USE OF RECOMBINANT PROTEIN FRAGMENTS FROM Mycoplasma mycoides subsp. mycoides SC TO IMPROVE CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP) T CELL AND ANTIBODY BASED ASSAYS

BY

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Abstract

Contagious bovine pleuropneumonia (CBPP), caused by the bacterium *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony type (*Mmm*SC), is a highly contagious respiratory disease that causes significant losses of cattle in more than 26 countries of sub-Saharan Africa. Development of reliable diagnostic tools is key to helping monitor disease prevalence and incidence. This study screened selected recombinant *Mmm*SC antigens for their potential in antibody based diagnostics and their ability to activate memory T cells. Gene fragments from the surface located *Mmm*SC molecules were expressed in *Escherichia coli* expression systems and the recombinant protein fragments purified for further usage. They were tested against ten sera from infected and uninfected cattle to evaluate their potential for improved CBPP diagnostic. They were also tested for their ability to stimulate memory T lymphocytes from experimentally *Mmm*SC infected cattle. Peripheral blood mononuclear cells (PBMCs) from ten cattle were stimulated with recombinant *Mmm*SC antigens for 72 hours and IFNγ secretion was measured using Bovigam kit (AgriQuality, Australia). The different responses elicited by the antigens were compared using student t-test. The results showed that none of the recombinant fragments induced IFNγ higher than the cut- off value of ($\geq$0.7). However, heat inactivated *Mmm*SC lysate induced IFNγ secretion ($P < 0.05$) but the recombinant fragments did not induce significant amounts of IFNγ secretion ($P > 0.05$) when preinfection and postinfection time points were compared. Also, none of the recombinant protein fragments was recognized by post infection sera. The results showed that antibody based diagnostics based on these particular recombinant protein fragments is not feasible. The study concluded that the recombinant protein fragments tested did not have strong T cell epitopes or T cells failed to migrate to circulation. Absence of antibodies suggested lack of B cell epitopes in the structure of these recombinant fragments.

CHAPTER ONE

1.0 **INTRODUCTION**

1.1 **Background Information**

Contagious bovine pleuropneumonia (CBPP), is a World Organization for Animal Health (OIE) notifiable disease and was included among the former list 'A' diseases (OIE, 2004), due to its significant impact on livestock. List A contains the most serious contagious animal diseases (Pilo *et al.,* 2003). Contagious bovine pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* small colony type (SC) is a severe lung disease affecting primarily cattle and rarely buffaloes (Provost *et al*., 1987). CBPP currently is among the economically most serious diseases of cattle in Africa, with estimated losses per annum at about $2 billion (Masiga *et al*., 1996). Economic losses are significant due to high infectivity and the presence of chronic sub clinical carriers. The disease is endemic in large areas of sub-Saharan Africa and can rapidly spread due to unrestricted cattle movements (March, 2002).

CBPP has been eliminated from Europe, North America and Australia mainly by slaughter of infected herds combined with movement restriction and vaccination campaigns. The application of a slaughter policy is not a likely possibility in Africa, due to budgetary constraints for refunding farmers and fragmented veterinary services (Jores *et al*., 2008). Among the potential control measures that have been given high priority by health organizations, the development of new generation vaccines and improved diagnostics, are recognized as the most appropriate and cost-effective CBPP-control measures (Thiaucourt, 2002).

1.2 **Statement of the Problem**

Serodiagnosis plays a key role in survey and control programs to combat CBPP (Bruderer *et al.*, 2002). Several serological tests have been described; these include slide agglutination, complement fixation test (CFT), agar gel precipitation, c-enzyme-linked immunosorbent assay, passive haemagglutination, western-blot and dot blot (Abdo *et al.*, 1998). CBPP control demands more sensitive tests. Currently the CFT is the OIE recommended test for the detection of CBPP. It is fairly sensitive in the acute phase, but becomes insensitive three months after infection (Poumarat *et al.*, 1989). The strong similarity in the components of the surface of *Mmm*SC with those of other, closely related, mycoplasmas causes serological cross-reactions which can lead to false positive diagnostic results (Stark *et al.*, 1995). In addition, the complexity of CFT makes it time consuming, expensive and difficult to accurately and reliably obtain results (OIE, 2004).

Successful estimation of vaccine efficacy depends heavily on correct classification of diseased (vaccine failure) and non diseased animals among the vaccinates (Plikaytis and Carlone, 2005) and this necessitates highly sensitive and specific diagnostic assays to monitor vaccination success and distribution of disease within the region. All together, the economic impact of CBPP urges the development of improved rapid, sensitive, and specific diagnostic assays for the detection of *M. mycoides* subsp. *mycoides* SC infections.

1.3 Research Questions

a). Which *Mmm*SC molecules are recognized by antibodies from infected animals and for how long after infection?

b). Which *Mmm*SC molecules have the potential to be recognized by *Mmm*SC- specific T cells and be used as tools for improved diagnosis of CBPP?

1.4 Objectives of the Study

1.4.1 General objective

To identify recombinant *Mmm*SC antigens with potential for the development of improved immunodiagnostic assays.

1.4.2 Specific objectives

a). To express and purify selected *Mmm*SC recombinant fragments cloned in *E. coli* expression system,

b).To screen selected *Mmm*SC recombinant fragments for their ability to detect memory T cell responses,

c).To screen selected recombinant *Mmm*SC recombinant fragments for their potential in antibody based diagnostics,

1.5 Study Hypotheses

a). *Mmm*SC recombinant protein fragments have ability to be recognized by memory T cells.

b). *Mmm*SC recombinant protein fragments are immunogenic and can be used as diagnostic target molecules for antibody detection.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Contagious Bovine Pleuropneumonia (CBPP)

Contagious bovine pleuropneumonia (CBPP) is a highly infectious respiratory disease characterized by the presence of sero-fibrinous, interstitial pneumonia and sequestra in the lungs of diseased cattle (Bashiruddin *et al.*, 2005). CBPP is characterized by low mortality and high morbidity in endemic areas. Acute inflammatory lesions in the lungs may lead to death due to respiratory distress but in most cases chronic infection occur causing weight loss and reduced fertility (Provost *et al.*, 1987). When the disease spreads for the first time within a sensitive cattle population, it generally causes high mortality. CBPP poses a threat to disease-free countries (FAO, 2003) and has been identified as a top priority transboundary animal disease (TAD) in Africa.

2.2 Historical Background and Distribution

Contagious bovine pleuropneumonia (CBPP) was first described by Gallo in Italy in 1550 under the name 'polmonera' (Newton and Norris, 2000). Epizootics occurred in 1828 in Belgium, in 1833 in the Netherlands, in 1840 the disease was reported in Britain and by 1884, CBPP was widespread in the United States of America (Newton and Norris, 2000). By the end of 19th century the disease had been eradicated, mainly by a policy of movement control and slaughter, from most of Europe and North America but was still present in Austria and Germany in 1920s and Spain and Portugal in 1990s (Newton and Norris, 2000). In Australia, CBPP was introduced

in 1858, by an English pedigree cow. However, the disease was not eradicated in Australia until 1973, more than a century since it was first reported, following an intensive ten year vaccination campaign (Newton and Norris, 2000; Thiaucourt *et al.*, 2004).

CBPP was introduced into South Africa from the Netherlands by a Friesian bull in 1853 and disseminated rapidly by trek oxen along the transport route. Within two years it had killed over 100 000 head of cattle resulting in starvation of tens of thousands of Xhosa people and devastation of the nation (Thiaucourt *et al.*, 2004). Other southern African countries such as Namibia, Angola, Zambia and Botswana reported the disease in late 1850s; introduced probably by infected cattle from trekkers who emigrated from South Africa. The disease was eradicated in South Africa and Botswana in 1924 and 1939, respectively. However, the disease was reintroduced in the North West district of Ngamiland in Botswana in 1995 from Namibia. CBPP was subsequently eradicated in Botswana in 1996 through slaughter of approximately 320 000 head of cattle in Ngamiland (Amanfu *et al.*, 2000; Windsor and Wood, 1998; Masiga *et al.*, 1996). The origin of the disease in West and Central Africa is obscure. CBPP is endemic in Nigeria but sporadic outbreaks occur in Cameroon. In Eastern Africa, the disease is thought to have been introduced from India during the Ethiopian invasion in 1867; although some authors believe it was introduced by British troops (Masiga *et al.*, 1996). The history of CBPP in Kenya dates back to 1901 when the first case was recorded in a bull at the Delamere farm in Naivasha (Masiga *et al.*, 1975; Wanyoike., 1999). By 1920s the disease had spread through the northern rangeland of Samburu, Marsabit, the entire North-eastern province, and Laikipia and Kajiado in the South (Wanyoike., 1999).

The joint CBPP/ Rinderpest mass vaccination campaigns in 1960s and 70s successfully controlled the two diseases in the African continent but CBPP was not completely eradicated. Unfortunately, since 1990s, there has been a significant re- emergence of the disease (Wanyoike, 1999).

Historically, CBPP has appeared almost in all parts of the world except South America and Madagascar (Thiaucourt *et al.*, 2004). Currently, CBPP is most widespread in Africa; present in Central, East, West and parts of Southern Africa but is absent in North Africa (Tambi *et al.*, 2006). In East Africa, countries such as Rwanda, Burundi, most parts of Tanzania, Southern Sudan, Ethiopia and Somalia have remained endemically infected with CBPP. In West Africa, CBPP is endemic in eastern Guinea, Mali, Niger and Mauritania. In Southern Africa, countries such as Angola and Zambia are endemically infected with CBPP while Zimbabwe, Lesotho, Swaziland, Malawi, Mozambique, Botswana and South Africa are at risk (Tambi *et al.*, 2006) and more than 26 sub-saharan countries reported cases of CBPP between 1995 and 2002. CBPP was eliminated in Europe in the 19th century, although the disease re-emerged more recently in Italy and southern France (1984-1993), and earlier in Portugal and Spain .These outbreaks were all brought under quick control and Europe remains free from CBPP (Newton and Norris, 2000). In Asia, CBPP has been reported recently in India, Bangladesh and Myanmar while in the Middle East, importation of African cattle is believed to be responsible for the sporadic outbreaks that have been identified (Hudson, 1971). CBPP was eradicated in the USA in 1892 by stamping out of infected herds (Provost *et al.*, 1987). The global distribution of CBPP,

although concentrated in Africa, clearly indicates that this disease remains an ever present threat to other parts of the world unless it is totally eradicated from Africa.

In Kenya, CBPP is endemic in the North and Northeast of the country from where it periodically escapes through uncontrolled cattle movement to cause epidemic outbreaks. Narok District in Kenya which neighbours Ngorongoro district in Tanzania is endemically infected (Kusiluka and Sudi, 2003; Wanyoike, 1999; Masiga *et al.*, 1996). In 1997, outbreaks occurred at two locations close to Nairobi city that were associated with the movement of trade stock (Wanyoike, 1999).

2.3 **Etiology and Taxonomy**

Mycoplasma mycoides subsp. *mycoides* small colony type (*Mmm*SC) is the etiological agent of CBPP (FAO, 2003). *Mycoplasma* species are the smallest free-living prokaryotes found on Earth (Razin *et al.*, 1998). *Mmm*SC belongs to the genus *Mycoplasma*, phylum Firmicutes, class Mollicutes, order Mycoplasmatales and family Mycoplasmataceae. *M. mycoides* subsp. *mycoides* SC belong to the *M. mycoides* cluster, a group of six closely related mycoplasmas (Walker, 2004), including *M. mycoides* subsp. *mycoides* Large Colony (*Mmm*LC), *M. mycoides* subsp. *capri, Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *Mycoplasma* sp. bovine serogroup 7 (Walker, 2004). They share biochemical, immunological and genetic characteristics (Johansson *et al.*, 1996).

2.4 Biology of *M. Mycoides* subsp. *mycoides*

The genus *Mycoplasma* is widely spread in nature as parasites of humans, mammals, reptiles, fish, arthropods, and plants (Razin *et al.,* 1998). Some close relatives of *Mmm*SC with medical importance include *Mycoplasma pneumoniae* that causes atypical pneumonia in humans, *Mycoplasma penetrans* that has the ability to penetrate mammalian host cells and *Mycoplasma genitalium* that preferably colonizes the genital tract (Waites *et al.*, 2005). Examples of mycoplasmas with veterinary importance include the porcine *Mycoplasma suis* (Nicholas, 2004) that infects red blood cells causing porcine eperythrozoonosis, *Mycoplasma bovis*, the most common pathogenic mycoplasma in Europe and North America that causes a wide range of bovine conditions, (Nicholas, 2004) *Mycoplasma gallisepticum*, the primary agent of chronic respiratory disease in chickens, and infectious sinusitis in turkeys causing important economic losses in poultry industry (Nicholas, 1998) and *Mycoplasma mycoides* subsp. *mycoides* large colony which causes septicaemia in goats and sheep (Nicholas, 1998). By 1998, close to 80 species of mycoplasma had been isolated (Razin *et al.*, 1998).

Mycoplasma mycoides subsp. *mycoides* SC PG1 strain has a genome of 1211kb (Westberg *et al.*, 2004), lacks a cell wall, is fastidious *in vitro* and has a tendency to form centered colonies on solid medium (Nicholas, 2004). Mycoplasmas are inherently resistant to those antibiotics that target the cell wall; in addition they have higher mutation rates than conventional bacteria thus they can rapidly develop resistance to drugs (Ayling *et al.*, 2000). They are sensitive to compounds that interfere with protein and nucleic acid synthesis. Since they lack a cell wall they are fragile organisms; consequently successful infection is restricted to close and repeated

aerosol transmission. Mycoplasmas grow slowly and generally require 3 to 7 days' incubation before colonies become apparent. Growth is best at 37 ^{0}C in an atmosphere of increased CO_2. Because of total or partial inability to synthesize fatty acids, exogenous sterols are required for their growth (Walker, 2004).

2.5 **Epidemiology**

2.5.1 **Reservoir**

The only reservoir for *Mmm*SC is the cattle itself, as some hosts recover from disease but remain potential carriers of the pathogen (FAO, 2003). Introduction of an infected animal into a naïve population accounts for dissemination of infection (Walker, 2004). Asymptomatic carriers, serve as the source for maintaining CBPP in a population. It has been reported that outbreaks occurred when healthy and carrier animals gather at river banks during the dry season (Nwanta and Umoh, 1992).

2.5.2 **Susceptible animals**

Cattle (*Bos taurus* and *Bos indicus*) are the main species susceptible to CBPP. The domestic buffalo (*Bubalus bubalus*) is also susceptible (Provost *et al.*, 1987).

2.5.3 **Transmission**

Direct contact is the principal mode of transmission although wind-borne and indirect transmission cannot be excluded (Regalla *et al.*, 1996). Transmission occurs primarily through the inhalation of infected droplets from a clinically sick or carrier animals (Kusiluka and Sudi,

2003). Infection can also be acquired from fodder and fomites contaminated with infected urine and fetal fluids (Masiga and Domenech, 1995).

