DETERMINATION OF *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* COMPONENTS THAT CONFER PROTECTION AGAINST CONTAGIOUS BOVINE PLEUROPNEUMONIA AND UNDERSTANDING OF IMMUNOLOGICAL RESPONSES

by

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DECLARATION

This Thesis is my original work and has not been presented for a degree or professional qualification in any other University.

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DEDICATION

To my dear wife Grace, children Victor and Angel and my parents Pricilla and Daniel Mwirigi.

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ABBREVIATIONS/ACRONYMS AND SYMBOLS

AU-IBAR	African Union-Interafrican Bureau for Animal Resources
APS	Ammonium persulphate
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CPS	Capsular polysaccharide
CCU	Colour changing unit
cELISA	Competitive enzyme-linked immunosorbent assay
CFT	Complement fixation test
CBPP	Contagious bovine pleuropneumonia
DNA	Deoxyribonucleic acid
ECaNEp	Embryonic calf nasal epithelial
EDC	1-ethyl-3(3-dimethylaminopropyl) carbodiimide
EU	European Union
FAO	Food and Agriculture organization
FI-MV	Formalin-inactivated measles virus
FI-RSV	Formalin-inactivated respiratory syncytial virus
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
GlpO	L-α-glycerophosphate oxidase
HEPES	N-[2-hydroxyethyl] piperazine N-[2-thanesulfonic acid]
ISCOM	Immunostimulating complex

IAEA	International Atomic Energy Agency
ILRI	International Livestock Research Institute
I ELISA	Indirect Enzyme Linked Immunosorbent Assay
IFN-γ	Interferon-gamma
KALRO	Kenya Agricultural and Livestock Research Organization
KEVEVAPI	Kenya Veterinary Vaccine Production Institute
LAT	Latex agglutination test
LAMP	Loop-mediated amplification
LppQ	Lipoprotein Q-enzyme-linked immunosorbent assay
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
Mmm	*Mycoplasma mycoides* subsp. *Mycoides*
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PPLO	Pleuropneumonia-like organism
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
SRBC	Sheep red blood cells
TEMED	Tetramethylethylenediamine
Th	T helper
TLR	Toll Like Receptors
TNF-α	Tumour necrosis factor-α
TX-114	Triton X-114
Vsp	Variable surface antigens

VSRI	Veterinary Science Research Institute
$\chi 2$	Chi-square
OIE	World Organization for Animal Health (Office International des Epizooties)

ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is a severe respiratory disease caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) which is widespread in Africa. The main control option is a live vaccine, with low efficacy and a short duration of immunity. Development of an efficacious CBPP vaccine requires understanding immunogenicity of the antigens and protective immune responses. A series of experiments were undertaken to establish whether components of the *Mmm*, including whole cell lysate, membrane proteins and capsular polysaccharide can induce protection. Three separate experiments were conducted to evaluate the capacities to protect and detect possible correlations with immunological responses. The first experiment examined the efficacy of inactivated vaccine formulations: heat inactivated and formalin inactivated *Mmm* were compared with the live attenuated vaccine. Second experiment involved evaluation of a vaccine formulation generated from the *Mmm* membrane protein components. The third experiment entailed vaccination of cattle with a conjugated Capsular Polysaccharide and subsequent experimental challenge with the infective Afadé strain. Disease outcome was analysed through clinical, pathological observations and immunological parameters. The protection levels were 31%, 80.8% and 74.1% for the formalin-inactivated, heat-inactivated and live attenuated preparations, respectively. Conjugated capsular polysaccharide produced a protection rate of 57%. The vaccine also elicited CPS-specific antibody responses with the same or a higher titer than animals vaccinated with the live vaccine. Interestingly, the animals immunised with membrane proteins had enhanced disease. These findings indicate that; i) low doses of heat-inactivated *Mmm* can offer protection to a level similar to the current live attenuated (T1/44) vaccine formulation, ii) vaccination with membrane proteins revealed enhanced inflammatory reactions after

challenge iii) capsular polysaccharide antigens conjugated to a protein is immunogenic and induces protective immunity in cattle and iv) high immunoglobulin (IgG and IgM) responses can be raised against the carbohydrate component of the CPS-based glycoconjugate. Future development for a vaccine against CBPP needs to focus on understanding of immunological reactions that leads to pathological conditions and those that lead to protection especially the innate immune response.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 CONTAGIOUS BOVINE PLEUROPNEUMONIA

1.1.1 Introduction

Contagious bovine pleuropneumonia (CBPP) is a highly infectious disease, primarily of cattle (both *Bos taurus* and *Bos indicus*), causing respiratory distress of pneumonia and occasionally the joints. It is caused by a bacterium, *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*). CBPP is invariably introduced into a herd by direct contact, inhalation of infective droplets from an infected animal or contact with contaminated water holes, dip tanks and grazing areas. Cattle of all types (both *Bos taurus* and *Bos indicus*) are susceptible with variations in breed susceptibility in cattle. For example, the N'Dama cattle of Guinea and imported cattle from Europe are more susceptible than the zebu (Masiga *et al.*, 1996). Age is also a determinant on the outcome of the disease. Calves of less than six months develop arthritis rather than the pulmonary disease (Masiga *et al.*, 1996). When the disease spreads for the first time in a naive cattle population, it generally causes high mortality. The disease was first described in Europe in the 16th Century (Provost *et al.*, 1987). It has been eradicated from most continents but still persists in Africa. Cattle production is affected through mortality and reduced productivity in countries that have the disease. World Organisation for Animal Health (Office International des Epizooties, OIE) has included the disease in the list of important notifiable diseases of trade (OIE, 2014) and hence affected countries are excluded from international trade. This is because of its high potential for transmissibility, irrespective of national borders and serious socioeconomic consequences. The disease may therefore

be considered as the most important bacterial disease that threatens the cattle industry in Africa.

1.1.2 Clinical signs

The incubation period of CBPP is poorly defined, clinical signs following infection may become apparent days to several months after the contact (Provost *et al.,* 1987) and hence become established in a herd before it is noticed. The disease manifests in different forms that range from hyperacute through acute and, subactute to chronic stages (Provost *et al.*, 1987). In the hyperacute form, death takes place after a week at most and is often without any other signs. The acute form, presents with sudden onset of fever of 40°C or more usually, after five to seven days. A typical respiratory disease then develops; breathing is laboured and hence the affected cattle stand with head and neck extended and forelegs spread apart, with dilated nostrils and mouth open panting for air. This is soon followed by a moist cough accompanied by a little foamy sputum, pronounced chest pain, sometimes restricted to certain regions of the ribs and extensive zone of dullness follow. The sub-acute form occurs most frequently in about 40 to 50 per cent of the animals affected. The symptoms resemble those of the acute form, but are less severe and fever is intermittent and never very high. This form usually develops into the chronic form. The chronic form presents with intermittent fever, together with loss of both appetite and weight. Calves of less than six months of life more often show lameness from swollen and painful limb joints. Up to 25 per cent of infected cattle can become chronic carriers of infection, often referred to as “lungers” and are believed to play a role in initiating new outbreaks when they are introduced into susceptible herds (FAO, 2002).

1.1.3 Pathology

CBPP is characterized by the presence of sero-fibrinous, interstitial pneumonia, interlobular oedema, hepatization and capsulated lesions termed as sequestra in the lungs and pleura in the posterior part of the chest. These pathological signs may not be exhibited by young calves. Usually one lung and pleura are affected by sero-fibrinous exudate, often of up to 30 litres containing clots of fibrin. Fibrin 'Omelettes' are often found floating in this fluid or attached to the parietal pleura and the lung surface (a pathognomonic sign). The chronic form of the disease presents with dry pleurisy that is characterised by adhesions joining the parietal membrane to the pulmonary membrane and no exudate in the thoracic cavity. Hepatisation of lobules normally commences at the periphery and proceeds towards the centre of the lobes. The lobes vary in colour from red, grey, and yellow depending on the stages of inflammatory lesions and often cover an extensive area of lung uniformly, giving it a characteristic marbled appearance. In animals during the chronic stage or after recovery, necrotic lesions in the lungs are surrounded by a capsule of fibrous connective tissue called sequestra. The size is variable from one to 30 cm in diameter and several can be found simultaneously in one lung. Small sequestras can be replaced by fibrous scars but large ones persist for years. A sequestrum may be drained by a bronchus, and become a source of infection. CBPP may also present with renal lesions, with well-defined yellowish infarctions, seen once the capsule is removed. Single or multiple renal infarcts are observed during different stages. The acute phase is represented by multiple infarcts while in subacute and chronic cases, infarcts progress to form large areas of fibrosis accompanied by tubular dystrophic calcification and tubular atrophy. The above description is adapted from FAO (2002),

Provost *et al.* 1987, Grieco *et al.,* 2001 and my observations during a CBPP vaccine trial (see figure 1.1)

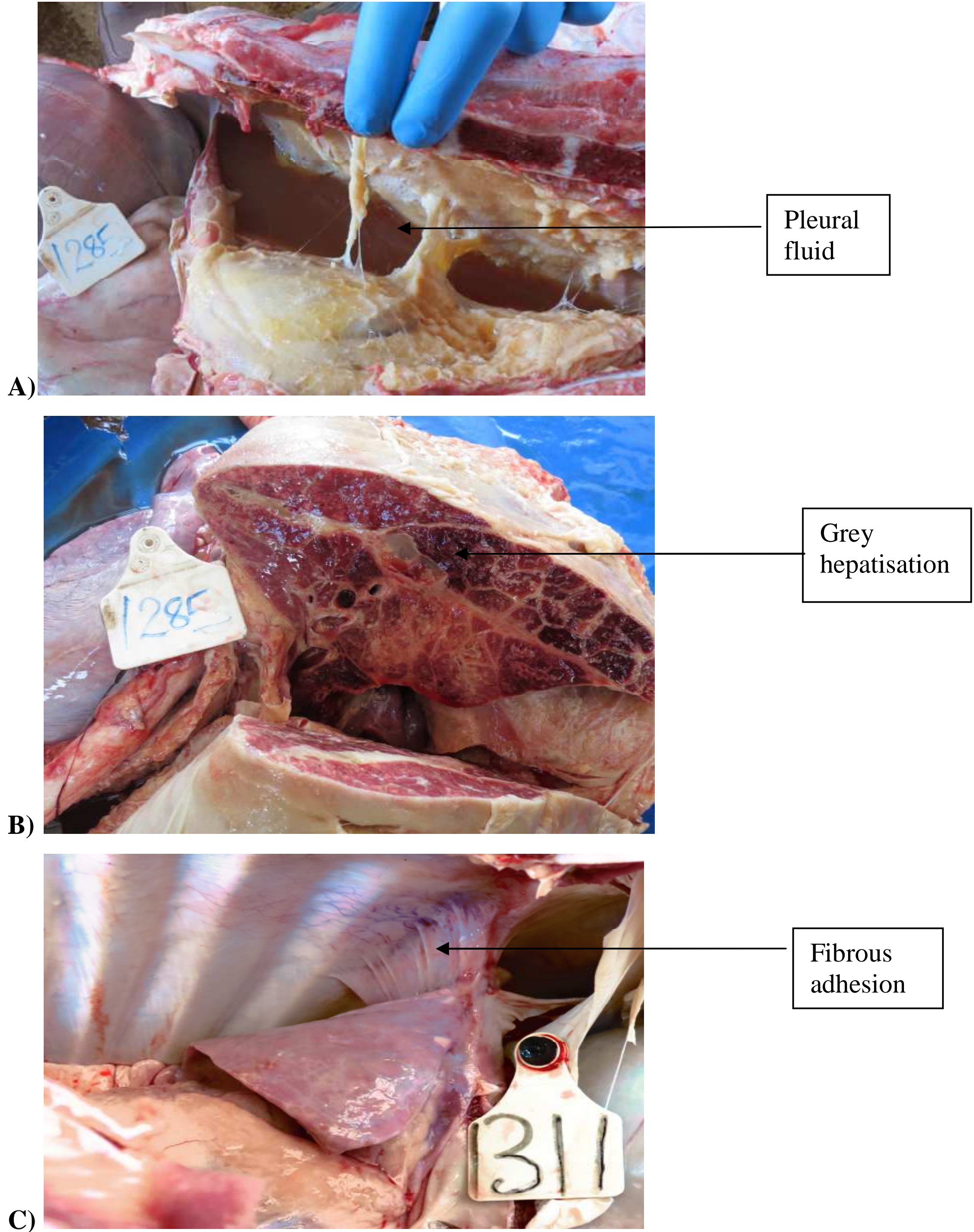


Figure 1.1 CBPP gross pathology, seen at post-mortem at examination. A shows the pleural cavity of a cow filled with pleural fluid and extensive fibrin deposits, B shows a marbled lung, thickened interlobular septae and grey hepatisation and C shows a lung adhering to the chest wall.

1.1.4 History and geographical distribution of CBPP

CBPP was first described in 1550 according to early writings about the disease. The disease was widespread over most of Europe due to wars and trade in the early 1800 and later introduced into United States from Britain via a dairy cow in 1843 but was eradicated by 1892. The disease later spread to Australia from Britain through trade. An eradication through "stamping out" campaign in 196Os steered the Australia declaration of being CBPP-free in 1973. It is claimed that CBPP was initially introduced in South Africa from Europe and spread by Boer settlers who trekked with their cattle up to the Kenyan highlands at the turn of the 20th century. (Description adopted from Windsor, 2000).

Although the disease has been eradicated in most parts of Europe, sporadic outbreaks exist, the latest being reported in 1990 in France, Portugal, Italy and Spain during the 1980s and 1990s but Europe has been CBPP-free since 1999 (Regalla *et al.*, 1996; Dupuy *et al.,* 2012). Data on prevalence of CBPP in Asia and the Middle East today is limited due to poor surveillance systems. The disease existed in China from 1919 until eradication in 1989 (Xin *et al.,* 2012). At present, CBPP is widespread in Africa, South of Sahara; from the Atlantic coast to the Indian Ocean, except for some countries in the very South, for example, South Africa and Botswana.

1.1.5 Diagnosis of CBPP

This section gives an account of diagnostic methods that are currently approved by OIE and recent methods that are not yet approved. The methods discussed involve; clinical, pathological, culture, serological and nucleic acid recognition.

1.1.5.1 Clinical, Pathological and Culture Diagnosis

Diagnosing CBPP using clinical signs is unreliable as early infection and those with mild disease may be asymptomatic or show signs indistinguishable from any severe pneumonia (Masiga *et al.* 1996; (OIE, 2008). Since the clinical appearance of the disease depends on the different stages of disease development and hence individual differences, examination of a large a number of animals is important (FAO, 2002). Examination for pathological lesions of CBPP is therefore the preferred method of disease monitoring. The lesions are distinctive and pathognomonic for the disease and can be used for disease surveillance in the abattoirs (OIE, 2008). Confirmation of the disease is best confirmed through isolation of the causative agent from affected cattle. The samples are taken from animals that are alive (nasal swabs or nasal discharges, pleural fluid and blood or at necropsy (lungs with lesions, lymph nodes of the broncho-pulmonary tract, and synovial fluid from those animals with arthritis) for culture (OIE, 2008). The isolated agent can then be identified by culture, biochemical, nucleic acid methods and immunological tests (Rice *et al.,* 2000; Rurangirwa *et al.,* 2000). However, the results vary depending on the stage of lesion development and if the animals had been treated with antibiotic, negative results are not conclusive.

1.1.5.2 Serological methods

A serological test encompasses detection of specific changes induced by a pathogen in the serum of an infected host. Serological tests which have been developed for the diagnosis of CBPP are generally classified into two groups on the basis of whether the test detects the presence of either specific antibodies or pathogen antigens. This method

of diagnosis is generally used for monitoring CBPP at herd level (Amanfu *et al.,* 2000; Bruderer *et al.,* 2002). Although different tests are in use in diagnosis of CBPP, a report by Muuka et al., (2011) after an experimental infection study indicate no single serological test can detect CBPP positive animals at all stages of infection. Complement fixation test (CFT) and a competitive enzyme-linked immunosorbent assay (cELISA) are the only recommended by OIE and widely used. Other tests used in the diagnosis of CBPP include: competitive ELISA, LppQ ELISA, Latex Agglutinations Tests, Immunoblotting Test and Polymerase Chain Reaction (PCR) based tests.

The CFT test involves incubation of diluted serum samples, *Mmm* as antigen and complement from normal guinea-pig serum in an uncoated 96 well microplate. This is followed by addition of a haemolytic system consisting of sheep red blood cells (SRBC) and rabbit hyperimmune serum to SRBCs and further incubation. If the serum sample contains antibodies to *Mmm*, they will bind the antigen and activate the compliment and hence consume the complement and prevent SRBC lysis. A negative result is indicated by heamolysis of SRBC. The results are then read as percentage of observed complement fixation (Campbell and Turner, 1953; OIE 2008). The sensitivity of CFT however is lower in the early or later stages of infection (three months after infection) (Bruderer *et al.,* 2002) therefore it is recommended that any observed complement fixation should be followed by additional investigations. Although the assay is straight forward, standardization of the mycoplasma and haemolytic system is considered difficult to perform and require skilled personnel (Bruderer *et al.* 2002; OIE, 2008).

The competitive ELISA (cELISA) offers some advantages in terms of ease of testing and standardisation of results, but it has sensitivity levels similar to CFT (Le Goff and Thiaucourt, 1998). cELISA also detects *Mmm* antibodies for longer, including during chronic phase of CBPP, but has lower sensitivity in detecting acute infections (Amanfu *et al.,* 2000; Niang *et al.,* 2006). The test involves competition between a monoclonal antibody (mAb) (3F3) and serum antibodies for an *Mmm* variable surface protein (Gaurivaud *et al.,* 2004). A CBPP positive sample reduces binding of the mAb and this results in subsequent colour reduction, while negative serum does not mask the epitope and the mAb is bound to the antigen and detected. The cut-off for the positive serum samples is set at 50% completion in comparison to a CBPP positive control.

An indirect ELISA based on the N-terminal half of Lipoprotein LppQ (Abdo *et al.,* 2000) was developed in 2002. The test is based on LppQ's ability to induce a strong and specific immune response to *Mmm* in infected cattle (Abdo *et al.* 2000; Bruderer *et al.* 2002). The LppQ-ELSA is comparable in sensitivity with cELISA and CFT (Bruderer *et al.* 2002; (Bruderer et al., 2002). However, the sensitivity of the LppQ-ELISA is low in the early phase of infection but higher after a longer time period post-infection when compared to the CFT (Bruderer et al., 2002). The LppQ-ELISA is currently under evaluation by the International Atomic Energy Agency (OIE, 2008). A recombinant antigen cocktail ELISA for diagnosis of CBPP has also been developed after selection of antigens among one-third of the surface proteome proteins of the infectious agent *Mmm* (Neiman *et al.,* 2009).

Two latex agglutination test (LAT) tests that are simple, easy to use and cheap have been developed. One is an antibody detection test based on a specific polysaccharide antigen extracted from the *Mmm* capsule which was bound to latex beads (Ayling *et al.*, 1999). The other is an antigen detection test and uses latex beads coated with a polyclonal antibody (pAb) specific for the capsular polysaccharide (CPS). However the latter test is not recommended due to false-positive reactions (March *et al.*, 2003).

An immunoblotting test (IBT) diagnostic test has been developed (OIE, 2008). The test analyzes the humoral immune responses in relation to the electrophoretic profile of *Mmm* antigens consisting of five immunodominant antigens with apparent molecular weights of 110, 98, 95, 62/60 and 48 kDa, (Gonçalves *et al.,* 1998). The IBT is highly specific and the most sensitive serological test described for CBPP (Schubert *et al.,* 2011). A field evaluation of the test revealed a sensitivity of up to 92.6% compared to 77.5% for CFT and specificity was 100% (Regalla *et al.,* 1996). However, the test is not suitable for mass screening and is used as a confirmatory test when false positive results in CFT are suspected (OIE, 2008).

1.1.5.3 PCR based molecular based tests

Polymerase Chain Reaction (PCR) based diagnostic tests offer rapid detection, specific identification and differentiation between members of the *Mycoplasma. mycoides* cluster (see section 1.2.1). The protocols are based on analysis of digested amplicons (Bashiruddin *et al.,* 1994; Persson *et al.*, 1999) and using primers that amplify genes specific to classical *Mycoplasma mycoides* cluster followed by species-specific amplification, example, Real-time PCR methods (qPCR) ant nested PCRs (Miserez *et al.*

1997; Bashiruddin *et al.,* 2005; Taylor *et al.,* 2008; Lorenzon *et al*. 2008). Use of PCR enables detection and identification of *Mmm* from culture and clinical materials including nasal mucous, tissue from lung, kidney, spleen, and semen from bovines (Bashiruddin *et al.,* 2005; Miserez *et al.,* 1997). A particular PCR assay able to differentiate between European and African/Australian *Mmm* isolates has been developed (Miles *et al.,* 2006). In Africa, especially in rural areas, use of the PCR is uncertain since the test depends on a laboratory infrastructure with sophisticated equipment and trained personnel that are not readily available. To counter these challenges an in-the-field diagnostic test based on loop-mediated amplification (LAMP) of DNA under isothermal conditions (Notomi *et al.,* 2000) has proved to be more specific and sensitive (Mai *et al.*, 2013).

1.1.5.4 Biochemical Tests

The biochemical tests are based on *Mmm* sensitivity to digitonin, its inability to produce 'film and spots', its capacity to ferment glucose, its reduction of tetrazolium salts (aerobically or anaerobically), its failure to hydrolyse arginine, its lack of phosphatase activity, and its weak or lack of proteolytic properties (OIE, 2008). These tests can be carried out if the immunological and molecular based tests are inconclusive and should be carried out by a reference laboratory.

1.1.6 Economic impact of CBPP in Africa

Economic losses due to CBPP can be massive, resulting from direct death of animals and indirect effects of decline in economic activities. Quantification of many direct and indirect costs is impossible to determine with any precision (Windsor and Wood, 1998). However, studies have been done to try and estimate the losses in Africa. Direct loss due

to death of livestock in Africa has been estimated to cost 2 billion US$ per year (Masiga *et al.,* 1998). A study by Tambi and others (2006) in 12 sub-Saharan African countries (Burkina Faso, Chad, Côte d'Ivoire, Ethiopia, Ghana, Guinea, Kenya, Mali, Mauritania, Niger, Tanzania and Uganda) estimates the cost of CBPP to 3.7 million Euros per country. The socioeconomic implications associated with the loss of the animals include; decline in economic activity in industries that depend on cattle and their products and disruption of trade. For example, in Botswana, closure of its access to the European Union (EU) market and the economy-wide effects of such a closure would be a 60% decline in beef and other export products (Tambi *et al.*, 2006). The financial return on investment in CBPP control is positive, an investment of 14.7 million Euros to control CBPP would prevent the loss of 30 million Euros (Tambi *et al.*, 2006). True cost of control and eradication require several considerations as described by Windsor and Wood, (1998). Some of the factors include; government costs during outbreaks (compensation, slaughter, testing, surveillance and tracing), loss of government income from taxes, cost of maintaining CBPP free zones and cost of control measures to local people.

1.2 BIOLOGY OF *MYCOPLASMA MYCOIDES SUBSP. MYCOIDES*

1.2.1 Taxonomy and phylogeny

Mycoplasmas are the smallest self-replicating organisms, free-living bacteria (Razin *et al.,* 1998) and have evolved from other bacteria by regressive evolution, leading to reduced genomes (Weisburg *et al.*, 1989) that span from 577-590 kb in *Mycoplasma genitalium* (Su and Baseman, 1990) to 1359 kb in *Mycoplasma penetrans* (Sasaki *et al.,* 2002). The 1212 kb genome of *Mmm* was published in 2004 (Westberg *et al.,* 2004).

Taxonomically, mollicutes are distinguished phenotypically from other bacteria by their small size and total lack of a cell wall (Razin *et al.,* 1998). "Mycoplasma", is the trivial name used to refer the species in the class Mollicutes. The Mollicutes are classified into five groups (hominis, pneumonia, anaeroplasma, spiroplasma and group known to contain only the isolated species *Asteroleplasma anaerobium*) based on the 16S rRNA sequencing, placing *Mmm* in the spiroplasma group (Weisburg *et al.,* 1989). Within that group, it is one of five closely related species that form the *Mycoplasma mycoides* cluster where all members are highly pathogenic and cause severe diseases in ruminants (see table 1.1).

