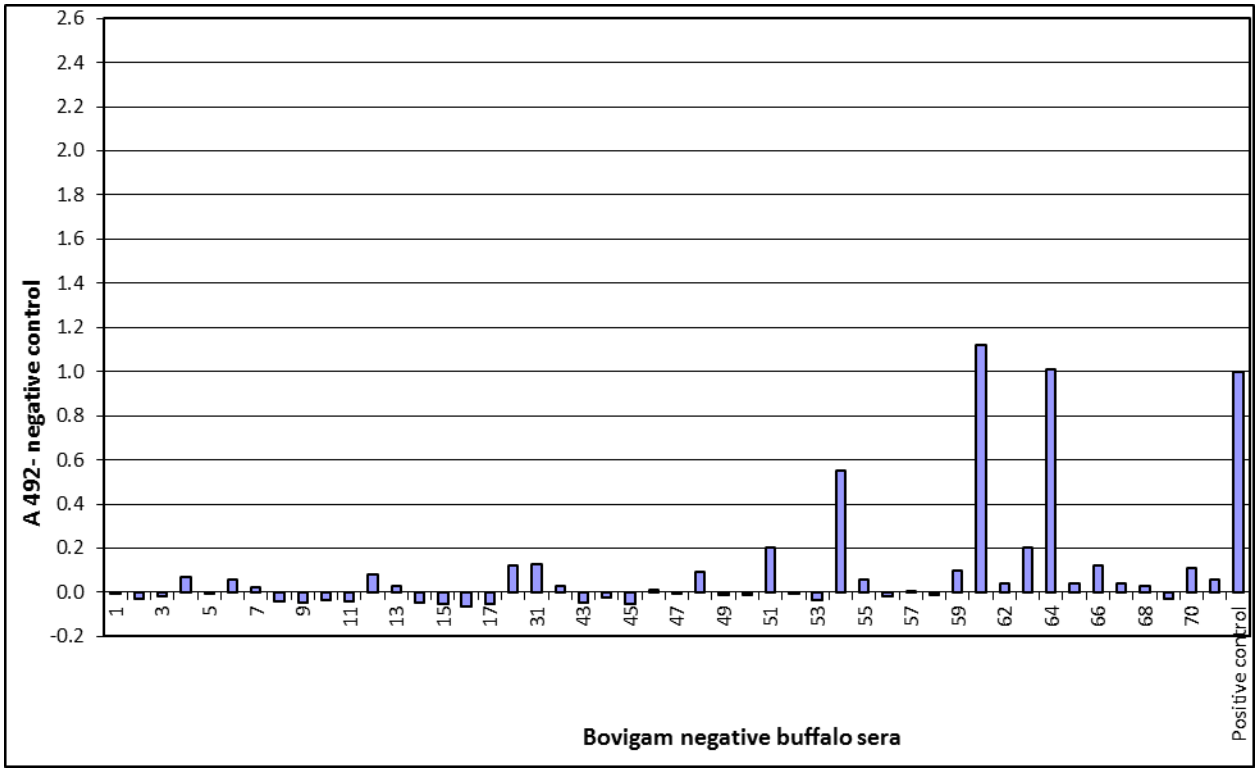


Figure 3.26: Results of the ELISA showing sera from the Bovigam negative buffalo reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

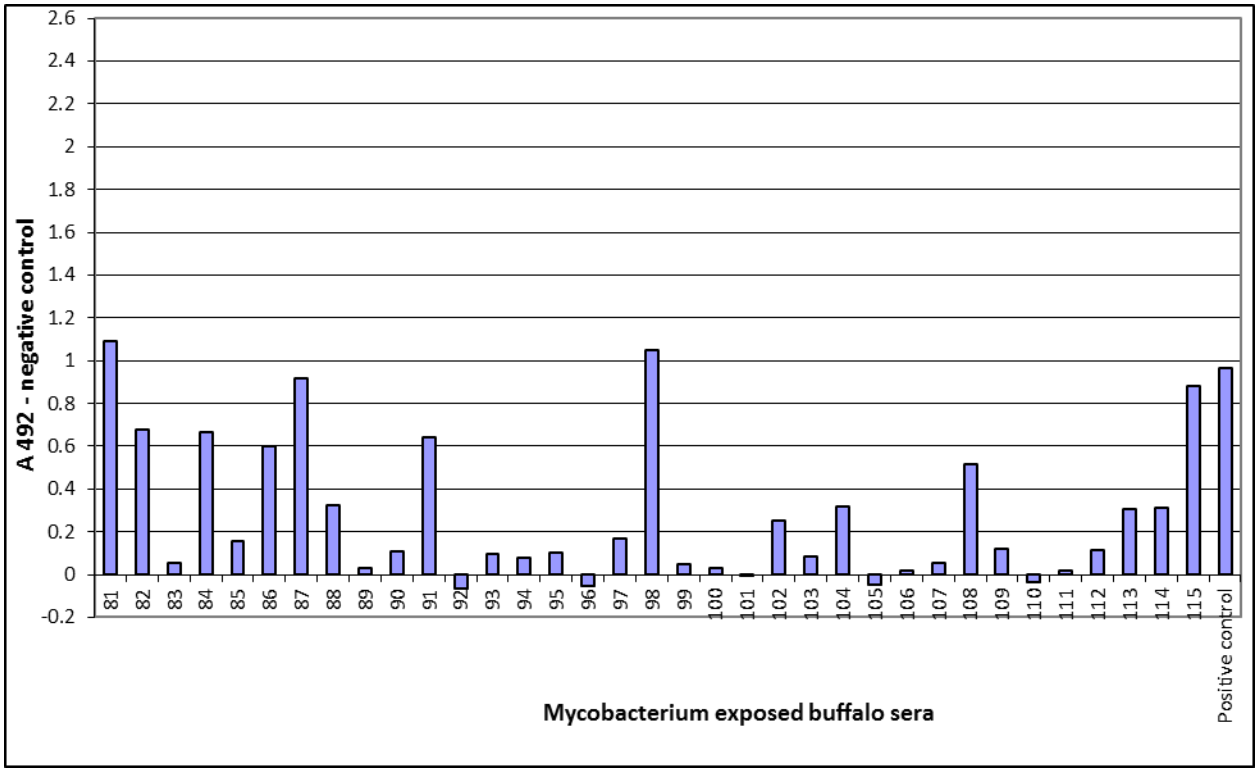


Figure 3.27: Results of the ELISA showing sera from the Mycobacterium exposed buffalo reacting with the Frag 2-MGFP fusion protein. The plotted OD-values are the average of duplicate readings of samples at absorbance 492 nm minus the OD of the negative control.

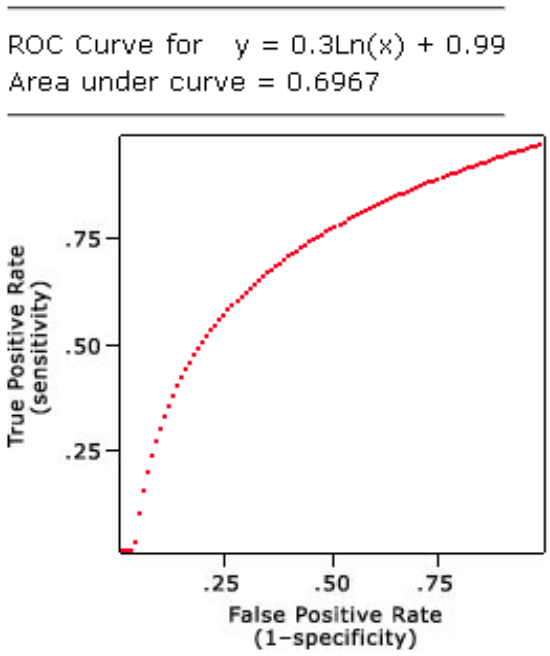
Table 3.2 Summary of the results of Frag 2-MGFP ELISA using panels of characterized buffalo and cattle sera

		OD range	Number of samples in OD range
Cattle sera	**Tuberculin skin test positive cattle**	0.2 - 0.5	50% (16/32)
		≤ 0.2	21.9% (7/32)
		≥ 0.5	28.1% (9/32)
	BTB free cattle	0.2 - 0.5	42% (21/50)
		≤ 0.2	46% (23/50)
		≥ 0.5	12% (6/50)
	Non-tuberculous Mycobacterium exposed cattle	≥ 0.5	80% (8/10)
Buffalo sera	**Buffaloes with tuberculous lesions**	≥ 0.2	22% (4/18)
		< 0.2	78% (14/18)
	Bovigam negative buffalo	≥ 0.2	10.4% (5/48)
		< 0.2	89.6% (43/48)
	Non-tuberculous Mycobacterium exposed buffalo	≥ 0.2	40% (14/35)

Table 3.3: The diagnostic performance of the ELISA using cattle and buffalo sera.

	Cattle sera		**Buffalo sera**	
Cut-off point	0.5	0.65	0.2	0.3
Sensitivity (%)	28	22	22	11
Specificity (%)	88	92	90	94
PPV (%)	60	64	44	40
NPV (%)	66	65	75	74
Area under ROC curve	0.6967		0.7161	

A



B

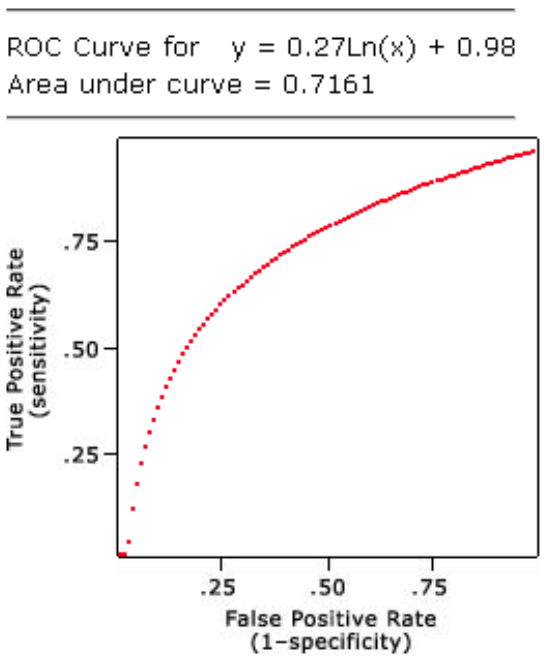


Figure 3.28: ROC curve analysis of the; A: Cattle sera; B: Buffalo sera.