2.5.4 **Clinical features**

There is considerable variation in severity of signs observed in cattle affected by CBPP, ranging from hyper acute through acute to chronic and subclinical forms. Respiratory distress, nasal discharge and coughing are the main signs of CBPP. The early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy. Animals show dullness, anorexia, reluctance to move, irregular rumination with moderate fever. Coughing is usually persistent. As lung lesions develop, the signs become more pronounced with increased frequency of coughing (Provost *et al*., 1987). The pathological signs are usually characteristic with marked pleural adhesion accompanied by exudative pericarditis (Regalla *et al*., 1996). Figures 2.1 and 2.2 shows the thoracic cavity of infected cattle yellow sero fibrinous exudate and marbled appearance of a cut lung surface respectively.

In the sub acute form, signs may be limited to a slight cough only noticeable when the animal is exercised. The outcome of CBPP in naïve herds ranges from deaths to total recovery of the affected animals (Bashiruddin *et al*., 2005). Lesions containing viable *Mmm*SC may remain in a number of cattle hence these become healthy carriers. CBPP in Europe, unlike that caused in Africa where mortality rates are typically 10-70% in epizootics, is characterized by low morbidity and low or non-existent mortality with the majority of infected cattle showing chronic lesions (Masiga *et al*., 1996).

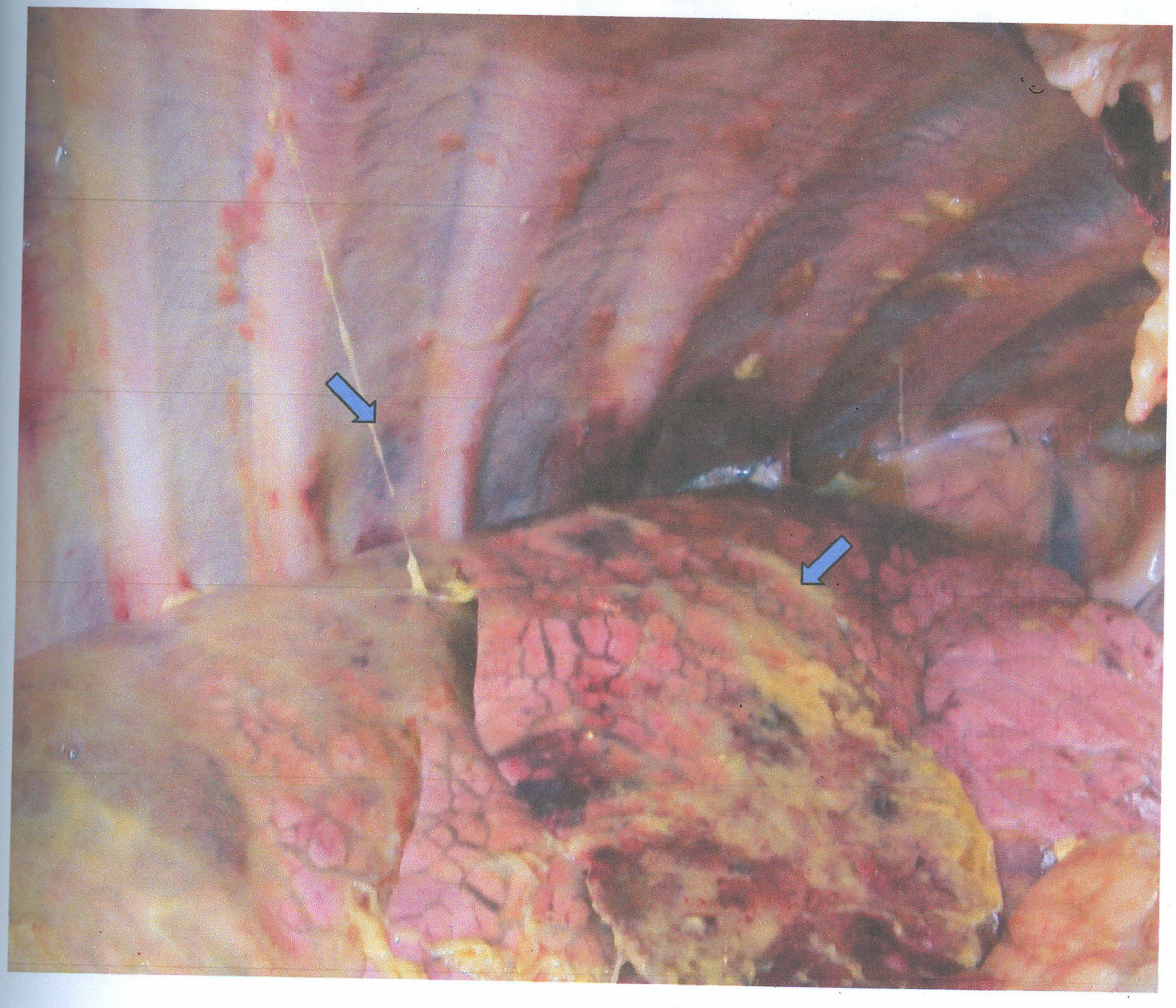


Fig 2.1 Thoracic cavity of cattle experimentally infected with *Mmm*SC arrows showing yellow sero fibrinous exudate and lung adhesion to the costal wall (Picture provided by Mulongo, ILRI Nairobi, Kenya).

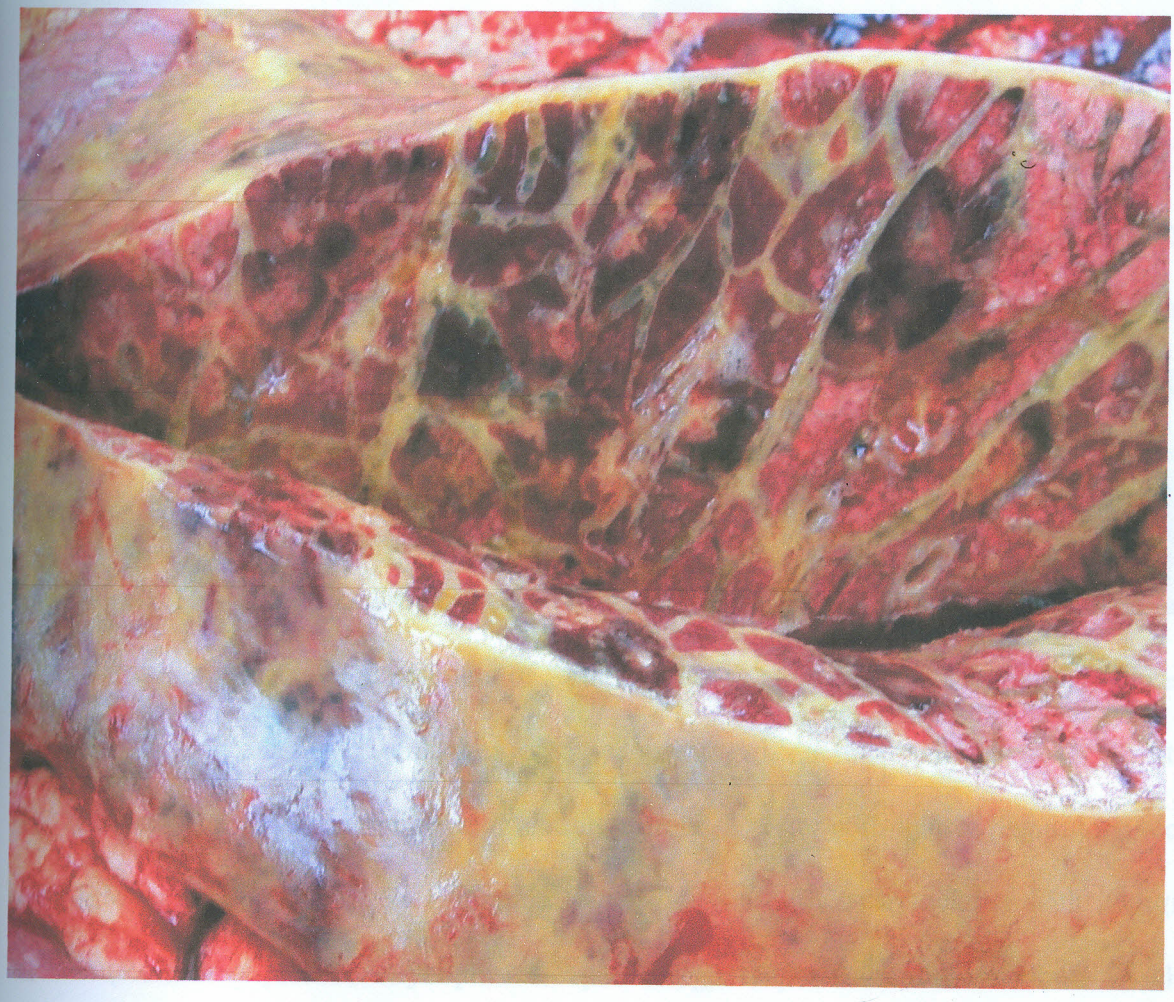


Fig 2.2 Cut surface of a lung in experimental *Mmm*SC infected cattle showing marbling and thickened interlobular septae (Picture provided by Mulongo, ILRI, Nairobi, Kenya).

2.6 Immunity and Immune Responses to *Mmm*SC

2.6.1 Humoral responses

IgM is the first antibody to be produced after mycoplasma infection of a naïve host (Cassell *et al.*, 1974). Since *Mmm*SC is a respiratory pathogen, it is expected that the local antibody response will play a major role by inhibiting the growth of the pathogen as well as its attachment to host cells (Niang *et al.*, 2006). Evidence of the important role of antibodies in local immune

responses has been shown in other mycoplasma species (Simecka, 2005). The protective role of antibodies during *Mmm*SC infection has been demonstrated by Masiga *et al*, (1975). These authors showed that transfer of sera from cattle that had recovered from CBPP was able to confer protective immunity to recipient calves. Molecular mechanisms that antibodies use to inhibit the growth of mycoplasma are not understood although it is likely that the surface exposed proteins of *Mmm*SC are likely targets of antibody responses (Kiarie *et al.*, 1996).

Plasma cells residing beneath the mucosa produce IgA in response to mucosal stimuli. The major function of IgA is to block attachment of a pathogen to the mucosal surface, but IgA can enhance the killing of organisms by enzymes or other agents within mucosal secretions (Roitt *et al.*, 2002). Resistance to *M. pneumoniae* infection correlates better with IgA in respiratory secretions than IgM or IgG serum levels (Fernald, 1979). IgE antibody is associated with allergies and asthma (Roitt *et al.*, 2002). Mycoplasma- specific IgE responses could contribute to wheezing and difficulty in breathing found in some mycoplasma respiratory disease (Seggev *et al.*, 1996).

2.6.2 **Cell mediated responses**

T cells are an important component of the inflammatory response during mycoplasma infection (Rodriguez *et al.*, 2000). A more recent study has shown no correlation between elevated IFNγ responses of PBMCs with mild disease or protection (Jores *et al.*, 2008). The study did not observe a correlation between IFNγ release of animals with and without pathomorphological gross lesions. Therefore, the study did not confirm a role for $CD4^+$ T- lymphocytes in protection. Earlier, the T cell compartment responsible for cellular responses had been identified as IFNγ

producing MHC-class II restricted $CD4^+$ T lymphocytes of the T-helper 1 (Th_1) phenotype. (Dedieu *et al.*, 2005). In animals infected either endo-bronchially or by contact, a strong correlation between the prolonged and persistent presence of these cells and survival of the animals had been observed. Conversely, cattle with acute disease or increased pathology showed lower levels of PBMC-derived IFNγ. Further evidence for the critical role of $CD4^+$ T cells is the observation that one year after recovery of naturally infected animals, IFNγ producing $CD4^+$ lymphocytes persisted in lymph nodes (Dedieu *et al.*, 2006). In cattle that recovered with accompanying sequestra formation, the magnitude of these responses was lower. This study also suggested that in recovered animals, a subset of IFNγ producing $CD4^+$ T cells home onto regional lymph nodes and are responsible for this memory response. Cellular immune responses mediated by $CD8^+$ T cells have not been characterized (Dedieu *et al.*, 2005).

2.7 **Treatment, Control, Prevention and Vaccination of CBPP**

Strategies to control CBPP mainly focus on three aspects: prevention of transmission by either quarantine or killing whole infected herds (stamping out), antibiotic treatment of infected cattle, and large scale vaccination (Thomson, 2005). Quarantine involves the restriction of movement of infected cattle and is one of the most difficult CBPP control measures since it involves close collaboration between communities and governments. In Africa, this is difficult to achieve due to political, socio-economic and logistical considerations (Windsor and Wood, 1998). In this region, control of cattle movement is rendered impossible by porous borders, the pastoralist and transhuman lifestyles of many ethnic groups, inter-tribal cattle thefts and the inability of government animal health authorities to enforce regulations that govern animal movement

(Thomson, 2005). Stamping out when coupled with other measures as a means of eradicating CBPP has historically been the main method resorted to in the event of outbreaks and has been shown to be the most effective (Provost *et al.*, 1987; Newton and Norris, 2000). Nonetheless, as in the examples of Botswana and South Africa destruction of infected herds always leave behind tragic socio-economic losses due to indiscriminate measures that are employed without consideration to the livelihoods of affected farmers (Thomson, 2005).

Some antimicrobials with mycoplasmidal activity include danofloxacin, tilmicosin, tetracycline, spectinomycin and florfenicol (Ayling *et al.*, 2000). In Africa, antibiotic therapy is discouraged by government veterinary departments although it is still widely practiced by farmers on an individual basis (Thomson, 2005). Studies with long-acting tetracycline and danofloxacin show reduction of both CBPP livestock losses and disease transmission from infected animals to healthy ones (Thiaucourt *et al.*, 2004; Hubschle *et al.*, 2002). Nonetheless, scientific opinion on this issue is still divided, with other experts insisting that antibiotic treatment of CBPP favours development of lung sequestra, carrier animals, and antibiotic resistant strains and complicates detection of clinical disease hence causing surveillance difficulties (Provost *et al.*, 1987; Thiaucourt *et al.*, 2003).

Vaccination is the most widely used measure of CBPP control in Africa (Thiaucourt *et al.*, 1998). Currently the authorized vaccines in use are the live attenuated T1/44 strain and the T1sr strains, both derived from an isolate from Tanzania that has reduced virulence due to many passages. Thiaucourt *et al,* (2004) estimated that the efficacy of T1/44 after primary inoculation

is 40-60% and increases to 80-95% on re-vaccination. However, the immunity against both T1/44 and Tsr is short lived and generally lasts for less than one year (OIE, 2004; Thiaucourt, 2003). Unfortunately, vaccination has numerous pitfalls that have made it ineffective in Africa (March, 2002). These include the nature of the vaccines, the breadth of immune responses stimulated (Thiaucourt *et al.*, 2004), low quality control of vaccines manufactured in Africa (Thomson, 2005), vaccine effects that vary according to virulence of mycoplasma strains, possibility of reversion to virulence (Mbulu *et al.*, 2004) as well as the occurrence of post vaccination reactions (Thiaucourt *et al.*, 2004).

2.8 Serological Diagnosis of CBPP

2.8.1 Complement fixation test (CFT)

The CFT is the approved World Organization for Animal Health (OIE) test, although specific, it lacks sensitivity, with a positive result being any reaction at 1/10 or higher and requires highly trained staff and laboratory facilities to perform it accurately and consistently and laboratoty facilities (Regalla *et al.*, 1996). In addition, it is less effective at diagnosing animals in the early stages of the disease or of animals with chronic lesions (OIE, 2002).