Initial classification within the *Mycoplasma mycoides* cluster using DNA–DNA hybridization and biochemical and serological methods as well as 2D Polyacrylamide gel electrophoresis (PAGE) patterns (Costas *et al.,* 1987) resulted in differentiation of the *M. mycoides* subspecies and the *Mycoplasma capricolum* subspecies. However, a phylogenetic tree of the *Mycoplasma mycoides* cluster was inferred from a set of concatenated sequences from five housekeeping genes (fusA, glpQ, gyrB, lepA and rpoB) led to amendment of the cluster (Manso-Silván *et al.*, 2007). The taxonomy of the *Mycoplasma mycoides* cluster was amended resulting in the combination of *Mycoplasma mycoides* subsp. *mycoides* large colony type and *Mycoplasma mycoides* subsp. *capri* into a single subspecies and the assignment of the *Mycoplasma* sp Bovine group 7 strains into the separate species *Mycoplasma leachii* (Manso-Silvan 2009). The classification of the bacterium is as outlined below;

Phylum: Firmicutes

Class: Mollicutes

Order: Mycoplasmatales

Family: Mycoplasmataceae

Genus: Mycoplasma

Species: mycoides subspecies mycoides

Table 1.1 Members of the *Mycoplasma mycoides* cluster of veterinary importance

Species	Host	Disease
M. mycoides subsp. *mycoides*	Cattle (Buffalo, goat, sheep)	Contagious bovine pleuropneumonia
M. *capri*	Goat, sheep (cattle)	Contagious agalactiae/pneumonia
M. capricolum subsp. *caprineumoniae*	Goat (sheep)	Contagious caprine pleuropneumonia (CCPP)
Mycoplasma leachii	Cattle (goat, sheep)	Arthritis, mastitis, calf pneumonia
M. capricolum subsp. *Capricolum*	Goat	Contagious agalactiae/pneumonia

Table adapted from (Cottew and Yeats, 1978; Thiaucourt and Bölske 1996; (Manso-Silván *et al.,* 2007; Thiaucourt *et al.,* 2011).

1.2.2 Host parasite interaction - host specificity

Mycoplasmas are widespread in nature as parasites of humans, mammals, reptiles, fish, arthropods, and plants (Razin *et al.,* 1998). They are mostly extracellular parasites (Razin *et al.,* 1998; Rosengarten *et al.,* 2000) but there are some exceptions: *Mycoplasma penetrans* can invade and survive in its human host cell and *Mycoplasma fermentans*, *Mycoplasma pneumoniae*, *Mycoplasma genitalium* as well as *Mycoplasma gallisepticum* sometimes reside in nonphagocytic cells (Rottem, 2003).

The genomic reduction in *Mollicute* evolution has led to a limited metabolic capacity hence most mycoplasmas are parasites exhibiting strict host and tissue specificities (Rottem, 2003). Nevertheless, some mycoplasmas can be found in hosts and tissues different from their normal habitats (Razin *et al.,* 1998). For example, respiratory infections to hamsters can be induced in hamsters by the human respiratory pathogen *Mycoplasma pneumoniae.* Organ and tissue specificity is also exhibited by mycoplasma. Thus, *M. pneumoniae* is found preferentially in the respiratory tract and *M. genitalium* is found primarily in the urogenital tract, but exceptions are possible as demonstrated by *M. genitalium* isolation from the respiratory tract and *M. pneumoniae* from the genital tract (Goulet *et al.*, 1995). *Mmm* has been shown to be significantly more adherent to bovine epithelial cells than caprine and porcine, suggesting that the cytoadherence reflects specificity for the bovine species (Aye *et al.,* 2015).

1.2.3 Major surface antigens and their potential impact in virulence of *Mmm*

Some of the prominent surface antigens detected on the cellular membrane of mycoplasmas, and in particular of *Mmm* include: lipoproteins, metabolic pathway enzymes, variable surface antigens, adhesins and capsular polysaccharide. Although in other pathogenic bacteria, virulence is determined mainly by toxins, cytolysins and invasins, no such typical primary virulence genes have been found on the genomes of the Mycoplasmas species that have been sequenced (Sasaki *et al.,* 2002; Vasconcelos et al. 2005; Jaffe *et al.,* 2004; Westberg *et al.*, 2004).

1.2.3.1 Lipoproteins

A number of *Mmm* lipoproteins have been described. Lipoprotein A (Monnerat *et al.*, 1999), has been demonstrated that is an important target for the CD4 T-cells (Dedieu *et al.,* 2010). The functions Lipoprotein B (Vilei *et al.,* 2000) and Lipoprotein C (Pilo *et al.,* 2003) are under investigation. Lipoprotein Q (LppQ) (Abdo *et al.,* 2000) is specific for *Mmm* and its strong antigenicity has been exploited for the development of a robust indirect ELISA test for serological diagnosis of CBPP (Bruderer *et al.,* 2002). Lipoproteins play a role in mechanisms of pathogenicity by inducing pro-inflammatory cytokines and might assume the function of lipopolysaccharides which are missing in mycoplasmas (Brenner *et al.,* 1997; Calcutt *et al.,* 1999; Marie *et al.,* 1999; Calcutt *et al.,* 1999). A study on the use of recombinant LppQ as a vaccine in adult cattle indicated enhanced disease that may be attributed to the protein (Nicholas *et al.,* 2003*;* Mulongo *et al.,* 2015).

1.2.3.2 Variable surface antigens

Variable surface proteins (Vsp) have been investigated extensively in mycoplasmas including; *Mycoplasma agalactiae*, *Mycoplasma hyorhinis*, *Mycoplasma penetrans* and *Mycoplama pulmonis* (Citti *et al.,* 2000; Glew *et al.,* 2000; Röske *et al.*, 2001; Flitman-Tene *et al.,* 2003). *Mmm* has been shown to have a variable surface protein of 16 kDa designated Vmm (Persson *et al.,* 2002). Its function is currently not known, but it is suspected to help *Mmm* escape the host's immune system. Vsp's have been shown to have a role in the evasion from the host's immune response by undergoing high-frequency phase and size variation (Denison *et al.*, 2005; Simmons and Dybvig, 2007).

1.2.3.3 ATP binding cassette (ABC) transporter proteins

In *Mmm* type strain, eight complete ATP binding cassette (ABC) transporters have been identified (Westberg *et al.,* 2004). The ABC transporters are involved in active glycerol uptake and glycerol phosphorylation. The system involved consists of the glycerol uptake system GtsABC (Vilei and Frey, 2001) and membrane bound L-α-glycerophosphate oxidase (GlpO) Pilo *et al.*, 2005). The glycerol is metabolized in *Mmm* after phosphorylation to dihydroxyacetone phosphate by an oxidative process leading to the release of the highly toxic compound H_2O_2 that cause oxidative damage to the host tissues. The study also indicated that antibodies against GtsB noncompetitively inhibits glycerol uptake resulting in a significant reduction of H_2O_2 production. This suggests that the glycerol uptake system GtsABC is indirectly involved in virulence of *Mmm*. The new emerging European strains of *Mmm* lack the *gtsB* and *gtsC* genes, hence these strains produce significantly lower amounts of H_2O_2 and also seem to be less virulent than African strains that possess the full *gtsABC* operon and are highly virulent. The L-α-glycerophosphate oxidase (GlpO) has been shown to play a direct role in cytotoxicity of *Mmm* strains towards embryonic calf nasal epithelial (ECaNEp) cells (Pilo *et al.*, 2005). Further studies on cytotoxic effects to ECaNEp showed that vaccine strain T1/44 that is low virulence also released high amounts of H_2O_2 an indication of high toxicity. Therefore there must be other factors contributing to pathogenicity of *Mmm.*

1.2.3.4 Adhesins

Adhesins play an important role in the primary steps employed by mycoplasmas while interacting with their host eukaryotic cells using specific mammalian membrane receptors (Rottem, 2003). The intimate interaction of mycoplasma with mammalian cells for long

periods is assumed to trigger a cascade of signals that cause inflammation (Razin *et al.,* 1998). Although several adhesins have been identified in other *Mycoplasma* species (Razin *et al.,* 1998; Belloy *et al.,* 2003; Seymour *et al.,* 2011), adhesins of *Mmm* have not yet been detected in spite of their potential role in immune protection.

1.2.3.5 Capsular polysaccharide

Mmm synthesises a galactan or capsular polysaccharide (CPS) consisting mainly of galacto-furanosyl units in 1-6 *p* linkage (Plackett and Buttery, 1964) located on the outer surface or in the plasma membrane and secreted in the blood stream of infected cattle (Bertin *et al.,* 2013). The CPS is presumed to give the mycoplasma physico-chemical resistance against the host, such as the bactericidal activity of complement and other host defence functions and to trigger the inflammatory process in the infected host (Pilo *et al.,* 2007). Studies have also attributed the capsular polysaccharide to virulence of *Mmm*. Lloyd and Titchen (1976) showed that intravenous injection of galactan from *Mmm* to calves produced transient apnoea, increased pulmonary arterial pressure and pulmonary oedema, leading to the contraction of blood vessels, which may initiate thrombosis. March and others (2000) demonstrated that a strain of *Mmm* that produces low amounts of capsular polysaccharide was much more sensitive to growth inhibiting antisera than strains that produced larger amounts of polysaccharide. Strains of *Mmm* that produced large amounts of capsular polysaccharide also generated a significantly longer duration of bacteraemia in a mouse infection assay than the strain with little capsular polysaccharide (March *et al.,* 2000). Genes associated with polysaccharide synthesis and forming a biosynthetic pathway are organized in clusters within two loci representing genetic variability hot spots acquired via horizontal gene transfer (Bertin *et al.,* 2015). The

redundancy capsule biosynthesis genes in the *Mmm* genome may reflect the importance of the capsule as a virulence factor (Westberg *et al.,* 2004).

1.3 IMMUNE RESPONSES TO *MMM*

1.3.1 Introduction

Animals that recover from CBPP infection are resistant to further challenge (Windsor and Masiga, 1977). Although this suggests immunity is induced following CBPP infection, there is limited understanding of the protective immune response. The immune mechanism against *Mmm* has been suggested to involve innate immunity as well as both humoral and cellular responses (Masiga *et al.,* 1975; Cartner *et al.,* 1998).

1.3.2 Innate immunity

Mycoplasma invasion of respiratory surfaces encounter resistance from non-specific defence mechanisms (Cartner *et al.*, 1998). Activation of innate immune response by inducing production of chemokines and cytokines through attraction and activation of neutrophils and mononuclear leukocytes is one of the mechanisms (Kaufmann *et al.,* 1999; Kauf *et al.,* 2007). Macrophages have also been shown to play a critical role in clearance of mycoplasma (Lai *et al.,* 2010). In *Mmm*, a study on plasma cytokine in two groups of cattle ($CD4^+$ T cell-depleted and non-depleted cattle) experimentally infected with *Mmm* showed that irrespective of the depletion status, high TNF-alpha levels correlated with more severe pathology accompanied by high IFN-gamma levels (Sacchini *et al.,* 2012).

Pattern recognition receptors (PRR) are important in host's innate immunity against pathogens. Toll-like receptors (TLR) are the best-characterized groups of PRR family. TLRs are receptors responsible for recognizing pathogens as diverse as gram-positive and gram-negative bacteria, viruses, and fungi, as well as protozoa (O'Neill and Bowie, 2007; Takeda and Akira, 2005). The TLRs recruit signalling molecules to their intracellular signalling domains, leading to the activation of the nuclear factor kappa beta (NF-κβ) and secretion of proinflammatory cytokines (Triantafilou *et al.*, 2006). TLRs also play an important role in activation and maturation of dendritic cells (Visintin *et al.*, 2001). Bovine TLR genes have been characterised, in bovine skin TLRs 2 and 7 are most abundant and believed to play a role in bacterial recognition (Menzies and Ingham, 2006). The role of TLRs in *Mmm* has not been studied. However, M161Ag, a 43-kDa surface lipoprotein of *Mycoplasma fermentans* have been shown to be important for TLR2-mediated cell activation and complement activation (Nishiguchi *et al.,* 2001). Studies indicate TLR2 plays a critical role in the ability of innate immunity to determine *Mycoplama pulmonis* numbers early after respiratory infection by triggering initial cytokine responses of host cells (Love *et al.,* 2010).

1.3.3 Humoral immune responses against *Mmm*

Antibody binding and antibody dependent immune mechanisms are important in response against *Mmm* as evidenced by the protection against CBPP following transfer of sera from cattle recovered from CBPP (Masiga *et al.,* 1975). MAbs targeting membrane epitopes in *Mmm* have been shown to have growth-inhibiting activity (Kiarie *et. al.,* 1996). The humoral immune response to *Mmm* studies have mainly focused on identifying the immunodominant surface proteins (Monnerat *et al.,* 1999; Abdo *et al.,*

2000; Pilo *et al.,* 2003; Hamsten *et al.*, 2010). Specific antibodies responsible for immunity have not yet been identified. Analyses of *Mmm*-specific IgM, IgG1 and IgG2 antibody responses done at the pulmonary (local) and systemic levels for animals with disease experimentally reproduced by contact and followed for over a year revealed no correlation between antibody titters and clinical signs or lung lesions (Niang *et al.,* 2006). However, IgA titers in lung or blood seemed to play a role in protection against CBPP. A study on kinetics of the humoral immune response against 65 *Mmm* surface antigens for an extended period in cattle that survived a primary infection with Mmm revealed no antigen-specific antibodies (Schieck *et al.,* 2014).

1.3.4 Cell-mediated immune responses to *Mmm*

Cell-mediated immunity has been postulated to be involved in protection against CBPP (Roberts *et al.,* 1973; Tulasne *et al.,* 1996). A study by Dedieu and others (2005) demonstrated that all *Mmm*-infected cattle developed a *Mmm*-specific cell-mediated immune response. In the experiment, endobronchially- and in-contact infected animals showed *Mmm*-specific immune response with the difference being the delay before the onset. The *Mmm*-specific CD4 Th1-like T-cell response was maintained until slaughtering whereas in animals with acute disease, progression of CBPP was associated with a decreased ability of the PBMC to produce IFN-γ. Further experiments on CBPP infection focusing on cell-mediated responses in lymph nodes reported a persistent *Mmm* specific response mediated by IFN- γ secreting CD4+ T cells in lymph nodes of all recovering cattle (Dedieu *et al.*, 2006). The responses were also significantly higher in completely recovering animals than in recovering animals with lung sequestra. CD4+ T cells have been shown to contribute to proliferation of cells collected from lymph nodes

draining the lungs of *Mmm*-infected cattle and stimulated with the pathogen in vitro and T1 biased cytokine recall responses observed in cattle that have recovered from infection but not in animals developing the acute form of the disease (Totté *et al.,* 2008). The two phenotypes of *Mmm*-specific memory CD4 were observed based on CD62L expression and proliferative capacities while recall proliferation of B cells occurred but was strictly dependent on the presence of CD4. Analysis of IFN-γ released and pathological changes at necropsy of naïve cattle treated with cyclosporine and exposed to in-contact infection showed a delay in events that follow infection, an indication of it's role in disease outcome (Scacchia *et al.*, 2007). However, in other studies the correlations of IFN-γ release and severity of disease was not as evident as described by Jores *et al.*, (2008) reported no correlations between IFN-γ release and presence or absence of pathological lesions after a studying IFN-γ released upon stimulation in peripheral blood mononuclear cells (PBMCs) collected from experimentally infected cattle.

Understanding of the role of IFN- release and CD4+ T cells may therefore play an important role in development of a vaccine against CBPP.

1.3.4 Immunopathology induced by *Mmm*

Generally, Mycoplasmas have evolved strategies to cause tissue injuries through stimulation of immune responses. Release of mediators by cells in response to mycoplasma-derived components induce recruitment and extravasation of leukocytes to the site of infection by mycoplasmas thereby causing tissue damage (Razin *et al.,* 1998). In *Mmm* infection, inflammation of the lung is the major pathological outcome (Provost *et al.*, 1987; Hübschle *et al.,* 2003). Kidney lesions have also been reported in cattle

infected with *Mmm* (Grieco *et al.,* 2001). The precise mechanism is not well understood. However, immune responses to the pathogen have been attributed to pathogenesis of the disease (Provost *et al.,* 1987; Dedieu and Balcer-Rodrigues, 2006). The kidney lesions observed in cattle infected has been attributed to immune complex deposition (Grieco *et al*., 2001; Mulongo *et al.*, 2015). Induction of autoimmune reactions (Gavanescu *et al.,* 2004) and mitogenic activation of B and T lymphocytes also trigger inflammatory signals (Razin *et al.*, 1998). In *Mmm*, live *Mmm* has been shown to trigger apoptosis of all bovine lymphocyte subsets as well as the monocyes/granulocyte subset while heat inactivated *Mmm* did not induce a similar effect, suggesting that secreted substances may play a role in the cytotoxic effect (Dedieu *et al.*, 2005). This has also been demonstrated in *Mycoplasma hominis* and *Mycoplamsa salivarium* (Zhang and Lo, 2007). The molecules responsible for apoptosis of infected cells by *Mmm* are yet to be identified (Pilo *et al.,* 2007). This demonstrates that development strategy of an efficient vaccine against CBPP will depend on understanding of the protective and immunopathological mechanisms.

1.4 CONTROL OF CBPP

CBPP has been successfully controlled in Botswana and developed world while in rest of Africa it has continued to spread and affect new areas (Muuka *et al.,* 2013). This has been attributed to uncontrolled or illegal movement of cattle from known infected cattle populations due to failure of surveillance systems, emergency preparedness and early reaction to outbreaks (Amanfu, 2009). The methods that have been used to control CBPP include; cattle movement control and quarantine, stamping out, test and slaughter, treatment and vaccinations.

1.4.1 Movement control, test and slaughter and stamping out

Adequate control of cattle movement is one of the most efficient methods of containing spread of CBPP within a country. In Africa, the method presents several challenges that include: inadequate veterinary personnel, transhumance lifestyle, trade, socio-cultural practices and civil strife (Provost *et al.*, 1987; Windsor and Wood, 1998; Msami *et al.*, 2001).

The test and slaughter method that involve testing of suspected herd or detection at meat inspection and positives slaughtered has been successful in the control of CBPP (Santini, 1998). However, the method is lengthy and need to be supported with adequate quarantine (Msami *et al.,* 2001; Kairu-wanyoike *et al.,* 2014).

Stamping out is considered to be the simplest and sure way to control CBPP. However, most African countries lack the resources for compensation of cattle owners (Okaiyeto *et al*., 2011). The method has also had serious negative socio-economic effects as experienced by a stamping-out eradication of CBPP in Botswana during 1996, which led to negative effects on short-term economics and increased malnutrition in children (Mullins *et al.,* 2000). The method should therefore be used as a last resort and should be considered in non-endemic areas (OIE, 1992).

1.4.2 Treatment using antibiotics

The use of antibiotics in CBPP treatment is controversial due to concerns of creation of chronic carriers arising from sequestra formation which are believed to be responsible for disease spread (Thiaucourt *et al.,* 2004; Amanfu, 2007). However, there is no sufficient

evidence that sequestra will break down to cause clinical disease emanating from such sequestra (Huebschle *et al.,* 2006). Experimental evaluation of a long-lasting oxytetracycline did not result in significant sequestra formation in CBPP-infected animals (Niang *et al.,* 2010). Even though treatment with antibiotic therapy is discouraged by OIE, they are routinely administered by farmers (Thiaucourt *et al.,* 2004). Studies of the tetracycline that is used most often in the field show reduced inflammation at the inoculation site but did not prevent infection and the pathogen was able to persist in the host (Thiaucourt *et al.,* 2004; Danbirni *et al.,* 2010). Therefore, may be it might have some benefit together with vaccination campaigns, for example, in the control of post-vaccination reactions to reduce mortalities and bacterial burden. Since mycoplasmas have no cell wall traditional antimicrobial targeting this structure such as penicillins have are not effective but antibiotics targeting protein or nucleic acid synthesis are effective. However, antimicrobials might have some benefit when used in combination with vaccination campaigns, for example, in the control of post-vaccination reactions to reduce mortalities and bacterial burden. However, recent *in vivo* studies have shown a reduction in CBPP transmission after oxytetracycline treatment (Niang *et al.,* 2010). *In vitro* experiments have also shown reduction in the number of mycoplasma by oxytetracycline, danofloxacin and tulathromycin (Mitchell *et al.,* 2013). Further research in this area is therefore important in enhancing reduction of the disease burden.

1.4.3 Vaccination

Vaccination against CBPP is the routinely used method of control in Africa due to its practicability (Tulasne *et al.,* 1996). The vaccines currently used are derived from the T1/44 strain of *Mmm* (OIE, 2008). This strain was derived from a Tanzanian isolate and

attenuated by passaging 44 times in embryonated eggs (March *et al.,* 2000). Despite its long use, it has several drawbacks that include: protection that lasts 12 months or less (Thiaucourt *et al.,* 2000), an efficacy level of only 40-60% and that can be increased to 80-95% upon revaccination (Thiaucourt *et al.,* 2004), severe post-vaccinal adverse reactions that result in lesions at the point of inoculation (Sori, 2005; Kairu-Wanyoike *et al.,* 2014) and the possibility of reversion to virulence (Thiaucourt *et al.,* 2004). Present CBPP vaccines also require to be kept cool during storage, transportation, reconstitution and use in the field, hence the absolute requirement of a cold chain (Tulasne *et al.,* 1996). In Africa, maintenance of a cold chain is an expensive undertaking. Other factors that have led to low success rate of vaccination include: inadequate funding of annual cattle mass vaccination programmes, availability of a rapid on farm screening test to aid sero monitoring and deficiency in awareness on the preventive measures and interventions (Kairu-wanyoike *et al.*, 2014).

In Africa, there has been no clear strategy to apply in control/eradication of CBPP. Many African veterinary services call for yearly repeated intensive vaccination campaign while experts claim that anything less than complete commitment, political social and economic, will result in failure (Thiaucourt *et al.,* 2004). However, several suggestions have been highlighted to counter the above challenges in the methods involved in control of CBPP. Experience and current data shows that the repeat vaccinations using the T1/44 vaccine significantly reduce the post-viccinal reactions (Thiaucourt *et al.*, 2004; Muuka *et al.,* 2014). Therefore, regular vaccination of the cattle at risk of contracting CBPP may enhance acceptance of the vaccine by farmers. The burden of compensation can be reduced by involving institutions involved in beef marketing that can compensate the

farmers through salvaging CBPP infected cattle when slaughtered (Muuka *et al.,* 2013). Since CBPP is a trans-boundary disease a high degree of cooperation is needed between neighbouring countries in the development and implementation of regionally coordinated CBPP prevention, preparedness and control/eradication programmes (Amanfu, 2009).

1.5 Research towards a better vaccines against CBPP

1.5.1 Introduction

The first crude vaccine against CBPP was first evaluated in 1852 by Louis Willems and consisted of pleural exudates from diseased animals (Blancou, 1996). Since then several forms of CBPP vaccines have been developed and used in the past, they include; T1 and its derivatives T1/44 and T1sr (Sheriff and Piercy, 1952) and KH3J strain and its streptomycin resistant KH3Jsr (Lindley, 1965). Pastoral communities in Africa have used (and still use) the Willem's method of subcutaneous inoculation of infective lung fluids into unaffected cattle (Blancou, 1996). Egg based vaccines were used extensively in East Africa in the 1960s (Rweyemamu *et al.,* 1995). Although they confer some protection they are discouraged due to severe adverse reactions (Willems' reaction) at the injection point. The gross lesions are characterised by an oedema that appears 10-20 days after inoculation. This oedema varies in size and, in some cases lead to loss of the tail or death of the animal. Inactivated vaccines have also been tested experimentally, but the protection levels reported were not satisfactory (Gray *et al.,* 1986). However, some of the experiments reported successful protection of cattle using an inactivated, oil adjuvanted CBPP vaccine (Garba and Terry, 1986).

