UNIVERSITY OF NAIROBI

DEVELOPMENT OF AN ANTIBODY BASED ASSAY DIFFERENTIATING CONTAGIOUS BOVINE PLEUROPNEUMONIA INFECTED CATTLE FROM THOSE VACCINATED WITH A SUBUNIT VACCINE

BY

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DECLARATION

I declare that this thesis is my original work and has not been submitted elsewhere for examination or award of a degree. Where other people's work has been used, this has been properly acknowledged and referenced in accordance with the requirements of the University of Nairobi.

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ABSTRACT

Mycoplasma mycoides subsp. *mycoides* (*Mmm*) is the causative agent of contagious bovine pleuropneumonia (CBPP) in cattle. The disease is primarily controlled by early diagnosis, quarantine, and vaccination using live, attenuated *Mmm* strains. Limitations to the use of *Mmm* live attenuated vaccines comprise the necessity of a cold chain for delivery, side effects induced at the inoculation site, and the short duration of immunity engendered. New subunit vaccines with superior qualities compared to the current live attenuated vaccines have recently been developed. The development of a diagnostic assay that differentiates naturally infected from vaccinated animals (DIVA) will be of great benefit to a CBPP eradication campaign and was the main aim of this study. To develop this assay, two methods of establishing infection in cattle namely endotracheal intubation and in-contact transmission were initially compared. In all the three infection trials, cattle manifested clinical CBPP at the same time using the endotracheal intubation model, but not by the in-contact transmission. Endotracheal intubation was thus determined as the better method of infecting animals in this study. The sensitivity of *Mmm* lipoprotein B (LppB) as a potential diagnostic antigen was also determined in an indirect ELISA (iELISA) using sera from naive and experimentally infected cattle. The iELISA using recombinant LppB detected significantly more positive cattle samples compared to the OIE recommended complement fixation test ($p = 0.023$). This study also evaluated 4 *Mmm* recombinant proteins (MSC_0397, MSC_0636, MSC_0653, and LppB) and identified two (MSC_0636, and LppB) that detected acute and chronic clinical stages of CBPP. Ten *Mmm* antigens (MSC_0136, MSC_0397, MSC_0431, MSC_0499, MSC_0636, MSC_0653, MSC_0775, MSC_0776, MSC_0957, and LppB) were further characterized as potential antigens for development of a DIVA assay. In this study, the most sensitive antigens in the subunit vaccine were MSC_0499 and MSC_0776 while the best non-vaccine antigens were MSC_0636 and LppB. Combined, these antigens could differentiate infected from subunit-vaccinated animals, but further studies are recommended to test and evaluate for sensitivity and the period after infection or vaccination that animals show up as positive.

DEDICATION

To my children: Stallon, Alvin, and Ashley for their support and endurance during my study and writing of this thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

Ag	Antigen
ANOVA	Analysis of Variance
APCs	Antigen Presenting Cells
APs	Ammonium Persulphate
ARC	Agricultural Research Council
AUIBAR	African Union Inter-African Bureau for Animal Resources
BSA	Bovine Serum Albumin
CBPP	Contagious Bovine Pleuropneumonia
CFT	Complement Fixation Test
CPs	Capsular Polysaccharides
DNA	Deoxyribonucleic Acid
DIVA	Differentiation of Infected from Vaccinated Animals
EU	European Union
FAO	Food and Agriculture Organization
GlpO	L-α-Glycerophosphate Oxidase
IAEA	International Atomic Energy Agency
iELISA	Indirect Enzyme Linked Immunosorbent Assay

LIST OF ABBREVIATIONS AND ACRONYMS CONT'

ILRI	International Livestock Research Institute
ISCOM	Immuno-stimulating Complex
KALRO	Kenya Agricultural and Livestock Research Organization
KEVEVAPI	Kenya Veterinary Vaccines Production Institute
LAT	Latex Agglutination Test
LppQ	Lipoprotein Q
MHC	Major Histocompatibility Complex
Mmm	*Mycoplasma mycoides* subsp. *mycoides*
OIE	Office International des Epizooties
OVI	Onderstepoort Veterinary Institute
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TEMED	Tetramethylethylenediamine
VIDO	Vaccine and Infectious Disease Organization
VSRI	Veterinary Science Research Institute