2.8.2 Competitive enzyme-linked immunorsorbent assay (cELISA)

cELISA was designated an alternative test by the OIE International committee in May 2000 and as a prescribed test for international trade in May 2004 (OIE, 2004). Compared to CFT, cELISA has equal sensitivity and greater specificity. It is a herd test that is easier to perform than CFT,

but its performance characteristics have not yet been fully assessed (Le Goff and Thiaucourt, 1998).

2.8.3 **Lipoprotein Q (LppQ) test**

LppQ has a strong antigenic N-terminal part which is located on the outer surface of the membrane, while its C-terminal is involved in membrane anchoring. The high specificity and strong antigenicity of LppQ have been exploited for the development of a robust indirect ELISA test for serological diagnosis and for epidemiological investigations of CBPP (Bruderer *et al.*, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

The *Mmm*SC strains were cultured as stationary cultures for 3 days at 37 ^{0}C in 1000 ml bottles in Eaton's growth media, containing 21 g of PPLO broth base, 20% horse serum, 10% yeast extract, 10 g of glucose, 0.5 ml of penicillin (200 000 IU/ml), and 12.5 ml of 0.2% phenol red. Mycoplasmas were collected in log phase growth by centrifugation at 10 000g for 30 minutes at 4 ^{0}C and washed three times in phosphate buffered saline buffer (PBS; 150 mM NaCl, 1.5 mM KH_2P0_4, 9 mM Na_2HPO_4 x 12 H_2O, 2.5 mM KCl pH 7.2). Washed cell pellets were stored at -20 ^{0}C. The PG1 strain was used for genomic DNA extraction while the Afade strain was used for experimental endobronchial infection. For *in vitro* antigen stimulation assays, Afade strain was resuspended in 1 ml of PBS in 2 ml Eppendorf tubes and ruptured using 350 mg of zirconium beads (0.1 mm diameter) in Fast prep Instrument four times. Cell debris was removed by centrifuging four times at 10 000g at 4 ^{0}C for 10 minutes each time collecting the supernatant. The Afade strain was heat-inactivated by boiling at 100 ^{0}C for 10 minutes in a water bath, aliquoted and stored at -20 ^{0}C.

3.2 Genomic DNA Extraction

Cell pellets that had been stored at -20 ^{0}C were washed once in 0.01 M Tris- HCl, pH 8.0, 0.01 M EDTA at 4 ^{0}C and the pellet used immediately. The cell pellet was resuspended in 2.5 ml of 0.01 M Tris-HCl, pH 8.0, 0.01 M NaCl, 0.01 M EDTA. To 100 µl aliquots, 500

µl of 5 M guanidium thiocyanate, 0.1 M EDTA, 0.5% sarcosyl was added and held at room temperature for 10 minutes. The tubes were placed on ice and 250 µl of 7.5 M ammonium acetate, pH 7.7, was added to the tubes. DNA from the lysate was extracted thrice with 0.5 ml of phenol: chloroform: isoamyl alcohol; each time the emulsion was centrifuged at 13 000g for 10 minutes and the upper aqueous phase transferred to another tube. To the final aqueous phase, 600 µl of 2- propanol was added and DNA pelleted by centrifugation at 13 000g for 15 minutes. The supernatant was discarded and the DNA pellet washed three times with 80% ethanol by centrifugation at 13 000g for 10 minutes. The supernatant was discarded and the DNA dried in air for 15 minutes. The DNA was resuspended in 100 µl water and stored at -20 ^{0}C.

3.3 **PCR Amplification of Mycoplasma Genes**

PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 9780, USA) in a 50 µl reaction mixture containing 5 µl of 25 mM of $MgCl_2$, 10 µl of 5 X PCR buffer, 1 µl of 10 mM of each dNTP, 3 µl of 10 pmol of forward and reverse primer, 0.25, µl of AmpliTaq DNA polymerase (Promega, USA) and 1 µl of genomic template DNA. A drop of mineral oil was used in each reaction tube. The DNA thermocyler was programmed to perform the following steps: 94 ^{0}C for 2 minutes followed by 94 ^{0}C for 1 minute, 53 ^{0}C for 1 minute, 72 ^{0}C for 90 seconds, this was repeated 34 times, with a final 7 minutes extension step at 72 ^{0}C before cooling to 4 ^{0}C. Negative controls consisted of PCR mix and water without the addition of DNA.

3.3.1 **PCR product purification**

PCR product was purified by QIAquick kit (Qiagen, Germany). The PCR product purification kit combines the convenience of the spin- technology with the selective binding properties of a uniquely designed silica membrane. Special buffers provided with the QIA quick PCR purification kit are optimized for efficient recovery of DNA and removal of contaminants. DNA adsorbs (pH $\leq$ 7.5) to the silica membrane in the presence of high concentrations of salt while contaminants pass through the column. Impurities are efficiently washed away, and the pure DNA is eluted (pH 7 – 8.5 and low salt concentrations) with Tris buffer or water.

250 µl of binding buffer PB1 was added to 50 µl of the PCR sample and mixed. A QIA quick spin column was placed in a 2 ml collection tube. The sample was applied to the QIA quick column and centrifuged for 60 seconds at 10 000g. The flow through was discarded and the QIA quick column placed into the same tube. 0.75 ml ethanol containing buffer PE was added to the QIA quick column to wash and centrifuged for 60 seconds at 10 000g. The flow through was discarded and the QIA quick column placed back into the same tube. The column was centrifuged for an additional 1 minute. The QIA quick column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl elution buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIA quick membrane and the column centrifuged for 60 seconds at 10 000g.

3.3.2 **Checking the PCR product**

One percent agarose gel electrophoresis was used to verify the quality and quantity of the PCR product. A clean gel tray was assembled and the appropriate size comb to contain the agarose gel on a flat, even surface set. To prepare a one percent agarose gel, one gram of agarose was weighed and put into a 250 ml conical flask, 100 ml of 1X TAE was measured and added into the flask. The mixture was heated to boiling point by a microwave oven to melt the agarose to a homogenous solution. The mixture was stirred by a magnetic stirrer until it stopped to steam and cooled to about 50 ^{0}C. Then, 3 µl of 10 mg/ml solution of ethidium bromide was added into the liquid. The solution was swirled to mix the ethidium bromide and the mixture cooled at room temperature until it could be held by hand. The gel was poured into the tray avoiding bubbles, and the solution given 30 minutes to gel. During this time, the PCR products to be analyzed were prepared by adding 5 µl of each reaction to 1 µl of gel loading dye into clean 96 well microtitre plate. 5 µl of Phi X marker was also added to 1 µl of gel loading dye. The gel was placed into the tank (Kodak Biomax, USA) with 1X TAE ensuring that the gel was submerged. 6 µl of the samples and molecular marker were loaded into the wells. The tank cover was replaced, the plugs attached to the power supply, and voltage applied at 80 V (Biorad Model 3000Xi, USA) for 45 minutes until the dye reached 2 cm from the bottom of the tray. Thereafter power was turned off, and the gel apparatus disconnected from the power supply. The gel tray was removed and taken to the transilluminator to view the fluorescent DNA bands with UV radiation. The results were documented by photography

of the gel. The amount of sample DNA loaded was estimated by visual comparison of the band intensity with that of the marker. The PCR products were adjusted to a concentration of 2ng/ul.

3.4 **Ligation of DNA into the Cloning Vector**

pBAD 102/D-TOPO expression vector (Invitrogen, USA) was used to obtain recombinant polyhistidine (HisTag) fusion proteins. Briefly, 0.5 µl of fresh PCR product was mixed with 0.5 µl salt solution, 1.2 M NaCl, 0.06 M $MgCl_2$, 0.5 µl pBAD102/D-TOPO vector and sterile water added to a final volume of 5 µl. The reactions were mixed gently and incubated for 5 minutes at room temperature (22-23 ^{0}C). The reaction tubes were placed on ice before transformation.

3.4.1 **Transformation of one shot TOP10 competent cells**

3 µl of the TOPO cloning reaction was added into a vial of one shot TOP10 chemically competent *E. coli* and mixed gently. This was incubated on ice for 10 minutes. The cells were heat shocked for 30 seconds at 42 ^{0}C without shaking. Immediately the tubes were transferred to ice. 250 µl of room temperature Super Optimal broth Catabolite repression (S.O.C) medium was added. The snap cap tubes were capped and horizontally shaken at 200 rpm at 37 ^{0}C for 1 hour to allow expression of the antibiotic resistance gene. 200 µl from each transformation was spread on a prewarmed selective plate and incubated overnight at 37 ^{0}C. The next day, total colonies were counted. Five colonies were picked

and labeled for analysis. Five single colonies were streaked and spread on a LB plate and incubated overnight at 37 ^{0}C.

3.4.2 **Analyzing transformants**

Five single colonies were picked and each inoculated overnight into 3 ml LB containing 50 µg/ml ampicillin for plasmid DNA isolation.

3.5 **DNA Isolation**

3.5.1 **Plasmid DNA isolation**

Plasmid DNA was extracted using QIAprep kit (Qiagen, Germany). The QIAprep miniprep procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt. The optimized buffers in the lysis procedure combined with the unique silica-gel membrane ensures that only DNA is adsorbed, while RNA, cellular proteins, and metabolites are not retained on the membrane but found in the flow through. Endonucleases are removed by wash buffer PB, to ensure that plasmid DNA is not degraded. Buffer PE removes salts while DNA is eluted using buffer EB or water.

E. coli overnight culture was spun at 3 000g for 5 minutes. Pelleted bacterial cells were resuspended in 250 µl resuspension buffer P1 and transferred to a microcentrifuge tube. 250 µl of buffer P2 was added and gently the tube was inverted 6 times to mix. 350 µl of buffer N3 was added and the tube immediately inverted gently 6 times. The tube was

centrifuged for 10 minutes at 10 000g and the supernatant applied to the QIA prep spin column by decanting. The spin columns were centrifuged for 60 seconds and the flow through discarded. The QIA spin column was washed by adding 0.5 ml buffer PB and centrifuging for 60 seconds. The flow through was discarded. The QIA prep spin column was washed by adding 0.75 ml buffer PE and centrifuging for 60 seconds. The flow through was discarded and centrifuged for an additional minute to remove residual wash buffer. The QIA prep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl buffer EB (10 mM Tris- Cl, pH 8.5) was added to the center of each QIA prep spin column, left to stand for a minute and centrifuged for 60 seconds at 10 000g.

3.5.2 **Agarose gel analysis of plasmid DNA**

1.5% agarose gel was used to visualiaze the plasmid DNA. Briefly, 2 µl of bromophenol loading dye was mixed with 10 µl of eluted pure plasmid DNA and loaded into the wells. 3 µl of Hind III DNA marker was loaded as a marker. The gel was illuminated with UV light to visualize the DNA.

3.5.3 **Measurement of DNA concentration**

Plasmid DNA concentration was determined by use of nanodrop ND- 1000 UV spectrophotometer. The concentration was then adjusted to 50 ng/ml before being sent for sequencing.

3.6 **Preparation of Chemically Competent *E. coli* Cells**

A single bacterial colony from a plate that had been incubated overnight at 37 ^{0}C was picked and transferred into a 25 ml of LB broth in a 250 ml flask. The culture was incubated for 6 hours at 37 ^{0}C with vigorous shaking at 225 rpm. 4 ml of this starter culture was used to inoculate 250 ml of super optimal broth (SOB) in 1 liter flask overnight at 18 ^{0}C with moderate shaking at 200 rpm. The following day, the OD_{600} was monitored up to 0.55 when the vessel was transferred to an ice-water bath for 10 minutes. The cells were harvested by centrifugation at 2 500g for 10 minutes at 4 ^{0}C. The medium was poured and the open centrifuge bottle was stored on a stack of paper towels for 2 minutes. The cells were resuspended by swirling in 80 ml of ice cold Inoue transformation buffer. Cells were harvested by centrifugation at 2 500g for 10 minutes at 4 ^{0}C. The medium was poured and the open centrifuge tube stack on paper towels for 2 minutes. The cells were resuspended gently in 20 ml of ice cold Inoue transformation buffer and 1.5 ml of DMSO added. The bacterial suspension was mixed by swirling and then stored on ice for 10 minutes. Working quickly, 100 µl aliquots of the suspensions were dispensed into chilled, sterile microfuge tubes. Immediately, the competent cells were snap-freezed by immersing the tightly closed tubes in a bath of liquid nitrogen. Then the tubes were stored at -80 ^{0}C until needed.

3.7 **Expression of Recombinant Protein Fragments**

Briefly, *E. coli* clones containing the desired recombinant plasmid (pBAD102/D- TOPO) were cultivated overnight in 4 ml of LB containing 50 µg/ml ampicillin at 37 ^{0}C in a

shaking incubator (New Brunswick, USA), 225 rpm to OD_{600} = 1 -2. The next day, 10ml of LB containing 50 µg /ml ampicillin was put in a 14 ml Sterilin^{Tm} tube and 0.1 ml of the overnight culture added. The cultures were grown at 37 ^{0}C with vigorous shaking to an OD_{600} = 0.5 (mid log phase). 1 ml aliquot of cells was removed from each tube, centrifuged at 10000g in an Eppendorf 5402 microcentrifuge for 30 seconds, and the supernatant discarded. The cell pellet was frozen at -20 ^{0}C and this was the zero time point sample. Four 10-fold serial dilutions of 20% arabinose were prepared with sterile water using aseptic techniques (2%, 0.2%, 0.02%, and 0.002%). Expression was induced with 90 µl different concentrations of arabinose added to the five 9 ml cultures. The cultures were grown at 37 ^{0}C with shaking for 4 hours; 1 ml samples would be removed, centrifuged at 10 000g for 30 seconds, supernatant aspirated and cell pellet frozen at -20 ^{0}C. These were the 4 hour time point samples. This was repeated at 6 hour time point.

After confirmation of the cells expressing the recombinant proteins and determination of the optimal conditions for expression, large scale growth was done. Briefly, 100 ml of LB broth containing 50 µg/ml ampicillin was added to 250 ml flat conical flask. 1 ml of the overnight culture was added to each of the flasks. The cultures were grown at 37 ^{0}C with vigorous shaking to an $O.D_{600}$ = 0.5 (mid-log phase). They were induced with the optimal arabinose concentration and incubated with vigorous shaking at 225 rpm for the optimal time. The bacterial cells were then harvested by centrifugation at 2 500g for 30 minutes in 50 ml Falcon^{Tm} tubes, and the pellets thawed for 15 minutes on ice and cells resuspended in 4 ml lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole pH

8.0). Lysozyme was added to a concentration of 1 mg/ml and incubated on ice for 30 minutes and lysed by sonication with six 10 second bursts in 50 ml Falcon^{Tm} tubes. The lysate was centrifuged at 10 000g for 30 minutes at 4 ^{0}C to pellet the cellular debris. 5 µl of 1X SDS sample buffer was added to 5 µl of supernatant for SDS-PAGE analysis.