1.5.1 T1/44 vaccine improvement

Attenuated broth cultures derived from passaged isolates of *Mmm* are the only vaccine types that have been in use within Africa (Rweyemamu *et al.,* 1995). The protection conferred by the CBPP vaccines is relatively of short duration (one year for strain T1 and six months for KH3J) implying that booster vaccinations are required. Unfortunately, even after attenuation the strains may still retain some residual virulence. This was demonstrated by the T1/44 vaccine strain capacity to cause CBPP following endobronchial inoculation of the vaccine (Mbulu *et al.,* 2004).

Failure by the T1sr vaccine during the campaigns in the 1990s as evidenced by the inability of the vaccine in Botwana in 1995 necessitated the need for improvement (Masupu *et al.,* 1997; Thiaucourt *et al.,* 2000). Following this event the use of T1sr was even discouraged. A study comparing T1/44 and T1sr vaccines conducted in three African countries revealed a protection rate of between 33% - 67% after three months, depending on the strain used in the challenge (Thiaucourt *et al.,* 2004). Since then there have been suggestions to change the composition and in maintaining the viability of the live vaccines to improve efficacy (Tulasne *et al.,* 1996). The OIE recommended the minimum dose for cattle is 10^8 viable mycoplasma for CBPP vaccine (OIE, 1992), allowing losses during lyophilisation, storage and transport. To maintain this high number the use of 75mM *N*-[2-hydroxyethyl] piperazine *N*-[2-thanesulfonic acid] (HEPES) was recommended to buffer the growth media since *Mmm* is pH sensitive (March *et al.,* 2002). An experimental evaluation of the buffered and the current vaccine however did not reveal differences in protection levels (Nkando *et al.,* 2012)

1.5.2 Novel approaches in developing a safe and efficacious CBPP vaccine

A CBPP vaccine that is thermostable and averts side reactions will play a key role in containing the disease, as long as it has an efficacy equal to or better than the current vaccine. This could be achieved through various approaches that eliminate elements acting as virulence factors while maintaining the same level of immunogenicity. An empirical approach that involves development of a better vaccine against CBPP through several methods in parallel, identification of the protective arm of immunity and understanding of pathological mechanism has been suggested (Jores *et al.,* 2013).

The use of a subunit vaccine would solve some of the problems encountered by the live culture vaccine. In general, a vaccine based on subunits facilitates the possibility of avoiding components which have a negative regulatory effect on the ensuing immune response.

1.5.2.1 Immunostimulating complex (ISCOM) vaccines

The ISCOM is formulated as a particle which combines a multimeric presentation of antigen with a built-in adjuvant. The ISCOM particle is a cage-like structure of about 40 nanometres (nm), composed of the saponin adjuvant Quil A, cholesterol, antigen and phospholipids (Morein *et al.,* 2004). ISCOM can be considered as a carrier for a combination of antigen and adjuvant, targeted at antigen-presenting cells (APCs) and the lymphatic system (Tulasne *et al.,* 1996). A vaccine against CBPP based on ISCOMs was first evaluated in mice and cattle in 1997 using detergent-solubilized *Mmm* cells to create a subunit vaccine with a high number of antigens (Abusugra *et al.,* 1997). Experimental trials in cattle revealed strong primary and long lasting secondary antibody responses in

similar magnitudes to naturally infected animals, as well as induced T cell responses (Abusugra et al., 1997). A field trial with the vaccine showed significantly reduced mortality compared with controls. However, there was no difference in the gross pathological and histopathological score between vaccinated and non-vaccinated animals (Hübschle *et al.*, 2003). No difference in the formation of sequestra was apparent between the groups. The vaccine was therefore considered as not to induce a protective response. Another experimental trial using an ISCOM formulation of recombinant LppQ (Abdo *et al.,* 2000) did not offer any protection to CBPP challenge and appeared to exacerbate the pathological lesions as compared to unvaccinated control animals (Nicholas *et al.,* 2003). The failures may be attributed to the *Mmm* components used in these formulations and use of ISCOMs is still a viable approach for delivery of a CBPP subunit vaccine (Dedieu-Engelmann, 2008).

1.5.2.2 DNA vaccine

Whole bacteriophage (phage) λ particles have been described as highly efficient vehicles for DNA vaccine delivery (Clark and March, 2004; Lankes *et al.,* 2007). In the context of CBPP vaccine development, a DNA vaccine by bacteriophage λ library screening was tested (March *et al.*, 2006). In this study, a whole genome library was cloned into a bacteriophage λ ZAP Express vector which contains both prokaryotic (Plac) and eukaryotic (PCMV) promoters upstream of the insertion site. Two strongly immunodominant clones were identified and subsequently tested for vaccine potential against *Mmm* biotype-induced mycoplasmemia. After challenge, mice vaccinated with clone (expressing MSC_0397) exhibited a reduced level of mycoplasmemia compared to controls, suggesting a protective effect when delivered as a bacteriophage DNA vaccine.

However, evaluation of the vaccine in cattle is needed to confirm these results. This method represents a new approach to immunization which is potentially less expensive, stable and easy to produce (March *et al.,* 2006; Mateen and Irshad, 2012).

Apart from having a high efficacy and thermostability, it would be useful that future vaccines allow discrimination between infected and vaccinated animals. A safe marker system must therefore be incorporated into new vaccines (Tulasne *et al.,* 1996).

1.6 PROBLEM STEMENT AND RATIONALE

At present, the protective antigens of the mycoplasma are not known. It is likely that protective antigens are located on the surface of the mycoplasma, where they are accessible to host antibodies. The main antigens on the surface are protein or carbohydrate in nature, and both can have essential functions for the cell, such as adherence to the host cell or uptake of nutrients. The purpose of the research reported in this thesis was to assess the nature of the protective antigens, by testing the immunity induced by inactivated mycoplasma, membrane proteins and the capsular polysaccharide.

1.6.1 Evaluation of inactivated vaccines

Inactivated vaccines against CBPP have been tested experimentally with varying results but such vaccines have not been tested in the field (Garba and Terry, 1986; Gray *et al.*, 1986). The experiments, however did not offer conclusive reports on the capacity of inactivated mycoplasma to protect against CBPP.

The present study therefore aimed at assessing protective capacity of formalin inactivated (Garba and Terry, 1986) and heat inactivated (Gray *et al.,* 1986) vaccines compared with the live attenuated T1/44 vaccine.

1.6.2 *Mycoplasma mycoides* subsp. *mycoides* membrane protein vaccine

Membrane proteins of mycoplasmas have been shown to play a role in adhesion to host cells (Razin *et al.,* 1998; Rottem 2003) and are immunogenic in nature (Kahane and Razin 1969; Droesse *et al.,* 1995; Svenstrup *et al.,* 2006). Some of the membrane lipoproteins from *Mmm* have been characterized in detail. Most of them are major antigens and are readily detected in the serum of infected cattle on immunoblots, including LppA (Monnerat *et al.,* 1999a; Monnerat *et al.,* 1999b), LppB (Pilo *et al.,* 2003) and LppQ (Abdo *et al*., 2000). Monoclonal antibodies (MAbs) against surface proteins of *Mycoplasma gallisepticum*, have shown to have capacity to inhibit both growth and metabolism *in vitro* (Yoshida *et al*., 2000). Thus given the potential of the membrane proteins as a vaccine a formulation using an oil adjuvant was developed and evaluated to determine their capacity to induce protection of cattle infected with CBPP. Part of the study also aimed at assessing the specific antibody responses following vaccination

1.6.2 Conjugated capsular polysaccharide vaccine

Conjugation of carbohydrates has made it feasible to develop carbohydrate-based vaccines (Peeters *et al.,* 2003). Immunization with neoglycoprotein consisting of capsule-derived carbohydrates coupled to an immunogenic protein provides a long lasting protection to encapsulated bacteria (Jones, 2005; Lucas *et al.,* 2005). A number of

glycoconjugate vaccines based on fragments of capsular polysaccharides have been developed against bacterial infections (Vliegenthart, 2006). Glycoconjugates vaccines containing CPSs from *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* have been successful in preventing infectious diseases in children (Jones, 2005)

Pure polysaccharides are poor immunogens, as they lack T cell epitopes needed for T cell help. Immunization with glycoconjugates, elicits T-cell help for B cells that produce IgG antibodies to the polysaccharide component (Avci and Kasper, 2010). The cellular and molecular mechanisms for adaptive immune responses mediated by glycoconjugate immunization has been demonstrated by (Avci1 *et al.,* 2012). The study showed that upon uptake by antigen presenting cells (APCs), glycoconjugate vaccines are depolymerized yielding glycan-peptide, a processed glycan chemically bound to a peptide fragment. The glycan-peptide is then displayed by an MHC-class II molecule on the surface of APCs (see Fig. 1.3).

The CPS is an important surface antigen and pathogenic factor in *Mmm.* Studies by Waite and March (2002) have demonstrated that vaccination of mice with a conjugated *Mmm* CPS significantly increased the antibody responses compared to what was produced by polysaccharide alone. Based on the ability of the conjugated CPS to induce high antibody response, I used a CPS conjugated with ovalbumin via a carbodiimide-mediated condensation was developed and tested in cattle.

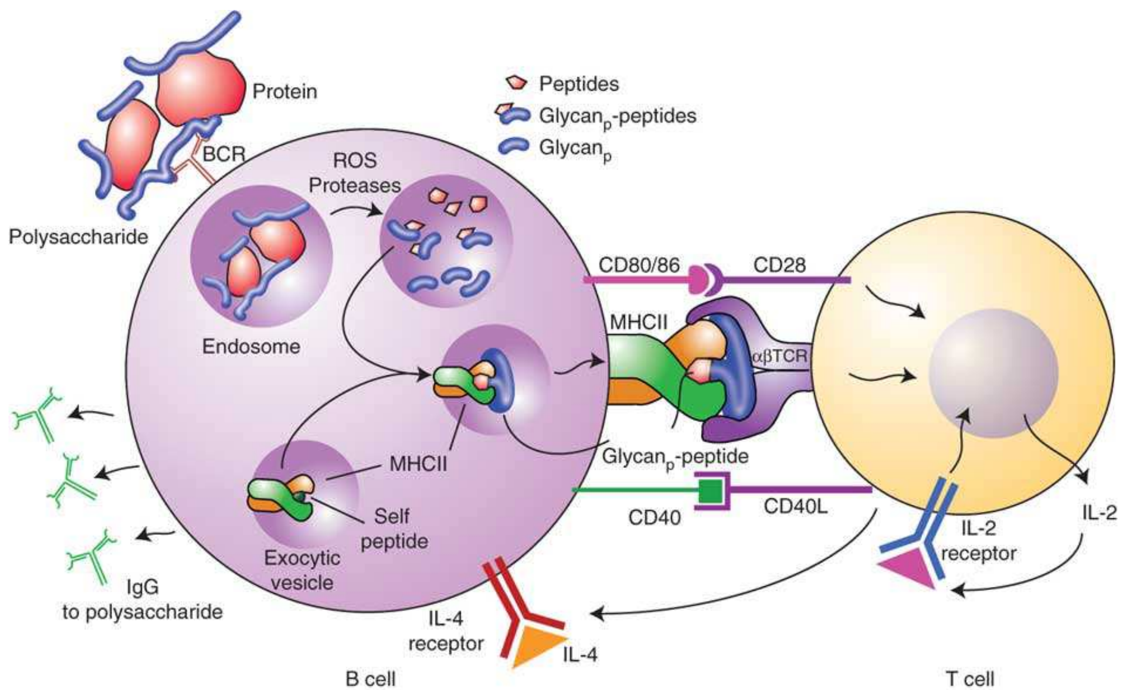


Figure 1.2 Mechanism of T-cell activation by glycoconjugate vaccine; schematic representation shows antigen processing and presentation of glycoconjugate vaccines resulting in helper CD4+ T-cell induction of B-cell production of IgG antibodies to the polysaccharide (Adapted from (Avci1 *et al.*, 2012)

1.6.4 Study of humoral immunity following vaccination

Vaccine efficacy normally relies on the type of immune response induced in the animal host by the introduction of a pathogen. Therefore, knowledge on the aspects of the immune response are responsible for protection and the onset and duration of protection is important. An immunological memory is defined by the acquired property of the immune system to respond more rapidly and more intensively to a second antigen stimulation. The development of this immunological memory is essential for good protection since, generally, resting memory cells have a long lifespan. Therefore, the improvement of vaccines for CBPP implies the need for studies to understand the immunological mechanisms that provide protection during this infection.

The immunological mechanisms involved during *Mmm* infection, or following vaccination against CBPP, are not fully elucidated. Consequently, there is no *in vitro* test at present that can be used to assess accurately the immune status of cattle with respect to CBPP infection. The OIE therefore requires that potency assessment of CBPP vaccines should be achieved by virulent contact challenge of vaccinated animals. The results obtained are then evaluated using the clinical, pathological and serological scoring system of Hudson and Turner (OIE, 1992; Tulasne *et al.,* 1996). This procedure is expensive, time-consuming and does not always give reproducible or consistent results.
A detailed understanding of the immune response following vaccination against CBPP might permit the design of *in vitro* immunological tests with a strong *in vivo* correlation, which can be used as cheaper and quicker methods for vaccine potency testing. One of the major themes in the study was to assess the humoral immune response after vaccination.

This thesis reports a series of experiments that focused on testing vaccine formulations that have a potential for generation of a safe and efficacious vaccine against CBPP. The project therefore aimed at carrying out a number of experiments to confirm that immunity can be induced by inactivated mycoplasma or components of the mycoplasma and assessed the immunological responses that follow immunization.. The protection by the vaccine formulations was defined by three distinct criteria:

1. Mild or no CBPP clinical symptoms that included; coughing, fever and recumbence

2. Absence of features of CBPP at post mortem examination that is characterized by; lung lesions (oedema, consolidation and thickened interlobular septa with sequestration), presence of pleural fluid and fibrous adhesions
3. No isolation of viable *Mmm* from lung tissues at necropsy

1.7 HYPOTHESIS

The study was guided by the hypothesis that vaccination with whole cell killed or components (membrane protein and capsular polysaccharide) of *Mmm* stimulate an immune response that protect cattle upon experimental challenge with an infective dose of *Mmm.*

1.8 MAIN OBJECTIVE

To determine *Mmm* structural components protective capacity and the type of immune response that confers protection against Contagious Bovine Pleuropneumonia.

1.8.1 SPECIFIC OBJECTIVES

1. To examine the efficacy of standardised amount of whole *Mmm* proteins in inactivated vaccine formulations (heat inactivated, formalin inactivated *Mmm* and compared with the live attenuated vaccine.
2. To evaluation protective capacity of a vaccine formulation generated from the *Mmm* membrane protein components.
3. To evaluate a Capsular polysaccharide (CPS) conjugated vaccine formulations protective capacity against CBPP.
4. To describe the correlation between antibody titres, isotypes with protection

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 *IN VITRO* CULTURE OF *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES*

The Afadé strain (obtained from International Livestock Research Institute (ILRI)) of *Mmm* was grown in pleuropneumonia-like organism media (PPLO broth; Difco). The Afadé strain had been used at ILRI in previous experiments and reliably produced severe CBPP (Sacchini *et al.*, 2011). The media was supplemented with thallous acetate 0.04%, penicillin G 500 Units/ml, horse serum 20%, glucose 0.5gm/ml and 5% fresh yeast extract. Mycoplasma were then grown in an incubator containing 5% CO_2 and harvested during mid-logarithmic phase at a pH of 6.8.

An estimation of viable mycoplasma content was determined based on colour changing units (CCU/ml) by a microtitration method described by FAO (Litamoi *at al.,* 1996). In short, a 10–fold dilution series from 10^{-1} to 10^{-10} of the growing mycoplasma was prepared in bijoux bottles containing 4.5 ml of growth medium. Then, 200µl of complete growth medium from each vial was dispensed into eight wells of a 96 well plate, sealed and incubated the plate at 37°C for 10 days. Growth of the cultures was evidenced by colour of the medium from pink to yellow using a plate reading mirror. The estimation of the *Mmm* concentration was based on the dilution that demonstrates appearance of yellowish colour in 50% of its replicates and is expressed as colour changing unit (CCU50)/ml**.**

2.2 CATTLE EXPERIMENTS

2.2.1 Experimental cattle

Currently, no small animal model for CBPP exists, the studies were therefore performed on the natural bovine host. Naive Boran cattle (*Bos indicus*) were obtained from the International Livestock Research Institute (ILRI) ranch in Kapiti, a CBPP-free region in Kenya, and transported to an isolation unit at the Kenya Agricultural and Livestock Research Organisation, Veterinary Science Research Institute (KALRO-VSRI) – Muguga (Fig. 2.1). The animals had not been vaccinated against CBPP. All the animals were tested for CBPP antibodies using the Compliment Fixation Test (CFT). Clinical signs for CBPP were also assessed by a qualified veterinarian. No animal was positive for CFT or had indications of CBPP prior to the start of the study. The animals were acclimatized for at least one month prior to the start of the experiment. Experienced husbandry staff observed the animals prior and during the experiment. During the whole period of the experiment, animals were fed on grass, hay and mineral supplements. Experiments were conducted following guidelines of Kenya legislation for animal experimentation and the VSRI Animal Welfare Committee regulations (Approval No. KARI/VRC/IACUC/2/00122010).

Figure 2.3 Experimental animals [Boran (*Bos indicus*)] in the isolation unit facility at KALRO-VSRI, Muguga. The cattle were grazed in the paddock, fed on hay, mineral supplements and water during the day while confined in a brickhouse at night.

2.2.2 Challenge

Cattle were challenged by endotracheal intubation using the method described by Nkando *et al.*, (2010). This method of inoculation has several advantages that include: known site of infection within the lung, disease onset is more rapid than when induced by contact, thereby reducing the number of sampling days and allowing cattle to be sampled as a cohort with a defined time after infection (Gull *et al.,* 2013). Briefly, cattle were sedated by intramuscular injections of 1ml 2% Xylazine solution. While the animal was in standing position, a bronchial rubber tube was inserted through the nostril into the larynx, deeply into the trachea and an infectious inoculum was introduced. Each animal received 60 ml of *Mmm* culture (10^9 CCU/ml) followed by 15 ml of 1.5% agar suspended in distilled water and then 30 ml of PBS.

2.2.3 Clinical observations

The cattle were restrained in a crush daily at the same hour (09:00 – 10:00 am) for clinical observation. Rectal temperatures and other clinical signs of illness (nasal discharge and coughing) and general body condition of the animal were recorded daily on observation sheets.

2.2.4 Collection of blood, serum preparation and storage

The animals were bled immediately before vaccination and at weekly intervals during the trial period. Blood samples were taken from the jugular vein into vacutainer tubes (without anticoagulant). Samples were then transported to the laboratory and allowed to clot at room temperature overnight, followed by centrifugation at 3000 x g to separate clots, red blood cells and other debri. The serum samples were then aliquoted into 1.5 ml Nunc® (Thermo Fisher Scientific, Rochester, NY, USA) tubes and stored at -20°C for analysis.

2.2.5 Autopsy and collection of tissue samples

Cattle were humanely euthanized using captive bolting and necropsied by a veterinary pathologist with assistance of a large animal internist. Post-mortem was carried out on all animals that died or were killed *in extremis* while those that remained were killed between 7 and 8 weeks after challenge. The cattle were euthanized by captive bolt and then exsanguinated. Particular attention was paid to the thoracic cavity, lungs and kidneys. Lungs were examined for CBPP lesions including encapsulation, consolidation, fibrous adhesion and sequestration. The type and size of the lesion was recorded.

Lung tissues were collected between the lesion and the grossly normal tissue and stored at -20°C until culturing for mycoplasma was done.

2.2.6 Isolation of *Mmm* from lung sections

Culturing of *Mmm* from the lungs was done by incubating a small piece of lung tisse in Gourlay's broth media (with penicillin and thallium acetate) at 37°C in a humidified 5% CO_2 incubator. One milliliter of the suspension was titrated in a dilution series (1:10, 1:20, 1:30 and 1:40) after one day of growth. From these dilutions, 0.2 ml was then dropped onto agar plate with Gourlay's media and incubated at 37°C. Morphological features and typical "fried egg" appearance were checked at days 1, 5 and 10 for *Mmm* colonies.

2.2.7 Lesion scoring and vaccine efficacy

Lesion scoring, as described by Hudson and Turner (1963) was used to determine the severity of the disease in each animal. This scoring was done as follows;

1) Presence of only encapsulated, resolving or fibrous lesion or pleural adhesion: score = 1
2) Presence of other type of lesion like consolidation, necrosis or sequestration: score = 2
3) *Mmm* isolated: score = 2

The resulting sum of the score was multiplied by a factor depending on the lesion size (1: <5 cm; 2: > 5 and < 20 cm; 3: > 20 cm in diameter).

Protection rate (vaccine efficacy) was calculated as follows: vaccine efficacy = 1 – [mean score of vaccinates/mean score of controls] × 100.

2.3 DETECTION OF IMMUNE RESPONSE

2.3.1 Complement Fixation Test

A modified complement fixation test (CFT) by Campbell and Turner, (1953) was used to screen and determine antibody titers. Briefly, 25 µl of test sample sera were diluted at 1/10 in Veronal buffer [VB; 0.85% NaCl (w/v); diethylmanolyurea ($C_4H_4N_20_3$) (w/v) Barbital; 0.0185% (w/v) $C_4H_4N_20_3$ Sodium salt; 0.0028% (w/v) anhydrous CaCl; 0.168% $MgCl_2.6H_2O$ dissolved in 1 L of distilled water] and dispensed in 96-well round bottomed plates (Costar, Corning, MA, USA). Twenty five µl of *Mmm* antigen in VB was added followed by 25 µl of appropriately diluted pig complement. The mixture was shaken vigorously and incubated at 37°C for 30 minutes with periodic shaking. Twenty five µl of haemolytic system [6% sheep red blood cells (SRBC) and haemolytic serum (hyperimmune rabbit serum against SRBC), pre-incubated for 30 min] was added and shaken vigorously before further incubation at 37°C for 30 minutes. Reading was done after centrifugation of the microplates at 125 x *g* for 2 minutes under an inverted mirror. The results were read based on the percentage of complement fixation observed. Positive results were recorded from wells with 100% inhibition at 1/10. To determine the antibody titres, the positive samples were serially diluted to endpoint (no haemolysis occured).

2.3.2 Antibody titres using Indirect ELISA

Cattle sera were analysed for CPS and *Mmm* membrane proteins specific IgG, IgM and IgA isotypes by ELISA three weeks post primary vaccination and three weeks post-

secondary vaccination. The antibody responses were determined as described by Abusugra and others (1997). Briefly, ELISA plates (Nunc, Copenhagen, Denmark) were coated with antigen at a concentration of 5µg/ml in coating buffer (50 mM carbonate buffer pH 9.6) and incubated overnight at 4°C. All washings were made with phosphate-buffered saline (PBS) (150 mM, pH 7.5) containing 0.5% Tween-20 (PBS-T). The plates were blocked with 5% skimmed milk powder in PBS-T (blocking buffer) for 1 h at 37°C. All incubations were carried out with gentle agitation and the plates were washed four times with PBS-T between incubations. Antisera were four-fold diluted in blocking buffer and incubated for 2 h at 37°C. The following dilution of antibodies against the bovine sera were used: Horseradish peroxidase (HRP) conjugated sheep anti-bovine IgM (Pierce) at 1:10,000, Alkaline Phosphatase conjugated rabbit anti-bovine IgG (whole molecule; Sigma) at 1:5,000 and HRP conjugated mouse anti-bovine IgA (AbD Serotec) at 1:2,000 respectively in blocking buffer and incubated for 1 h at 37°C. The enzyme reactions were visualized by addition of substrate buffer. For the peroxidase reagents, the substrate was Tetramethylbenzidine (TMB). After 15 to 20 min the reaction was stopped by 2MH_2SO_2 for TMB and the absorbance was measured at 450 nm. The enzymatic reaction of alkaline phosphatase was visualised by addition of p-Nitrophenyl Phosphate (PNPP), H_2O, diethanolamine, 500mM $MgCl_2$, pH 9.8, and the reaction was stopped by 50ml 1MNaOH and absorbance measured at 405nm. A BioTek Synergy HT Multi-Mode Microplate Reader (BioTek, USA) was used in the measurement of absorbance.

The cut-off was determined as the mean OD value of the negative reference serum plus 2 SD and the antibody titre (end point) was expressed as the last dilution giving an OD

value higher than the cut-off. The negative reference sera used for calculating the cut-off were for cattle collected prior to vaccination.