CHAPTER 1: GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Background

Mycoplasma mycoides subsp. *mycoides* (*Mmm*), the causative pathogen of contagious bovine pleuropneumonia (CBPP) in cattle, water Buffalo, Yak, and Bison is a transboundary cattle disease of financial significance in Africa (Tambi *et al.,* 2006). Both borans and zebus are susceptible to the disease with variations in breed susceptibility in cattle. For example, the N'Dama cattle from Guinea are more susceptible than the zebu (Masiga *et al.,* 1996). The *Mmm* is significantly more adherent to bovine epithelial cells than caprine and porcine, thus cytoadherence reflects specificity for the bovine species (Aye *et al.,* 2015). The disease has been listed as a severe bacterial disease with notifiable outbreaks (OIE, 2010) and affected countries are excluded from trade in live cattle and their products. Due to the deteriorated quality of veterinary services and lack of operational funds to compensate livestock owners if procedures such as stamping out of affected herd are adapted, CBPP has persisted in many African countries. Also, the combination of high cost and the logistical difficulties of movement control between and within pastoral communities and small-scale farmers, animal welfare considerations, and the potential loss of a valuable genetic resource base, all make the available CBPP control measures extremely difficult to implement (Windsor, 2000; Kusiluka and Sudi, 2003). To help combat the disease, two live attenuated vaccines namely T1/44 and T1/SR, are currently in use. A complement fixation test (CFT) and a competitive ELISA (c-ELISA) are the only prescribed World Organization for Animal Health (OIE) serological diagnostic tests to work with the T1/44 and T1/SR live-attenuated vaccines. The approved live attenuated vaccines and prescribed diagnostic tests have shortcomings, which necessitates the development of more effective vaccines and diagnostics.

1.1.1. Clinical symptoms and transmission of CBPP

In adult cattle, CBPP manifests as acute, subacute, or chronic forms while calves of less than six months develop arthritis rather than the pulmonary disease (Masiga *et al.,* 1996). The acute phase is at the onset of an outbreak with about 10% of infected cattle dying within 7 days of the start of the initial clinical symptoms (Provost *et al.,* 1987). During the acute stage, cattle develop signs that include fever $>40^0$ C, isolate from the herd and develop a dry cough, difficulty in breathing, nasal discharge, frothy salivary exudates, standing with forelegs apart, with extended head and neck. The acute form is followed by early subacute clinical signs that include; dullness, respiratory distress, fever, anorexia, and uneven chewing. The early subacute CBPP affects about 20% of infected cattle. In the late subacute stage, animals develop painful labored breathing and a severe cough. This late subacute stage eventually progresses into chronic CBPP, with animals seemingly in good physical shape though chronic lung lesions could be existing. The chronic carriers of the disease are estimated to be about 25% of infected cattle, they are infectious and play a role in disease transmission among cattle herds (EMPRES, 2002; Sacchini *et al.,* 2011).

CBPP transmission occurs when a naïve animal inhales infectious aerosols from an infected animal (Mariner *et. al.,* 2006). Transmission of up to a distance of 137m has previously been reported (Lloyd and Etheridge, 1983). The leading reservoir of *Mmm* contagion is cattle and apart from sequestra in the lungs, the organisms have also been isolated from nasal swabs (Kusiluka *et al.,* 2000b; Windsor and Masiga, 1977a). Trans-placental spread of the bacteria has since been suggested after the separation of *Mmm* from the fetus of a disease-ridden cow (Stone *et al.,* 1969; Masiga *et al.,* 1972). The *Mmm* have also been isolated from urine and semen of acutely infected animals, with titers of between 10 to 10^8 bacteria/ml (Scudamore, 1976; Gonçalves, 1994). Previously, transmission through dirtied feed has been proposed under on-station settings (Windsor and Masiga, 1977b).

1.1.2. History and classification of *Mmm*

Mycoplasmas are tiny self-replicating microorganisms (Razin *et al.,* 1998; Pilo *et al.,* 2007). These microorganisms progressed from other bacteria through degenerative evolution to reduced genomes of 1212 kb (Woese *et al.,* 1980; Weisberg *et al.,* 1989; Westberg *et al.,* 2004). CBPP-causing bacteria was first isolated by Nocard and Roux, (1898), classified as Mycoplasma in 1956 (Edward and Freundt, 1956) and given a name *Mycoplasma mycoides* subsp. *mycoides* Small Colony type in 1978 (Cottew and Yeats, 1978). *Mmm* belongs to the genus Mycoplasma, Family Mycoplasmataceae, Order Mycoplasmatales, and Class Mollicutes (Edward *et al.,* 1967; Nicolet, 1996).