3.7.1 **SDS-PAGE**

The crude induced and uninduced lysates of the samples were resuspended in 80 µl of 1 X SDS-PAGE sample buffer, boiled for 5 minutes and centrifuged at 10 000g for 15 seconds. Glass plates were prepared by scrubbing with lint-free tissue paper wetted with 70% ethanol. The gel sandwich was assembled by placing the spacers at either side of one glass plate and placing the second plate on top of the spacer. The spacer and the glass plates were aligned at both ends and a clamp applied on either side of the sandwich. The clamped sandwich was placed onto the pouring stand. Resolving and stacking gel mixtures were prepared without adding ammonium persulfate. 100 µl of 10% ammonium persulfate was added to the resolving gel mixture and gently swirled to mix and then poured into the gel using a pipet. The resolving gel was poured to a height of 1 cm below the teeth of the comb when fully inserted. The resolving gel was overlaid with 200 µl of 95% ethanol and allowed to polymerize. The stacking gel was poured on top using a pipet and immediately the comb was inserted. The gel sandwich was placed in an assembly apparatus. The upper buffer chamber was filled with 1 X electrode buffer. The frozen time point pellets were resuspended in 80 µl of 1 X SDS sample buffer. The proteins were resolved on a 10% SDS-PAGE (water, 1.5 M Tris buffer, 0.5 M Tris buffer, 10%

SDS, Temed, 30% acrylamide and 10% APS) in a gel apparatus (ATTO corporation, Japan) and stained with Coomassie brilliant blue, destained and photographs of the gels taken.

3.7.2 **Purification of recombinant protein fragments**

The recombinant proteins were purified under native conditions by metal affinity chromatography with nickel- nitriloacetic acid resin (Ni-NTA), (Qiagen, Germany). Briefly, 1 ml of the 50% Ni-NTA slurry was added to 4 ml of the cleared lysate and mixed gently by shaking at 200 rpm on a rotary shaker at 4 ^{0}C for 60 minutes. The lysate-Ni-NTA mixture was loaded into a column with the bottom outlet capped. The bottom cap was removed, column flow-through collected for SDS-PAGE analysis. The column was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0) and wash fractions collected for SDS-PAGE analysis. Elutions were carried out three times each time with 0.5ml elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 8.0).The elutes were collected in three tubes and analyzed by SDS-PAGE. The recombinant protein were aliquoted and stored in 100 ul aliquots at -80 ^{0}C.

3.7.3 **Measurement of protein concentration**

Protein concentration was determined by MicroBCA Protein assay reagent kit (Pierce, USA). The BCA assay measures the formation of Cu^{+} from Cu^{2+} by the Biuret complex in alkaline solutions of protein using bicinchoninic acid (BCA). As a result of the interaction of copper and BCA with cysteine, cystine, tryptophan, and tyrosine residues

in the protein a rection occurs. The peptide bond is responsible for colour development at elevated temperatures. The BCA reagent forms a complex with Cu^{+}, which has a strong absorbance at 562 nm.

Fresh micro-BCA assay solution was prepared and 1.5 ml Eppendorf tubes set up containing samples and known amounts of BSA standard protein in the range of 0.5 to 16 ug with each final sample volume of 500 µl. 500 µl of micro-BCA assay solution was added to each tube, vortexed and incubated at 60 ^{0}C for 60 minutes. The samples and the BSA standards were cooled and vortexed and absorbances read at 562 nm. A micro-BCA BSA standard curve was drawn and the concentration of the unknown samples determined from the standard curve.

3.8 **Immunoblotting**

Immunoblotting is an analytical technique used to detect specific proteins in a given sample or extract. It uses gel elecrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane, typically nitrocellulose or polyvinylidene fluoride (PVDF), where they are probed (detected) using antibodies specific to the target protein.

Briefly, four pieces of filter paper and a piece of nitrocellulose membrane were cut as the same size as the gel. They were incubated for 10 minutes in tank-blotting 1 X Western

transfer buffer. The gel was also immersed in 1 X Western transfer buffer. A blotting sandwich was assembled by first placing two pieces of wet filter on a sponge pad, and then placing the gel and the two pieces of the filter paper, taking care to squeeze out any air bubbles between the gel and the filter paper with gentle rolling a pasteur pipette over each layer in the sandwich. This was then topped with the second sponge pad. The sandwich was secured and inserted into an electro-eluter blotting chamber (Biorad model No.422, Italy) taking care to ensure that the membrane side of the gel was closest to the positive electrode (anode). The chamber was filled with 1 X Western transfer buffer. The apparatus was cooled throughout the transfer by placing it in the cold room at 4 ^{0}C. Buffer was circulated in the chamber by using a magnetic stirring rod. A constant potential difference of 30 V was applied overnight (Crosspower 150, Japan). After transfer was complete, the sandwich was disassembled and the membrane washed in distilled water. The orientation of the gel on the membrane was also marked. To monitor the transfer effiency, the membrane was incubated in Ponceau S staining solution with gentle agitation for 2 minutes. It was then destained in distilled water until the bands were visible. The orientation of the membrane on the gel was marked.

Immunodetection with anti-hisantibodies was carried out. Briefly, the membrane was washed twice for 10 minutes each time with TBS buffer at room temperature. The membrane was incubated for 1 hour in blocking buffer (3% BSA (w/v) in TBS buffer) at room temperature. The membrane was washed twice for 10 minutes each time in TBS-Tween /Triton buffer at room temperature. The membrane was washed again for 10

minutes with TBS buffer at room temperature and incubated in anti-His antibody in blocking buffer (3% BSA (w/v) in TBS buffer) in a dilution of 1:1000 at room temperature for 1 hour. The membrane was subsequently washed twice for 10 minutes each time in TBS-Tween / Triton buffer at room temperature. The membrane was washed for 10 minutes in TBS buffer at room temperature and incubated with a secondary antibody solution (Alkaline phosphatase (AP) conjugated anti-mouse IgG diluted in 3 % BSA (w/v) in TBS for 1 hour, diluted in 1: 5000. The membrane was finally washed four times for 10 minutes each time in TBS-Tween/Triton buffer at room temperature. The bound conjugated antibody was detected by the addition of chromogen solution (AP staining solution) for 15 minutes. The chromogenic reaction (colour development) was stopped by rinsing the membrane twice with distilled water. The membrane was dried and photographed immediately.

3.8.1 **SDS-PAGE and Western blot analysis**

The purified recombinant proteins were separated on 10% polyacrylamide gels (Laemmli, 1970) and electro-transferred to polyvinyldifluoride (PVDF) membranes overnight at 30 V using a full tank (Biorad model No.422, Italy). The membrane was blocked with 0.5% gelatin in PBST, pH 7.4; 0.05% Tween 20 on a shaker at room temperature for 1 hour. The blocking buffer was discarded and the membrane washed thrice with wash buffer PBS, 0.1% Tween 20 each time 5 minutes. The membrane was cut into 3 mm wide strips, and the strips were dipped into 250 µl of cattle preinfection and post infection sera at a dilution of 1: 100 for 1 hour in a plastic trough with separate

wells. Five preinfection sera and five postinfection sera (from a previous study) were used. The sera had tested positive for CBPP using CFT and LppQ test (Appendix 6). Anti-HisTag mouse monoclonal antibody was used as positive control at a dilution of 1: 5000 at room temperature with gentle shaking. The membrane was washed three times and then incubated with AP conjugated antibovine IgG diluted to 1: 5000 for 1 hour at room temperature with shaking. The membrane was washed twice with wash buffer and once with substrate buffer 5 minutes each time. Colour reaction was developed with the addition of 33 µl BCIP (5- bromo- 4- chloro-3- indolyl phosphate and 66 µl of NBT (nitro blue tetrazolium, Sigma-Aldrich) to 10 ml substrate buffer. The blot was left to develop for 5- 60 minutes and reaction stopped by washing with distilled water. The membrane was put onto a whatman paper and let to dry. The blot was documented by taking a photo.

3.9 **Experimental Cattle**

Cattle experimentation was reviewed and approved by the International Livestock Research Institute (ILRI) Institutional Animal Care and Use Committee. A group of 10 boran cattle (*Bos indicus*) provided by Dr. Joerg Jores were used for the study. A summary of the cattle used is shown in Table 3.2. All the cattle were screened for antibodies against *M. mycoides* subsp. *mysoides* using LppQ ELISA assay (Bommeli diagnostics, Switzerland) and found to be sero-negative.

Table 3.2 Identification codes and age of cattle used in the study

Cattle ID	Age (Months)
BD92	18
BD95	18
BD99	18
BD102	18
BD105	18
BD106	18
BD107	18
BD111	17
BD115	17
BD116	17

3.9.1 **Chekit-CBPP enzyme immunoassay (EIA)**

The CHEKIT-CBPP enzyme immunoassay kit (Bommeli diagnostics, Switzerland) provides a rapid, simple, sensitive and specific method for detecting antibodies against *M. mycoides subsp. mycoides* SC type.

Briefly, reagents were allowed to equilibrate to room temperature. 90 µl of Chekit-CBPP Sample-Diluent was dispensed into each well of the microtiter plate. 10 µl of the undiluted serum and controls were added into the appropriate wells of the microtiter plate

at a final dilution 1: 10. The contents of each well were mixed gently by shaking the microtiter plate briefly. The microtiter plate was covered with a lid and incubated for 60 minutes at 37 ^{0}C in a humid chamber. The microtiter plate was washed thrice using 300 µl for each well with CHEKIT- Washing- Solution. The microtiter plate was shaken thoroughly to empty the wells, and then tapped on an absorbent paper. 100 µl of anti-ruminant IgG conjugate was dispensed into each well, plate covered and incubated for 60 minutes at 37 ^{0}C in a humid chamber. The microtiter plate was washed thrice using 300 µl of washing solution per well. 100 µl of CHEKIT- TMB- Substrate, into each well. The substrate was incubated at room temperature for 15 minutes. Colour reaction was stopped by addition of 100 µl of CHEKIT- Stop- Solution- TMB into each well. The results were read using a spectrophotometer at wavelength of 450 nm.

3.9.2 **Interpretation of the CBPP screening results**

The OD of the duplicates was averaged. The OD of the positive control (OD_{pos}) as well as the OD of the samples (OD_{sample}) were corrected by subtracting the OD of the negative control (OD_{neg}).The samples were analyzed in relation to the negative and the positive controls with the formula: (See table 3.3 for interpretation of the results)

$$\text{Value (\%)} = \frac{OD_{sample} - OD_{neg}}{OD_{sample} - OD_{neg}} \times 100$$

Table 3.3 Interpretation of CBPP screening results

Value	< 35 %	≥ 35
Interpretation	Negative	positive

3.9.3 **Experimental cattle infection**

The cattle were infected with *Mmm*SC Afade strain. Briefly, boran cattle (*Bos indicus*) were sedated with 0.05 mg/kg body weight of xylazine hydrochloride (250 kg weight of animal) by intravenous injection. A gastric tube was then inserted into the trachea of the animals. The successful insertion of the tube to the bifurcation of the trachea was controlled by means of breathing sound heard. Then, 50 ml of culture of *Mmm*SC Afade strain (5×10^7 colony forming units/animal) was instilled followed by 20 ml of 1.5% warm agar. The agar suspension was flushed down to the instillation site with 30 ml of PBS. These animals were maintained in-doors, fed on hay and water. The animals were monitored daily for clinical signs, body temperatures and sacrificed 30 days post infection. Serum samples were taken on a weekly basis, and post mortem examination was performed after 30 days.

3.10 **Preparation of Peripheral Blood Mononuclear Cells (PBMCs) by Density Gradient Centrifugation**

In order to test recombinant *Mmm*SC specific proteins for their ability to trigger IFNγ secretion of PBMCs from experimentally infected cattle, bovine PBMCs were provided by Dr. Jores and Dr. Naessens generated from their experimental infection trial. Briefly PBMCs purification was carried out as outlined below:

Blood was collected from the jugular vein using a gauge- 16 needle into a syringe containing an equal volume of Alsever's solution (0.55g citric acid; 20.5g D-glucose; 4.2 g of sodium chloride, 8 g of dehydrate trisodium citrate; all mixed in 1 litre of distilled water) and mixed gently. 30 ml blood was layered onto 20 ml ficoll paque solution-1077 in a 50 ml polypropylene tube and centrifuged at 1 500g for 30 minutes at room temperature, with centrifuge brakes off. Through the use of a sterile pipette PBMC from the interface were aspirated and transferred to a sterile tube and topped up with warm Alsever's solution to 50 ml. The PBMCs were centrifuged at 1 000g for 10 minutes at room temperature. Cell pellets were broken and washed three times with Alsever's solution by centrifugation at 500g for 10 minutes at room temperature, to remove platelets. After the third wash, the pellet was resuspended in 10 ml of RPMI 1640 with HEPES supplemented with 10% fetal bovine medium, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 200 IU of penicillin per ml, 150 μg of streptomycin per ml. An aliquot of the PBMC was then diluted to 10% in trypan blue dye (Sigma- Aldrich) and viable cells counted by trypan blue exclusion on an improved Neubaeur Chamber (Profondeur, Germany). The cell density was determined using the following formula:

Cell density = Number of cells counted x $1x10^4$ x dilution factor.

3.11 **Lymphostimulation Assays**

Lymphostimulation used in this study was adapted from Dedieu *et al*, (2005). Cells were first diluted to a concentration of 10^7. One hundred microlitres of PBMCs (2 $x10^5$/well) were distributed into each well of a 96-well flat-bottomed microtitre plates for stimulation. The PBMCs were incubated with *Mmm*SC recombinant fragments at 5ug/ml, Concanavalin A (St. Quentin, France) (con A) at 5 µg/ ml was used as a positive control, RPMI media as a negative control and heat inactivated *Mmm*SC Afade strain at a final concentration of 5 ug/ml. All tests were performed in duplicate. Cultures were incubated for 72 hours at 37 ^{0} C under 5% atmospehere CO_2 in an incubator. Supernatants were harvested after centrifugation and stored at -20 ^{0}C until use.

3.12 **IFNγ ELISA Analysis**

Supernatant was harvested after centrifugation and used for IFN-γ ELISA (Bovigam ™, AgriQuality Pty Ltd, Australia). Briefly, freeze dried components were reconstituted while other reagents were equilibrated. 50 µL of the Green Diluent were added to the required wells. 50 µl of test and control samples were added to the appropriate wells containing Green Diluent. Control samples were added after test samples. Mixing was done by pipetting up and down 5 times. Each of the plate was covered with a lid and incubated at room temperature for 60 minutes. The contents were shaked out and the trays washed 6 times. Care was taken not to cross contaminate adjacent wells. After the sixth wash, trays were tapped face down several times on absorbent paper to remove as

much remaining wash buffer as possible. 100 µL of freshly prepared conjugate reagent was added. The plates were covered and incubated for 60 minutes at room temperature. The plates were washed and the enzyme substrate solution was prepared after this wash step. 100 µL of freshly prepared enzyme substrate solution was added to the wells and each plate covered with a lid and incubated for 30 minutes away from direct sunlight. 50 µL of enzyme stopping solution was carefully added to each well and mixed by gentle agitation. The absorbance of each well was read within 5 minutes of terminating the reaction using a 450 nm filter spectrophotometer. The absorbance values were then used to calculate results. All assays were done in duplicate.

3.13 **Data Presentation and Analysis**

Data management was done on an Excel spreadsheet. All entries were checked for any keyboard errors. Statistical analyses were conducted in GenStat 9th edition discovery version. The cut-off value for IFNγ was taken as an absorbance of 0.7. Student t-test was used for comparative analysis between preinfection week and the 3rd post infection week. Photographs of blots and gels were also taken and documented.