3.3 Fluorescence polarization assay

3.3.1 rMPB70-FITC

For the FPA, the tracer must be able to fluoresce, hence the purified rMPB70 was labeled with NHS-fluorescein and the degree of labeling was calculated to be 3.82 moles fluorophore per mole protein. The labeled rMPB70 was tested in the ELISA to check if the epitopes were still intact. The presence of the ELISA signal after labeling the rMPB70 (Figure 3.29) indicated intact epitopes even though it was less than that of the unlabeled rMPB70. This slightly lower signal could be due to discrepancies in quantitation of rMPB70 versus rMPB70-FITC.

For a test result to be valid in FPA, an antibody that binds the tracer must yield a higher mP value than a negative antibody which does not bind the tracer. The rMPB70-FITC was tested in the FPA (Table 3.4) using the control chicken anti-rMPB70 IgY antibodies The tracer alone gave the correct mP value of 35 (for FITC

fluorophore), but when the chicken anti-rMPB70 IgY antibodies were added the expected results were not observed: duplicate and / or triplicate measurement readings were not the same and the calculated mP value of the positive chicken anti-rMPB70 IgY antibodies (D80) were expected to have been higher than that of the negative chicken anti-rMPB70 IgY antibodies (D0). The anti-*M. bovis* serum was expected to have a higher mP value at a dilution of 1:125 than at 1:250, but this was not the case. Different strategies were employed to try to solve the problem by:

- using sample buffer with different LiDS concentrations (PBS with 0.4%, 0.2% or 0.1% LiDS, and PBS without LiDS),
- a range of tracer concentrations from 0.66 µM to 3.3 µM,
- different batches of microtiter plates and
- changing FPA reader settings (gain adjustments and number of flashes),

None of these interventions improved the outcome of the FPA. Therefore it was decided to make smaller tracers by fragmenting the *mpb70* gene into three regions.

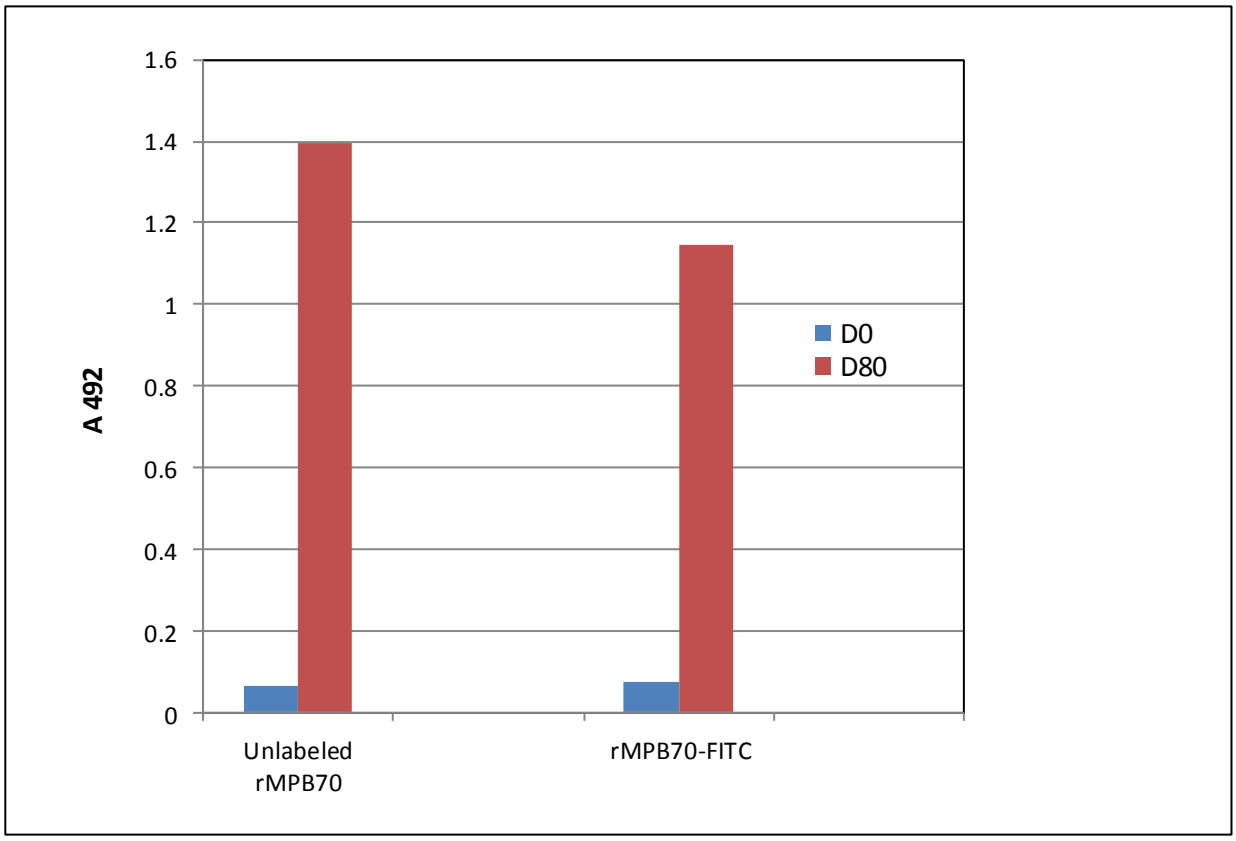


Figure 3.29: Results of the ELISA showing chicken anti-rMPB70 IgY antibodies (IgY = 60 µg/ml) reacting with labeled and unlabeled rMPB70. D0, anti-rMPB70 IgY antibodies isolated from the eggs before immunization with the rMPB70; D80, anti-rMPB70 IgY antibodies isolated from the eggs on day 80 after immunization with the rMPB70. The plotted OD-values are the average of duplicate readings of labeled and unlabeled rMPB70 at absorbance 492 nm.

Table 3.4: FPA results using rMPB70-FITC tracer with the chicken anti-rMPB70 IgY and rabbit anti-*M. bovis* antibodies

Tracer concentration	mP value set at 35 Duplicates and/or Triplicates			Antibodies
1.32 µM	11.54	20.40		IgY Day 0: 1 mg/ml
	24.19	19.89		IgY Day 80: 1 mg/ml
	13.39	23.25		Anti-*M. bovis* 1:250
	23.81	24.26		Anti-*M. bovis* 1:125
0.66 µM	15.85	-0.72	14.07	IgY Day 0: 1 mg/ml
	26.55	12.94	1.05	IgY Day 80: 1 mg/ml
	-2.74	-5.98	-10.98	Anti-*M. bovis* 1:250
	5.00	-7.08	-1.62	Anti-*M. bovis* 1:125
3.3 µM	36.68	35.86	44.32	IgY D0: 1 mg/ml
	42.73	35.53	33.37	IgY D80: 1 mg/ml
	26.81	27.32	28.65	Anti-*M. bovis* 1:250
	30.53	32.16		Anti-*M. bovis* 1:125

3.3.2 MPB70 fragment MGFP fusion proteins

The Frag 2-MGFP fusion protein which was recognized by BTB infected buffalo sera was tested in the FPA (Table 3.5) using control chicken anti-rMPB70 IgY antibodies with the tracer settings at 400 mP value for the green fluorescent fluorophore. The results were not satisfactory as was seen with the rMPB70-FITC. Signals produced by the positive chicken anti-rMPB70 IgY antibodies (D80) were expected to have been higher than that of the negative chicken anti-rMPB70 IgY antibodies (D0) and duplicates results were different, therefore smaller tracers were made by synthesizing peptides.

Table 3.5: FPA results using Frag 2-MGFP fusion protein tracer with the control chicken anti-rMPB70 IgY antibodies

Tracer concentration	mP value set at 400 Duplicates		Antibodies
7.95 µM	-1696.77	319.89	IgY D0: 1 mg/ml
	982.30	1427.97	IgY D80: 1 mg/ml
0.795 µM	1054.76	535.56	IgY D0: 1 mg/ml
	1866.03	537.54	IgY D80: 1 mg/ml
0.0795 µM	567.20	688.78	IgY D0: 1 mg/ml
	288.63	80.00	IgY D80: 1 mg/ml

3.4 Peptides

Fifteen peptides (Figure 12 of Appendix 4) of 15 amino acid residues which overlapped by 5 residues were synthesized by GenScript and had fluorescein (FITC-Ahx) attached on the N-terminus so that they could be used as tracers in the FPA. They were based on the MPB70 predicted epitopes (Section 2.4.4) and from the previous studies that defined antigenic regions of the MPB70 protein with sera from *M. bovis* infected bovine (Radford *et al*., 1990; Wiker *et al*., 1998; Lightbody *et al*., 2000). Figure 3:30 compares the peptides synthesized with these previously identified antigenic regions.