The members of class mollicutes and genus Mycoplasma are highly pathogenic in ruminants and form the classical '*Mycoplasma mycoides* cluster' (Taylor *et al.,* 1992; Pettersson *et al.,* 1996). Confusion in serological diagnosis due to cross-reactions between different species in this cluster was observed (Cottew and Yeats, 1978; Rurangirwa *et al.,* 2000). Phylogenetic studies of 16S rRNA sequences enabled reclassification of *Mollicutes* that occasioned the renaming of *Mycoplasma mycoides* subsp. *mycoides* Large Colony (*Mmm*LC) type and *Mycoplasma mycoides* subsp. *capri* (*Mmc*) and the assignment of the *Mycoplasma sp.* Bovine group 7 strains into the separate species, *Mycoplasma leachii* (Manso-Silvan *et al.,* 2009). Table 1.1 shows the current nomenclature of five members of the '*Mycoplasma mycoides* cluster', the disease they cause, and the main host that they infest.

Table 1.1: Members of '*Mycoplasma mycoides* cluster', disease they cause and host they infest

Bacteria	**Disease**	**Host**
M. mycoides subsp. *mycoides*	CBPP	Cattle, Bison, Yak
M. leachii	Arthritis, mastitis, calf pneumonia	Cattle
M. capricolum subsp. *capripneumoniae*	CCPP	Goats, Gazelle, Mouflon
M. mycoides subsp. *capri*	Mastitis, arthritis, keratitis, pneumonia, and septicaemia (MAKePS)	Goats, Barbary sheep
M. capricolum subsp. *capricolum*	MAKePS	Goats, Sheep

(Adapted from Manso-Silvan *et al.*, 2007; Manso-silvan *et al.*, 2009; Fisher *et al.*, 2012).

1.1.3. Geographic distribution of CBPP

Worldwide distribution of CBPP ensued during the 19th century due to intercontinental trade in live cattle (Thiaucourt *et al*., 2000). The sequence of events of CBPP occurrences and extermination in various countries was documented previously. Due to uncontrolled cattle movements as a result of war, transhumance, and trade, CBPP was prevalent in Europe in the 18th century. The disease spread into Asia, Australia, southern Africa, and the USA in the 19th century through the importation of infected animals from Europe. CBPP was introduced into east, central, and west Africa in the 19th century by the sick animals from India (Provost *et al.*, 1987). CBPP is widespread in 26 Africa countries. In western Africa, CBPP is mainly sporadic (Masiga *et. al.*, 1996). Figure 1.1 shows the distribution of the disease in Africa.

Figure 1.1: Distribution of CBPP in Africa (Adapted from OIE, 2016).

1.1.4. Epidemiology and control of CBPP

The epidemiology of CBPP in Africa is subject to the following factors: i) manifestation of sub-acute symptomless contagions and perseverance of chronic cases in cattle and ii) difficulties in controlling cattle movements. These factors are partly the cause and maintenance of the disease (Masiga, 1995). While the bacteria have been isolated from sheep and goats, no study has shown that *Mmm* cause disease in these ruminants (Brandao, 1995; Thomson, 2005). Transmission between goats and sheep harbouring the infection and cattle has never been documented (Kusiluka *et al.*, 2000).

Historically, CBPP eradication was achieved in Europe and North America by end of the 19th and the start of the 20th century through the slaughter of infected herds, isolation from the rest of the herd, controlled travels, and the use of available basic vaccines (Egwu *et al.*, 1996; Provost *et al.*, 1987). The reappearance of the disease in southern Europe during the 1980s and 1990s was controlled by well-organized disease surveillance, isolation, and slaughtering of sick cattle (Egwu, *et al.*, 1996; Nicholas *et al.*, 2000). Australia used the same approaches used by Europe and north America to eradicate CBPP in 1973 (Kusiluka and Sudi, 2003). Although the killing of sick animals is an option to eradicating CBPP (Windsor and Wood, 1998), the program cannot be readily applied in Africa due to limited financial resources to compensate for the slaughtered cattle (Windsor, 2000). In Africa, countries such as Namibia, Botswana, Zimbabwe, South Africa, and Senegal the disease has been eradicated. Namibia had a successful control campaign based on monitoring cattle movement and vaccination with T1/44 (Huebschle et al., 2003) while Botswana, managed to eradicate the disease at a significant cost in 2008 by stamping out infected cattle (Masupu et al., 1997). Zimbabwe reported the last occurrences of CBPP in 1904, while South Africa last reported

the disease in 1924. Senegal followed OIE guidelines to conditionally declare itself free of CBPP in 2009.