CHAPTER FOUR

RESULTS

4.1 Cloning of *Mmm*SC Protein Fragments

Previous studies identified new immunogenic proteins from *Mmm*SC that were potential candidate molecules for improved diagnostics. The gene sequences could be derived from published genome sequence (Westberg *et al.*, 2004). Four immunogenic fragment genes were PCR amplified and cloned into pBAD 102/ D – TOPO vector in the directional cloning site upstream of the 6 X His-tag sequence (figure 4.1). The gene fragments were cloned in the correct reading frame, under the control of the *ara*BAD promoter (P_{BAD}). All the resultant recombinant clones were analyzed by PCR and then by restriction enzyme digestion fragment mapping.

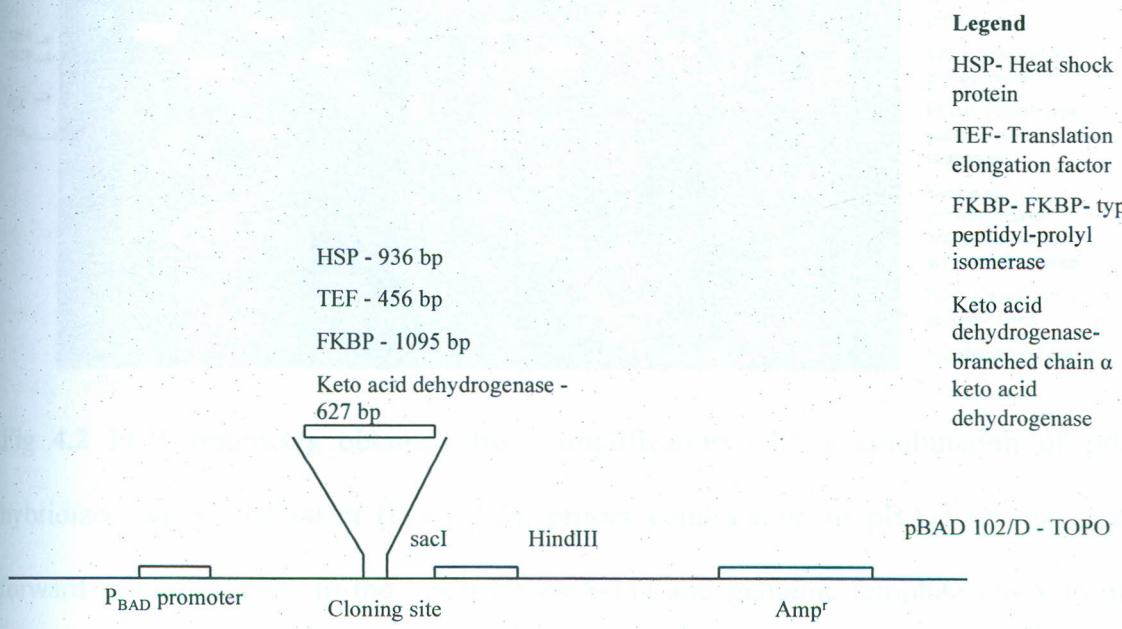
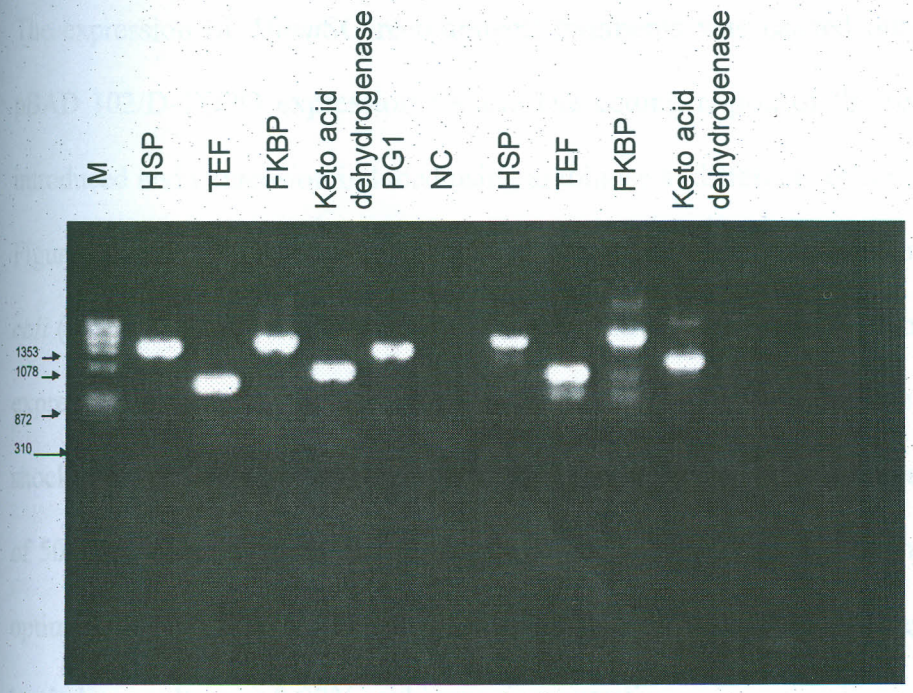


Fig 4.1 Cloning of genes in pBAD 102/D- TOPO. Genes were PCR amplified and cloned in the cloning site of the vector

DNA sequencing analysis of the genes fragment integrated into pBAD 102/D- TOPO vector cloning site demonstrated that heat shock protein-70 consisted of 936 bp encoding 312 amino acids; translation elongation factor Tu, consisted of 456 bp encoding 152 amino acids; FKBP consisted of 1095 bp encoding 365 amino acids; and branched chain α keto acid dehydrogenase consisted of 627 bp encoding 209 amino acids (figure 4.2). Clones with the correct sizes were selected for protein expression analysis.



Legend

M- Molecular marker

HSP- Heat shock protein

TEF- Translation elongation factor

FKBP- FKBP- type peptidyl prolyl isomerase

Keto acid dehydrogenase- branched chain α keto acid dehydrogenase

PG1- *Mmm*SC PG1 genomic DNA

NC- Negative Control

Fig 4.2 PCR fragments obtained from amplification with a combination of primers that hybridized within the insert (lanes 2-5); primer combination of pBAD reverse primer and a forward primer specific to the insert (lanes 8-11) and genomic template DNA from *Mmm*SC PG1.PhiX174/Hae III DNA size marker was used. Their sizes are given in basepairs on the left side.

The plasmid pBAD 102/ D- TOPO containing the genes were transformed into the *E. coli* expression host TOP10 and stable transformants were screened for the expression of heat shock protein, translation elongation factor Tu, FKBP peptidyl prolyl isomerase and branched chain α keto acid dehydrogenase.

4.2 **Expression and Purification of recombinant proteins**

The expression of *Mmm*SC recombinant fragments was carried out in *E. coli* TOP10, using pBAD 102/D-TOPO expression vector. The coding region of the recombinant fragments was introduced and expression induced using arabinose at different concentrations and time periods. Figure 4.3 shows SDS- PAGE analysis of whole cell extract of those of the arabinose induced *E. coli* (lanes 1-6), in comparison with non-induced *E. coli* (lane 7) harbouring heat shock protein expression plasmid. Arabinose induced bacteria cells, showed high level expression of the heat shock protein fragment and was detected at the desired band corresponding to the predicted size of 50 kDa. Uninduced bacteria cells expressed the heat shock protein fragment minimally. The optimal conditions for maximum expression of heat shock protein fragment were determined to be six hour culture at 0.02% arabinose concentration.

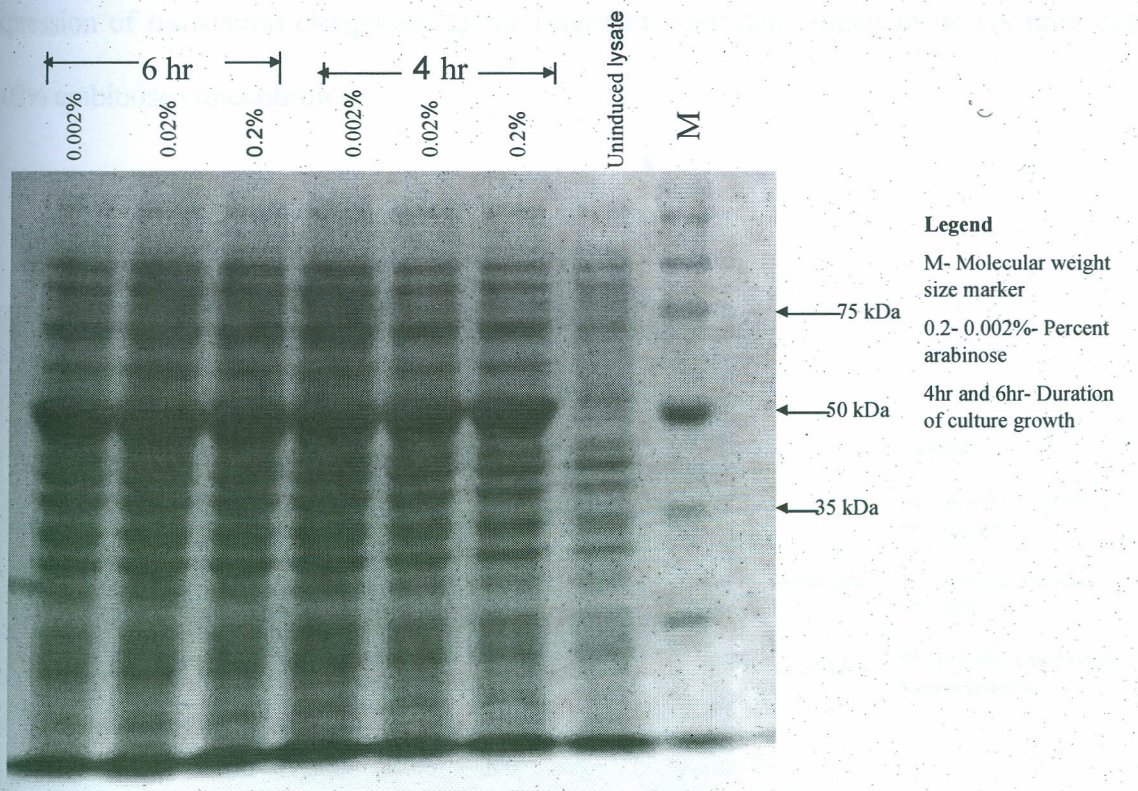


Fig 4.3 Ten percent SDS-PAGE for determination of optimal conditions needed for maximum expression of heat shock protein fragment, lane (7) uninduced bacterial lysate and lane (8) molecular size marker.

Figure 4.4 shows SDS- PAGE analysis of whole cell extract of those of the arabinose induced *E. coli* (lanes 1 -6), in comparison with non-induced *E. coli* (lane 8) harbouring translation elongation factor expression plasmid. As expected arabinose induced bacteria cells, showed high level expression of the translation elongation factor fragment and was detected at the desired band corresponding to the predicted size of 33 kDa. Uninduced bacteria cells expressed the translation elongation factor fragment minimally. The optimal conditions for maximum

expression of translation elongation factor fragment were determined to be six hour culture at 0.02% arabinose concentration.

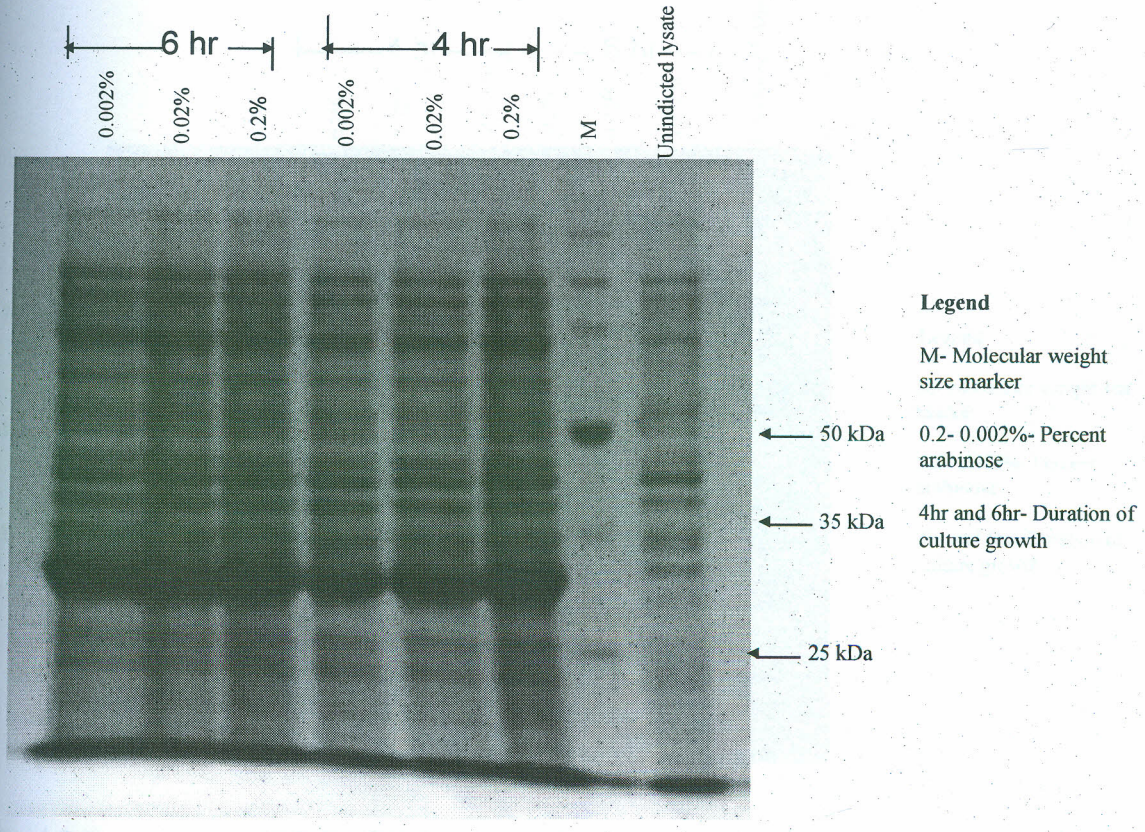


Fig 4.4 Ten percent SDS-PAGE for determination of optimal conditions needed for maximum expression of needed for maximum expression of translation elongation factor fragment, lane (7) molecular size marker and lane (8) uninduced bacterial lysate.

Figure 4.5 shows SDS- PAGE analysis of whole cell extract of those of the arabinose induced *E. coli* (lanes 2-7), in comparison with non-induced *E. coli* (lane 8) harbouring FKBP expression plasmid. Arabinose induced bacteria cells, showed high level expression of the FKBP protein fragment and was detected at the desired band corresponding to the predicted size of 57 kDa. Uninduced bacteria cells expressed the FKBP fragment minimally. The optimal conditions for

maximum expression of heat shock protein fragment were determined to be four hour culture at 0.02% arabinose concentration.