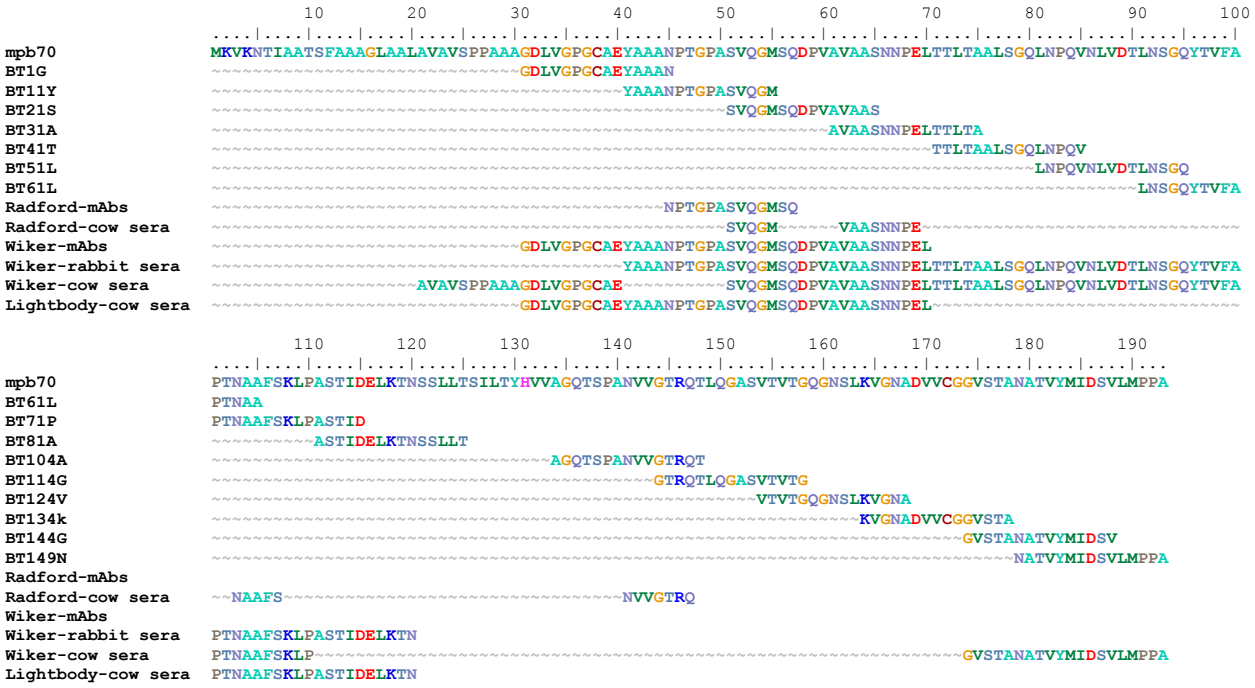


Figure 3.30: Comparison of position of peptides synthesized in the present study (BT1G, BT11Y, BT21S, BT31A, BT41T, BT51L, BT61L, BT71P, BT81A, BT104A, BT114G, BT124V, BT134K, BT144G & BT149N) with the MPB70 antigenic regions shown by different researchers: Radford *et al.* (1990) using monoclonal antibodies and cow sera; Wiker *et al*. (1998) using monoclonal antibodies, polyclonal rabbit sera and cow sera; Lightbody *et al*. (2000) using cow sera.

The peptides were characterized in the ELISA (Figure 3.31). The positive control chicken anti-rMPB70 IgY antibodies, D80 gave the highest signal of OD_{492} of 2.5 with peptide 1 (BT1G), followed by peptide 6 (BT51L) with OD_{492} of 0.7. The remaining peptides gave low signals below OD_{492} of 0.5 The position of peptide BT1G falls within the MPB70 fragment 1, while peptide BT51L sequences lie within the MPB70 fragment 2 (Figure 3.32). Sequence comparison showed that peptide BT1G has residues similar to those already identified by Lightbody *et al*. (2000) using bovine sera and Wiker *et al*. (1998) using monoclonal antibodies while residues from peptide BT51L are similar to those identified by Wiker *et al*. (1998) using bovine and rabbit sera.

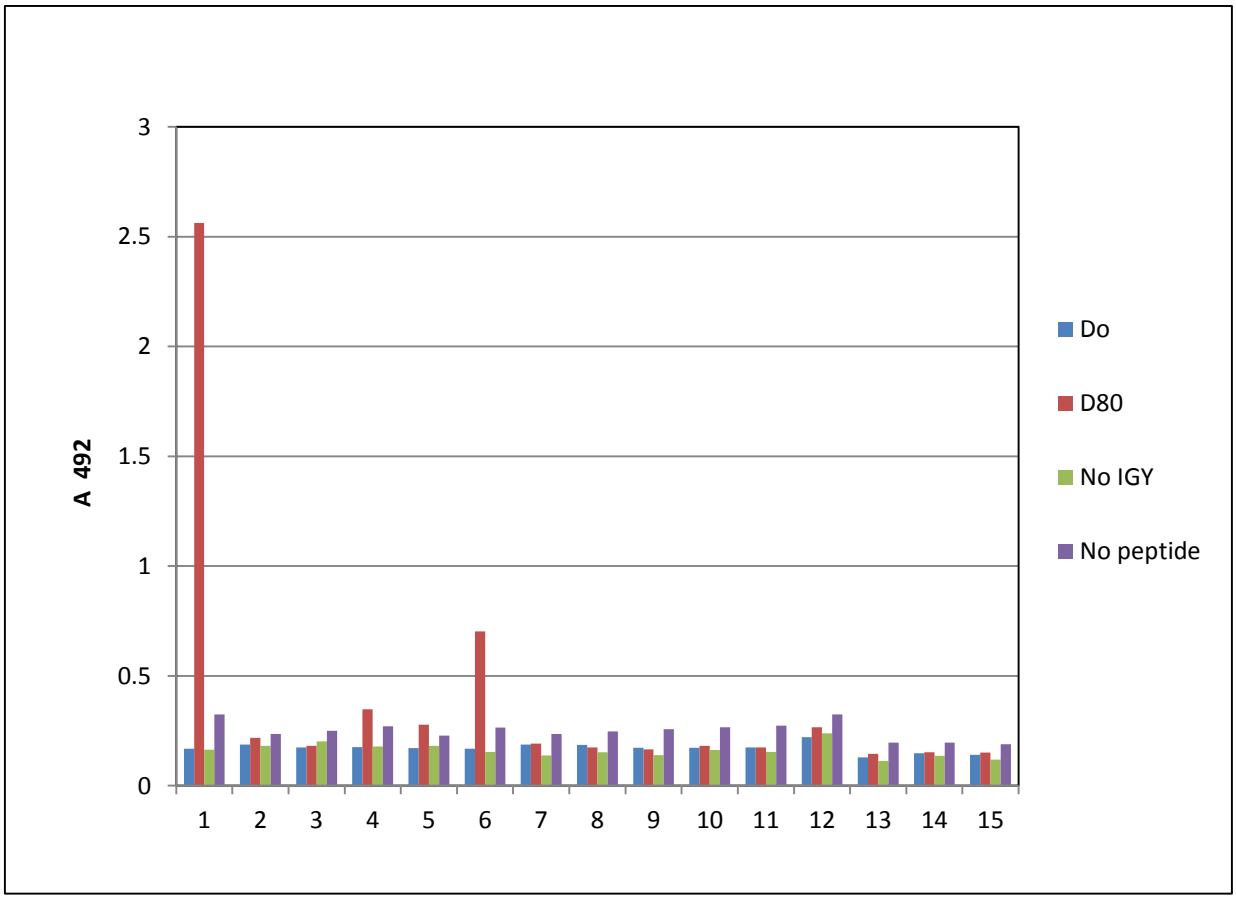


Figure 3.31: Results of the ELISA showing chicken anti-rMPB70 IgY antibodies (IgY = 60 µg/ml) reacting with the MPB70 peptides. D0, anti-rMPB70 IgY antibodies isolated from the eggs before immunization with the rMPB70; D80, anti-rMPB70 IgY antibodies isolated from the eggs on day 80 after immunization with the rMPB70; No IgY, a control containing all reagents except chicken anti-rMPB70 IgY antibodies; No peptide, a control containing all reagents except the peptides. The plotted OD-values are the average of duplicate absorbance readings of peptides at 492 nm.

```
                    10        20        30        40        50        60        70        80        90       100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
mpb70               MKVKNTIAATSFAAAGLAALAVAVSPPAAAGDLVGPGCAEYAAANPTGPASVQGMSQDPVAVAASNNPELTTLTAALSGQLNPQVNLVDTLNSGQYTVFA
Frag 1              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GDLVGPGCAEYAAANPTGPASVQGMSQDPVAVAASNNPELTTLTAALSGQL
Frag 2              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ALSGQLNPQVNLVDTLNSGQYTVFA
BT1G                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GDLVGPGCAEYAAAN
BT51L               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~LNPQVNLVDTLNSGQ
```

Figure 3.32: The deduced amino acid sequences of the MPB70 Fragments 1 & 2 aligned to the first 100 amino acid residues of MPB70. The position of peptides identified with the chicken antibodies, peptide1 (BT1G) and peptide 6 (BT51L) are shown.

The two peptides that reacted with the chicken anti-rMPB70 IgY antibodies (peptides 1 & 6) were tested in the FPA using the control chicken anti-rMPB70 IgY antibodies

with the tracer settings at 35 mP value. The results were not satisfactory as it was seen with the rMPB70-FITC and the Frag 2-MGFP proteins. Duplicates measurement readings were different and the calculated mP value of the positive chicken anti-rMPB70 IgY antibodies (D80) were not higher than that of the negative antibodies as expected (Tables 3.6 & 3.7), therefore the FPA was stopped here for the purposes of this study. A panel of infected and uninfected cattle and buffalo sera could not be tested with the FPA as this technique could not differentiate between a well-defined BTB positive and a negative control serum.

Table 3.6: FPA results using MPB70 peptide BT1G tracer with the control chicken anti-rMPB70 IgY antibodies

Tracer concentration	mP value set at 35 Duplicates		Antibodies
1420 nM	68.2	31.1	IgY D0: 1 mg/ml
	78.3	-307.7	IgY D80: 1 mg/ml
142 nM	68.9	1444.4	IgY D0: 1 mg/ml
	105.3	46.9	IgY D80: 1 mg/ml
14.2 nM	18.9	88.2	IgY D0: 1 mg/ml
	33.8	56.8	IgY D80: 1 mg/ml

Table 3.7: FPA results using MPB70 peptide BT51L tracer with the control chicken anti-rMPB70 IgY antibodies

Tracer concentration	mP value set at 35		Antibodies
1420 nM	28.2	60.5	IgY D0: 1 mg/ml
	29.6	-407.4	IgY D80: 1 mg/ml
142 nM	700	-904.8	IgY D0: 1 mg/ml
	-147.1	144.8	IgY D80: 1 mg/ml
14.2 nM	-13.3	-41.7	IgY D0: 1 mg/ml
	91.4	115.0	IgY D80: 1 mg/ml

CHAPTER 4

4.1 DISCUSSION

The MPB70 protein has been widely used in the serodiagnosis of bovine tuberculosis (Cho *et al*., 2007; Lightbody *et al*., 2000). In this study, it was chosen as a marker to detect late *M. bovis* infections. The gene encoding the mature MPB70 was cloned, the protein expressed as a histidine tagged protein and purified. The molecular weight of rMPB70 was 22 kDa which is within the predicted 16 and 23 kDa range (Nagai *et al*., 1991; Surujballi *et al*., 2002). Immunoblot analysis revealed that the polyclonal rabbit anti-*M. bovis* antibodies reacted with the 22 kDa rMPB70, but there were non-specific background reactions with *E. coli* proteins. This is not surprising as Munk & coworkers (1988) in their study using polyclonal antibodies observed high levels of non-specific reactions when using proteins produced in *E. coli*.