1.2. Literature review

The available inoculation models for cattle with *Mmm* during vaccine efficacy studies are invasive and laborious and may interfere with the trial outcomes (Paul *et al.,* 2019). Antibiotic treatment, increased emphasis on field testing with the slaughter of reactors, rigid control over moving out of the enzootic areas and extensive vaccination are options for CBPP control in Africa (Windsor, 2000; March, 2004; Wanyoike *et al.,* 2004). The available CBPP vaccines and diagnostic tests have several pros and cons. The current live-attenuated vaccines, based on the African *Mmm* strain T1, confer limited protection, generally lasting less than one year and rather low efficacy (Egwu *et al.,* 1996; Jores *et al.,* 2013). Also, the requirements for high doses (Tulasne *et al.,* 1996; Nicholas *et al.,* 2000; Thiaucourt *et al.,* 2000), cold chain, and the reported adverse effects at the site of injection (Provost *et al.,* 1987) are disadvantages of the current vaccines. The CFT and c-ELISA serological assays, prescribed by OIE, are recommended at herd level only (Bashiruddin *et al.,* 1994; Amanfu *et al.,* 2000). The drawbacks to the isolation of *Mmm* by culture and identification include low sensitivity caused by bacterial contamination, low specificity due to cross-reactivity of closely related species, and labour-intensive laboratory procedures (Rice *et al.,* 2000; Le Grand *et al.,* 2004). The introduction of PCR since 1994 provided a much quicker and more sensitive diagnosis of CBPP (Bashiruddin *et al.,* 1994; Dedieu *et al.,* 1994). However, identification by conventional PCR protocols remains problematic due to the close phylogenetic relatedness among and other members of the *M. mycoides* cluster (Le Grand *et al.,* 2004). Real-time PCR formats using SYBR green detection of PCR products compensated some of the disadvantages connected to conventional PCR (Fitzmaurice *et al.,*

2008) but are less specific than real-time PCR assays that include specific probes (Gorton *et al.,* 2005; Vilei and Frey, 2010). A multiplex rt-PCR assay using TaqMan®-labelled locked nucleic acid (LNA) probes, targeting LppQ (MSC_1046) and lipoprotein (MSC_0136) genes was described (Schnee *et al.,* 2013). The duplex rt-PCR is intended for confirmatory diagnosis of clinically or pathologically suspected cases, however, most African countries lack well-trained personnel and equipped laboratories to perform the assay.

1.2.1. Models of inoculating cattle with *Mmm* during vaccines efficacy studies

The studies describing intubation and contact models of challenging cattle with *Mmm* are limited. The intra-tracheal intubation method commonly used calls for the usage of sedatives and anesthetics that may cause ruminal stasis (Taylor, 1992). The contact transmission method is costly because it utilizes an extra group of donor cattle during trials and transmission of *Mmm* occurs at different times in different individual animals (Dedieu *et al.,* 2005). Pathogenesis studies of *Mmm* call for a suitable inoculation model that provides an exact infection dose and precise time of disease manifestation to allow for a better comparison of clinical and pathological signs in individual cattle (Nkando *et al.,* 2010).

1.2.2. Antibiotic treatment of cattle infected with CBPP

Antibiotic treatment is not an endorsed CBPP control approach due to the fear that the use of antimicrobials increases the number of disease carriers that disguise clinical symptoms (Provost *et al.,* 1987). Symptomless animals result in a problematic diagnosis, causing unrecognized incidences. There is also a fear that the uncontrolled use of antibiotics can promote the re-emergence of resistant *Mmm* strains and residues in human food (Wesonga and Thiaucourt, 2000). However, antibiotic treatment against CBPP is commonly used by pastoralists in Africa (Mariner *et al.,* 2006; Twinamasiko *et al.,* 2004; Msami *et al.,* 2001). Some trials have found antibiotics such as danofloxacin and oxytetracycline to be effective

against CBPP (Ayling *et al.,* 2000; Twinamasiko *et al.*, 2004; Yaya *et al.*, 2004; Huebschle *et al.,* 2006; Muuka *et al.,* 2018).

1.2.3. Quarantine of cattle infected with CBPP

In Africa, it is difficult to implement quarantine because the control measure involves the restriction of movement of infected cattle. Political, socio-economic, and logistical concerns also deter the application of quarantine as an approach for CBPP control (Masiga *et al.,* 1996; Windsor and Wood, 1998). Cattle movement as a control measure is hindered by regional porous borders, transhuman routines of pastoralists, inter-ethnic cattle robberies, and the incapability of state and regional authorities to enforce rules that govern the movement of cattle (Windsor and Wood, 1998; Thomson, 2005).

1.2.4. Vaccination of cattle against CBPP infection

Several vaccines have been used to control CBPP. The first simple vaccine was developed in 1852 by Louis Willems and contained pleural fluid from sick cattle (Egwu *et al.,* 1996). Vaccination was done by placing pleural fluid or lung's soft tissue below the skin at the tip of the tail or nose of naïve animals; that elicited some level of protection (Provost *et al.,* 1987). However, this vaccine was discouraged since it gave severe adverse reactions at the point of attachment, called the Willems' reaction because it often led to severe reactions and sometimes loss of tail (Blancou, 1996), as shown in figure 1.2.