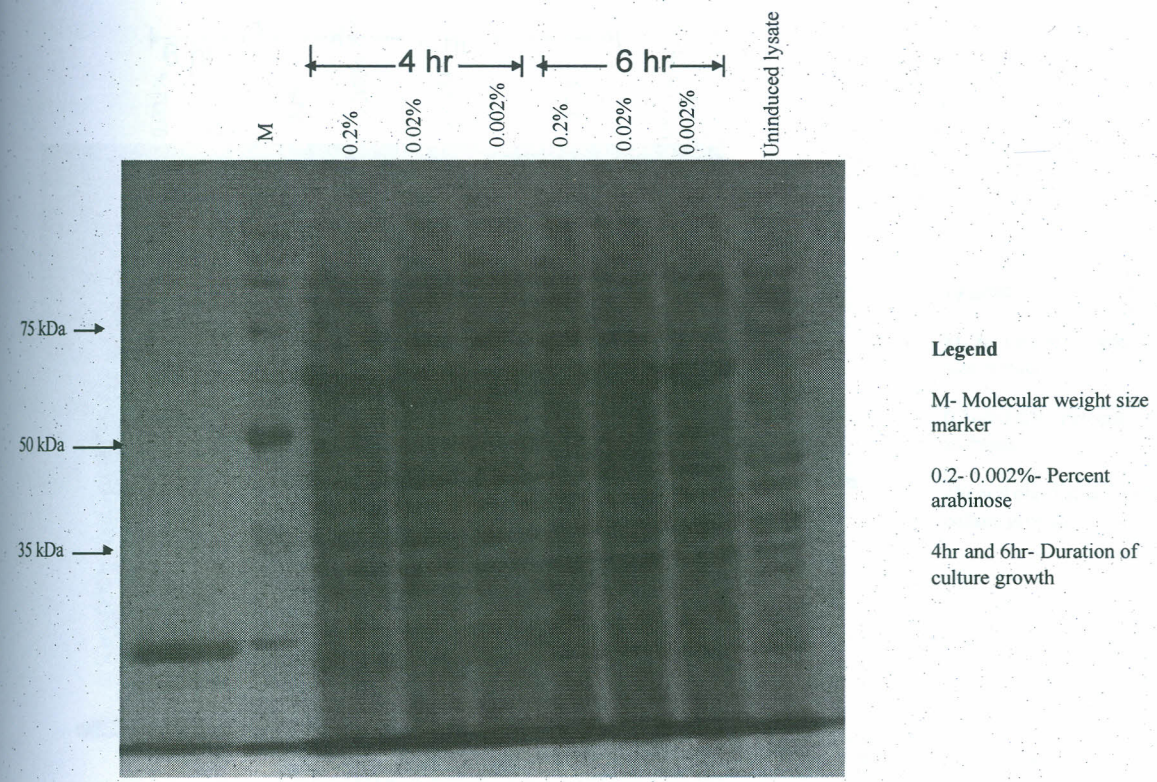


Fig 4.5 Ten percent SDS-PAGE for determination of optimal conditions needed for maximum expression needed for maximum expression of FKBP fragment, lane (1) molecular size marker and lane (8) uninduced bacterial lysate.

Figure 4.6 shows SDS- PAGE analysis of whole cell extract of those of the arabinose induced *E. coli* (lanes 1-5,7), in comparison with non-induced *E. coli* (lane 8) harbouring keto acid dehydrogenase expression plasmid. Arabinose induced bacteria cells, showed high level expression of the branched chain α keto acid dehydrogenase fragment and was detected at the desired band corresponding to the predicted size of 35 kDa. Uninduced bacteria cells expressed the branched chain α keto acid dehydrogenase fragment minimally. The optimal conditions for

maximum expression of heat shock protein fragment were determined to be four hour culture at 0.02% arabinose concentration.

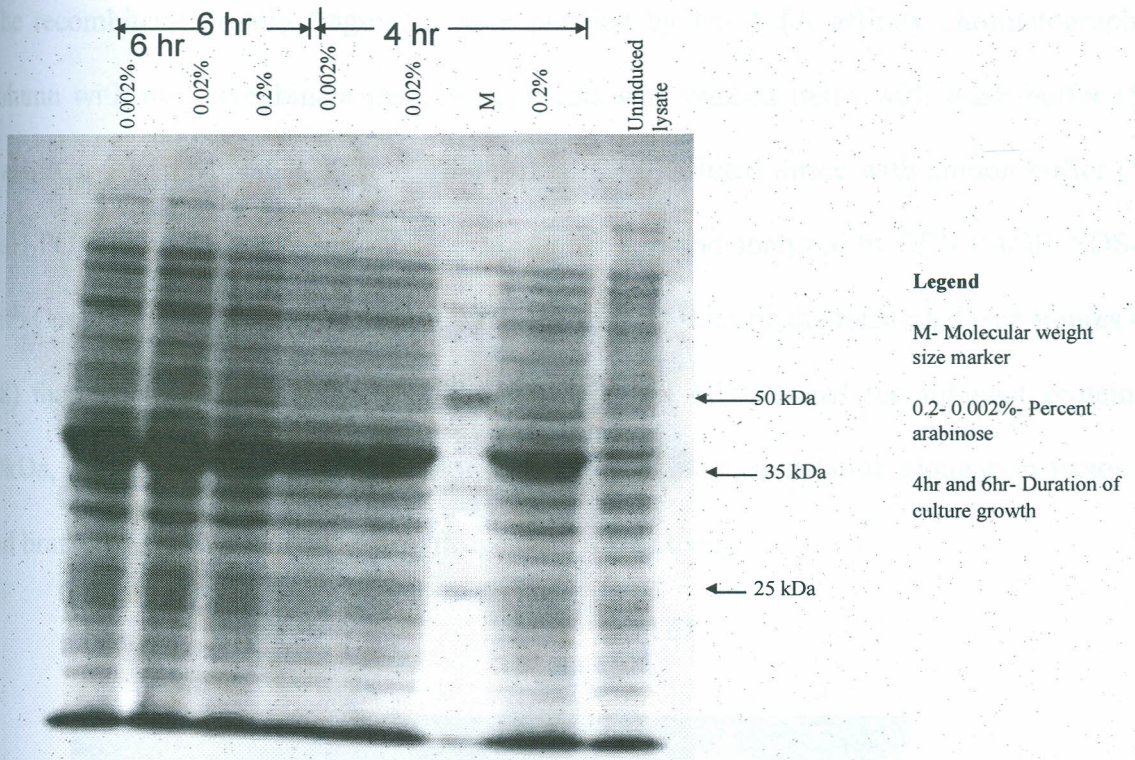


Fig 4.6 Ten percent SDS-PAGE for determination of optimal conditions needed for maximum expression of Keto acid dehydrogenase fragment, lane (6) molecular size marker and lane (8) uninduced bacterial lysate.

In all the arabinose induced bacteria cells, high level expression of the recombinant proteins was detected and all with the desired band corresponding to the predicted sizes (kDa) of the recombinant fragments. Non-induced bacteria cells expressed the recombinant proteins minimally. The optimal conditions for maximum expression of the *Mmm*SC recombinant fragments was determined to be 6 hour at 0.02% arabinose concentration for heat shock protein

and translation elongation factor, 4 hour at 0.02% arabinose concentration for FKBP and keto acid dehydrogenase.

The recombinant protein fragments were purified by Ni- NTA affinity chromatography. The column with the recombinant protein fragments was washed twice with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole pH 8.0), eluted thrice with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole pH 8.0) and analyzed by SDS-PAGE. SDS-PAGE with Coommasie blue staining and 6 X His protein tag staining demonstrated (see figures 4.7 and 4.8) that the molecular weights of the purified 6 X His- tagged recombinant proteins were 50kDa, 33kDa, 57kDa and 35 kDa for heat shock protein, translation elongation factor, FKBP and branched chain α keto acid dehydrogenase, respectively.

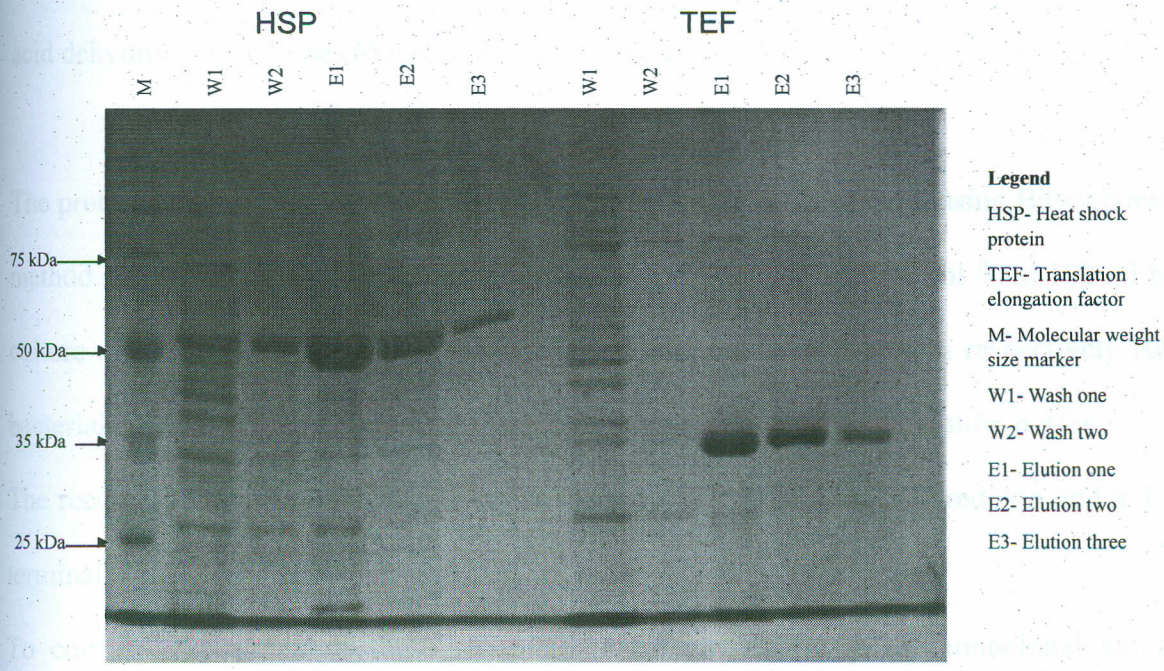


Fig 4.7 Ten percent SDS-PAGE of expressed and purified recombinant *Mmm*SC heat shock protein and translation elongation factor fragments. Lane (1) molecular size marker

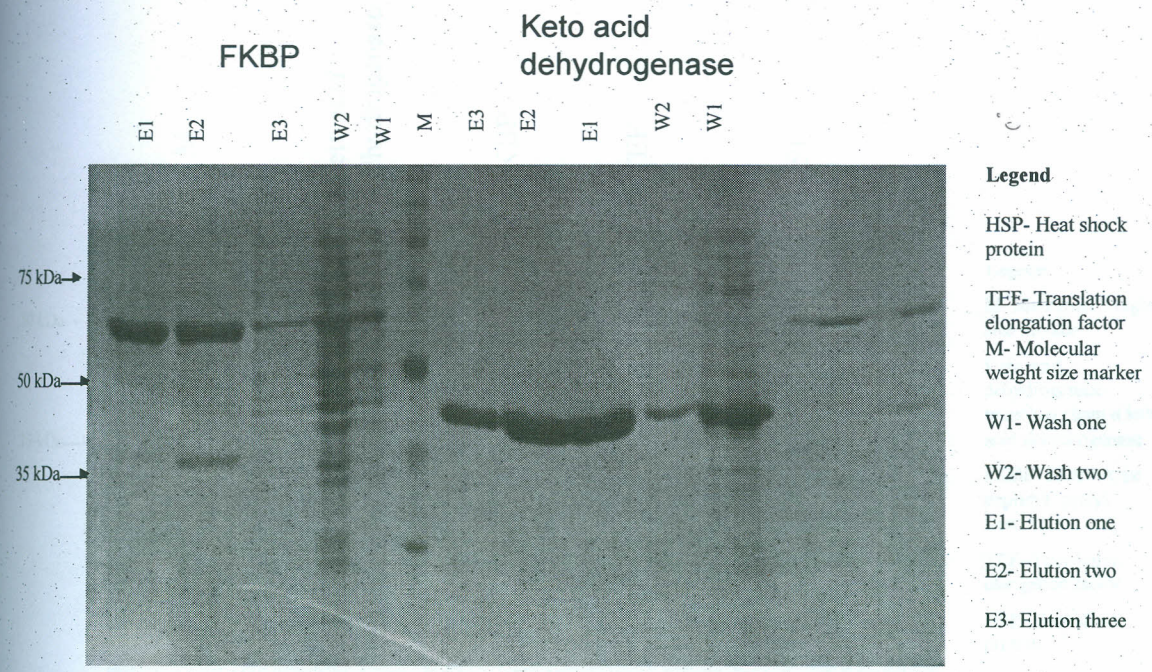


Fig 4.8 Ten percent SDS-PAGE of expressed and purified recombinant *Mmm*SC FKBP and keto acid dehydrogenase. Lane (6) molecular size marker

The protein concentrations of the fractions eluted (E1s), were measured using Bicinchoninic acid method and yielded protein concentrations of 0.5, 0.5, 0.9 and 1.2 mg/ ml from 100 ml bacterial culture of TEF, FKBP, keto acid dehydrogenase and heat shock protein respectively.100 ml of bacterial culture yielded 650 µl of the recombinant protein eluted with elution buffer.

The recombinant protein fragments contained a 13 kDa N-terminal thioredoxin and a 3 kDa C-terminal V5, 6 X His peptide tags.

To confirm expression of these recombinant protein fragments, a monoclonal anti-His tag antibody recognizing six histidine residues was used in western blot analysis (figure 4.9). Monoclonal anti-His tag antibody strongly reacted with protein bands of 35 kDa, 57 kDa, 33 kDa and 50 kDa, corresponding to the recombinant protein fragments.

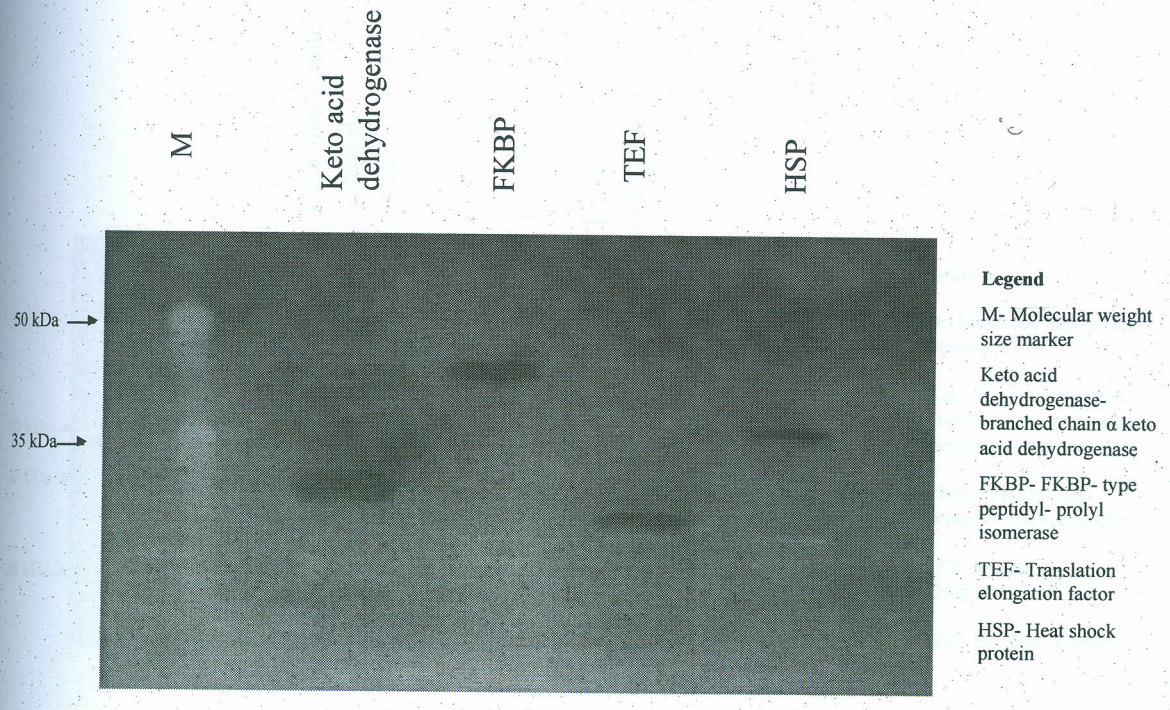


Fig 4.9 Immunoblotting with anti-His-tag monoclonal antibody. The recombinant protein fragments were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Lane 1, rainbow marker; lane 2, keto acid dehydrogenase; lane 3, FKBP; lane 4, TEF and lane 5, HSP.