The rMPB70 was found in the insoluble fraction possibly due to the high levels of protein expression which leads to the accumulation of aggregated, insoluble protein which forms inclusions bodies. However, the inclusion bodies were useful because they aided in the purification and isolation of the expressed protein (Speed *et al*., 1996). Solubilisation of inclusion bodies was achieved by the use of 6 M urea for purification on Ni-affinity column and the protein was refolded by dialysis in PBS at 4°C.

Mycobacterial proteins contain species-specific as well as cross-reactive epitopes (Buchanan *et al*., 1987; Kingston *et al*., 1987; Lightbody *et al*., 2000). The characterization of species-specific epitopes on the MPB70 protein using monoclonal antibodies has been reported (Wood *et al*., 1988). Most of the epitopes were found in the N-terminal region of the protein when linear B-cell epitopes on MPB70 were

mapped (Radford *et al*., 1990; Wiker *et al*., 1998). In the present study, an MPB70 fragment 2 was generated and identified as an epitope containing region (residues 76-135) using anti-MPB70 chicken antibodies and sera from BTB infected buffaloes. The epitopes recognized by the sera from the *M. bovis* infected cattle and rabbit antibodies (Wiker *et al*., 1998) fall in the fragment 2 region of MPB70. Moreover, the MPB70 fragment 2 region includes residues 103-107 (NAAFS) which was found to be either a cross-reactive epitope or it reacted non-specifically with bovine antibodies (Radford *et al*., 1990) and this might be the reason why there were a lot of non-specific, false positive reactions to the fragment 2-MGFP fusion protein using ELISA on the BTB free cattle and Bovigam negative buffalo sera in this study.

Smaller antigenic regions were also identified using the MPB70 synthetic peptides. The peptides BT1G (amino acid residues 31-45) and BT51L (amino acid residues 81-95) were recognised by the anti-MPB70 chicken antibodies and fall within fragments 1 and 2, respectively. This means that fragment 1 may be an important region and requires further investigation. Other *E. coli* strains or a different expression system could result in the successful protein expression of this fragment. It is noteworthy that peptide BT1G has a high level of identity with MPB83 peptide 5 identified during a similar study using the same approach (by Dr Fehrsen, ARC-OVI; results unpublished, Figure 4.1). That study used a protein very similar to the MPB70, namely MPB83, which is expressed early in BTB infections and the same region was identified. The chicken anti-rMPB70 IgY antibodies did not react with the MPB83 peptide 5 and vice versa. This suggests that this region is antigenic in both proteins and if the peptides are combined they could be of diagnostic use. Future work may include testing the fifteen peptides in ELISA with well characterized immune sera from either cattle or buffaloes. The sensitivity and specificity of the ELISA might be improved in this way.

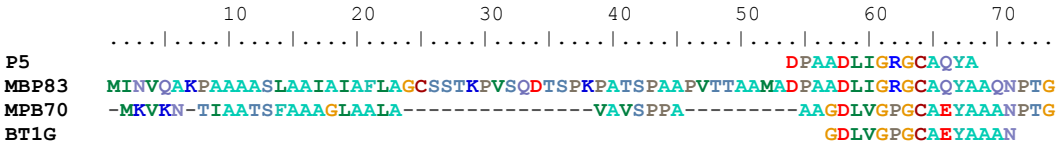


Figure 4.1: The first 74 residues of the deduced amino acid sequence of MPB83 aligned to MPB70. "–" indicates spaces to allow alignment. The position of peptides identified with chicken antibodies, peptide 5 (P5) and the MPB70 peptide (BT1G) are shown.

Fluorescence polarization assay (FPA) has previously been utilized as a serological test and has shown potential for use in the diagnosis of various veterinary diseases (Nasir & Jolley, 1999; Jolley & Nasir, 2003). Its simplicity, rapidity and ability to be used in the field offers advantages over other serological tests. As it does not require species-specific reagents, it is therefore a suitable test for BTB in wildlife. The FPA has previously been applied to detect *M. bovis* antibodies in different animals including bison, elk and llamas (Lin *et al*, 1996), cattle (Surujballi *et al*., 2002) and cervids (Surujballi *et al*., 2009). The FPA utilizing fluorescein-labeled MPB70 protein (Surujballi *et al*., 2002) and MPB70 peptide F-733 (Jolley *et al*., 2007) as tracers was described and the results were not satisfactory because of a lack in the sensitivity and specificity of the assay, hence this study was aimed at identifying additional MPB70 specific epitopes which have the potential to improve the sensitivity and specificity of the FPA in diagnosing bovine tuberculosis.

In the FPA, the tracer must be able to fluoresce; hence rMPB70 was labeled with FITC. The FITC labeling did not alter the structural integrity of the MPB70 epitopes. This was determined by comparing the reactivity of the labeled and unlabeled rMPB70 to the chicken antibodies raised against the rMPB70. The FITC-rMPB70 protein was able to bind the chicken antibodies. According to Jolley & Nasir (2003), proteins of up to 50 kDa can be used as tracers. Even though the rMPB70 (22 kDa) was suitable for use as a tracer, when tested in the FPA, the results failed to distinguish between the positive and negative chicken antibodies. Possibly the tracer (22 kDa) was not small enough for the FPA. Smaller tracers were made by synthesizing peptides derived from the MPB70 sequence. The peptides that reacted with the chicken anti-MPB70 IgY antibodies were tested in the FPA but still there was difference between the positive and negative chicken antibodies. Therefore, the

FPA protocol and the use of the PHERAstar micro plate reader may need to be further investigated.

Even though the MPB70 Frag 2-MGFP fusion protein did not perform well in the FPA, it was recognized by at least two sera from BTB infected buffaloes in ELISA indicating a potential for use in serological tests like ELISA. The ELISA, being a simple, rapid and low cost test (Hu *et al*., 2011), it can be used for high throughput testing. Therefore this study further investigated the Frag 2-MGFP fusion protein in an ELISA using panels of infected and uninfected cattle and buffalo sera, respectively.

The Frag 2-MGFP fusion protein ELISA showed a specificity of 88% and sensitivity of 28% with cattle sera at a cut-off point of OD_{492} = 0.5. For surveillance of BTB in large populations with unknown BTB status, a test with high specificity is needed to minimise unnecessary culling of valuable animals. Therefore, the specificity was maximised to 92% at a cost to the sensitivity (22%) by increasing the cut-off point to 0.65, but still the specificity did not increase significantly.

The study by Wood *et al*. (1992) found specificity and sensitivity of ELISA in cattle to be 96.4% and 18.1% respectively and Sudgen *et al*. (1997) found the specificity and sensitivity to be 75% and 65% both using the native MPB70 (Appendix 5). The specificity and sensitivity was much higher at 100% and 89.7% when Cho (1998) used rMPB70. However, later studies also employing rMPB70 were not able to reproduce these results. A study by Yearsley *et al*. (1998) found a specificity of 82.6% and sensitivity of 31%. Farias *et al. (*2012) found specificity of 94.6% and this is more comparable to the 92% findings of this study. The variation in the specificity and sensitivity might be because of the different forms of the antigen used (fragment of MPB70, native and rMPB70), assay formats and probably most importantly, the sera selected or available from different populations and the stages of the disease (McNair *et al*., 2001).

For the buffalo sera, MPB70 Frag 2-MGFP fusion protein ELISA showed a specificity and sensitivity of 90% and 22 %, respectively, at a cut-off point of OD_{492} = 0.2. However, by establishing the cut-off at higher value of 0.3 the specificity increased to 94% accompanied by a decrease in sensitivity to 11%. The 94% specificity is higher than 73.7% reported by Sudgen *et al*. (1997) testing bison sera using native MPB70 at a cut-off point 0.170.

The area under the ROC curve measures a diagnostic test's discriminatory power (Amadori *et al*., 1998; Faraggi *et al*., 2002) and in this study it measures the ability of the ELISA to correctly classify the sera with and without antibodies to *M.bovis* infection. An area under the curve (AUC) between 0.90-1 represents an excellent test, 0.80-0.90 is a good test, 0.7-0.8 is a fair test, 0.6-0.7 is a poor test and 0.5-0.6 indicates that the test is worthless (Fan *et al*., 2006; http://gim.unmc.edu/dxtests/roc3.htm). The larger the AUC the better the test discriminates the positive sera from the negative ones, but in this study the AUCs for cattle and buffalo sera were 0.6963 and 0.7161, respectively, which indicated that the ELISA discriminated poorly *M. bovis* infected buffaloes and cattle from non-infected ones.

Some researchers reported MPB70 to be species-specific (Nagai *et al*., 1981; Harboe *et al*., 1990) however later studies found MPB70 not to be entirely *M. bovis*-specific (Wood *et al*., 1992; Fifis *et al*., 1992) due to the interferences of non-tuberculous mycobacteria (NTM) with the host immune response which have been implicated as a complicating factor (Woods & Washington, 1987; Waters *et al*., 2006). In the present study, false-positive reactions with MPB70 Frag-2 MGFP fusion protein were obtained even after maximising specificity in BTB free cattle sera (8%) and in negative Bovigam buffalo sera (6.25%). This could possibly be due to exposure to a rich flora of environmental mycobacteria in southern Africa (Michel *et al.*, 2007; Tschopp *et al*., 2010) to which animals elicit an immune response resulting in cross-reacting antibodies to MPB70 (Farias *et al.,* 2012).

In another study, Fifis *et al*. (1992) reported cross-reaction of MPB70 in ELISA with 9% of sera of animals not infected or infected with other mycobacterial species, resulting in false positive reactions. However, Wood *et al*. (1992) also detected some

cross-reactivity (3.6%) of MPB70 in their study. Both studies used purified native MPB70 protein. In the first study, three groups of serum samples were tested, the infected group which consisted of 19 sera from *M. bovis* culture-positive cattle; the negative group with 17 sera from cattle in uninfected herds and from culture-negative herds from infected herds; the cross-reactor group with 24 sera from (a) cattle naturally infected with either *M. paratuberculosis* or *M. avium* or atypical mycobacterial infections, (b) cattle that had 'skin tuberculosis' lesions and (c) two cows experimentally infected with either *Rhodococcus equi* or *Nocardia asteroids*. In the second study, the serum samples came from herds with a history of bovine tuberculosis, thereby causing an underestimation of the true specificity as the appropriate way to determine specificity is to use samples from an area where the disease existed.