2.4 PROTEIN CONTENT DETERMINATION

Proteins were quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Santa Cruz Biotechnology, Inc., U.S.A) as described by the manufacturer (Appendix 1). Briefly, 8 dilutions of bovine serum albumin (BSA) standards (5 µg/ml – 250 µg/ml) were prepared in PBS (pH 7.4). Twenty five µl of each BSA standard and the sample were loaded into microplate wells followed by addition of 200 µl of prepared reagent (solution of sodium bicinchoninate (BCA) and cupric sulfate in the ratio of 50:1 respectively) to every well. The mixture was incubated 37º C for 30 minutes and allowed to cool at room temperature for 5 minutes. Absorbance of all wells was measured in a microplate reader at 562 nm using BioTek Synergy HT Multi-Mode Microplate Reader. A standard curve using the data from the standard sample dilutions was drawn and the concentration of the protein was then calculated based on their optical density values.

2.5 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins as previously described (Laemmli, 1970). Briefly, a discontinuous gels made of a 5% stacking gel [3.1 ml dH_2O; 0.7 ml 30 % acrylamide-bisacrylamide; 1.25 Tri-HCL pH 6.8; 25 1 of 10% ammonium persulphate and 5 µl Tetramethylethylenediamine (TEMED) and 12 % separating gel [2.45 ml dH_2O; 1.75 ml, 4.0 ml 30% acrylamide-bisacryamide; Tris-HCL pH 8.8; 25 1 ammonium persulphate

(APS); 5 µl TEMED]. Electrophoresis was conducted using the Serva Electrophoresis GMbH, Heildelberg, Germany] under constant current of 25 mA for 3 hours.

2.6 DETECTION OF PROTEINS IN POLYACRYLAMIDE GEL

To visualise the proteins, gels were stained using a method described by (Johnstone and Thorpe, 1987). Briefly, after electrophoresis, the gel was transferred into a container and 5 times gel volume of 0.025% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid added. Staining was carried out overnight at room temperature with gentle agitation. The staining solution was then poured and the gel rinsed with dH_2O. Distaining solution (5% methanol and 7.5% acetic acid) was added and replenished several times until background of the gel was fully distained. Protein bands were conspicuously visualised against a clear background.

2.7 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) was used to determine if there were differences in CFT titres between the three vaccines. Pearson correlation coefficient was used to analyse relationship between CFT and Indirect ELISA titres and pathology score. Pathology scores were evaluated to determine if there was a difference in protection afforded by the vaccine formulations. Differences between mean pathology scores in the three groups (vaccinates and controls) were also analysed using ANOVA. Evaluation of differences between antibody responses as revealed by CFT was performed using the Chi-square (χ^2) test. Prism statistical software (6th edition GraphPad Software, San Diego California USA, www.graphpad.com) was used in the analysis

CHAPTER 3: EVALUATION OF INACTIVATED AND LIVE ATTENUATED VACCINES AGAINST MYCOPLASMA MYCOIDES SUBSP. MYCOIDES

3.1 INTRODUCTION

Vaccinations using inactivated vaccines have been successful in a number of mycoplasma diseases including contagious agalactia (Buonavoglia *et al.,* 2008) and contagious caprine pleuropneumonia (Rurangirwa *et al.,* 1987). Similar trials for CBPP with a saponin-inactivated vaccine (Nicholas *et al.* 2004) and with an Immunostimulating Complex (ISCOM) formulation (Hübschle *et al.,* 2003) have not been successful. However, large quantities of heat-inactivated mycoplasma formulated with a suitable adjuvant have been shown to induce immunity against CBPP (Gray *et al.,*, 1986; Garba and Terry, 1986). This suggests that mycoplasma may have to be present in large numbers, either alive or dead, to induce a sufficient protective response. Although this was based on the use of an extreme method of vaccination in which cattle were inoculated twice with very large doses of inactivated mycoplasma, it confirms that an inactivated vaccine can confer immunity.

The method of inactivation may play an important role in immunogenicity. Inactivation by heat or by sodium hypochlorite can substantially alter the immuno-proteins of *Mycoplasma agalactiae* and hence reduce the immunogenicity (Tola *et al.,* 1999). Protection by the live T_1 strain of *Mmm* has also been shown to be dose dependent, with a low dose of 10^5 mycoplasma conferring low protection, while there was no significant difference between doses of 10^7 and 10^9 (Gilbert and Windsor, 1971; Masiga *et al.,* 1978; Thiaucourt *et al.,* 2004).

Owing to the discrepancies in reports on the capacity of inactivated mycoplasma to protect against CBPP, three vaccines formulations were assessed for their protective capability: formalin inactivated (Garba and Terry, 1986) and heat-inactivated (Gray *et al.,* 1986) vaccines were compared with the live attenuated T1/44 vaccine. The main purpose of the study was to evaluate the efficacy of inactivated mycoplasma and compare formalin-fixed and heat-inactivated formulations, using equal quantities of antigen.

The hypothesis that was guiding the study was that vaccination with killed whole cell *Mmm* stimulates an immune response that can protect cattle upon experimental infection with infective dose of *Mmm.* Three experiments were therefore established to address this hypothesis. The specific objectives were:

1. To prepare vaccine formulations by inactivating whole cell *Mmm* through heat and formalin
2. To describe clinical and pathological outcomes in cattle vaccinated with inactivated mycoplasma and compare with animals vaccinated live attenuated *Mmm*
3. To describe antibody response in cattle vaccinated with inactivated *Mmm* and live attenuated *Mmm*

3.2 MATERIALS AND METHODS

3.2.1 Inactivated and live attenuated vaccine preparation

Two inactivated vaccine formulations were prepared and T1/44 live attenuated vaccine purchased from Kenya Veterinary Vaccine Production Institute (KEVEVAPI).

a) Formalin inactivation

The vaccine was prepared as previously described (Garba and Terry, 1986). Briefly, the vaccine was prepared from a pure 4-day old culture of the Afadé strain using pleuropneumonia-like organism media (Difco™ PPLO Broth) and harvested at a concentration of 10^{10} colony changing units (CCU) per millilitre (Litamoi *et al.* 1996). The culture was inactivated by adding 0.7% (v/v) of formaldehyde (BDH Chemicals Ltd, Poole, UK) and incubated overnight at 37°C. The suspension contained 3 mg/ml of protein as determined using the Bicinchoninic Acid method described in chapter 2, section 2.4. The preparation was then stored at +4°C until used. The final product was obtained by emulsifying equal volumes of inactivated culture and Freund's Incomplete Adjuvant (FIA; Difco).

b) Heat inactivation

Preparation of the vaccine was done as described (Gray *et al.*, 1986). The Afadé strain of *Mmm* was grown for 4 days in PPLO as described in chapter 2, section 2.1 to a concentration of 10^{10} CCU/ml and centrifuged at 12,000g. The pellet was washed three times in phosphate buffered saline (PBS; 150 mM NaCl, 1.5 mM KH_2PO_4, 9 mM $Na_2HPO_4 \times 12H_2O$, 2.5 mM KCl [pH 7.2]) and re-suspended in 10 ml of the same

solution. Mycoplasma viability was checked by growing 10 ml of 1:10 dilution in of inactivated *Mmm* in PPLO media for seven days. Protein concentration was then determined using the Bicinchoninic Acid Assay described in chapter 2, section 2.4. This suspension was adjusted to contain 3 mg/ml of mycoplasma protein. Mycoplasma were killed by heating in a water bath at 56 ^{0}C for 30 min and stored at -20°C until the day of immunization. On the day of vaccination, an equal volume of Freund's Complete Adjuvant (FCA; Difco) was added and mixed by means of an emulsion mixer.

c) Live attenuated (T1/44)

Contavax (KEVEVAPI; B/No. 01/2012) at a concentration of 10^7 live Mycoplasma was used. Immunization was done as instructed by the manufacturer.

3.2.2 Cattle and intradermal inoculation

Forty Boran cattle (*Bos indicus*) between 8 and 10 months of age were obtained and maintained according to the procedures described in chapter 2, section 2.2.

Cattle were then randomly divided into four groups of ten animals, one of which comprised the non-immunized control group. Group 1, 2 and 3 were inoculated with heat-inactivated, formalin-inactivated and live attenuated (T1/44) vaccine, respectively. Each animal was subcutaneously vaccinated with 2 ml of the vaccine formulation on the neck. Animals received a primary immunization with Freund’s complete adjuvant for the heat-inactivated mycoplasma or Freund’s incomplete adjuvant for the formalin-fixed mycoplasma. After three weeks, two booster injections, separated by 2 months, were delivered with the inactivated mycoplasma mixed with Freund’s incomplete adjuvant.

The live T1/44 vaccinated group received a single dose. The non-immunized (no vaccination) control group 4 was introduced at the time of challenge.

3.2.3 Challenge and clinical observations

Three weeks post the second booster administration, cattle were infected with live *Mmm* through endotracheal intubation as described in chapter 2, section 2.2.2.

Rectal temperatures and other clinical signs were recorded as described in chapter 2, section 2.2.3.

3.2.4 Autopsy, cultural examination and lesion scoring

Post-mortem was carried out after 7 weeks on all the animals after challenge based on the procedure described in chapter 2, section 2.2.5.

3.2.5 Serological examination

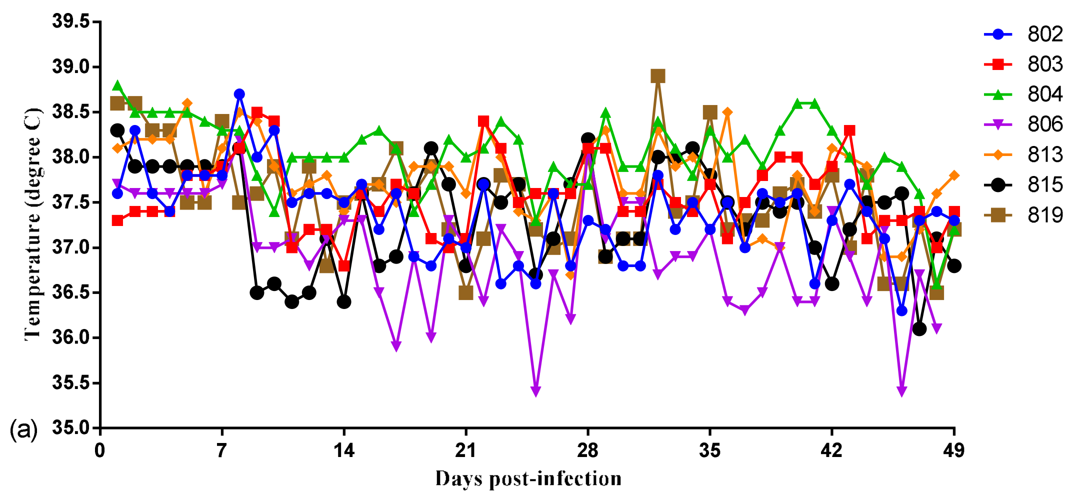
The animals were bled immediately before vaccination and at weekly intervals during the trial period and antibody responses determined using CFT as described in chapter 2, section 2.3.1.

3.3 RESULTS

3.3.1 Clinical observations

Animals under study had no clinical signs prior to challenge. Clinical responses observed after challenge included cough and nasal discharge. Only one animal in the control group developed fever (temperature 39.5^{o}C) for five days. Some animals also experienced low temperatures, suggesting infection may not have been severe (Fig. 3.1).

All experimental animals experienced respiratory distress characterized by coughing (Figure 3.2). This was observed from the second week post intubation except for the group that received formalin-fixed *Mmm* that experienced symptoms from the fourth week onward. No significant differences among groups were observed (P=0.65).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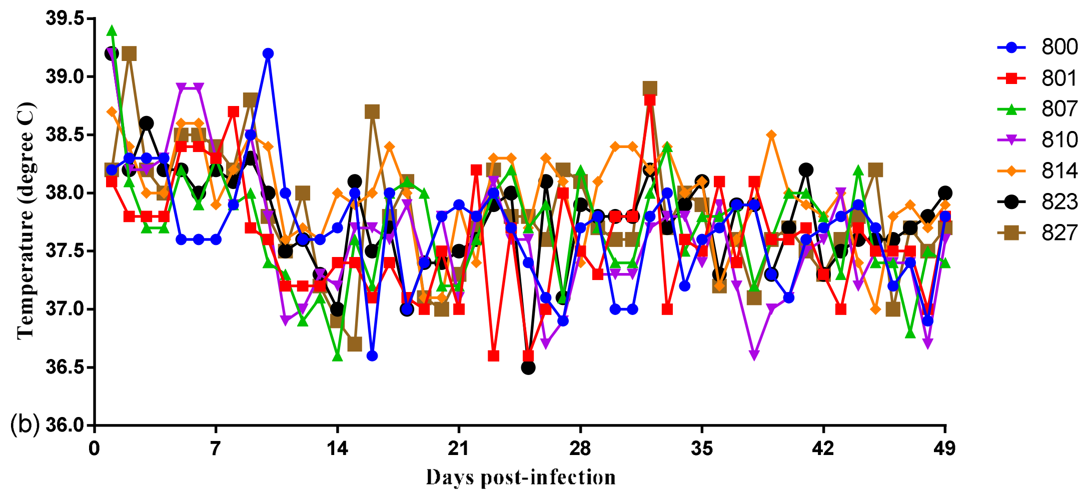
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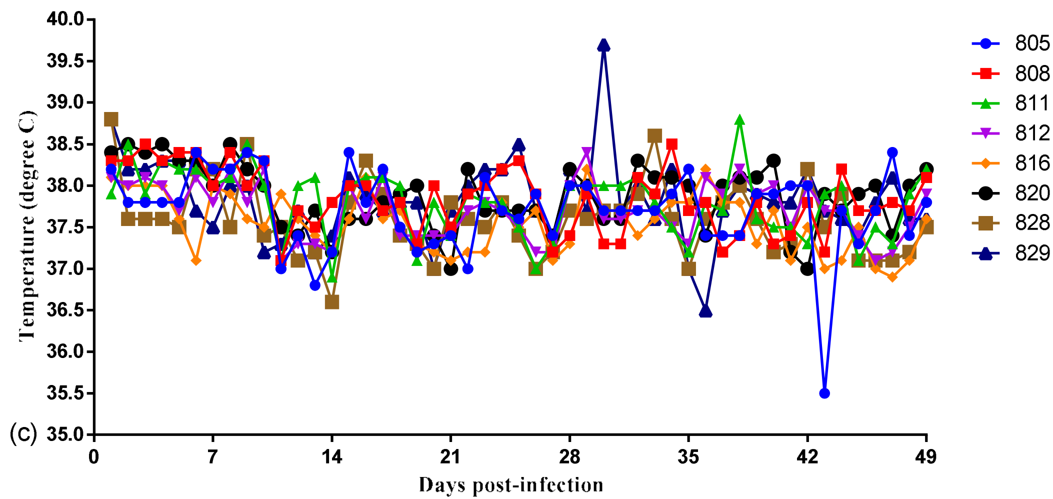


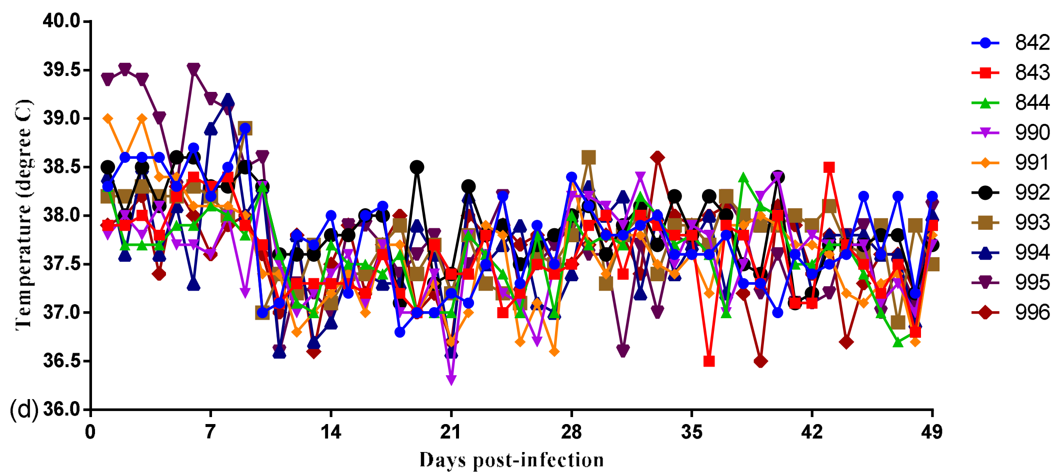


Figure 3.4 Summary of rectal temperatures from inactivated and T1/44 vaccines experiment; (a) Heat inactivated; (b) Formalin inactivated; (c) Live attenuated T1/44; (d) unvaccinated cattle after challenge. Fever was defined as occurrence of a rectal temperature of $\geq 39.5^{o}$C. Cattle in the vaccinated group did not experience fever after challenge. Only one animal in the control group experienced fever after challenge. Some animals also experienced low temperatures. The final data points on each plot correspond to the day on which the corresponding animal was euthanized. During the experiment six animals from the vaccinated groups died of causes not related to CBPP.

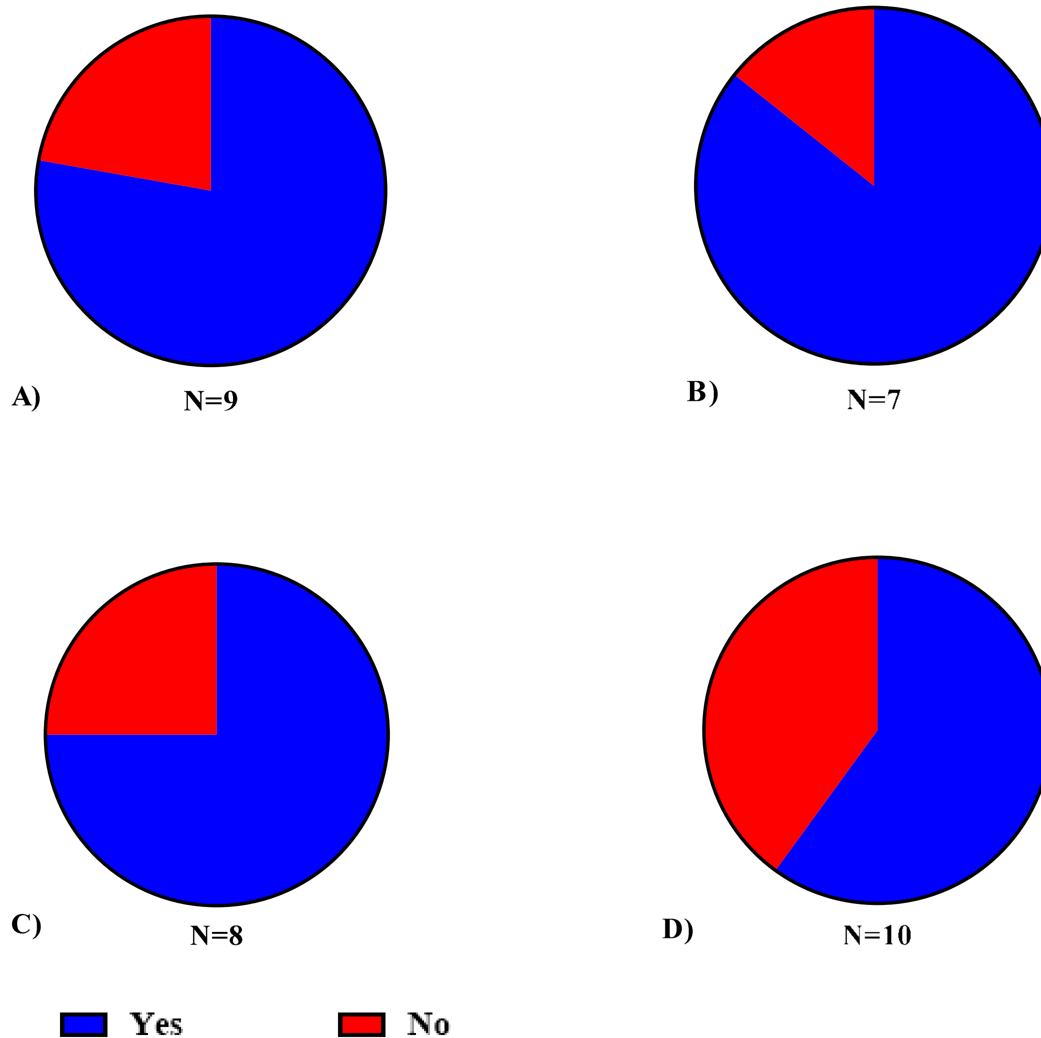


Figure 3.2 Summary of respiratory distress (coughing) experienced by the cattle after infection (challenge) with live *Mmm*. Proportion of respiratory distress in the groups; A) Heat-inactivated; B) Formalin-inactivated; C) Live attenuated (T1/44) and D) Control group. All the groups experienced respiratory distress following challenge. During the experiment six animals from the vaccinated groups died of causes not related to CBPP.

3.3.2 Antibody responses post vaccination and challenge

Antibody (Ab) responses following vaccination and after challenge were measured using CFT. The highest Ab titres recorded during vaccination and after challenge are shown in Table 3.2. Following vaccination and challenge, seroconversion was observed in 9/9 (100%), 5/7(71%) and 3/8 (37.5%) in heat inactivated, formalin inactivated and live attenuated (T1/44) respectively (Table 3.1).

Seroconversion was first detected one week post vaccination for the animals that received inactivated vaccines while three weeks post vaccination for the group receiving the live attenuated vaccine. The CFT titre levels were significantly higher after vaccination for the inactivated formulations as compared to the live attenuated. No significant antibody titre differences were observed after vaccination and post challenge ($P>0.05$). No correlation between antibody levels following vaccination and lesions after challenge was observed ($P>0.05$). Interestingly, only two animals (991 and 995) seroconverted following challenge (Table 3.1).

Vaccination group	Animal No.	Highest CF titre after vaccination and before challenge	Highest CF titre after challenge
Heat-inactivated	819	1:20	1:80
	802	1:40	1:20
	815	1:40	1:40
	813	1:40	1:20
	803	1:40	1:20
	824	1:40	1:20
	804	1:40	1:20
	806	1:20	1:20
Formalin-inactivated	807	1:40	1:40
	827	1:40	1:40
	814	1:10	1:40
	800	0	0
	801	1:80	1:40
	823	1:20	1:40
	810	0	0
Live attenuated (T1/44)	808	1:10	0
	812	0	0
	811	0	0
	805	1:20	1:40
	816	0	1:20
	828	0	1:40
	820	0	0
	829	1:20	0
Control group	842	-	0
	843	-	0
	844	-	0
	990	-	0
	991	-	1:80
	992	-	0
	993	-	0
	994	-	0
	995	-	1:160
	996	-	0

Table 3.1 CFT titres results of cattle following vaccination with inactivated and live attenuated vaccine and challenge.

3.3.3 Pathological investigations and vaccine efficacy

The experiment was terminated 7 weeks after challenge. Table 3.4 summarises the pathological observations. Post-mortem examination exposed pathological lesions typical of CBPP including consolidation and hepatization of the lung parenchyma and pleuritis, well-developed sequestra and fibrous adhesion of the parietal and visceral pleurae were observed.

Mycoplasma were only isolated from animals in the non-vaccinated (7/10), formalin inactivated (2/7) and live attenuated vaccinated (3/8) groups. The mycoplasma had typical "Fried Egg" morphology of mycoplasma colonies (Figure 3.3). High pathological scores were observed in the control group, followed by the formalin-inactivated group, the heat-inactivated and live attenuated groups respectively. Average scores for lesion size were higher in non-vaccinated animals (Table 3.2). No gross lesions were observed in one animal that had pyrexia and serological response. Sequestra formation was recorded only in two animals that had been vaccinated using the formalin-inactivated vaccine.

Vaccine efficacy for the three vaccines, calculated using average Hudson scores, is shown in Table 3.5. The protection rates for the formalin-inactivated, heat-inactivated and live attenuated groups were 31.0, 80.8 and 74.1 respectively.