Fig 4.10 shows a SDS-PAGE analysis that was carried out to show all the recombinant protein fragments. Crude *Mmm*SC Afade lysate and heat inactivated *Mmm*SC Afade lysate were also ran on the gel for comparison purposes. The recombinant protein fragments and the heat inactivated *Mmm*SC Afade lysate were used in subsequent IFNγ and antibody assays.

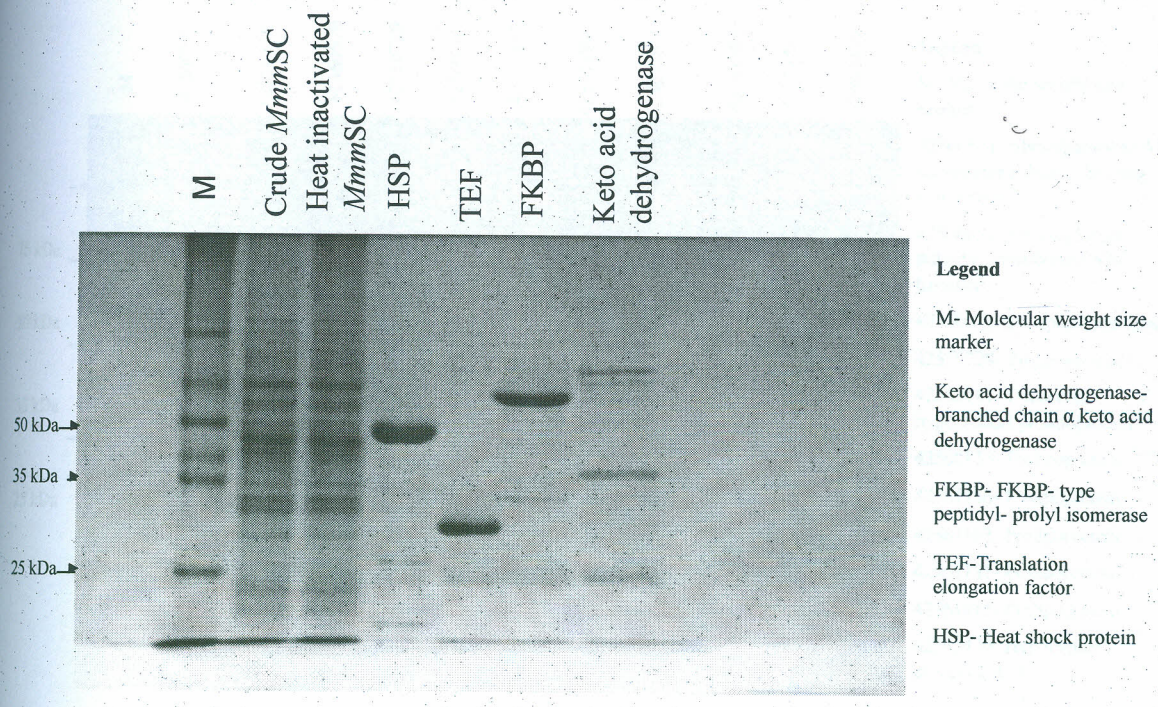
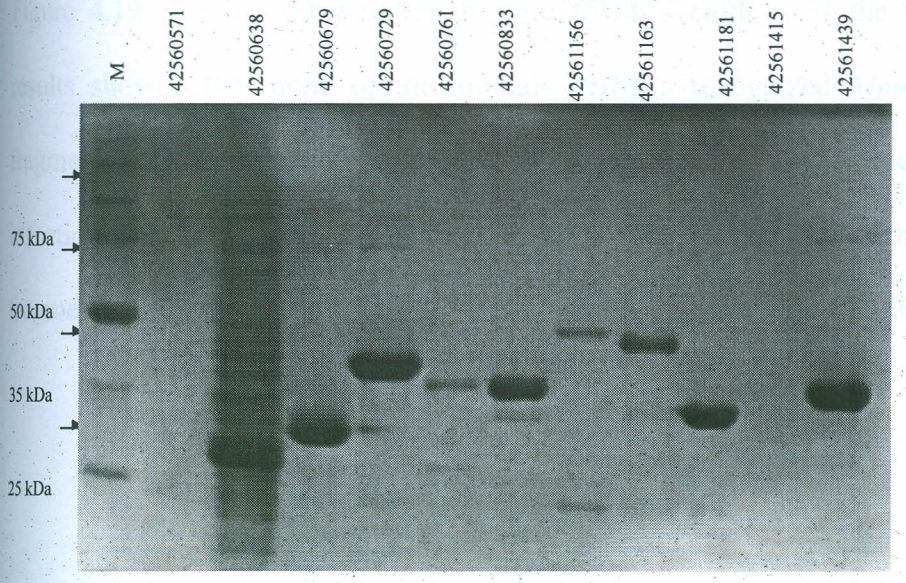


Fig 4.10 Ten percent SDS-PAGE, lane 1 molecular size marker; lanes 2 and 3 crude and heat inctivated *Mmm*SC lysate respectively; lanes 4-7 recombinant protein fragments

4.3 **Expression of Lipoproteins**

In addition to potential adhesion molecules, lipoproteins were also expressed (figure 4.11) but were not used for subsequent IFNγ gamma and western blots antibody responses assays. The different lipoproteins were expressed, purified and stored at -80^{0}C for later usage in antibody assays.



Legend

M- Molecular weight size marker

42560571- ribose/galactose ABC transporter-substrate binding component

42560638- Prolipoprotein putative phosphonate ABC transport

42560679- Prolipoprotein lppC

42560729- Prolipoprotein C

42560761- Conserved hypothetical prolipoprotein

42560833- Prolipoprotein

42561156- Prolipoprotein

42561163- Prolipoprotein

42561181- Prolipoprotein

42561415- Prolipoprotein

42561439- Hypothetical prolipoprotein

Fig 4.11 Ten percent SDS-PAGE of expressed and purified lipoproteins; lane 1, molecular size marker; lane 2, 42560571; lane 3, 42560638; lane 4, 42560679; lane 5, 42560729; lane 6, 42560761; lane 7, 42560833; lane 8, 42561156; lane 9, 42561163; lane 10, 42561181; lane 11, 42561415 and lane 12, 42561439

4.4 IFNγ Production

To investigate whether memory T cells from infected cattle were able to recognize recombinant *Mmm*SC protein fragments, PBMCs culture supernatants were collected three days after stimulation with the recombinant protein fragments and IFNγ production was analyzed by ELISA. IFNγ secretion was assessed by comparison with the positive control and results were deemed positive when the optical density of the *Mmm*SC recombinant protein fragments-stimulated cell supernatant was ≥ 0.7.

Figure 4.12 presents IFNγ secretion from PBMCs cultures of the ten infected animals. The results showed that none of the animals PBMCs recognized *Mmm*SC recombinant protein fragments. IFNγ production was found to be below the cut- off value over the four weeks post infection duration for all the animal PBMCs after stimulation with the recombinant protein fragments. Stimulation with Con A yielded positive signals with PBMCs from all animals.

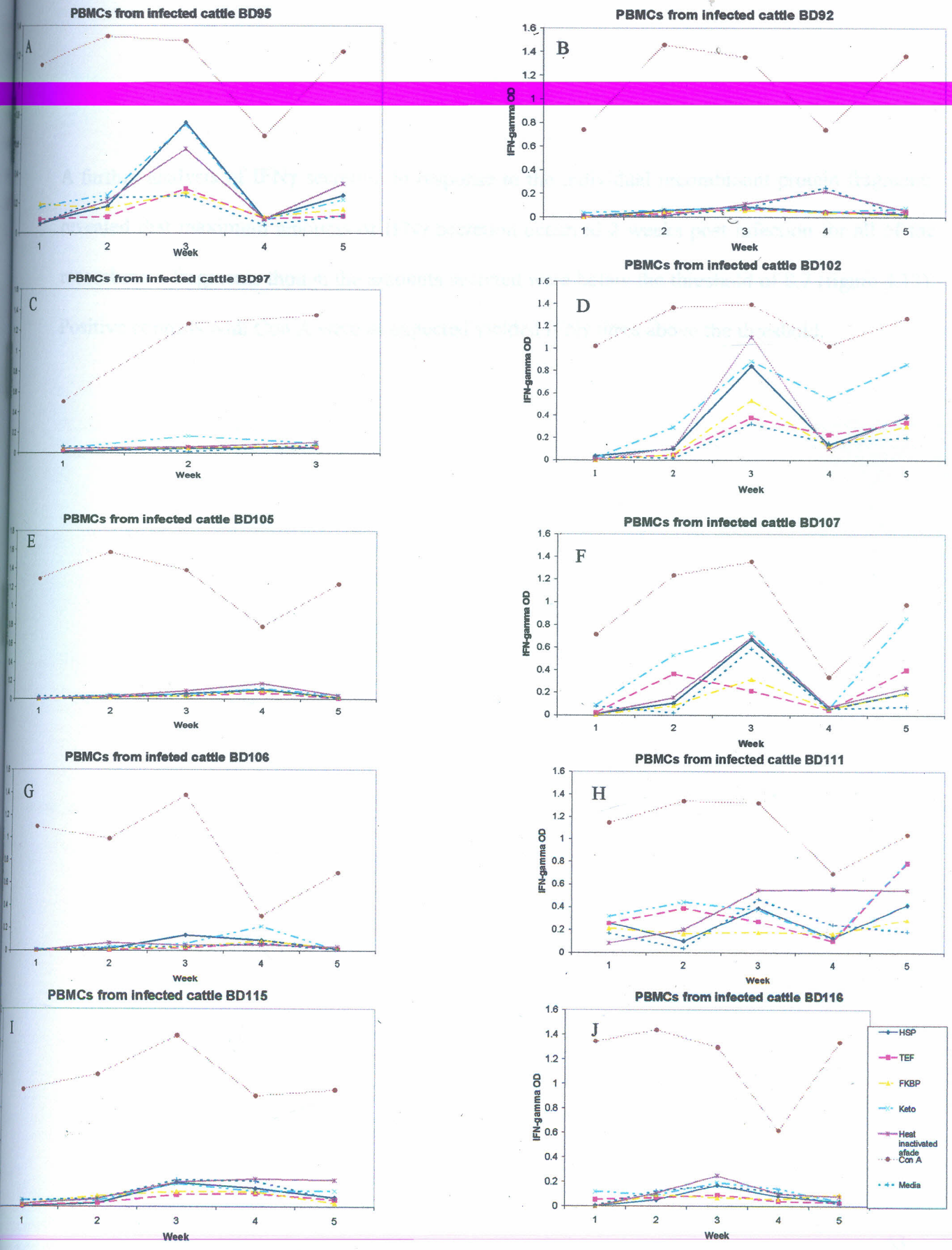


Fig 4.12 IFNγ response from PBMCs isolated from *Mmm*SC– infected cattle after stimulation with *Mmm*SC recombinant protein fragments, media, con A and heat inactivated *Mmm*SC Afade lysate

A further analysis of IFNγ secretion in response to the individual recombinant protein fragments revealed that maximum amounts of IFNγ secretion occurred 2 weeks post infection for all of the recombinant fragments though the amounts secreted were below the threshold of 0.7 (figure 4.13). Positive controls with Con A were as expected yielded IFNγ titres above the threshold.

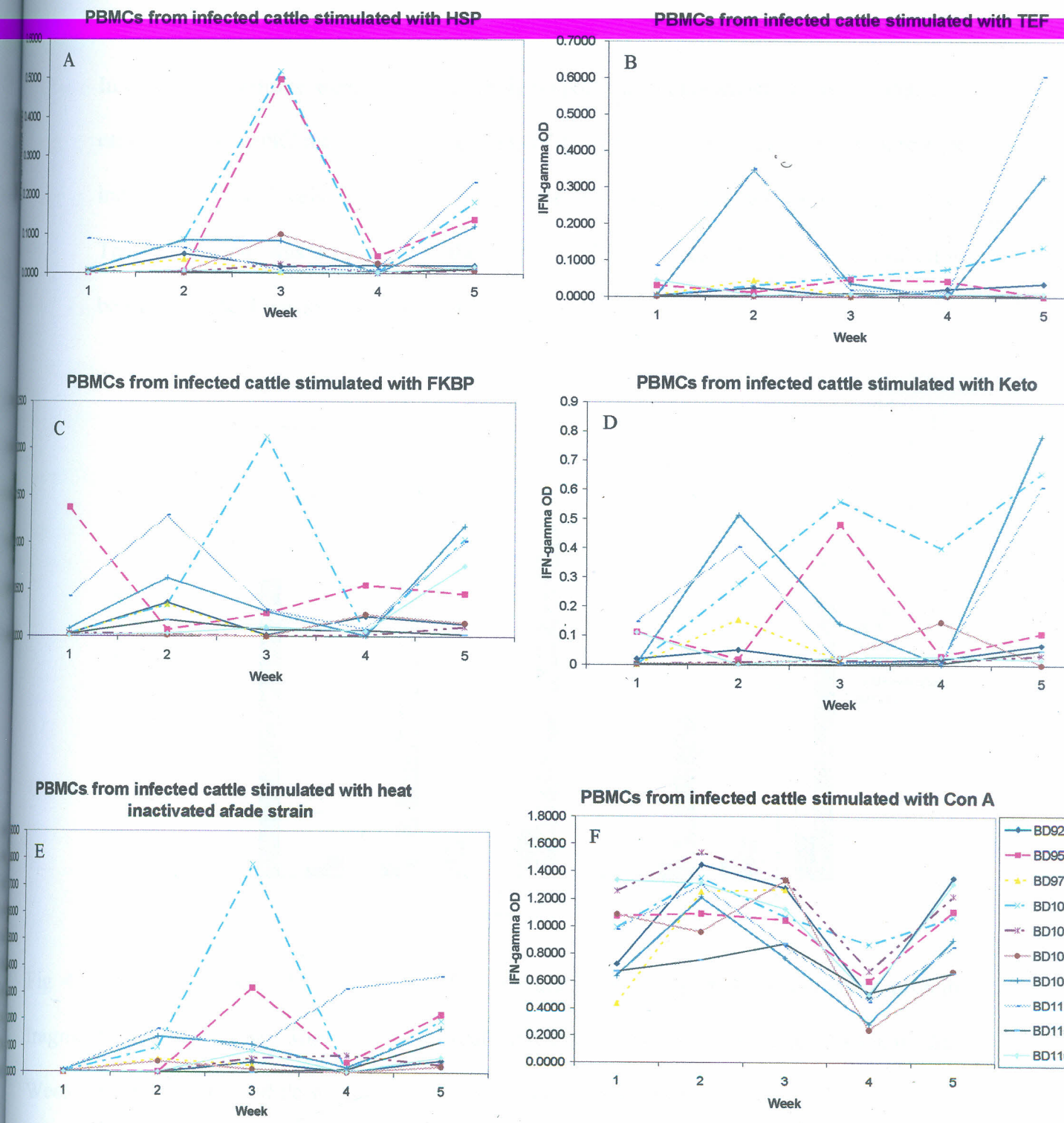


Fig 4.13 IFNγ responses from *Mmm*SC infected cattle after stimulation with *Mmm*SC recombinant protein fragments, heat inactivated *Mmm*SC Afade and Con A after being corrected for the media.