Ten sera from non-tuberculous Mycobacterium exposed cattle which were tested using the MPB70 Frag 2-MGFP in the ELISA (Figure 3.24) were previously characterized using different PPDs in the Bovigam test (by Akin Jenkins, DVTD, UP, results not published). The cattle were from a farm that had no history of BTB. Cattle 12-11 and 13-11 reacted strongly to PPDB while cattle 15-11 and 22-11 reacted strongly to PPDA. These results indicate that there are antigens present in the PPDs that can stimulate cells which were previously exposed to non-tuberculous Mycobacterium. These non-specific reactions were also observed in the MPB70 Frag 2-MGFP ELISA as false positive results were seen in the BTB free cattle which could also be due to exposure to non-tuberculous Mycobacteria.

Bovine tuberculosis is a chronic infectious disease among cattle, other domesticated and some wildlife animals (OIE Manual, 2009). The transmission of infection from affected to susceptible animals is highest when animals are kept in close contact and has been reported where domestic animals and wildlife share pasture or territory (O'Reilly *&* Dabon, 1995; Renwick *et al*., 2007). An observation in this present study was that there was reactivity to Frag 2-MGFP fusion protein in a high percentage of the non-tuberculous Mycobacterium exposed buffalo herd compared to the

unexposed buffalo. As the animals were from one game farm, close contact might have promoted respiratory route or alimentary mode of transmission via excretion of *M. bovis* in nasal and oral discharges which may in turn contaminate the drinking water. These pathways could possibly have caused the infection to spread between the herd resulting in a high level of non-specific reactions to MPB70 Frag 2-MGFP fusion protein.

Even though the results in present study were not ideal, the recombinant MPB70 is promising for use as diagnostic target to detect antibodies against *M. bovis.* Lilenbaum *et al*. (2011) detected positive cattle (5/18) that were negative by both comparative cervical tuberculin test and IFN-γ test. The ability of the ELISA using MPB70 to detect anergic animals have been reported before (Lilenbaum *et al*., 1999). It remains to be determined whether the success of their study was due to a difference in flora of environmental mycobacteria in South America compared to southern Africa.

There are many possible ways to improve on what has already been achieved, during the present study, in the application of the MPB70 assay. Future work could include developing an inhibition ELISA using rMPB70 and chicken anti-MPB70 IgY antibodies in order to improve the diagnostic performance of the ELISA.

A study by Waters *et al*. (2011), using a blend of MPB70 and MPB83 in a commercial IDEXX *M. bovis* antibody ELISA, obtained a high specificity of 98%. In that study minimal cross-reactive responses were elicited by infection or sensitization with non-tuberculous *Mycobacterium* spp. This indicate that MPB70 ELISA would be of value if combined with other antigens. Future work could combine the results of the current sudy with the results by Dr Fehrsen, ARC-OVI (results unpublished) using MPB83 which is expressed early in BTB infections. A cocktail using a protein that is expressed early and the other late would cover a larger window of the immune responses resulting in the detection of a higher percentage of animals with bovine tuberculosis (Kwok *et al*., 2010; Maas *et al*., 2012).

A different approach would be to use different antigens from MPB70, like recombinant ESAT-6 and CFP-10 as they are highly specific toward *M. tuberculosis* complex. Both proteins have been used as poly-epitope fusions to develop a serodiagnostic test for TB in cervids (*Cervus elaphus*) (Harrington *et al*., 2008). Kwok *et al*. (2010) has shown in a rabbit model challenged experimentally with different mycobacteria that ESAT-6 and CFP-10 can elicit humoral response. These proteins provide the necessary basis for a highly specific TB serodiagnostic test. In addition, the two recombinant antigens can help discriminate between *M. bovis* BCG vaccinated; environmental, non-tuberculous mycobacteria.

4.2 CONCLUSION

An antigenic region of MPB70 recognized by buffalo immune sera was narrowed down to one third of the protein. When serum samples from uninfected and naturally *M. bovis* infected buffaloes and cattle were tested with this MPB70 fragment in the ELISA, the diagnostic performance was, however, overall unsatisfactory and hence of very limited use as a serological test to detect antibody responses to BTB as a stand-alone assay. Probably a cocktail using more than one antigen is needed to have a good test. In addition, when the same fragment was tested in the FPA, we could not get the technique to work.

Smaller epitopes were identified using synthetic peptides. Peptides BT1G and BT51L were identified using chicken anti-MPB70 antibodies. When the two peptides were tested in the FPA, the technique failed to produce valid results.

Irrespective of how the MPB70 protein is approached, whether the whole protein or fragment thereof (in this study), the MPB70 was not found specific enough for serodiagnosis of *M. tuberculosis* complex infections.

APPENDICES

Appendix 1: Buffer Preparation and Solutions

1M IPTG stock solution

IPTG	1.19 g

Dissolve in dH_2O to a final volume of 5 ml. Filter sterilize and aliquot. Store at -20°C.

Luria broth

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g

Dissolve in dH_2O to a final volume of 1L. Autoclave at 121°C & 100 kPa for 20 min and store at RT.

Luria broth agar

Bacteriological agar no 1	1.5 g
Tryptone	0.8 g
Yeast extract	0.5 g
NaCl	0.5 g

Dissolve in dH_2O to a final volume of 100 ml. Autoclave at 121°C & 100 kPa for 20 min and cool to 55 °C. Add 60 µl of 50 mg/ml kanamycinamycin to a final concentration of 30 µg/ml and / or 2500 µl of 20% glucose to make a final concentration of 0.5% and pour into petri dish plates. Store plates at 4°C.

SDS-PAGE loading buffer

1.5 M Tris-HCl, pH 6.8	1 ml
20 % SDS	0.6 ml
Glycerol	3 ml
ß-mercaptoethanol	1.5 ml
18 mg/ml bromophenol blue	10 µl

Dissolve in dH_2O to a final volume of 10ml.

10 x SDS PAGE running buffer

SDS	10 g
Tris base	30.3 g
Glycine	144.1 g

Dissolve in 800 ml of dH_2O and make up to a final volume of 1L. Store at RT

12.5% Separating gel

Acrylamide	4.17 ml
1.5M Tris pH8.8	2.5 ml
dH_2O	3.23 ml
10% APS	100 µl
TEMED	6.7 µl

Mix.

4% Stacking gel

Acrylamide	1.33 ml
1M Tris pH6.8	2.5 ml
dH_2O	6 ml
10% APS	100 µl
TEMED	6.7 µl

Mix.

50 x TAE buffer

Tris base	242 g
Na_2EDTA	37.2 g
Glacial acetic acid	57.1 ml

Make up to a final volume of 1L with dH_2O. Store at RT.

TE buffer

1M Tris-HCl, pH 7.4	5 ml
0.5M Na_2EDTA, pH 8.0	1 ml

Mix and adjust to a final volume of 500 ml with dH_2O. Autoclave at 121°C & 100 kPa for 20 min and store at RT.

Towbin buffer

25 mM Tris base	3.02 g
192 mM Glycine	14.41 g

Dissolve in 800 ml of dH_2O and make up to a final volume of 1L.

IM Tris-HCl, pH 6.83, 7.4

Tris base	12.12 g
dH_2O	800 ml

Adjust pH with HCl to 6.83 or 7.4. Make up to 100 ml with dH_2O. Autoclave at 121°C & 100 kPa for 20 min and store at RT.

1.5M Tris-HCl, pH 6.8, 8.87

Tris base	18.18 g
dH_2O	800 ml

Adjust pH with HCl to 6.8 or 8.87. Make up to 100 ml with dH_2O. Autoclave at 121°C & 100 kPa for 20 min and store at RT.

Appendix 2: Information on the panel of serum samples used in ELISA

Table 1: Identification and origin of sera from BTB free cattle.

Sample identification	No. of samples	Location (Province)
1, 2, 3, 4 & 5	5	Gauteng
6, 7, 8, 9 & 10	5	Gauteng
11, 12, 13 & 14	4	Gauteng
15, 16, 17, 18 19 & 20	6	Gauteng
21, 22, 23, 24 & 25	5	Gauteng
26, 27, 28, 29, 30 & 31	6	Limpopo
32, 33, 34, 35 & 36	5	Mpumalanga
37, 38, 39, 40 & 41	5	Gauteng
42, 43, 44, 45 & 46	5	Gauteng
47, 48, 49 & 50	4	Gauteng

Table 2: Identification of sera from infected cattle that tested positive with the tuberculin skin test.

Sample identification	Tuberculin skin test
51-82	positive

Table 3: Identification and origin of sera from non-tuberculous Mycobacterium exposed cattle and Bovigam test result.

Sample identification	Location	Bovigam test
14-11	Gauteng	negative
22-11		reacted to PPD A
15-11		reacted to PPD A
9-11		negative
13-11		reacted to PPD B
11-11		negative
20-11		negative
3-11		negative
7-11		negative
12-11		reacted to PPD B

PPDA - Purified protein derivative produced from *Mycobacterium avium*

PPDB - Purified protein derivative produced from *Mycobacterium bovis*

Table 4: Identification and origin of sera from BTB negative buffalo sera and Bovigam test results.

Sample identification	No. of samples	Location	Bovigam
1-17	17	Free State	Negative
30, 31 & 32	3	Limpopo	Negative
43-55	13	Limpopo	Negative
56-59	4	Limpopo	Negative
60-72	13	Limpopo	Negative

Table 5: Identification and origin of sera from buffaloes with tuberculous lesions and histopathology test results.

Sample identification	No. of samples	Location	Histopathology
120-135	16	Herd B	tuberculous lesions
136-137	2	Herd C	tuberculous lesions

Table 6: Identification and origin of sera from non-tuberculous Mycobacterium exposed buffaloes and Bovigam test results.