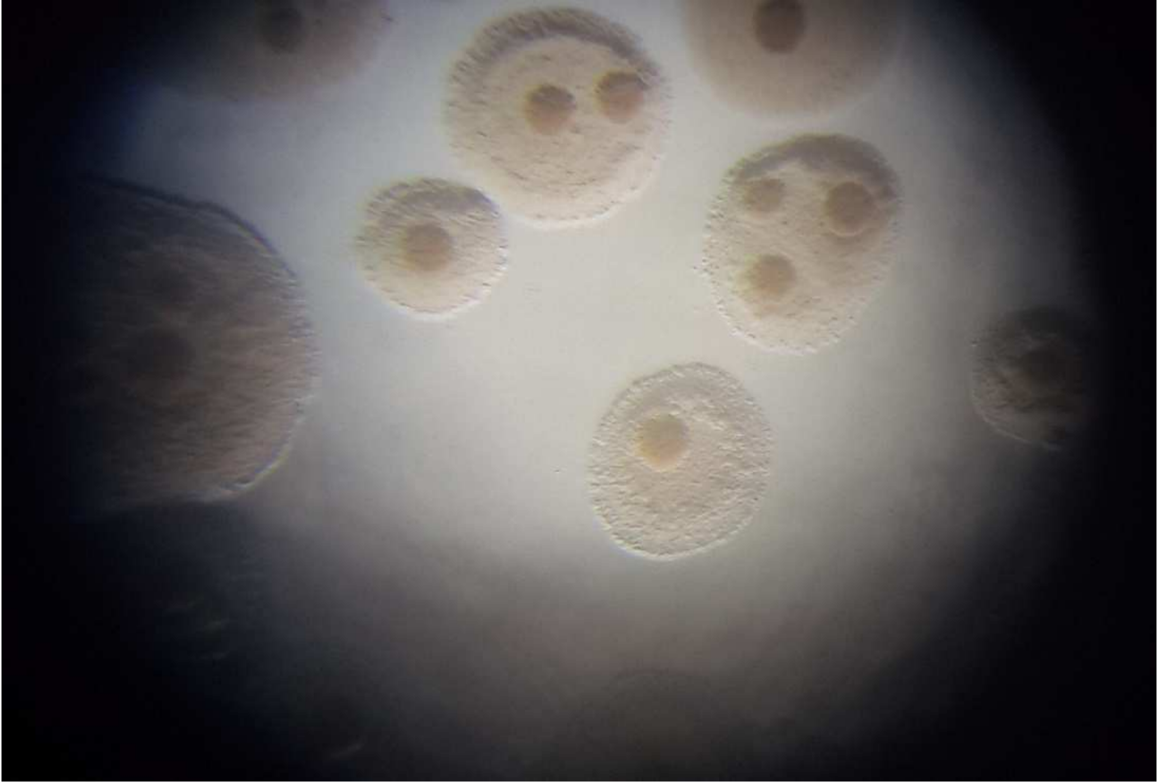


Figure 3.3 Magnified stained *Mmm* colonies in the surface of agar plate. The colonies have the characteristic 'Fried Egg' morphology. Viewed under a binocular microscope with 100X magnification

Vaccination group	Animal No.	*Mmm* isolated	Lung/Thoracic cavity lesions (cm)	Pathology Score
Heat-inactivated	819	-ve	No lesion	0
	802	-ve	No lesion	0
	825	-ve	No lesion	0
	815	-ve	Cons	2
	813	-ve	No lesion	0
	803	-ve	Fib adhe	1
	824	-ve	No lesion	0
	804	-ve	Fib tags	1
	806	-ve	Fib adhe	1
Formalin-inactivated	807	+ve	Seq (3x3)	4
	827	-ve	No lesion	0
	814	-ve	Cons	2
	800	-ve	No lesion	0
	801	-ve	Cons	2
	823	+ve	seq (10x7)	6
	810	-ve	No lesion	0
Live attenuated (T1/44)	808	+ve	No lesion	2
	812	-ve	Cons	2
	811	-ve	No lesion	0
	805	-ve	No lesion	0
	816	+ve	No lesion	2
	828	-ve	No lesion	0
	820	-ve	No lesion	0
	829	+ve	No lesion	2
Control group	842	+ve	Cons	4
	843	+ve	No lesion	2
	844	+ve	Fib adhe, hep	4
	990	+ve	Fib adhe, cons	4
	991	+ve	Cons	4
	992	+ve	Cons	4
	993	+ve	No lesion	2
	994	-ve	Res les	1
	995	-ve	No lesion	0
	996	-ve	Cons	2

Table 3.2 Summary of lesions and pathological results in animals vaccinated with inactivated and T1/44 vaccines. Cons = consolidation; fib adhe = fibrous adhesion; res adhe = resolving adhesion, Res les = Resolving lesion; cong = congestion; Seq = sequestration.

Vaccination group	Vaccine formulation			
	Heat-inactivated	Formalin-fixed	Live attenuated (T1/44)	Control
Number of cattle	9	7	8	10
Average lesion score	0.56	2	0.25	1.3
Average Hudson score	0.56	2	0.75	2.9
Vaccine efficacy (%)	80.8	31	74.1	

Table 3.3 Protective capacity of inactivated and live attenuated vaccines as measured by challenge trial. Vaccine efficacy = 1 − [mean score of vaccinates/mean score of controls] × 100.

3.4 DISCUSSION

3.4.1 Introduction

This study compared the protective capacity of the live T1/44 vaccine with two inactivated preparations of *Mmm* strain Afadé, inoculated with adjuvants. The two formulations, heat (Gray *et al.* 1986b) and formalin-inactivated (Garba and Terry 1986) vaccine formulations had prior been shown to have complete protective capacity. The heat-inactivated mycoplasma emulsified with complete Freund's adjuvant had been used at a concentration of 14.5 mg of protein/ml and 10ml were administered during vaccination. The formalin-inactivated dose of 0.5 ml of 10^{10}/ml mycoplasma was protective against CBPP. In the present experiment a dose of 3 mg/ml of proteins was used in the heat and formalin-inactivated vaccine formulations while T1/44 was used as instructed by the manufacturer.

3.4.2 Efficacy of the vaccines

All the vaccines tested offered partial protection against CBPP. Bronchiopneumonia was observed in the non-vaccinated group, an indication that severity of the disease was less in vaccinated animals. In the non-vaccinated group, more animals had severe pathology and incidence of mycoplasma isolation from tissues. Significant protection was offered by heat-inactivated (80.8%) and live attenuated (74.1%). The protection rate of the live vaccine in this experiment was found to be comparable to those observed previously (Gilbert F. R and Windsor R. 1971; Thiaucourt *et al.* 2000) of 50-80%. Although formalin-inactivated *Mmm* had shown protection previously, protection achieved in this experiment was very low (31%). We used the Afadé strain for immunization instead of

Gladysdale, but this is unlikely to be the reason for the different outcome with the previous studies (Garba and Terry, 1986), since *Mmm* strains show little heterogeneity (Fischer *et al.,* 2013.)

Use of adjuvant in producing a protective immune response to CBPP by killed mycoplasma is critical. In one experiment (Garba *et al.,*., 1989), animals immunised with incomplete Freund's adjuvant were significantly less protected after challenge compared to animals immunized with complete Freund's adjuvant. The improvement on the level of the vaccine may therefore depend on the type of adjuvant used. Although Freund's adjuvant enhances protection, it is not recommended for animals intended for human consumption since they cause spoilage of meat, chronic inflammation and sterile abscess (Murray and others 1972 in Rurangirwa *et al.,* 1987).

3.4.3 Antibody Response

The fact that animals immunized with formalin-fixed mycoplasma had higher serum titers of specific antibodies than animals immunized with the heat-inactivated mycoplasma, yet was less protected, suggest that the method of formulation plays an important role. We do not know at this stage the nature of the protective antigens, and it seems likely that what we measure in CFT does not correspond to protection. No correlation was evident between Ab titers and the severity of CBPP clinical signs or the types and intensity of lung lesions observed at necropsy ($P>0.05$), as already reported by others (Mamadou *et al.* 2006, Nkando *et al.* 2012; Sacchini *et al.* 2012). Only two animals in the control group had seroconverted but showed resistant on challenge. This has also been observed in other experiments o vaccination with T1/44 (Gilbert *et al.,* 1970; Windsor *et al.,* 1972).

3.4.4 Conclusion

In conclusion, this study demonstrates that a vaccine dose of 3mg of heat inactivated whole cell *Mmm* with Freund's complete adjuvant vaccination followed by two boosts can offer significant protection from *Mmm* infection. The protection capacity of inactivated vaccine indicates that a subunit vaccine or an inactivated vaccine against CBPP in a commercially acceptable adjuvant may be possible. However, careful selection of adjuvant should be done taking into consideration the potency and ease of application of the vaccine.

CHAPTER 4: EVALUATION OF THE CAPACITY OF *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* MEMBRANE PROTEINS TO PROTECT AGAINST CONTAGIOUS BOVINE PLEUROPNEUMONIA

4.1 INTRODUCTION

The success of heat-inactivated CBPP vaccine as evidenced in the previous chapter suggested that subunit vaccine development may be possible. Although protective immunity against *Mmm* can be induced, the specific antigens responsible have not yet been identified. The surface-exposed proteins have been targets for investigation because antibody production is one mechanism of protective immunity. Protection by antibodies has been demonstrated by conferring protective immunity on recipient calves following passive transfer of sera from cattle recovered from CBPP (Masiga *et al.,* 1975). Monoclonal antibodies (MAbs) against membrane proteins in other mycoplasmas have been shown to have a killing and growth inhibition effect. For example, MAbs against an integral membrane protein could kill *Mycoplasma hyorhinis* (Riethman *et al.,* 1987) and isolated membranes of *Mycoplasma pulmonis* elicited antibodies that had mitogenic activity (Lapidot *et al.,* 1995).

The major components of the outer membrane proteins of *Mmm* including ; Lipoprotein A (Monnerat *et al.*, 1999), Lipoprotein B (Vilei *et al.,* 2000), Lipoprotein C (Pilo *et al.,* 2003) and Lipoprotein Q (LppQ) (Abdo *et al.,* 2000) have been characterised. A LppQ vaccine formulated using Immunostamulating Complex (ISCOM) indicated enhanced pathology following immunisation of cattle (Abusugra *et al.,* 1997). Use of a purified recombinant LppQ as a vaccine also showed that the vaccinates were more susceptible to challenge with *Mmm* than cattle that were not vaccinated with LppQ (R. a. J. Nicholas et al., 2003). L-α-glycerophosphate oxidase (GlpO), a membrane-located enzyme that is involved in the metabolism of glycerol has been tested. The enzyme is able to cause host

cell death as demonstrated by killing of embryonic calf nasal epithelial cells infected with *Mmm* in the presence of physiological amounts of glycerol (Pilo *et al.,* 2005). However, immunization of cattle with recombinant GlpO did not show protection upon experimental challenge (Mulongo *et al.,* 2013). Given the importance of membrane proteins to induce strong antibody production and their accessibility by antibodies in vivo, it was important to assess their capacity to induce protection against CBPP. The extraction of outer membrane proteins using Triton X-114 (TX-114) has facilitated the production of pure membrane proteins free from other Mycoplasma antigens for investigation as potential vaccine candidates (Kiarie *et al.*, 1996; Jan *et al.*, 1996; Corona *et al.,* 2013).

The work carried out in this chapter was guided by the hypothesis that vaccination with membrane proteins dissolved in an oil adjuvant can stimulate a protective immune response in cattle upon experimental challenge with an infective dose of *Mmm.*

The objectives of the study were;

1. To extract and purify membrane proteins of *Mmm*
2. To describe clinical and pathological outcome in cattle vaccinated with *Mmm* membrane proteins and challenged with an infective dose of *Mmm*
3. To describe antibody responses in cattle vaccinated with *Mmm* membrane proteins and challenged with an infective dose of *Mmm*

4.2 MATERIALS AND METHODS

4.2.1 Extraction of membrane proteins

Afadé strain (obtained from International Livestock Research Institute (ILRI)) of *Mmm* was grown in pleuropneumonia-like organism (PPLO broth; Difco) media as described in chapter 2, section 2.1.

Enrichment of *Mycoplasma* membrane proteins using Triton X-114 was carried out as described previously (Duffy *et al.,* 1998 and Riethman *et al.,* 1987). Proteins embedded in the cell membrane, or anchored to it by an acryl moiety can be selectively solubilised using surfactants due to their amphiphilic nature. Briefly, 10 ml Triton X-114 was pre-washed in 500 ml of PBS by incubating the mixture at 37°C for 18 h and discarding the upper aqueous phase. This phase was repeated twice. The lower phase was decanted and used as a stock solution of 11.4% (v/v) Triton X-114. *Mmm* from 3 L culture was washed by centrifugation at 12,000 x g for 45 minutes. Cell pellets were re-suspended in ice-cold PBS followed by the addition of ice cold 10% (v/v) TX-114 (Sigma-Aldrich, UK) to a concentration of 1 mg of *Mmm* proteins/ml. The pellets were thoroughly dissolved by vortexing at 4°C for 1 hour. After centrifugation at 12,000 x g at 4°C for 5 minutes the supernatants were transferred to a new tube, incubated at 37°C for 5 min and then centrifuged at 8,000 x g at room temperature for 3 min to remove the insoluble components. The supernatant was carefully loaded onto a 6% (w/v) sucrose solution containing 0.006 (v/v) Triton X-114 in PBS and incubated in a water bath at 37°C for 9 minutes followed by centrifugation at 300 x g for 7 minutes. The supernatant was aspirated, which contained hydrophilic component and set aside, the lower (TX-114) phase was brought to the original volume with PBS. The suspension was dissolved by

incubating in ice for 15 min and phase partitioning repeated three times. Proteins were then precipitated by addition of methanol/chloroform and centrifuging at 9000 x g for 1 minutes at 4°C.

4.2.2 Purification of membrane proteins

The extracted proteins were purified by removal of the TX-114 from hydrophobic phase using Extracti-Gel® D (Pierce, Rockford, USA) detergent removing gel as described by the manufacturer. Briefly, Extracti-Gel® packed columns provided with the kit were washed and equilibrated with PBS buffer. The samples were then passed through the gel several times to remove all of the detergent.

4.2.3 Sodium dodecyl sulphate - polyacrlamide gel electrophoresis (SDS-PAGE)

Protein concentration from the extract and mycoplasma cells for SDS-PAGE was estimated as described in chapter 2, section 2.4. *Mmm* whole cell samples were mixed with lysis buffer (500 mM Tris/HCl pH 6.8, 4.6% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.004% bromophenol blue) and boiled for 5 minutes. The SDS-PAGE was then performed as described in chapter 2, section 2.5.

4.2.4 Vaccine formulation

The purified membrane proteins were solubilized in PBS (pH 8.0). Then, 6.6 ml of Emulsigen® (MVP Technologies, Omaha, USA; Oil-in-Water Emulsified Adjuvant) was added to 13.4 ml of solubilised proteins to make a stock of 20 ml containing 2.2 mg/ml. The mixture was thoroughly mixed prior to use.

4.2.4 Cattle Experiment

4.2.4.1 Treatment of groups and challenge

Two to three-year-old boran (*Bos indicus*) cattle were purchase and handled as described in the procedure in chapter 2, section 2.2.1. Twenty cattle were randomly divided into two groups. One group was vaccinated with membrane protein vaccine (1ml membrane protein and 1 ml adjuvant) and the other with the adjuvant (placebo) only. Each of the animals was subcutaneously inoculated on the neck with 2 ml (2.2 mg of membrane proteins) of the vaccine formulation. Secondary inoculation was carried out after 6 weeks. The animals were fed and housed in a confined paddock. Challenge was carried out four weeks after secondary inoculation by endobronchial inoculation using a procedure described in chapter 2, section 2.2.2. Blood was collected daily and serum preparation was done as described in chapter 2, section 2.2.4.

4.2.4.2 Clinical observations

Rectal temperatures, nasal discharge coughing and general attributes of the animals were monitored daily as described in chapter 2, section 2.2.3.

4.2.4.3 Necropsy, Mycoplasma and Lesion scoring

Cattle were humanely euthanized and necropsied by a veterinary pathologist 8 weeks after infection described in the procedure in chapter 2, section 2.2.5.

Mycoplasma culturing from the lungs was carried out to verify the presence of *Mmm* as described in chapter 2, section 2.2.6.

Determination of the lung pathology score of the pleuropneumonia infection and efficacy of the vaccine was done as described in chapter 2, section 2.2.7.

4.2.4.4 Detection of immune response

CFT and an Indirect ELISA was used to determine antibody titres against *Mmm* membrane proteins

i) CFT

Compliment Fixation Test (CFT) was carried out as described in chapter 2, section 2.3.1 to determine the antibody titers.

ii) Specific immunoglobulin isotype ELISA

Anti-membrane proteins antibody titres for specific IgG, IgM and IgA subclasses by ELISA three weeks post primary vaccination and three weeks post-secondary vaccination as described in chapter 2, section 2.3.2.

4.3 RESULTS

4.3.1 Analysis of *Mycoplasma mycoides* subsp. *mycoides* membrane proteins

Mmm membrane proteins were generated through Triton X-114-phase protein partitioning. Most of *Mmm* membrane proteins were extracted by Triton X-114, as expected of amphiphilic polypeptides. Comparison of protein profiles of *Mmm* aqueous-phase, Triton X-114-phase integral membrane and whole-organism lysate proteins using SDS PAGE showed *Mmm* proteins partitioned exclusively into the Triton X-114 phase, while the majority were in the aqueous phase.

4.3.2 Antibody responses

4.3.2.1 Complement Fixation Test (CFT)

For detection of antibody responses to membrane proteins, CFT was carried out for sera from the individual animals during all the days of the experiment. Only two of the vaccinated animals had antibodies to the membrane proteins following vaccination with peak CF antibody titres of 1:40. However, after challenge 5 animals seroconverted with antibody titres of between 1:10 and 1:640. Interestingly, one animal (# 1304) that had seroconverted after vaccination did not show presence of antibodies after challenge. In the group that had been vaccinated with a placebo, no specific membrane protein responses were detected before challenge. After challenge, five of the animals in the control group seroconverted and titres of between 1:10 and 1:640 are observed. Table 4.1 summarizes CFT responses in vaccinated and unvaccinated animals.

4.3.2.2 Indirect ELISA

Serum antibody responses of cattle immunized as described in section 4.2.4.1 were tested individually for IgG, IgM and IgA antibody isotypes using an indirect ELISA. After the first immunization, IgM subclass titres of about 20,000 – 100,000 were recorded three weeks later. However, the levels had decreased significantly after six weeks (Fig. 4.1). The IgG subclass to membrane proteins were elevated only after secondary immunization with the highest titre being 25,000 (Fig. 4.1). IgA isotype responses were not detected by the assay.

		CFT maximum Ab titres	
Membrane protein vaccinated group	Animal Number	after vaccination	after challenge
	1267	-	-
	1285	-	640
	1265	-	640
	1282	20	320
	1271	-	10
	1304	40	-
	1288	-	-
	1284	-	-
	1287	-	-
	1306	-	10
Unvaccinated group (control)	1279	-	10
	1283	-	320
	1263	-	-
	1293	-	-
	1298	-	640
	1274	-	40
	1290	-	-
	1289	-	-
	1272	-	20
	1294	-	-

Table 4.2 CFT antibody titres following vaccination with membrane proteins and challenge live *Mmm*.

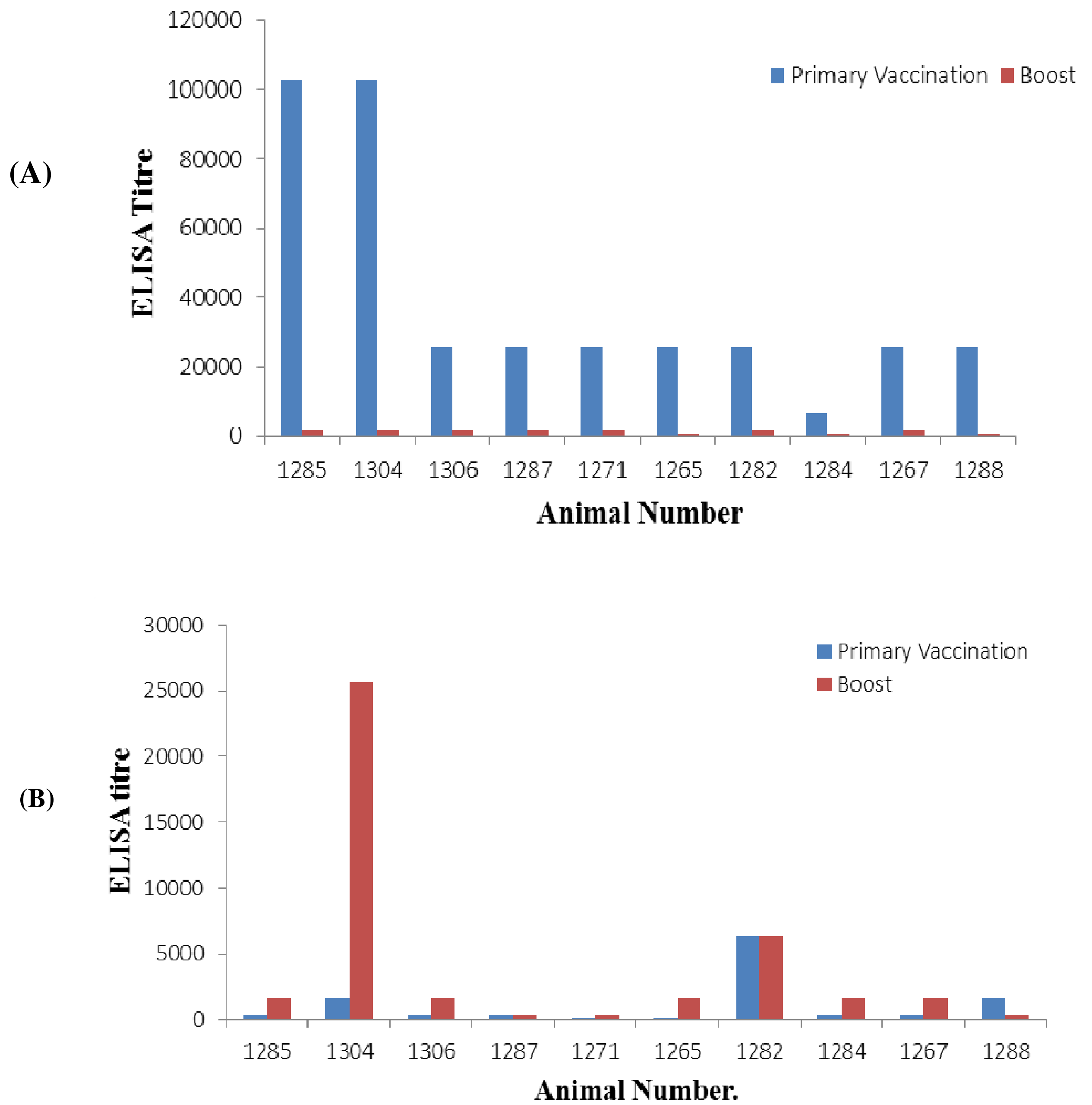


Figure 4.1 Antibody titers of cattle immunised twice three weeks apart with 200 µg Membrane proteins measured by ELISA. (A) IgM antibody titres (B) IgG antibody titres. The cut-off was determined as the mean OD value of the negative reference serum plus 2 SD and the antibody titre (end point) was expressed as the last dilution giving an OD value higher than the cut-off.

4.3.3 Clinical signs

The cattle under study displayed signs of sub-acute CBPP that were prominent between the second and third weeks after challenge. Animals with fever (≥39.5°C) were observed in both vaccinated and unvaccinated groups after three weeks (Fig. 4.2). In the vaccinated group, three animals had fever that lasted between 6 and 11 days while in the control group three animals had fever that lasted between 1 and 3 days (Table 4.2). One animal in the vaccinated group exhibited mild increase in temperature (39°C). Although there was a rise in temperature over time there was no significant difference between the means of the two groups (p=0.1). Respiratory distress (coughing) was prominent both in the vaccinated and control group. In the vaccinated and control groups 9/10 and 8/10 animals exhibited coughing respectively. One animal (# 1285) in the vaccinated group died of CBPP before the termination of the experiment as assessed at post-mortem examination and isolation of the aetiological agent. One vaccinated animal (#1282) was killed on *extrimis* after having fever for 8 consecutive days.