In order to determine weekly trend of IFNγ responses, weekly means were calculated of each of the *Mmm*SC recombinant fragments (Fig 4.14). The results showed a progressive increase on the levels of IFNγ from preinfection week to 2 weeks post infection. Thereafter, there was a decrease in the amounts of IFNγ released 3 weeks postinfection before peaking 4 weeks post infection.

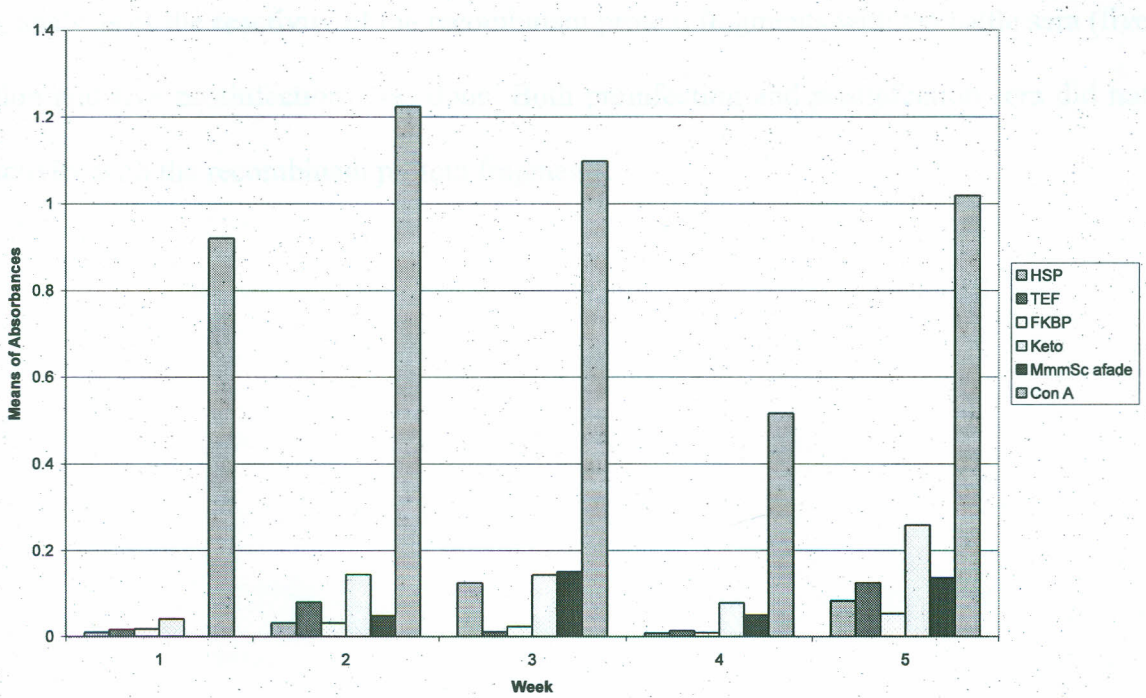


Fig 4.14 Weekly mean + SEM of IFNγ after stimulation with *Mmm*SC recombinant protein fragments, heat inactivated *Mmm*SC Afade lysate and Con A after being corrected for the media. Week 1 is preinfection, while weeks 2-5 were postinfection time points.

Using student t-test, a comparison of preinfection means and 3 weeks postinfection means for the *Mmm*SC recombinant protein fragments showed that there was no significant difference between the two time points for HSP, TEF, FKBP, keto acid dehydroganase and con A ($P > 0.05$).

However, there was a significant increase of IFNγ production for *Mmm*SC Afade lysate ($P < 0.05$). Based on this data, there was no increase of IFNγ at the fourth post infection week solely due to HSP, TEF, FKBP, Con A and keto acid dehydrogenase recombinant protein fragments.

4.5 Antibody Responses to the Recombinant Fragments in CBPP Diseased Cattle

To know whether the recombinant protein fragments induced an immunological antibody response, analysis of the reactivity of the recombinant protein fragments with ten cattle sera (five preinfection and five postinfection) was done. Both preinfection and postinfection sera did not show reactivity with the recombinant protein fragments.

CHAPTER FIVE

5.0 **DISCUSSION**

The development of an improved diagnostics against CBPP remains a challenging goal. The OIE- recommended diagnostic tests, complement fixation test (CFT) and the competitive ELISA (cELISA) are hampered by a limited sensitivity (Marobela *et al.,* 2003). The prerequisite for an improved diagnostic is the identification of *Mmm*SC antigens which are still unknown in spite of the vast genetic information on the *Mmm*SC type strain PG1 (Westberg *et al.*, 2004). Proteins with capability to be used in diagnostic assays should react with sera from infected animals in order to result in an appropriate diagnostic sensitivity and for specificity the proteins should not react with sera from uninfected animals or from animals infected with related organisms (Jores, personal communication).

In the present study, gene fragments were cloned into an expression vector, expressed and the recombinant protein fragments purified. Memory T cell and antibody responses towards these recombinant protein fragments were investigated. Immunostaining of the recombinant protein fragments in purified form with monoclonal anti- His tag confirmed the expression of the recombinant protein fragments derived from *Mycoplasma mycoides* subsp. *mycoides*. The expressed recombinant molecules were: heat shock protein, translation elongation factor, FKBP peptidyl prolyl isomerase, branched chain α keto acid dehydrogenase and lipoproteins. Heat shock protein, translation elongation factor, FKBP peptidyl prolyl isomerase and branched chain α keto acid dehydrogenase recombinant protein fragments were selected for use in subsequent immune responses studies since they had initially reacted in two dimensional gel electrophoresis

and Western immunoblotting suggesting they were immunogenic (Jores *et al.,* 2008). Lipoproteins were not selected for further immune response studies.

Expressed recombinant 6X His- tagged protein fragments and lipoproteins were soluble and were purified under native conditions. It was observed that during purification, considerable amounts of nontagged proteins interacted with the Ni- NTA resin. This was reflected in the larger number of proteins that appeared in the first wash. The bacterial expression system was an efficient and suitable expression system for *Mmm*SC genes as it allowed the production of substantial amounts of recombinant protein fragments. In *E. coli,* UGA codon is a stop codon while in Mycoplasma it codes for tryptophan (Minion, 1998). This could result in premature truncation of the polypeptides during translation in *E. coli* hosts leading to lack of expression of cloned mycoplasma gene fragments. Mycoplasma gene fragments were already lacking UGA in their nucleotide sequences.

The recombinant protein fragments final yield ranged between 0.5 – 1.2 mg/ml of the initial bacterial culture. The purity of the recombinant protein fragments was judged by SDS- PAGE and immunoblotting with anti-his tag antibody. The results indicated that anti-his tag antibody reacted with single bands of 33 kDa, 35 kDa, 50 kDa, and 57 kDa, suggesting that the recombinant fragments had been successfully purified. HSP protein fragment had the highest level of expression followed by keto acid dehydrogenase, FKBP and TEF respectively. Lipoproteins also had varying levels of expression.

Heat shock proteins (HSPs) function as intra-cellular chaperones for other proteins (Walter *et al.*, 2002). They play an important role in protein-protein interactions such as folding and assisting in the establishment of proper protein conformation and prevention of unwanted protein aggregation. By helping to stabilize partially unfolded proteins, HSPs aid in transporting proteins across membranes within the cell (Walter *et al.,* 2002; Borges *et al.,* 2005). In other mycoplasmas HSP has been reported to be surface localized and mediates binding to host receptors (Boulanger *et al.,* 1995).

Proteome analyses have revealed that translation elongation factor-Tu (TEF-Tu) is associated with cytoplasmic membranes of Gram-positive bacteria and outer membranes of Gram-negative bacteria (Kolberg *et al.,* 2008). EF- Tu has been shown to be immunogenic and binds to fibronectin in *M. pneumoniae* (Dallo *et al.*, 2002).

FKBP-type peptidyl-prolyl isomerase is a trigger factor. Trigger factors assists in the folding of newly synthesized proteins (Ludlam *et al.,* 2004). Trigger Factor's N-terminal domain (N) mediates ribosome binding while the middle domain (P) harbors peptidyl-prolyl isomerase activity.

Heat inactivated *Mycoplasma mycoides* subsp. *mycoides* Afade strain was a crude mycoplasma antigen. A previous study had shown that no correlation was obtained with crude antigen in the IFN-γ release with and without pathomorphological gross lesions (Jores *et al.*, 2008). It was not a good diagnostic, maybe because crude antigen is a mixture of antigens, and would not give good correlations with infection.

Lipoproteins are among the surface antigens found on the cellular membrane of *Mmm*SC. They are involved in mechanisms of pathogenicity since they are known to induce pro-inflammatory cytokines (Muhlradt and Frisch, 1994; Herbelin *et al*., 1994; Brenner *et al*., 1997; Marie *et al*., 1999; Calcutt *et al*., 1999). Currently, a few lipoproteins have been characterized extensively and most of them are readily detected in serum of infected cattle on immunoblots. Lipoproteins play critical roles in the interactions between mycoplasmas and eukaryotic cells, chief among them being adhesion (Pilo *et al*., 2007).

According to cut-off values of the bovigam gamma interferon test, 60% of the cattle were completely negative, therefore had no response to any of the four molecules. Two of the ten animals (20 %) (BD107 and BD 111) showed moderate IFNγ secretion, but in both cases they also responded to the negative control (RPMI medium). This suggests that when the PBMCs were taken from the animal, they were already some cells that were secreting IFNγ, and they continued to secrete when in culture. Another likely explanation was the possibility of the negative control being contaminated and that these contaminants acted as stimulants for IFNγ release when cells were cultured with the media. The two remaining animals (BD95 and BD102) secreted IFNγ particularly against HSP and keto acid dehydrogenase, two weeks postinfection. These two animals had low responses to the negative control.

Most of the IFNγ release occurred two weeks post infection, occasionally also at the third week post infection. Two weeks post infection was also the time point that most severe clinical symptoms (fever, coughing) were observed and the first acute case had to be killed at this time

point, so the worst time in CBPP disease. All the animal PBMCs had high responses (means absorbance of OD > 1.0) towards the positive control (Con A), though there was a decrease in the mean absorbance optical density of 0.55, 3 weeks post infection. A possible explanation is that on that day less PBMCs were seeded into the wells.

Several PBMCs from infected animals reacted to the heat inactivated *Mmm*SC afade, but always at two weeks post infection. Again, these IFNγ secretions were not a result of specific stimulation, as the negative control also showed IFNγ secretion. Based on this data, the IFNγ assay therefore, did not reveal specific IFNγ secretion upon stimulation with any of the four recombinant molecules, nor with whole heat inactivated *Mmm*SC Afade. Therefore, the assay cannot be used to identify *Mmm*SC specific memory cells, as they were not found in the blood four weeks postinfection. The possible explanations for this could be that no *Mmm*SC specific T cells were produced during the infection, or memory cells were induced, but none of them against the four recombinant antigens. Further explanations need to be sought why whole heat inactivated *Mmm*SC Afade showed little IFNγ release in the animals. Another possible explanation could be that memory cells were induced, but they did not migrate in the blood during the 4 weeks and a likely assumption is that at a later time after 4 weeks this population of cells may migrate to the blood. Finally, it is possible that the recombinant fragments used did not contain T cell epitopes or epitopes strong enough to induce T cell immunity.

The peaks of IFNγ released during the peak of disease suggested that there are other cells in the blood besides memory T cells that secreted the IFNγ, but they were not memory T cells. They may be natural killer cells that were induced as a consequence of the severe inflammatory

conditions, or gamma delta T cells; since both cell types are known to be able to produce IFNγ (Janeway *et al.*, 2005).

From the results of this study, these fragments are not suitable to be used as target antigens in the development of an IFNγ diagnostics prototype that would avoid inter-species cross-reactivity frequently observed with whole cell, their extracts or surface molecules harbouring identical antigenic determinants (Noormohammadi *et al.*, 1998; Bencia, 2002; Feberwee *et al.*, 2005).

As expected, preinfection sera did not produce positive results for any of the four recombinant fragments in immunoblots analysis. Post infection sera also did not react to any of the recombinant fragments. These results indicate absence of antibodies to these recombinant fragments in the sera sampled though a previous study had shown that they were immunogenic using two dimensional gel electrophoresis and western blots (Jores, personal communication). These results indicate that *Mmm*SC infected cattle did not have antibodies against these recombinant protein fragments, thereby suggesting that the recombinant protein fragments did not induce antibodies formation in the cattle. Absence of antibodies to these recombinant fragments suggested that they probably did not have B cell epitopes in their structure which needs to be confirmed through B cell epitope predicting bionformatics softwares. Alternatively, the recombinant protein fragments did not fold in the same way as the native protein and the conformational epitopes recognized by antibodies were not present in the recombinant protein fragments.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The results reported in this thesis have demonstrated that the four recombinant protein fragments that were tested were not able to induce IFNγ levels above the cut off value of (> 0.7), the peaks of IFNγ observed suggested that memory T cell were not responsible for the IFNγ production, and thus IFNγ ELISA assay was not a suitable diagnostic tool for *Mmm*SC using these *Mmm*SC recombinant fragments. Heat inactivated *Mmm*SC Afade lysate induced significant amount of IFNγ production at the third week postinfection compared to preinfection time point, this suggested the existence of T cell epitopes in the heat inactivated *Mmm*SC Afade lysate. In addition the four recombinant fragments were not recognized by sera from infected cattle a likely indication of the absence of B cell epitopes on their structure. The results indicate that the recombinant protein fragments were not immunogenic.

6.2 Recommendations and suggestions for future studies

These results need to be reproduced to either confirm that the fragments are immunogenic as an early study (Jores, personal communication) had shown or to be nonimmunogenic as this study indicates. Secondly, synthetic genes should be made especially to those genes with critical functions necessary for survival of *Mmm*SC, and investigated for their potential role in immunodiagnostics and other fragments within these recombinants, together with lipoproteins with vital functions to the pathogen should be investigated as likely candidates for immunodiagnostics. Further, being that the fragments were not immunogenic, these should

provide a stimulus for greater efforts by scientists to search and identify other recombinant molecules in *Mmm*SC using other closely related Mycoplasma species where a large pool of information about immunogenicity of their molecules exists. It is also good to note that plans are underway to utilize and examine the potential application of the lipoproteins expressed in this thesis in immunodiagnostics of CBPP at International Livestock Research Institute.

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