Sample identification	No. of samples	Location	Bovigam
81, 83, 84, 85, 86, 87, 90, 91, 92, 93 & 94	11	North West	negative
82	1		multiple reactor*
88 & 89	2		reacted to PPD A
95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 107, 108, 109, 32, 33, 34 & 35	18	North West	negative 2 avian reactors
106 & 110	2		reacted to PPD A
111	1		equal reactor[#]

*reacts with both PPD A and PPD B

[#]reacts with PPD B and PPD Fortuitum only or PPD B with both PPD Fortuitum and PPD A

Appendix 3: DNA and amino acid sequences of MPB70, MGFP and fragment MGFP fusions

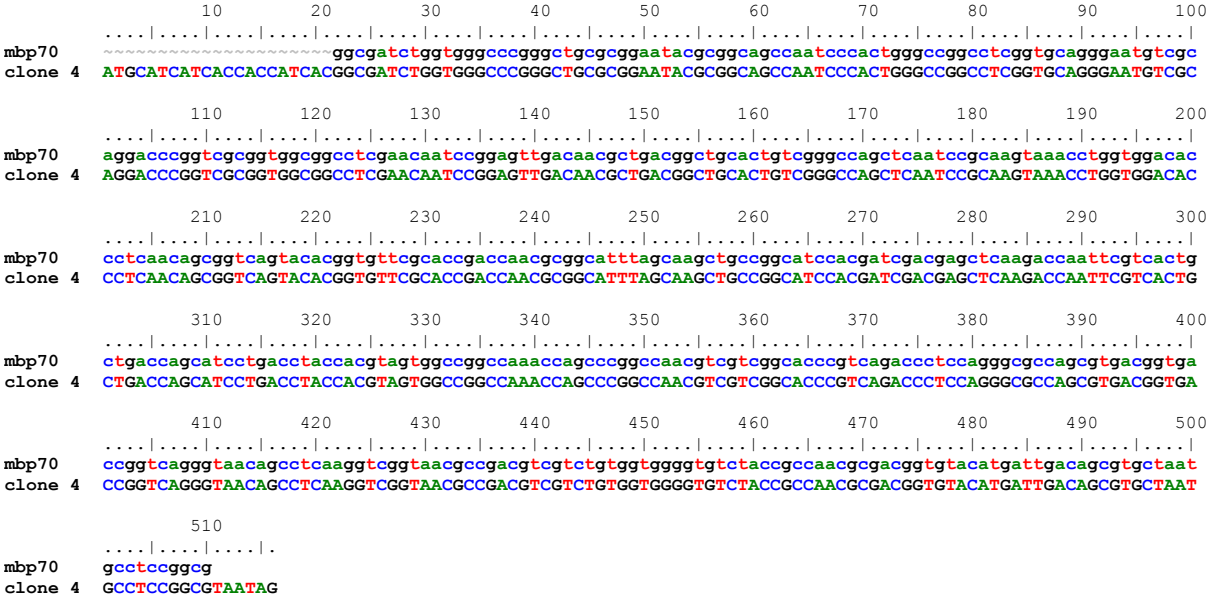


Figure 1: DNA sequences of rMPB70 clone 4 with forward primer, aligned to MPB70 (GenBank, D38230). Clone 4 has a start codon ATG that precedes six histidine codons and two stop codons TAA & TAG follows immediately after the cloned *mpb70* gene

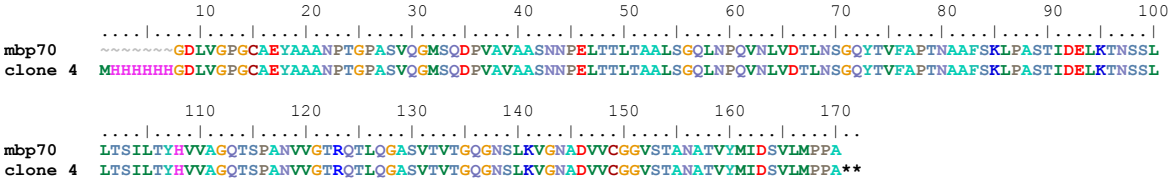


Figure 2: Translation of MPB70 clone 4 compared to that of MPB70. Clone 4 has a start codon methionine (M) that precedes six histidine (H) and two stop codons TAA & TAG (**) follows immediately after the cloned *mpb70* gene

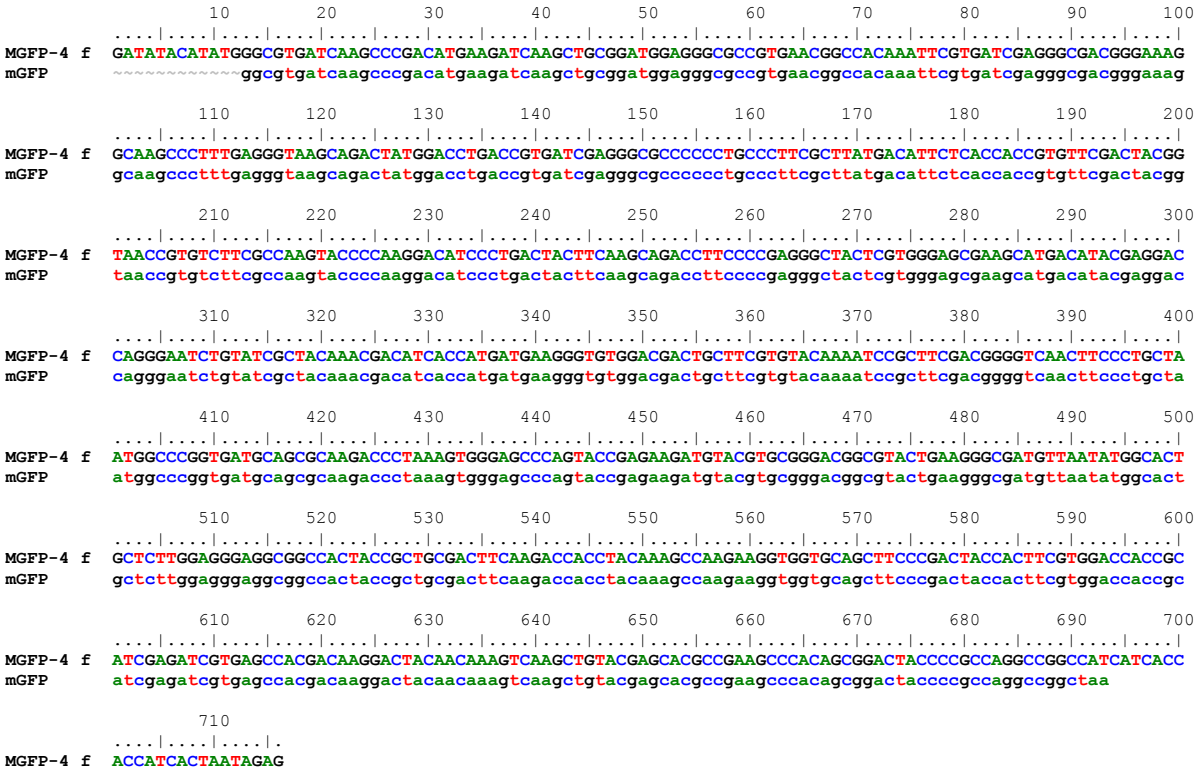


Figure 3: DNA sequences of rMGFP clone 4 with forward primer, aligned to MGFP. A start codon ATG precedes rMGFP clone. Six histidine codons follow immediately after the cloned *mgfp* gene but precede two stop codons TAA & TAG.

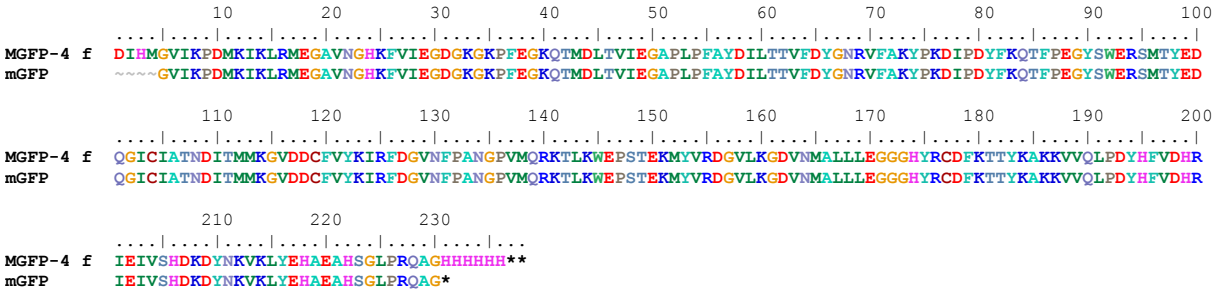


Figure 4: Translation of MGFP clone 4 compared to that of MGFP. A start codon M precedes rMGFP clone. Six H codons follow immediately after the cloned *mgfp* gene but precede two stop codons (**).

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                  10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
mbp70     gcggccgcaggcgatctggtgggcccgggctgcgcggaatacgcggcagccaatcccactgggccggcctcggtgcagggaatgtcgcaggacccggtcg
F1-1 rev  ATACATATGGGCGATCTGGTGGGCCCGGGCTGCGCGGAATACGCGGCAGCCAATCCCACTGGGCCGGCCTCGGTGCAGGGAATGTCGCAGGACCCGGTCG

                 110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
mbp70     cggtggcggcctcgaacaatccggagttgacaacgctgacggctgcactgtcgggccagctc~~~~~~~
F1-1 rev  CGGTGGCGGCCTCGAACAATCCGGAGTTGACAACGCTGACGGCTGCACTGTCGGGCCAGCTCGGCGTGATCAAGCCCGACATGAAGATCAAGCTGCGGAT
mGFP      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ggcgtgatcaagcccgacatgaagatcaagctgcggat

                 210       220       230       240       250       260       270       280       290       300
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  GGAGGGCGCCGTGAACGGCCACAAATTCGTGATCGAGGGCGACGGGAAAGGCAAGCCCTTTGAGGGTAAGCAGACTATGGACCTGACCGTGATCGAGGGC
mGFP      ggagggcgccgtgaacggccacaaattcgtgatcgagggcgacgggaaaggcaagccctttgagggtaagcagactatggacctgaccgtgatcgagggc