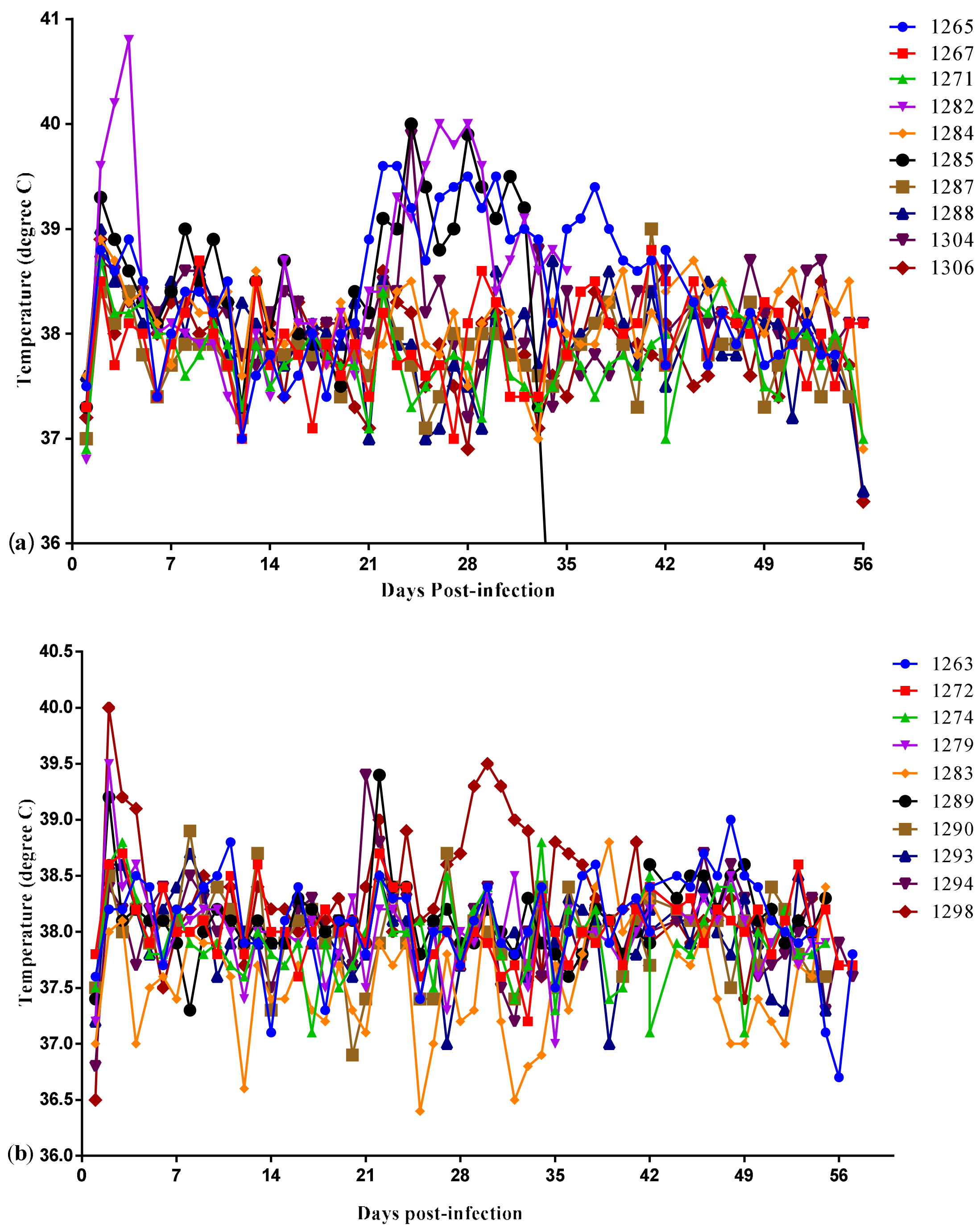


Figure 4.2 Rectal temperature for membrane proteins vaccination experiment; (a) Vaccinated; (b) unvaccinated cattle after infection. A rectal temperature of ≥ 39.5°C was regarded as fever. The readings terminate on the respective day for euthanasia of each animal.

	Animal number	Clinical observations	
		Respiratory distress	Pyrexia (days) or death
Vaccinated group	1267	Yes	-
	1285	Yes	6, died
	1265	Yes	11
	1282	Yes	8, killed on *extremis*
	1271	Yes	-
	1304	Yes	-
	1288	No	-
	1284	Yes	-
	1287	Yes	-
	1306	Yes	-
Unvaccinated group	1279	No	-
	1283	Yes	-
	1263	Yes	-
	1293	No	-
	1298	Yes	3
	1274	Yes	-
	1290	Yes	-
	1289	Yes	1
	1272	Yes	-
	1294	Yes	1

Table 4.3 Summary of clinical observations of cattle vaccinated with *Mmm* membrane proteins vaccine formulation and challenged with live *Mmm.*

4.3.4 Gross pathology findings and *Mmm* isolation

CBPP lung lesions were observed in both vaccinates (5/10) and control (3/10) groups following challenge. Only one animal (#1285) in the vaccinated group died before the end of the experiment. The lesions were confined to the right lung, leaving the left lung grossly normal (Fig. 4.3). Lesions were mainly found on the dorsal and lateral surface of the caudal lobe, with some extending to the cranial and accessory lobes. The tissues where the lesions were found exhibited oedematous and consolidation and exhibited red hepatisation, thickened interlobular septae and marble appearance upon excision. Pleural adhesion ranging from 18 to 40 cm was observed in the vaccinated (# 1285, 1265 and 1282) and 2 to 14 cm for control (# 1283, 1298 and 1274) group. Diaphragmatic adhesion was observed in all the animals with lung lesions except in one control animal (#1274). These pathological features are consistent with fibrous bronchopneumonia typical of acute and sub-acute CBPP. Different stages of sequestration was evident in one vaccinated (#1265) and in two control (#1274 and 1298) animals. The sequestra were clearly demarcated from normal tissue and encapsulated in fibrous tissue. Pleural fluid was observed only in one vaccinated animal (#1285) that measured to about 8 litres, but was absent in the control group (see fig. 4.4). Kidney lesions were observed only on one unvaccinated animal (#1293) that had liquefied fat and hemorrhage. The summary of the gross pathology is shown in Table 4.3.

	Animal No.	Post-mortem findings	*Mmm* isolated	Total pathology score
Vaccinated group	1267	No lesion	+ve	2
	1285	Pleural fluid 8 litres, fibrous adhesion thoracic wall (40 cm), diaphragmatic adhesion, Consolidation whole lung	Pleural fluid +ve Lung +ve	12
	1265	Adhesion on both lungs to the thoracic wall (30 cm), diaphragmatic adhesion (7 cm), sequestra (4X5, 6X7, 2X2 cm), pericardial adhesions	+ve	12
	1282	Adhesion lung to thoracic wall (18 cm), diaphragmatic adhesion, consolidation (22x22 cm)	+ve	12
	1271	No lesion	+ve	2
	1304	No lesion	+ve	2
	1288	Consolidation (2x2.5 cm)	+ve	4
	1284	No lesion	+ve	2
	1287	No lesion	+ve	2
	1306	Consolidation (7x2 cm)	+ve	8
		Mean score		5.8±4.661
Unvaccinated group	1279	No lesion	+ve	2
	1283	Adhesion to thoracic wall (12 cm), diaphragmatic adhesion, pericardial adhesion, sequestra (8 cm)	+ve	12
	1263	No lesion	+ve	2
	1293	Kidney – liquefied fat and haemorrhage	-ve	0
	1298	Adhesion to thoracic wall (14 cm), diaphragmatic adhesion, Sequestra (17x14 cm)	+ve	12
	1274	Adhesion to thoracic wall (2 cm), sequestra (1x1)2, kidney – liquefied fat	+ve	8
	1290	No lesion	+ve	2
	1289	Heart – increased pericardial fluid	+ve	2
	1272	No lesion	+ve	2
	1994	No lesion	-ve	0
		Mean score		4.2±4.422

Table 4.4 Gross pathology and *Mmm* isolation outcome of cattle vaccinated with *Mmm* membrane proteins and challenge with live *Mmm*.

Mmm was isolated from lung tissues of all the vaccinated and unvaccinated cattle except in two animals (#1294 and 1293) in the experiment. Mycoplasma was isolated in all the lung tissues that had lesions. Interestingly, one animal (#1294) that had kidney lesions did not have any mycoplasma isolated. Gross pathology and *Mmm* isolation findings are summarized in Table 4.3.

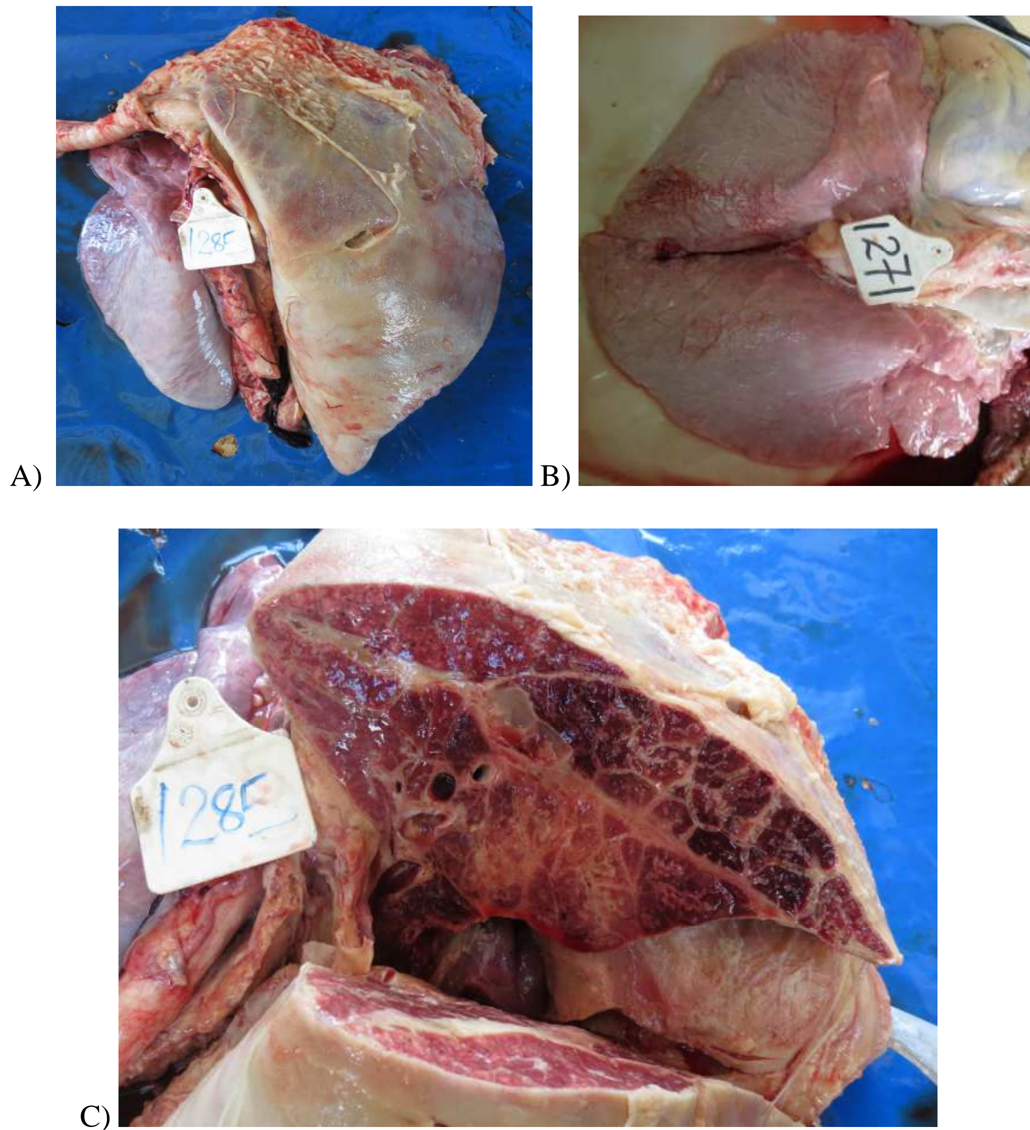


Figure 4.3 CBPP lung pathological appearance; unilateral chronic fibrinous pleuropneumonia. A) The right lung was enlarged, covered by a sheet of fibrin and firm. The left lung had no lesion. B) Normal lung (no lesion). C) The hardened lung has been dissected and reveals the marbled appearance of the fleshy and diseased areas.

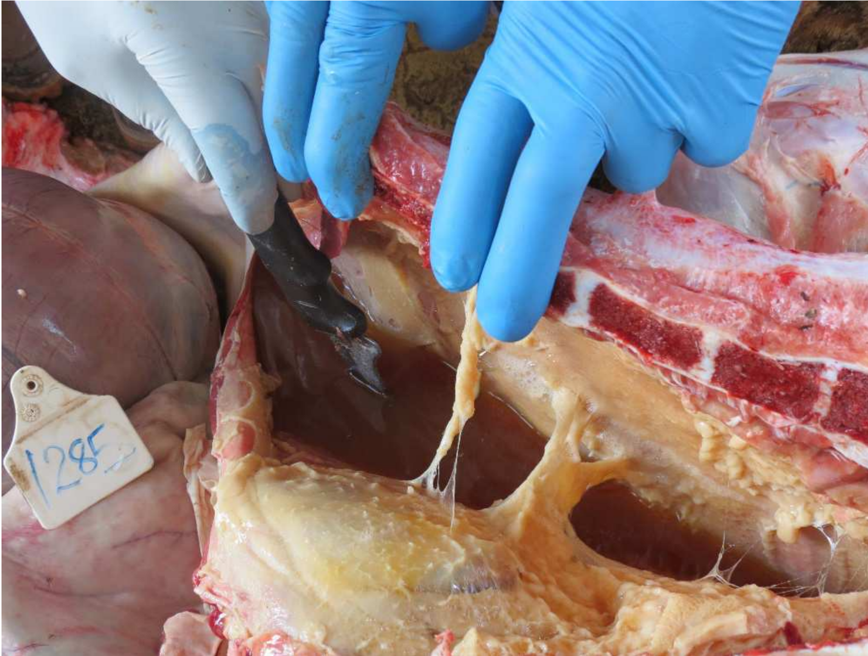


Figure 4.4 CBPP gross pathology. Severe fibrino-effusive pleuropneumonia in chronic stage. The thorax was occupied with large amounts of a translucent yellow fluid with strands and sheet of fibrin that adhere to the visceral pleura. The lung also adhered to the diaphragm and rib cage.

4.4 DISCUSSION

4.4.1 Introduction

The aim of this experiment was to determine whether immune responses induced by vaccination of cattle with membrane protein in emulsigen are protective upon challenge. *Mmm* membrane proteins induced specific antibodies as confirmed by CFT and indirect ELISA after vaccination. Post infection with live *Mmm* observations indicated that vaccinated animals developed enhanced disease.

The proteins extracted were amphiphilic in nature as confirmed by Triton X-114 extraction and phase partitioning. The membrane protein vaccine formulation contained a high number of proteins as demonstrated by the about 35 bands in the SDS-PAGE in comparison of 60 bands in the whole cell lysate. It is worth noting that mycoplasma lack a cell wall and the lipid membrane of the *Mmm* KH3J strain contain about 160 polypeptides with molecular masses ranging from 14 to 125 kDa (Jan *et al.,* 1996).

4.4.2 Clinical and gross pathology

There were no detectable clinical changes in cattle following vaccination, which means that vaccination with membrane proteins did not induce a reaction of clinical relevance. Clinical features in the non-vaccinated were less pronounced than in the vaccinated animals although the challenge was not severe. Pathological scores after infection were also higher in the vaccinated animals than in non-vaccinates as indicated by the extensive lung lesions, fibrous adhesions and presence of pleural fluid. Two animals in the vaccinated animals suffered severe disease with one succumbing and the other killed on *extremis* five weeks after infection. However, no significance difference ($P>0.01$) in

pathological lesions score were seen between the vaccinated and control group. Hence the vaccine formulation tested cannot be considered to induce a protective immune response.

4.4.3 Antibody responses

Post-vaccination responses showed that membrane proteins exhibited detectable antibody responses by CFT and indirect ELISA. CF antibodies were detected in only two animals (#1282 and 1304) after vaccination by CFT. The responses however seem not to be related to protection since the two animals exhibited CBPP clinical and pathological lesions. One animal (#1282) had a titre of 1:20 but developed severe CBPP and was killed on extremis. This has also been observed in other CBPP vaccine trials (Gilbert *et al.,* 1970; Windsor *et al.,* 1972). The other animal (#1304) produced a titre of 1:40 but did not have CBPP lesion, although the animal experienced respiratory distress and mycoplasma was isolated from the lung tissues.

The serum IgM antibody titres in cattle immunized with membrane proteins measured by ELISA after the first dose increased significantly. However, low levels were detected three weeks after the boost. A second dose administered after the three weeks resulted to a 3 fold increase in IgG titres. Thus, the IgG response may be indicating that Th1 type of response since it is upregulated by IFN-γ and IL-2 as indicated in previous immunization studies with ISCOM vaccines (Abusugra *et al.,* 1997). It is noteworthy that IgA was not detected in the serum. This may be due to the sensitivity of the indirect ELISA used to assay the antibodies or the subcutaneous injection may not lead to the production of the isotype antibodies.

Correlations between antibody specificities and pathological score were assessed. Correlation between the antibodies against membrane proteins and outcome of disease such as reduced disease severity or absence of pathological lesions would suggest the presence of a protective antigen. Although antigen specific responses were significant, indicating differing levels of responses, no significant correlation between the specific antibody response and presence/absence of lesions ($P>0.01$) was observed. The data from this study indicate that higher specific antibody titres do not lead to less severe pathological symptoms. Interestingly, animals that demonstrated severe clinical symptoms and pathological lesions had high antibody titres after vaccination. Since immunological responses have been attributed to pathogenesis of CBPP (Provost *et al.,* 1987; Dedieu *et al*., 2006), the results to can be interpreted to mean that the adverse clinical and pathological outcome was due to a robust memory response to the membrane proteins.

The relationship between antibody responses and pathology lesions raises an interesting question whether high titres might contribute in one way or the other to pathology as shown in previous vaccination trials using a membrane proteins LppQ (Pilo *et al.,* 2007) and GlpO (Mulongo *et al.,* 2013). The presence of pulmonary vasculitis that is pathologically characterized by the destruction of blood vessels in CBPP (Thiaucourt *et al.,* 2004) has been attributed to deposition of antigen-antibody complexes. A recent study by Mulongo and others (2015) has also shown that immunized cattle with purified recombinant LppQ and challenged with *Mmm* showed a strong seroconversion to LppQ and exhibited significantly enhanced post infection glomerulonephritis that suggested the development of type III immune complex disease.

4.3.4 Enhanced disease following vaccination and challenge

Rather than mediating clearance of mycoplamsa, vaccination with *Mmm* membrane proteins instead seemed to contribute in enhancement of CBPP. Previous studies in viral (Haynes *et al.,* 2003) and mycoplasma (Szczepanek *et al.,* 2012) have demonstrated that vaccination can lead to enhanced disease. Several vaccine trials conducted in military personnel during the 1960s using inactivated bacteria resulted in minimal efficacy and in some cases vaccination appeared to exacerbate disease upon subsequent challenge with virulent *M. pneumonia (*Smith 1967). In the infection experiment vaccination led to more severe illness than in the control volunteers. A study involving vaccination of P-130 P30 mutant in mice against *M. pneumoniae* showed evidence of exacerbated disease upon subsequent challenge with the wild-type strain PI1428, which appears to be driven by a Th17 response and corresponding eosinophilia (Szczepanek, 2012). Formalin-inactivated measles virus (FI-MV) vaccine (Peter, 2005), formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine or RSV glycoprotein (Haynes 2003) indicated enhanced disease following infection. The enhanced disease and pulmonary eosinophilia associated with formalin-inactivated respiratory syncytial virus vaccination were shown to be linked to glycoprotein CX3C-CX3CR1 interaction and expression of substance P (Haynes 2003). Vaccination with Dengue virus in humans has also been shown to enhance vascular permeability, extensive plasma leakage in various tissue spaces and serous cavities of the body, including the pleural, pericardial and peritoneal cavities in patients with haemorrhagic fever, may result in profound shock (Basu and Chaturvedi, 2008). Several mechanisms may be involved including immune complex disease, T-cell-mediated, antibodies cross-reacting with vascular endothelium, enhancing antibodies, complement

and its products, various soluble mediators including cytokines, selection of virulent strains and virus virulence (Basu and Chaturvedi 2008; Hughes *et al.,* 2012).

4.4.5 Conclusion

In conclusion, vaccinated and non-vaccinated animals revealed strong inflammatory reactions after challenge, and a Th1 response may be involved in the pathogenesis of CBPP. Future studies should focus on the Th1 response in the immune reactions. Future development of a vaccine against CBPP need to focus on understanding of immunological reactions that leads to pathological conditions and innate immune response.

CHAPTER 5: EFFICACY OF A CAPSULAR POLYSACCHARIDE CONJUGATE VACCINE TO PROTECT AGAINST CONTAGIOUS BOVINE PLEUROPNEUMONIA

5.1 INTRODUCTION

The capsular polysaccharide (CPS) also known as galactan is an important antigen and pathogenic factor in *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) located on the outer surface or in the plasma membrane. Several studies have linked the CPS to protection and virulence. CPS is assumed to protect the pathogen from the bactericidal activity of complement and other host defence functions, and to trigger the inflammatory process in the infected host (Pilo *et al*., 2007). Lloyd and Titchen (1976) showed that intravenous injection of CPS from *Mmm* to calves produced transient apnoea, increased pulmonary arterial pressure and pulmonary oedema, leading to the contraction of blood vessels, which may initiate thrombosis. March and others (2000) have demonstrated that a strain of *Mmm* that produces low amounts of capsular polysaccharide was much more sensitive to growth inhibiting antisera than strains that produced larger amounts of polysaccharide. Strains of *Mmm* that produced large amounts of capsular polysaccharide also generated a significantly longer duration of bacteraemia in a mouse infection assay than the strain with little capsular polysaccharide (March *et al.,* 2000).

Both humoral and cell mediated responses may contribute to immunity to CBPP as demonstrated by (Dedieu *et al.*, 2005; and Totté *et al.*, 2008). They showed a correlation between severity of disease and lower numbers of IFN- γ-secreting cells in a primary infection**.** But as *Mmm* is not an intracellular pathogen, it is likely that antibodies are the major protective response. An inverse correlation between specific IgA titres, but not IgG titres, was found with severity of symptoms (Niang *et al.,* 2006). *In vitro* studies on

monoclonal antibody PK-2 against *Mmm* capsular polysaccharide (Mwirigi *et al.*, 2010) has been shown to inhibit mycoplasma adhesion to bovine lung epithelial cells. Antibody PK-2 and several other CPS-specific monoclonals (Kiarie *et al.,* 1996; Aye, personal communication) also block mycoplasma growth. Adhesion of *Mmm* to host cells is thought to be an important step in the establishment of disease (Aye *et al.,* 2015). This attribute of the CPS therefore makes it a promising vaccine candidate.

The ability of carbohydrates to produce antibodies and induce memory is very low, unless they can be covalently coupled to a carrier protein (Peeters *et al.*, 2003). Studies by Waite and March (2002) have shown that vaccination of mice with a conjugated *Mmm* CPS significantly increased the antibody responses compared to what was produced by polysaccharide alone. We therefore hypothesized that a CPS conjugate vaccine might protect against CBPP.

This chapter focuses on determining the efficacy of CPS conjugated with ovalbumin via a carbodiimide-mediated condensation with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) (Waite & March, 2002). The method has been used previously to conjugate the O-specific polysaccharide of the lipopolysaccharide of *Escherichia coli* O157 to bovine serum albumin (Konadu *et al.,* 1994).

The work carried out was guided by the hypothesis that vaccination with a conjugated CPS dissolved in an oil adjuvant stimulates an immune response in cattle and protects them against live *Mmm* upon experimental challenge.