                 310       320       330       340       350       360       370       380       390       400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  GCCCCCCTGCCCTTCGCTTATGACATTCTCACCACCGTGTTCGACTACGGTAACCGTGTCTTCGCCAAGTACCCCAAGGACATCCCTGACTACTTCAAGC
mGFP      gcccccctgcccttcgcttatgacattctcaccaccgtgttcgactacggtaaccgtgtcttcgccaagtaccccaaggacatccctgactacttcaagc

                 410       420       430       440       450       460       470       480       490       500
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  AGACCTTCCCCGAGGGCTACTCGTGGGAGCGAAGCACGACATACGAGGACCAGGGAATCTGTATCGCTACAAACGACATCACCATGATGAAGGGTGTGGA
mGFP      agaccttccccgagggctactcgtgggagcgaagcatgacatacgaggaccagggaatctgtatcgctacaaacgacatcaccatgatgaagggtgtgga

                 510       520       530       540       550       560       570       580       590       600
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  CGACTGCTTCGTGTACAAAATCCGCTTCGACGGGGTCAACTTCCCTGCTAATGGCCCGGTGATGCAGCGCAAGACCCTAAAGTGGGAGCCCAGTACCGAG
mGFP      cgactgcttcgtgtacaaaatccgcttcgacggggtcaacttccctgctaatggcccggtgatgcagcgcaagaccctaaagtgggagcccagtaccgag

                 610       620       630       640       650       660       670       680       690       700
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  AAGATGTACGTGCGGGACGGCGTACTGAAGGGCGATGTTAATATGGCACTGCTCTTGGAGGGAGGCGACCACTACCGCTGCGACTTCAAGACCACCTACA
mGFP      aagatgtacgtgcgggacggcgtactgaagggcgatgttaatatggcactgctcttggagggaggcggccactaccgctgcgacttcaagaccacctaca

                 710       720       730       740       750       760       770       780       790       800
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  AAGCCAAGAAGGTGGTGCAGCTTCCCGACTACCACTTCGTGGACCACCGCATCGAGGTCGTGAGCCACGACAAGGACTACAACAAAGTCAAGCTGTACGA
mGFP      aagccaagaaggtggtgcagcttcccgactaccacttcgtggaccaccgcatcgagatcgtgagccacgacaaggactacaacaaagtcaagctgtacga

                 810       820       830       840       850       860
          ....|....|....|....|....|....|....|....|....|....|....|....|....
F1-1 rev  GCACGCCGAAGCCCACAGCGGACTACCCCGCCAGGCCGGCCATCATCACCACCATCACTAATAG
mGFP      gcacgccgaagcccacagcggactaccccgccaggccggctaa
```

Figure 5: DNA sequences of MPB70 fragment 1 clone 1 with reverse primer, aligned to the sequences of MPB70 and MGFP. A start codon ATG precedes the cloned MPB70 fragment-MGFP fusion. Six histidine codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons TAA & TAG.

```
                  10        20        30        40        50        60        70        80        90       100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
mbp70     AAAGDLVGPGCAEYAAANPTGPASVQGMSQDPVAVAASNNPELTTLTAALSGQL
F1-1 rev  IHMGDLVGPGCAEYAAANPTGPASVQGMSQDPVAVAASNNPELTTLTAALSGQLGVIKPDMKIKLRMEGAVNGHKFVIEGDGKGKPFEGKQTMDLTVIEG
mGFP      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GVIKPDMKIKLRMEGAVNGHKFVIEGDGKGKPFEGKQTMDLTVIEG

                 110       120       130       140       150       160       170       180       190       200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
F1-1 rev  APLPFAYDILTTVFDYGNRVFAKYPKDIPDYFKQTFPEGYSWERSTTYEDQGICIATNDITMMKGVDDCFVYKIRFDGVNFPANGPVMQRKTLKWEPSTE
mGFP      APLPFAYDILTTVFDYGNRVFAKYPKDIPDYFKQTFPEGYSWERSMTYEDQGICIATNDITMMKGVDDCFVYKIRFDGVNFPANGPVMQRKTLKWEPSTE

                 210       220       230       240       250       260       270       280
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
F1-1 rev  KMYVRDGVLKGDVNMALLLEGGDHYRCDFKTTYKAKKVVQLPDYHFVDHRIEVVSHDKDYNKVKLYEHAEAHSGLPRQAGHHHHHH**
mGFP      KMYVRDGVLKGDVNMALLLEGGGHYRCDFKTTYKAKKVVQLPDYHFVDHRIEIVSHDKDYNKVKLYEHAEAHSGLPRQAG*
```

Figure 6: Predicted amino acid sequence of MPB70 Fragment 1 clone 1 compared to MPB70 and MGFP, the proteins it was derived from. A start codon M precedes the cloned MPB70 fragment-MGFP fusion. Six H codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons (**).

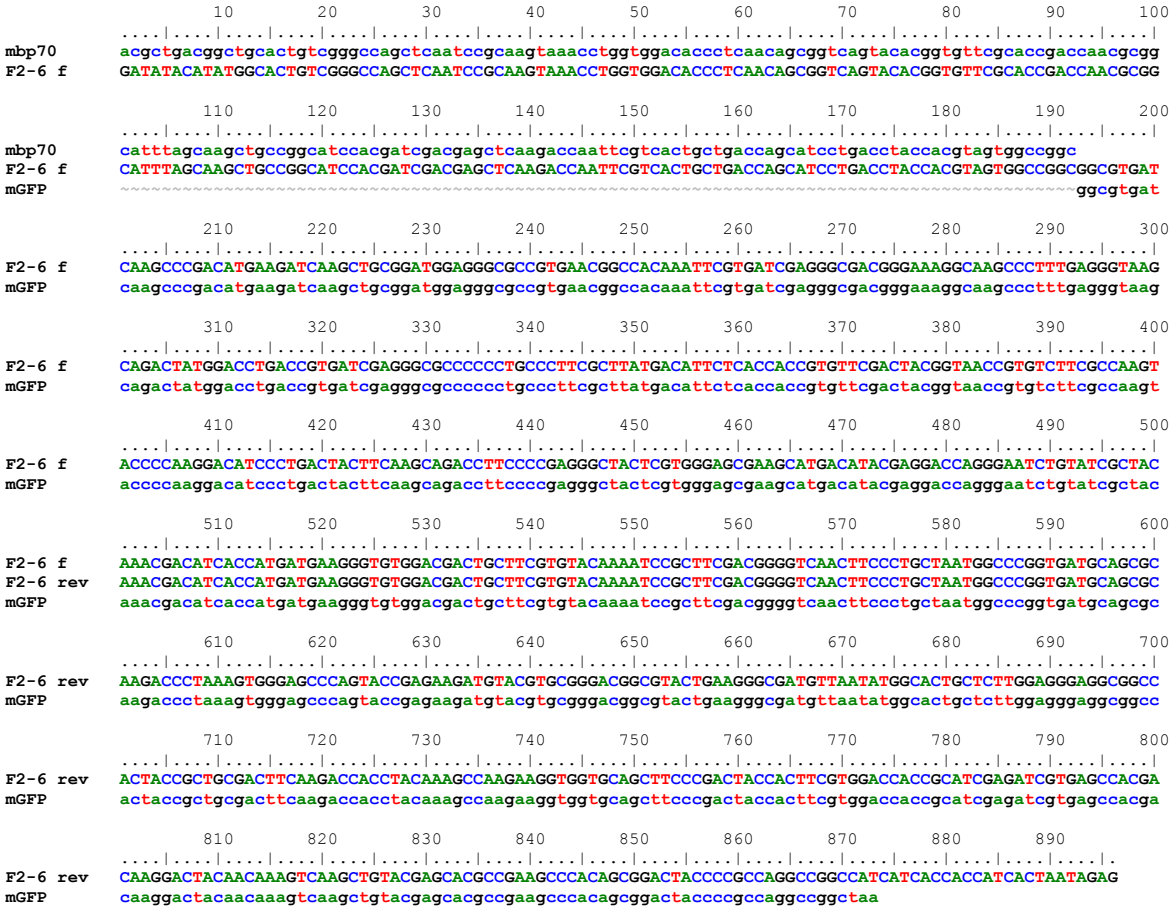

Figure 7: DNA sequences of MPB70 fragment 2 clone 6 with forward and reverse primers, aligned to the sequences of MPB70 and MGFP. A start codon ATG precedes the cloned MPB70 fragment-MGFP fusion. Six histidine codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons TAA & TAG.

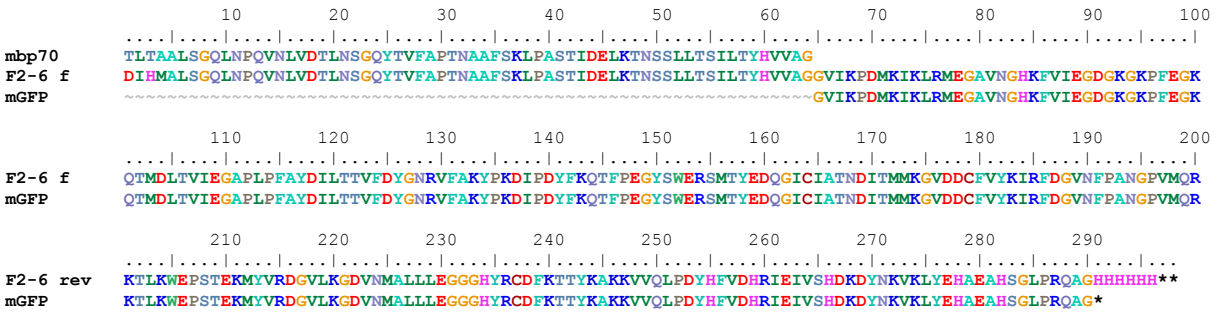

Figure 8: Predicted amino acid sequence of MPB70 fragment 2 clone 6 compared to MPB70 and MGFP, the proteins it was derived from. A start codon M precedes the cloned MPB70 fragment-MGFP fusion. Six H codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons (**).

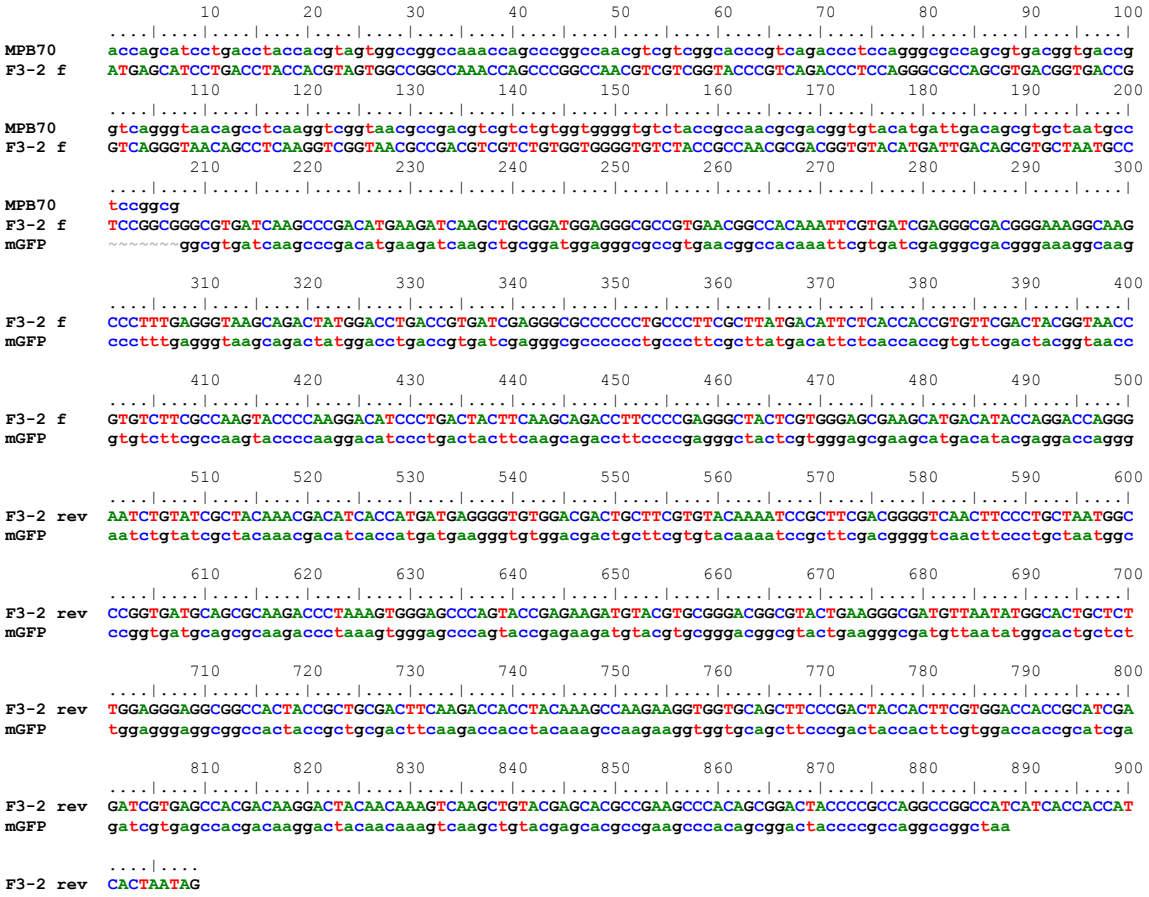

Figure 9: DNA sequences of MPB70 fragment 3 clone 2 with forward and reverse primers, aligned to the sequences of MPB70 and MGFP. A start codon ATG precedes the cloned MPB70 fragment-MGFP fusion. Six histidine codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons TAA & TAG.

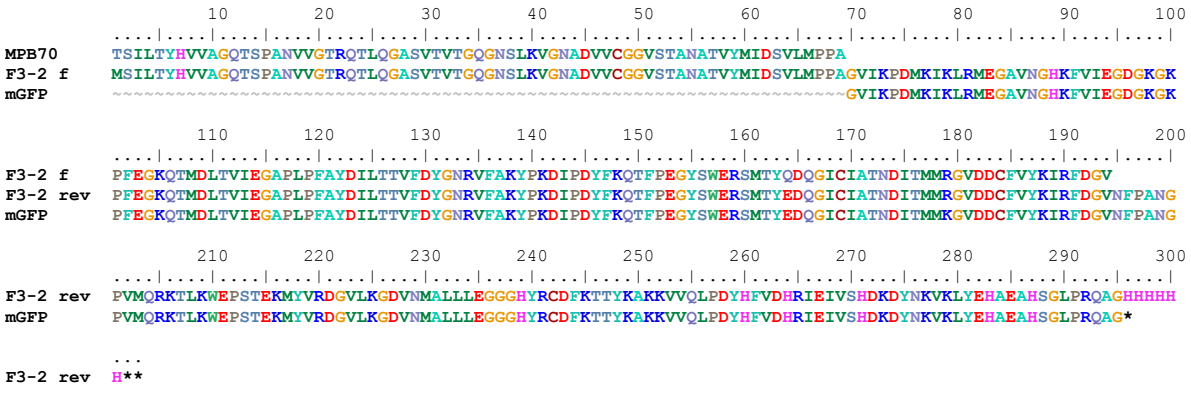

Figure 10: Predicted amino acid sequence of MPB70 fragment 3 clone 2 compared to MPB70 and MGFP, the proteins it was derived from. A start codon M precedes the cloned MPB70 fragment-MGFP fusion. Six H codons follow immediately after the cloned MPB70 fragment-MGFP fusion but precede two stop codons (**).

Appendix 4: Fragments and peptides derived from *mpb70* gene

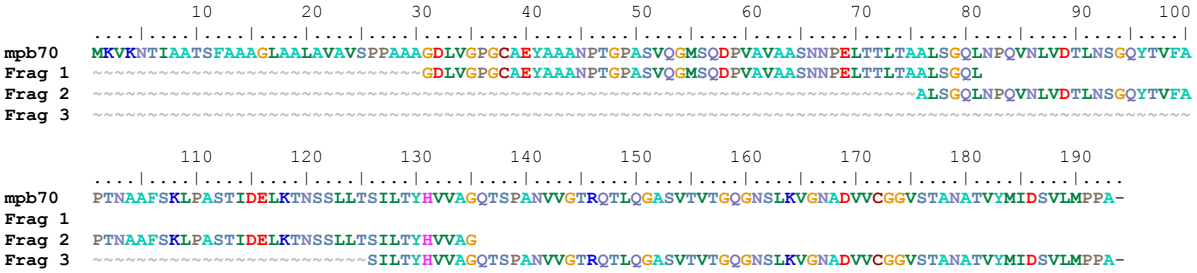

Figure 11: Deduced amino acid sequences of MPB70 fragments 1, 2 & 3 aligned to amino acid sequence of MPB70 protein

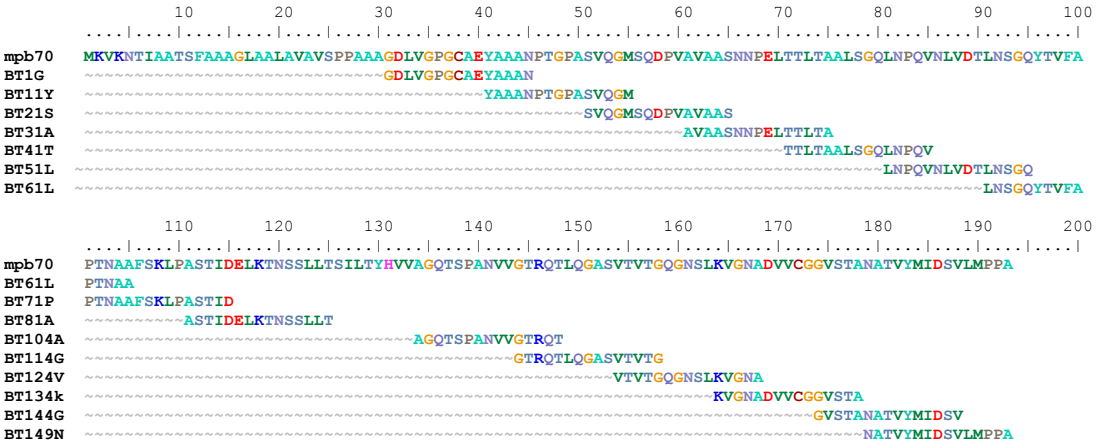

Figure 12: Synthetic peptides derived from MPB70 aligned to the deduced amino acid sequence of *mpb70*. The peptides overlap by five amino acid residues except peptide BT149N. There gap between peptide BT81A & BT104A indicates that there were no peptides synthesises because there were no epitopes predicted in that region.

Appendix 5: Comparison between MPB70 assays

	Antigen	Test	Origin of sera	Samples tested by ELISA (criterion)	Sensitivity (%)	Specificity (%)	Other comments
Current study 2012	**MPB70 Frag 2 - MGFP fusion protein**	**Indirect ELISA**	**South Africa (southern Africa)**	**Cattle** **1. Tuberculin skin test positive (32)** **2. BTB free (50)** **3. Non-tuberculous Mycobacterium exposed (10)** **Buffalo** **1. Tuberculous lesions (18)** **2. Bovigam negative (48)** **3. Non-tuberculous Mycobacterium exposed (35)**	**22** **11**	**92** **94**	False positive reactions: Catle (4/50) 8% Buffalo (3/48) 6.25%
Farias *et al.,* 2012	rMPB70	**Indirect ELISA**	Brazil (South America)	Cattle 1. Comparative intradermal tuberculin test (CITT) positive (53) 2. CITT negative (37)	88.7	94.6	False positive (2/37) 5.4% False negative (6/53) 11.7%
Cho, 1998	rMPB70	**Indirect ELISA**	Korea	1. Tuberculin skin test positive (29) 2. Tuberculin skin test negative(30)	89.7	100	
Yearsley *et al*., 1998	rMPB70	Indirect ELISA	Ireland	1. Lesion positive (32) 2. Lesion negative (1490)	31	82.6	False positive reactions 17.4%
Sudgen *et al.,* 1997	Native MPB70	Indirect ELISA	Canada & USA	1. Animals provided were from a variety of sources including USA and grouped into cattle, bison, llamas, fallow deer, elk, other Bovidae, Camelidae & Cervidae 2. Animals were considered either positive, negative or suspicious based on: Tuberculin kin test, culture, gross lesions, histopathology & experimental infection Cattle Bison	 65 78.9	 75 73.7	.
Wood *et al*., 1992	Native MPB70	Indirect ELISA	Australia, Wales & New Zealand	Cattle with a history of BTB	18.1	96.4	

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