The objectives of the study were;

1. To extract and purify CPS of *Mmm*
2. To describe the clinical and pathological outcome in cattle vaccinated with *Mmm* CPS conjugated with ovalbumin and challenged with an infective dose of *Mmm*
3. To assess correlation between antibody responses in CPS-vaccinated cattle and pathology after challenge with an infective dose of *Mmm*

5.2 MATERIALS AND METHODS

5.2.1 Preparation of CPS conjugate vaccine

5.2.1.1 Mycoplasma growth and Extraction and purification of CPSs

The Afadé strain (obtained from International Livestock Research Institute (ILRI)) of *Mmm* was grown in pleuropneumonia-like organism (PPLO broth; Difco) media as described in chapter 2, section 2.1.

CPS was extracted from the supernatant of mycoplasma cultures as previously described (Rurangirwa *et al.*, 1987). Briefly, 5 litres of mycoplasma culture was grown for 3 days and centrifuged at 8,000 g for 30 minutes. The supernatant pH was adjusted to 5.0 with glacial acetic acid and heated at 68°C for 2h in a water bath and filtered using Whatman paper to denature and remove the proteinaceous material. The removal of proteins was repeated until the aqueous phase appeared transparent. Two volumes of ethyl alcohol was added and kept at 4°C overnight. The mixture was then centrifuged at 1000 g for 15 min. The pellet was then suspended in 50 ml distilled water and stirred at room temperature for 2 h, centrifuged at 3,000 g for 30 min and the sediments discarded. The mixture was held overnight and dialysed against tap water for 2 days. Two volumes of ethyl alcohol was added and kept overnight at 4°C to precipitate the carbohydrate. The mixture was then centrifuged at 1000g for 15 min, the pellet dissolved in 10 ml of distilled water and dialysed for two days against distilled water with four changes per day. The precipitation and dialysis was repeated once more.

5.2.1.2 Determination of extracted polysaccharide concentration

Purified CPS concentration was determined using the Phenol-Sulphuric acid method for total carbohydrate as described by (Dubois *et al.,* 1956). Briefly, serial dilutions of glucose standards (1.5 – 12.5 mg/ml) were prepared in dH_2O. Fifty µl of the glucose standards and sample were loaded into a microtiter plate (Nunc, Copenhagen, Denmark). Then, 150 µl of concentrated sulfuric acid (Sigma-Aldrich, USA) was added. This was followed immediately with addition of 30 µl of 5% phenol (Sigma-Aldrich, USA). The mixture was incubated for 15 min. at 37^oC and left to cool at room temperature. The OD was measured at 490 nm using a microplate reader (Bio Tek, USA). A standard curve was plotted from the values observed for glucose standards and was used to calculate the concentration of the extracted carbohydrate based on OD values of the sample dilutions.

5.2.1.3 SDS PAGE and staining of the polysaccharide

Purity of the extracted carbohydrate was verified by SDS-PAGE as described in chapter 2, section 2.5. Visualization of the carbohydrate was achieved by staining with the Periodic Acid Schiffs method as described by (Johnstone and Thorpe, 1987). Briefly, the polyacrylamide gel was fixed in 5x volume of destaining solution (5% methanol and 7.5% acetic acid) in a sandwich box overnight at room temperature. An equal vol. of 7% cold acetic acid for 1 h at 4^oC was used to wash the gel. The fluid was poured off, and incubated with 5x the gel volume of freshly prepared 1% (w/v) periodic acid in 7% Cold acetic acid for 1h in the dark at 4^oC. The gel was washed with 7% acetic acid in the dark (several changes at 4^oC for 24 h). Colour was developed by replacing wash solution with Cold Schiff reagent and the gel held at 4^oC for 1h in the dark. Red diffuse bands signified presence of carbohydrate with discreet bands aligning with the protein bands suggesting

glycoprotein. Periodic acid reacts with vicinal hydroxyl groups commonly found in sugar molecules to generate aldehydes that are detected by their colour reaction with reduced Schiff's reagent.

5.2.1.4 Protein content determination

To determine the purity of the extracted carbohydrate, the amount contamination by proteins was carried out using a protein estimation assay as described in chapter 2, section 2.4.

5.2.1.5 Coupling of CPS to Ovalbumin and vaccine formulation

Conjugation of the ovalbumin (OVA) to the CPS was achieved through an established methodology (Waite and March, 2002). Briefly, 0.4 mg/ml of ovalbumin was dissolved in 5 ml 1% MES buffer (4-morpholinoethanesulfonic acid). Two ml of CPS (2 mg/ml) was added to the OVA solution. Then 5 ml of EDC (ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride) (10mg/ml in dH_2O) was added and stirred gently and reaction was left to take place for 2 h at room temperature. The mixture was dialyzed against sterile PBS overnight. The conjugation reaction is illustrated below (Fig 5.1).

Ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) Reaction Chemistry

Coupling of the CPS to the ovalbumin was facilitated by use of EDC as a cross-linker. The EDC reacts with carboxylic acid groups of glucosamine in the CPS to form an active *O*-acylisourea intermediate that is easily displaced by nucleophilic attack from primary amino groups in the reaction mixture. Primary amine forms an amide bond with the original carboxyl group, and an EDC by-product is released as a soluble urea derivative. The EDC crosslinking is most efficient in acidic (pH 4.5) conditions and must be performed in buffers devoid of extraneous carboxyls and amines therefore MES buffer (4-morpholinoethanesulfonic acid) is a suitable carbodiimide reaction buffer.

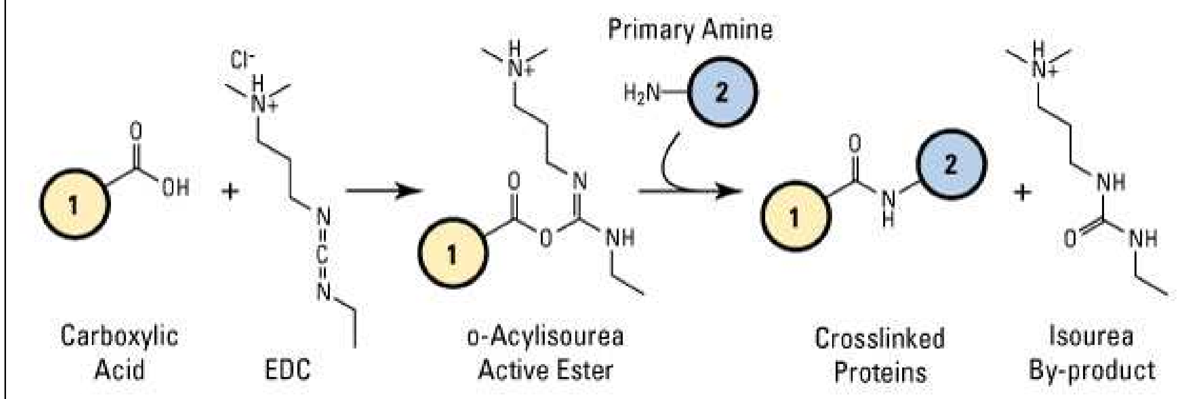


Figure 5.5 Carboxyl-to-amine crosslinking with the carbodiimide, EDC. Molecules (1) and (2) can be peptides, proteins or any chemicals that have respective carboxylate and primary amine groups. When they are peptides or proteins, these molecules are tens-to-thousands of times larger than the crosslinker and conjugation arms diagrammed in the reaction.

Above description adapted from https://www.thermofisher.com/ke/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/carbodiimide-crosslinker-chemistry.html

The vaccine was then prepared by adding an adjuvant, 30% Emulsigen® (MVP Technologies, Omaha, USA; Oil-in-Water Emulsified Adjuvant) to make a total of 20mls (CPS concentration: 100µg/ml). The mixture was thoroughly mixed prior to use.

5.2.2 Cattle experiment

5.2.2.1 Cattle

Two- to three-year boran (*Bos indicus*) cattle were purchase and handled as described in the procedure in chapter 2, section 2.2.1.

5.2.2.2 Treatment of groups and challenge

The 20 cattle were randomly divided into two groups. One group was vaccinated with CPS conjugated vaccine and the other with the adjuvant (placebo) only. Each of the animals was subcutaneously inoculated on the neck with 2 ml of the vaccine formulation containing 100µg of CPS. Secondary inoculation was carried out after 6 weeks. The animals were fed and housed in a confined paddock. Challenge was carried out four weeks after the secondary inoculation by endobronchial inoculation carried out using the procedure described in chapter 2, section 2.2.2.

5.2.2.3 Clinical observations

Clinical signs (rectal temperatures, coughing, nasal discharge and general behaviour of the animal) were monitored daily as described in chapter 2, section 2.2.3.

5.2.2.4 Autopsy and collection of tissue samples

Cattle were humanely euthanized and necropsied by a veterinary pathologist as described in the procedure in chapter 2, section 2.2.5.

5.2.2.4 Mycoplasma isolation

Mycoplasma culturing from the lungs was carried out to verify the presence of *Mmm* as described in chapter 2, section 2.2.6.

5.2.2.5 Lesion scoring and vaccine efficacy

Determination of the lung pathology score of the pleuropneumonia infection and efficacy of the vaccine was done as described in chapter 2, section 2.2.7.

5.2.3 Detection of immune response

Antibody responses following vaccination and challenge were monitored using the Compliment Fixation Test (CFT). Anti-Capsular polysaccharide antibody titres for specific IgG, IgM and IgA antibody isotypes by an Indirect ELISA three weeks post primary vaccination and three weeks post-secondary vaccination. CFT and Indirect ELISA were carried out as described in chapter 2, section 2.3.1 and 2.3.2 respectively

The CPS Ab titres were then compared with CPS Ab titres of cattle from a previous experiment that were vaccinated with live attenuated vaccine and protected after challenge.

5.3 Results

5.3.1 Confirmation of extracted polysaccharide identity

The CPS was well identified in the SDS-PAGE gel stained with Acid Schiff Stain (Fig. 5.2) as indicated by the purple colour characteristic of carbohydrate.

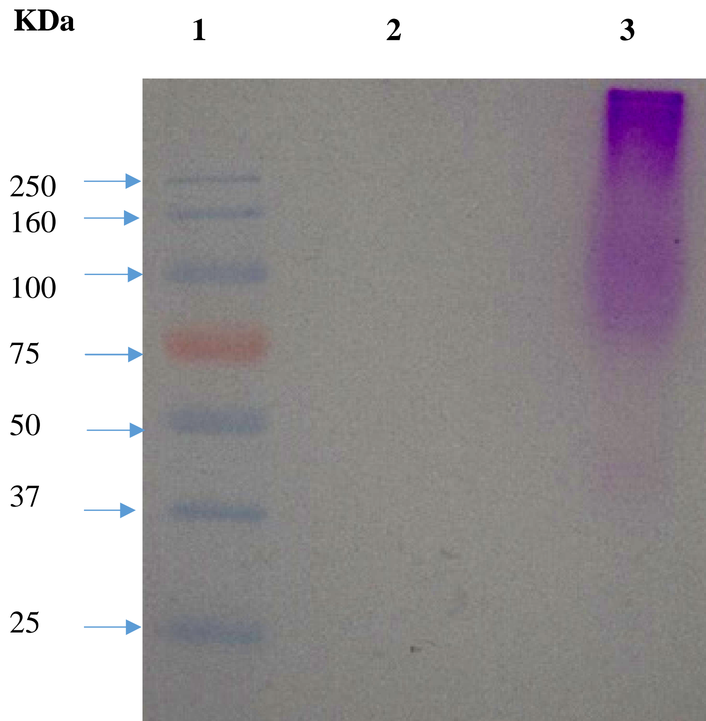


Figure 5.6 Gel stained for polysaccharide (Acid Schiff stain).

Lane 1 contain pre-stained molecular weight markers, lane 2 contained *Mmm* whole cell lysate and lane 3 contained *Mmm* extracted polysaccharide. Extracted CPS was observed as a purple smear on the gel. Whole *Mmm* lysate did not stain for carbohydrate.

5.3.2 Clinical observations following challenge

Figure 5.2 represents the rectal temperature following infection with the *Mmm*. Temperatures exceeding 39.5°C were recorded between day 17 and 19 post-intubation and persisted over a period varying between 2 and 3 days. Of the 10 vaccinated animals two (1299, 1260) had fever that lasted one day while one (1311) lasted for three days. In the control group, one (1298) had fever lasting three days and two (1289 and 1294) only one day. Respiratory distress that was assessed through coughing was observed in 5/10 vaccinates and 8 /10 animals in control group. No mortality was observed during the observation period (Table 5.1).

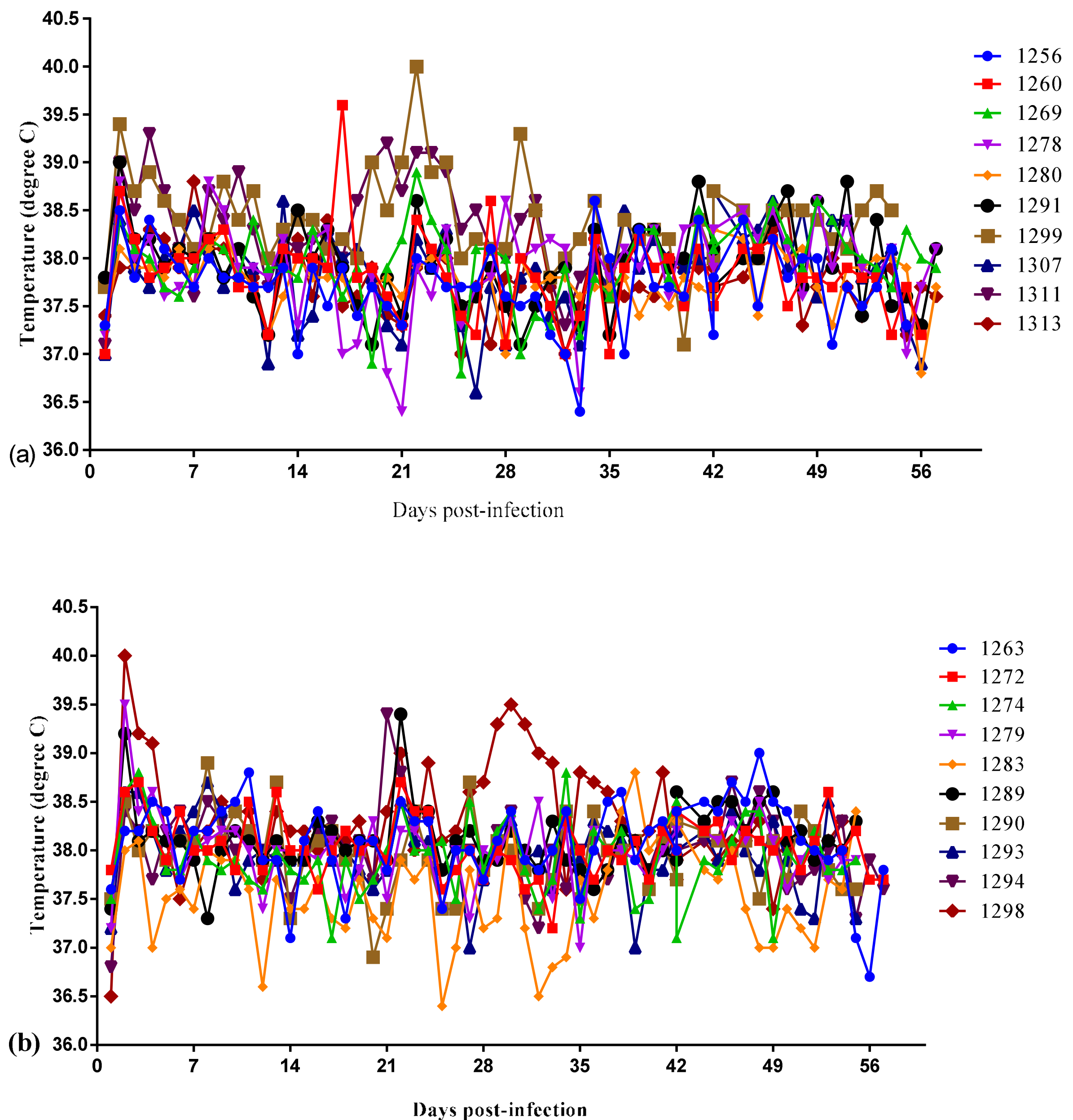


Figure 5.7 Rectal temperature for Conjugated CPS cattle vaccination experiment; (a) vaccinated with CPS conjugate vaccine; (b) unvaccinated cattle after infection. A rectal temperature of $\geq 39.5^{o}C$ was regarded as fever. The readings terminate on the respective day for euthanasia of each animal.

	Animal Number	Clinical observations	
		Respiratory distress	Pyrexia (above 39°C) (days)
Vaccinated group	1260	Yes	1
	1307	No	-
	1269	Yes	-
	1256	No	-
	1311	Yes	3
	1299	Yes	1
	1278	Yes	-
	1291	No	-
	1313	No	-
	1280	No	-
Un-vaccinated group	1279	No	-
	1283	Yes	-
	1263	Yes	-
	1293	No	-
	1298	Yes	3
	1274	Yes	-
	1290	Yes	-
	1289	Yes	1
	1272	Yes	-
	1294	Yes	1

Table 5.5 Result of clinical observations of CPS conjugate vaccinated cattle and challenged with live *Mmm* experiment.

5.3.3 Gross pathology and Mycoplasma isolation

The results of the pathology examination following exposure of vaccinated and control cattle to infection are summarized in table 5.2. Characteristic gross CBPP lesions were observed at necropsy on day 56 post-infection that were confined to the right caudal lung. Only two (1311, 1299) of the 10 vaccinates had typical CBPP lesions; fibrous adhesions to thoracic and diaphram and sequestra (see Fig. 5.4 and 5.5) when examined post mortem. Only one had severe lesions. No kidney lesion was observed in this group. Four (1260, 1269, 1278, 1313) of the ten animals had no CBPP lesions and also no mycoplasma was isolated from the lung tissues. The mean pathology score was 1.8. There was substantial evidence of CBPP lesions in the unvaccinated controls. Three (1283, 1298, 1274) of the 10 unvaccinated animals had severe CBPP lesions when examined post-mortem. Fibrous adhesion to the rib cage was common in these three animals. Severe kidney lesions were observed in three animals (1263, 1293 and 1274) in the control group. Mycoplasma was isolated from eight animals in the group. The mean pathology score was 4.2. The vaccinated and the non-vaccinated animals did not show gross lesions of the lymph nodes or joint involvement.

	Animal number	Post-mortem findings	*Mmm* isolated	Total pathology score
CPS Conjugate vaccinated group	1260	No lesion	-ve	0
	1307	No lesion	+ve	2
	1269	No lesion	-ve	0
	1256	No lesion	+ve	0
	1311	Fibrous adhesion (4 cm), diaphragmatic adhesion, sequestrum in remission	+ve	4
	1299	Fibrous adhesion to the thoracic cavity, sequestrum (10x10 cm)	+ve	8
	1278	No lesion	-ve	0
	1291	No lesion	+ve	2
	1313	No lesion	-ve	0
	1280	No lesion	+ve	2
		Mean score		18±2.57
Unvaccinated group	1279	No lesion	+ve	2
	1283	Adhesion to thoracic wall (12 cm), diaphragmatic adhesion, pericardial adhesion, sequestra (8 cm)	+ve	12
	1263	No lesion	+ve	2
	1293	Kidney – liquefied fat and haemorrhage	-ve	0
	1298	Adhesion to thoracic wall (14 cm), diaphragmatic adhesion, Sequestra (17x14 cm)	+ve	12
	1274	Adhesion to thoracic wall (2 cm), sequestra (1x1)2, kidney – liquefied fat	+ve	8
	1290	No lesion	+ve	2
	1289	Heart – increased pericardial fluid	+ve	2
	1272	No lesion	+ve	2
	1294	No lesion	-ve	0
		Mean score		4.2±4.66

Table 5.6 Results of gross pathology and *Mmm* isolation outcome of CPS-conjugate vaccinated cattle and challenged with live *Mmm* experiment

Figure 5.8 CBPP gross pathology. Fibrous strands of fibrin were adherent to the parietal and visceral pleura. The lobes were adherent to the diaphragm, rib cage and adjacent lobes of the lung.

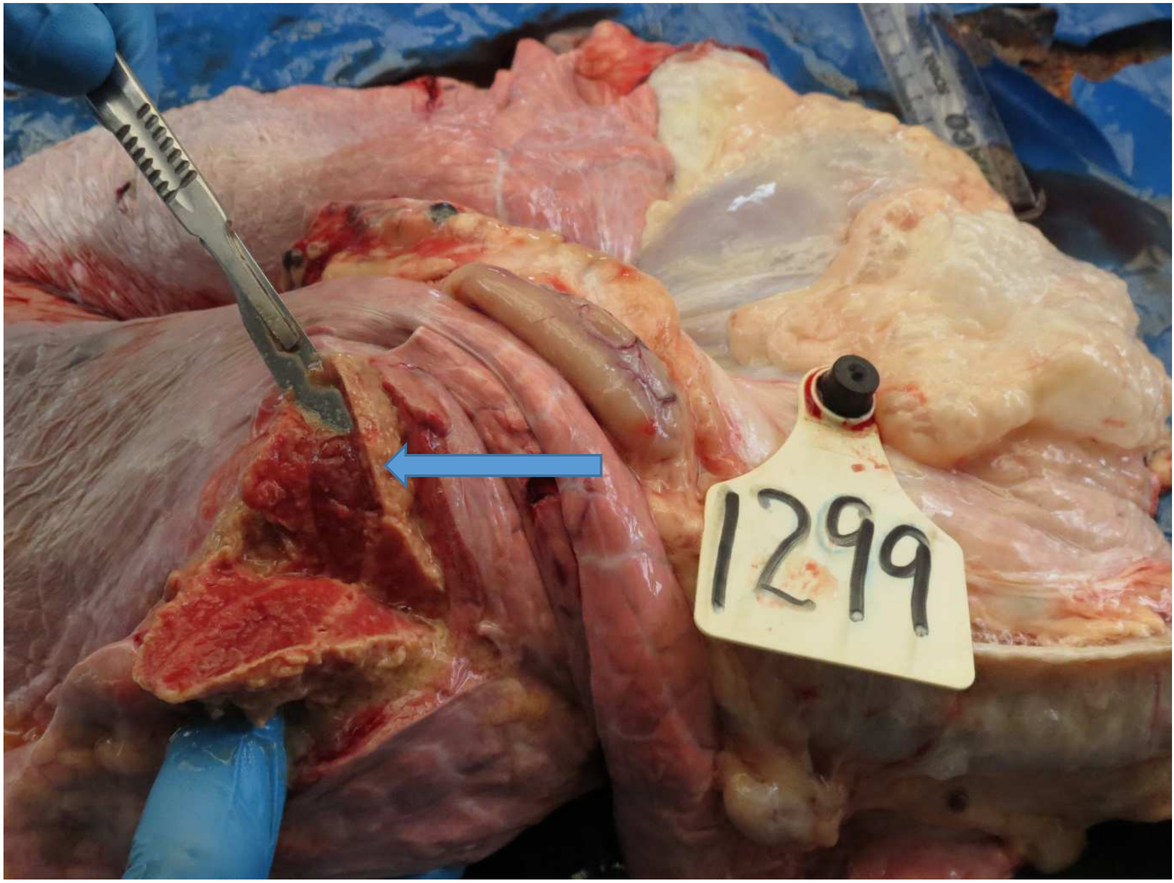


Figure 5.9 Encapsulated sequestraum with necrotic tissue is shown by the arrow.

5.3.4 Immunological response to vaccination and challenge

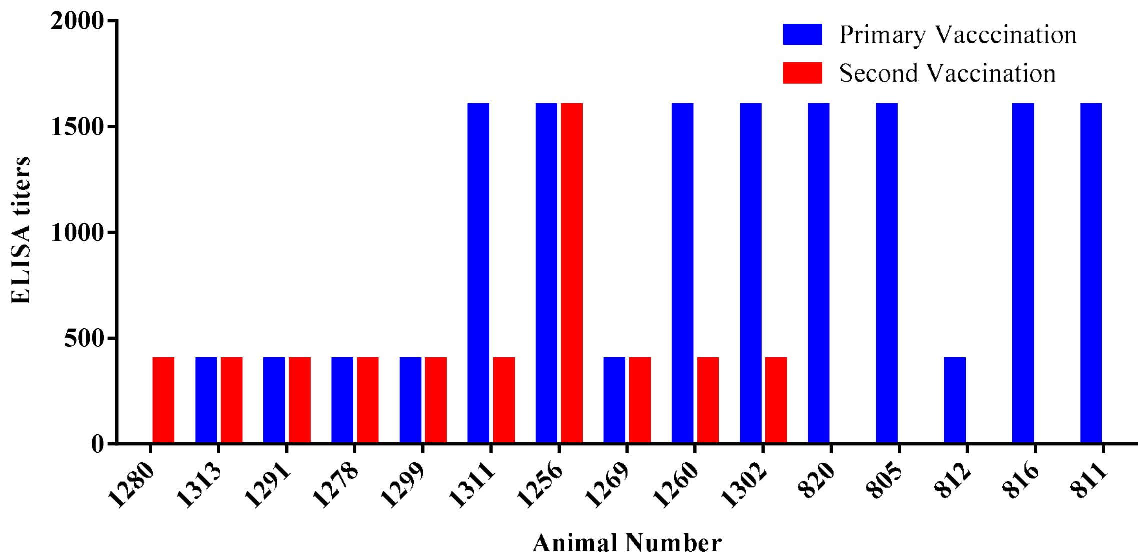
The results of CFT serological responses following vaccination are recorded in Table 5.3. Only one (1256) animal seroconverted following CFT after vaccination with a titre of 1:10. This animal did not have CBPP lesions when slaughtered at the end of the trial. None of the animals in the control group were found to be positive during this experimental period. Only two (1311, 1299) of the 10 vaccinates reacted to CFT following challenge and had typical CBPP lesions. The control group had more animals (5/10) seroconverting after challenge with three (1283, 1298, 1274) of them developing typical CBPP lesions. Two animals (1279 and 1272) that had low titres did not show CBPP lesions.

	Animal number	CFT maximum Ab titres	
		After vaccination	After challenge
Vaccinated with CPS conjugated vaccine	1260	-	-
	1307	-	-
	1269	-	-
	1256	10	10
	1311	-	320
	1299	-	160
	1278	-	-
	1291	-	-
	1313	-	-
	1280	-	-
Unvaccinated group (Control)	1279	-	10
	1283	-	320
	1263	-	-
	1293	-	-
	1298	-	640
	1274	-	40
	1290	-	-
	1289	-	-
	1272	-	20
	1294	-	-

Table 5.7 CFT antibody titres following CPS conjugate vaccine vaccination and challenge.

Determination of the IgM, IgG and IgA titres against the CPS was done using an Indirect ELISA. Anti-CPS IgM titres were low following primary vaccination. Analysis of the resulting serum samples indicated that cattle immunized with the glycoconjugate exhibited significantly higher anti-CPS IgG than IgM Ab titers. After the secondary immunization high antibody titres were detected using indirect ELISA. The average Ab titres for IgM and IgG had an increase of two fold following the boost (Fig. 5.6). IgA Abs may have been too low to be detected by the method used. The CPS conjugate vaccine produced high titres of IgG following secondary vaccination. Only one animal (805) of live attenuated vaccinated group produced titres of IgG equal to the CPS conjugate vaccinated group.

A) IgM antibody titres



B) IgG antibody titres

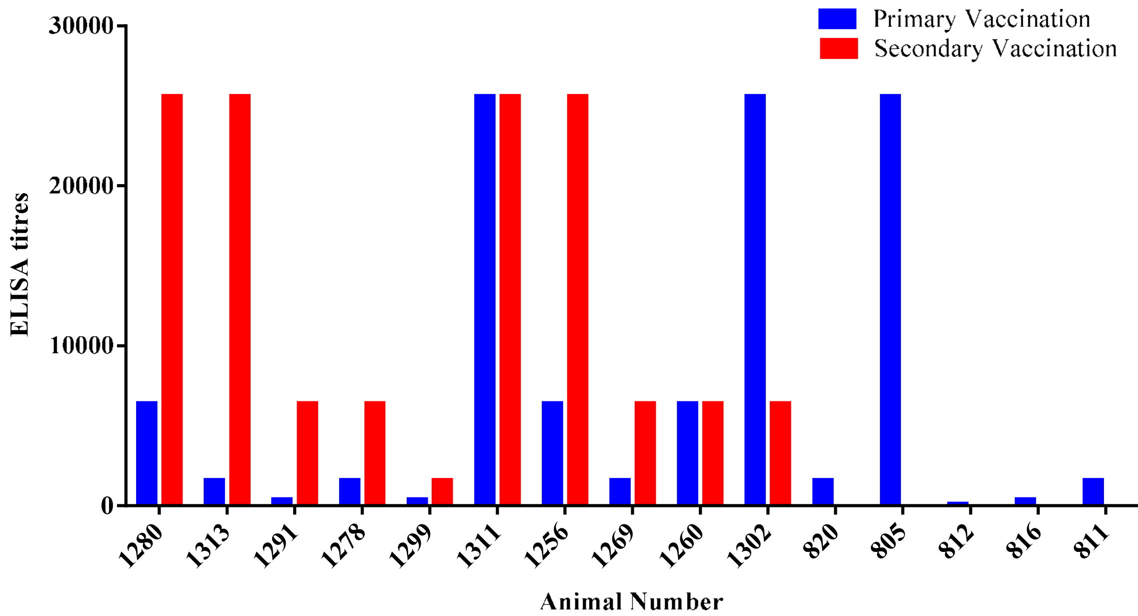


Figure 5.10 Antibody titres of cattle immunised twice three weeks apart with 200 µg CPS conjugate vaccine measured by ELISA. Animals (820, 805, 812, 816 and 811) were vaccinated with T1/44 live attenuated vaccine and had no lesions

5.4 DISCUSSION

5.4.1 Introduction

The study demonstrates that conjugated capsular polysaccharide vaccine mixed with Emulsigen as adjuvant induces partial protection in cattle against challenge with contagious bovine pleuropneumonia. The data presented here demonstrate that CPS conjugate from Afadé strain vaccine is immunogenic and efficacious against *Mmm*.

It has been described in the previous studies (Lloyd *et al.*, 1971) that CPS alone did not induce protective immunity. Lesions in joints and kidneys and a prolonged mycoplasmaemia were observed. Waite and March (2002) demonstrated that mice immunized with the CPS ovalbumin conjugate exhibited a significant antibody response against CPS but did not exhibit a reduction in mycoplasmaemia. However, the capacity of CPS conjugate experimental vaccines to induce protective immune responses has been demonstrated with a number of bacteria (Baraldo *et al.,* 2005; Burtnick *et al.*, 2012) . Given the general success of CPS conjugate vaccines, the study sought to evaluate the capacity of CPS ovalbumin conjugate vaccine in cattle.

5.4.2 Clinical observations

The clinical signs in the vaccinated and non-vaccinates groups appeared 3 weeks post-inoculation. Although no statistical difference was observed (P>0.05), the non-vaccinate seemed to have developed more severe clinical than vaccinates. More animals in control group (8/10) experienced respiratory distress compared to the vaccinated (5/10). Fever was inconsistent as some of the animals with lesions at necropsy did not show

temperatures of above 39.5°C. Fever may therefore not be a reliable indicator of CBPP infection as also demonstrated by Gull and others (2013).

5.4.3 Gross pathology findings

Differences in gross necropsy lesions between the vaccinated and non-vaccinated groups were evident, with lung lesions and adhesion to the ribcage being more extensive in the non-vaccinated animals. Interestingly, kidney lesions were only observed in the non-vaccinated animals. The kidney lesions were indicative of progression of disease to chronic phase (Grieco *et al.,* 2001). In general, infection was not very severe as the chronic disease developed in the non-vaccinate. A possible explanation would be the animals used in the experiment were resistant. However, endobronchial inoculations method used may have resulted in chronic disease pattern than natural exposure (Scacchia *et al.,* 2011; Gull *et al.*, 2013).

The vaccine did not prevent *Mmm* lung colonization in all the challenged animals. Mycoplasma was recovered in 5 vaccinated animals and 8 in the control group. All lung tissues with gross lesions were positive on mycoplasma isolation.

5.4.4 CPS conjugate vaccine efficacy

Efficacy of the vaccine as calculated using Hudson and Turner lesion scores CPS conjugate vaccine was 57%. Similar protection rates of 50-80% have been observed previously in experimental studies with live vaccines (Gilbert and Windsor, 1971; Thiaucourt *et al.* 2000; Nkando *et al.,* 2012).

5.4.5 Antibody responses and protection

The serological response, as assessed by CFT and Indirect ELISA reveal that the vaccine presented *Mmm* specific circulating antibodies. Only one out of 10 animals produced CFT Ab titres of 1/40 following revaccination. Interestingly, the Abs lasted only three weeks after challenge. Thus the majority of cattle did not respond serologically to revaccination. On challenge however, eight out of 10 vaccinated animals were resistant to infection. This has also been observed in other experiments of vaccination with T1/44 in which animals did not seroconvert at immunization but were resistant on challenge (Gibert *et al*., 1970; Windsor *et al.,* 1972) Windsor 1972). The antibody levels of IgM may have been low as the CFT has been shown to primarily detect IgM (Barber *et al.,* 1970).

Indirect ELISA indicated production of serum immunoglobulin IgM and IgG two weeks post vaccination. A second dose administered resulting in a two-fold increase of the antibody responses. Higher levels of antigen specific IgG were detected compared to IgM. This ability to switch from IgM to IgG has been shown in other experiments involving CPS-conjugate vaccines (Burtnick *et al.,* 2012). Vaccination with glycoconjugates as opposed to pure polysaccharides, prompts T-cell help for B cells to produce IgG antibodies against the polysaccharide component (Avci and Kasper 2010). According to Avci1 and others, (2012) the carbohydrate/major histocompatibility class II (MHCII) activates carbohydrate-specific CD4+ T-cell clones to produce cytokines (interleukins 2 and 4) that are essential for providing T-cell help to antibody-producing B cells. The switching of polysaccharide-specific IgM-to-IgG, glycoconjugate immunization induces memory B-cell development and T-cell memory (Avci1 *et al.*,

2013). Although secretory IgA against *Mmm* infection correlates with protection following experimental infection of cattle (Niang *et al.,* 2006), there was not a similar increase in serum IgA titres following the immunization. Two doses of CPS-conjugate vaccine elicited anti-CPS antibody titres comparable to or higher than those induced after immunization with live attenuated vaccine

5.4.6 Conclusion

In conclusion, the results of this experiment show that capsular polysaccharide antigens conjugated to a protein is immunogenic and induce protective immunity in cattle. The study has also demonstrated that high immunoglobulin (IgG and IgM) responses can be raised against the carbohydrate component of the CPS-based glycoconjugate. However, Ab titres did not correlate with clinical or pathology signs of CBPP, as already described (Garba and Terry 1986; Niang *et al*., 2006).

Epidemiological data suggest CBPP disease in a given geographical area is caused by a limited number of *Mmm* serotypes (March *et al.,* 2000). Since the CPS structure is maintained in all serotype, a conjugate vaccine would therefore offer protection in diverse geographical areas.

CHAPTER 6: GENERAL DISCUSSION, CONCLUSION AND RECOMENDATIONS

This thesis describes a series of experimental evaluation of whole *Mmm* antigen, membrane proteins and capsular polysaccharide (CPS) antigens to establish whether they can induce protective immunity and therefore constitute vaccine candidates. It also reports on the humoral immune responses following vaccination with the antigens. After experimental vaccination of cattle with heat killed whole *Mmm* cell lysate with Freud's complete adjuvant and CPS conjugate vaccine in Emulsigen adjuvant significant partial protection was observed. Live attenuated T1/44 vaccine also offered partial protection while membrane protein vaccine in Emulsigen adjuvant resulted in enhanced disease. Correlations between the specific immune responses elicited following vaccination and protection were not conclusive. These findings are discussed in this chapter in the context of identifying the protective components of *Mmm* that might aid in development of an efficacious vaccine against CBPP. It also presents recommendations for follow-up studies.

In chapter 3 and 5, evidence is presented that killed *Mmm* and CPS conjugate vaccinated animals could partially protect from CBPP infection following challenge. These findings support the idea of developing a killed mycoplasma vaccine with an appropriate adjuvant or sub-unit vaccine against CBPP. CBPP vaccine research using inactivated formulations (Garba and Terry 1986) concluded that inactivated *Mmm* induce poor protection, although (Gray *et al.,* 1986) demonstrated complete protection of animals exposed to natural infection following vaccination a high dosage of *Mmm* lysate mixed with Freund's complete adjuvant. The result in chapter 3 indicates that the type of adjuvant

may be playing a critical role in determining the protective capacity of the antigens. The complete Freund's adjuvant offered significantly better protection than the Freund's incomplete adjuvant. Previous investigations into the influence of adjuvant in developing a CBPP vaccine revealed comparative results (Garba *et al.*, 1989). The experiment by Garba *et al.* showed that Freund's incomplete and liquid paraffin adjuvants containing Arlacel A were more efficient than Aluminium hydroxide gel and Sodium alginate.

Studies tend to point that many adjuvants work by activation of innate immune response that create a local immune-competent environment at the site of injection (Awate *et al.,* 2013; Didierlaurent *et al.,* 2009). Several mechanisms have been put forward for the way adjuvants act, including formation of 'depot' at the injection site for sustained release of antigens (Herbert, 1968; Didierlaurent *et al.,* 2009), up-regulation of cytokines and chemokines that lead to cellular recruitment at the site injection site (Goto and Akama 1982; Mckee *et al.,* 2009; Seubert *et al.,* 2008), enhanced antigen uptake and presentation by antigen presenting cells (APC) (Guéry *et al.,* 1996; Flach *et al.,* 2011), stimulation of dendritic cells (DC) maturation that is required for efficient T cell activation (De Smedt *et al.,* 1996; De Becker *et al.,* 2000; Fujii *et al.,* 2003) and activation of inflammasomes that trigger non-specific activation of the innate immune system (Didierlaurent *et al.,* 2009; Seubert *et al.,* 2011). The water-in-oil emulsions (Freund's complete adjuvant) has been shown to act by its depot effect that generates prolonged and sustained high antibody titres (Herbert, 1968) and activation of DC (De Smedt *et al.,* 1996; De Becker *et al.,* 2000). This observation was realised in the present experiment as animals vaccinated using Freud's complete adjuvant had the highest number of animals that seroconverted following vaccination. Previous studies had also shown that when the CPS was given

alone it does not induce production of agglutination or CF antibody. However, when the CPS is given together with Freund's complete adjuvant, a low level of antibodies are detected (Hudson *et al.,* 1967). Emulsigen adjuvant was used in this study based on ability to elicit a robust production of humoral antibodies (Van Donkersgoed *et al.,* 1996; Levast *et al.,* 2014). Since adjuvants skew the immune response toward promotion of a Th1-type immune responses or induce a Th2 response (Millan et al., 1998; Ioannou *et al.,* 2002; Parameswaran *et al.,* 2014), an understanding of mechanisms of adjuvants to be used in the design of CBPP vaccine is important.

The CPS conjugate vaccine gave partial protection although previous studies performed in the 1960s and 1970s where CPS derived from *Mmm* induced lesions comparable to those produced by CBPP, and aggravated the disease when administered together with *Mmm* (Hudson *et al.,* 1967; Lloyd *et al.,* 1971). Intravenous injection of the CPS also led to the occurrence of a prolonged mycoplasmemia after subsequent inoculation of *Mmm* (Hudson *et al.,* 1967). Since mycoplasmas are predominantly extracellular parasites a distinct antibody response would be expected to be of particular importance in protection by preventing colonization of lung tissues. It has been proposed that an effective vaccine against CBPP should induce both local antibody and Th1 interferon-gamma (IFN-γ) responses (Dedieu *et al*., 2006; Dedieu, 2008). Immunization with the CPS conjugated vaccine elicited an elevated IgG response that is an indication of up-regulation of IFN-γ and IL2 and hence a Th1 type of response may be involved (Abusugra *et al.,* 1997; Ada and Isaacs 2003; Chen *et al.,* 2003; Wang *et al.,* 2015). Other *Mmm* antigens that have also been shown to stimulate CD4+ T cells proliferation and IFN-y responses *in vitro* are lipoprotein A (LppA) and glucose-specific IIBC component (PtsG) (Totte *et al.*, 2010).

The switch to from IgM to IgG-secreting B-cell normally requires interaction with an activated, antigen-specific Th lymphocyte (Ada and Isaacs 2003). IFN-γ is one of the major cytokines involved in the immune response to counter mycobacteria infection, and its main function is to activate macrophages thus allowing them to exert there microbicidal functions (Cavalcanti *et al.,* 2012). Understanding of the immune response following CPS conjugate vaccines will be important on designing an effective vaccine against CBPP.

The CPS-ovalbumin vaccine results reported in this thesis indicated that the mice model may not be appropriate for testing CBPP vaccine. The experiment by Waite and March, (2002) had shown that mice immunized with the CPS-ovalbumin conjugate did not induce antibodies that could reduce mycoplasmemia following challenge. The absence of an animal model for CBPP as demonstrated in chapter 5 is a serious drawback to "proof-of-concept" experiments as observations in murine may not correspond to bovine host. Therefore considerations should be given to the use of cattle for such experiments.

The antibody titres or specificities investigated following vaccination with *Mmm* membrane proteins in chapter 4 did not correlate with protection. However, this does not necessarily mean that antibodies have no role in protection. Since the whole *Mmm* cell lysate induced protection when used in the heat inactivated with Freud's complete adjuvant formulation several immune responses may be required to work in tandem. This has also been observed by Schieck *et al.,* 2014, high antibody titres against selected 65 *Mmm* surface proteins did not have antigen-specific antibodies correlating with protection. The enhanced diseased observed after vaccination of cattle with *Mmm*

membrane proteins and challenge with live *Mmm*, provides a mirror of how immune responses can enhance pathology. Immune complex disease seems to be a feature in the lung vasculature. A recent study on vaccination of cattle with the N Terminus of LppQ of *Mmm* resulted in Type III Immune complex disease upon experimental infection (Mulongo *et al.,* 2015). The result in this thesis is also consistent with other reports that point to uncontrolled inflammation as the major manifestation of CBPP (Provost *et al.,* 1987). Unregulated immune response has also been suggested as a mechanism by which *Mmm* ensure its propagation and dissemination (Dedieu *et al.,* 2006; Pilo *et al.,* 2007). There is need therefore to investigate how immunosuppressive therapy would affect the clinical outcome of CBPP. These challenges also indicate that as more antigens are being identified that may induce protection, it's important that they be evaluated to see if they predispose cattle to accentuated disease.

The result of the current work indicates that future CBPP research may need to focus on development of a CPS-based conjugate vaccine, determining proteins that may be protective and identification of an appropriate adjuvant to be used. Although polysaccharide-protein conjugates as vaccines have been shown to be very effective, cost-effective technologies for production still need to be developed to increase their availability. Partially synthesized antigens are therefore desirable in production of these vaccines. This has been demonstrated in the study of the antigenicity and immunogenicity of several *H. influenza* type b synthetic oligosaccharide-protein conjugates in laboratory animals and clinical evaluation in humans that showed adequate long-term protection (Ferna´ndez-Santana *et al.,* 2004; Vliegenthart, 2006). The commercial vaccine consisted of eight repeated units conjugated to human albumin (Vliegenthart, 2006). Production of

a vaccine through synthesis of oligosaccharides is therefore, at least in some cases, commercially feasible.

The clinical course of infection following endobronchial inoculation presented some notable observations. First, fever seemed to be an unreliable indicator of CBPP infection as animals without fever had gross necropsy lesions. This has also been observed in previous experiments (Gull *et al.*, 2013). Secondly, cough also appears to be an inconsistent clinical sign, as animals with lesions of moderate to severe disease at necropsy had no noticeable cough. Thirdly, a proportion of cattle in the experiments were resistant as observed in previous experimental infection with *Mmm* (Nkando *et al.*, 2010; Gull *et al.*, 2013). An innate mechanism may be playing a big role in control of disease during a primary challenge, as also suggested by a CD4-depletion experiment that showed that CD4+ T cells contribute little to the outcome of disease in a primary infection (Sacchini *et al.,* 2011). There is therefore a need to improve on the experimental model for testing vaccines against CBPP.

In conclusion, the work presented in this thesis has demonstrated that conjugated CPS vaccine in Emulsigen adjuvant and heat-inactivated whole *Mmm* in complete Freund's adjuvant offer partial protection against CBPP. The *Mmm* membrane proteins vaccine in Emulsigen adjuvant is not a suitable vaccine target for improved CBPP vaccines. There is therefore a need to improve on the CPS conjugate vaccine by evaluating different proteins and dosages that may be more effective. Identification of adjuvants needs to be assessed based on proper understanding of the protective immune responses against *Mmm*

infection. The work presented in this thesis presents an understanding that will set the stage for further investigation into vaccine development as a means of controlling CBPP.

The following are the recommendations and future research perspective from the study:

1. Future research should focus on identification of a potent and safe adjuvant that can be used to develop an inactivated vaccine.
2. Development of a sub-unit vaccine is feasible and proteins that may be involved in immunopathology should be eliminated from the formulation.
3. There is need to understand the immune reactions especially the role of Th1 mechanism and innate immunity.
4. Specific epitopes on the capsular polysaccharide that have protective capacity need to be identified. This may enable synthetic production of the carbohydrate components and hence a commercially feasible vaccine.
5. Future studies may assess different dosage of the CPS conjugate vaccine, evaluate coupling of the CPS to different carrier proteins effect, stability and immunogenicity and explore use different types of adjuvants.

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CHAPTER 8.0 APPENDICES

Appendix 1

PROTEIN CONCENTTRATION USING BCA PROTEIN ASSAY PROTOCOL

The microplate procedure requires a smaller volume (200 µl) of protein sample, and because the sample to Reagent AB ratio is 1:8; substances in the Substance Concentration Tolerance Chart on the first page of this protocol are not diluted out as much as with the cuvette procedure.

Preparation of BSA standard

The table below was used to prepare a set of BSA standards to make a standard curve.

Stock BSA standard is 2 mg/ml.

125-2,000 µg/ml

VIAL µl PBS µl	SOURCE CONCENTRATION
A 0 100 µl	stock 2,000 µg/ml
B 50 150 µl	stock 1,500 µg/ml
C 100 100 µl	stock 1,000 µg/ml
D 100 100 µl	Vial B 750 µg/ml
E 100 100 µl	Vial C 500 µg/ml
F 100 100 µl	Vial E 250 µg/ml
G 100 100 µl	Vial F 125 µg/ml
H 100 0 0 µg/ml	

Preparation of unknown samples

Unknown samples were tested in triplicate and the results averaged.

Two dilutions in triplicate were done (1:10 and 1:100)

Preparation of reagent AB

To determine how much of Reagent of AB that was required for 200 µl per well. **NOTE:** a standard is required on each plate. # unknown × 8 dilutions × # replicates = unknown wells (standard wells + unknown wells) × 0.2 = ml of Reagent AB

Example: 1 standard and 3 unknowns in triplicate: 8 dilutions × 3 = 24 wells of standard 3 unknown × 8 dilutions × 3 = 72 wells of unknown (24 + 72) * 0.2 = 19.2 ml for one full 96 well plate. Mix Reagent A with Reagent B in a 50:1 ratio In this example, at least 19.2 ml is needed: 20 ml Reagent A + 0.4 ml Reagent B. When Reagent A and B are mixed, the solution will turn light green. This is the expected result. Reagent AB must be prepared immediately before use.

Preparation of microplate

Add 25 µl of each BSA standard into microplate wells, columns 1-3. Vial A goes into row A, Vial B goes into row B, and so on. Add 25 µl of unknown 1 into microplate wells, columns 4-6. Vial A goes into row A, Vial B goes into row B, and so on. In the next columns, repeat for unknown 2 and 3, if applicable. Add 200 µl of Reagent AB to every well containing a sample.

Reading the microplate

Incubate plate at 60º C for 15 minutes or 37º C for 30 minutes. Remove plate from incubator and let cool at room temperature for 5 minutes. Measure absorbance of all wells in a microplate reader at 562 nm. Plot a standard curve using the data from the

standard sample dilutions and use the curve to determine the concentrations of each unknown.