Since then, CBPP vaccination was switched to the broth T1 vaccine (Davies *et al.,* 1968; Gilbert *et al.,* 1970) and was later on substituted by cold-chain dependent *Mmm* T1/44 live attenuated vaccine (OIE, 2008). The *Mmm* T1 bacteria were isolated in Tanzania and partially-attenuated by 44 passages in eggs to make the T1/44 live attenuated vaccine (Egwu *et al.,* 1996). In addition to T1/44, T1/SR was established (Rweyemamu *et al.,* 2001). Though both vaccines confer defense against CBPP, they have limitations. They cause reactions at the

site of inoculation of up to 25% of vaccinated animals (Masiga *et al.*, 1996; Mbulu *et al.*, 2004). They also induce short-lived protection and require icy packaging for delivery (Nicholas *et al.*, 2000; Thiaucourt *et al.*, 1998; Thiaucourt *et al.*, 2000). The T1/SR was eventually discarded after failed vaccination campaigns in the 1990s (Tulasne *et al.*, 1996; Nicholas *et al.*, 2000).

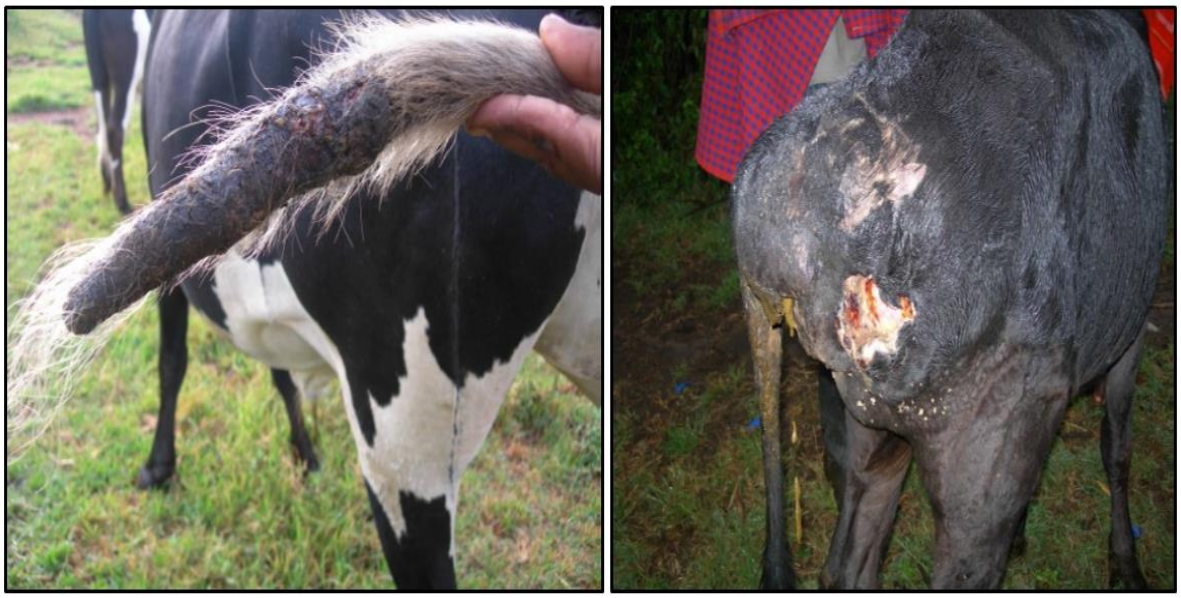

Figure 1.2: Willems' reaction and loss of tail in cattle vaccinated with T1/44 *Mmm* live-attenuated vaccine. The Necrotic and infected wound which may end up with sloughing-off of the tail tip (A), and Perineal involvement (B). Adapted from Mtui-Malamsha, (2009).

1.2.5. Research progress on the development of non-live strain vaccines for CBPP

The development of inactivated or subunit vaccines would solve several limitations of the current live-attenuated CBPP vaccines. Several attempts to develop alternative and improved CBPP inactivated and DNA vaccines were not successful.

1.2.5.1. Inactivated vaccines for CBPP

An immuno-stimulating complexes (ISCOMs) vaccine was first tested in mice and cattle in 1997 (Abusugra *et al.,* 1997). This ISCOM vaccine consisted of an *Mmm* solubilized in Decanoyl-N-methylglucamide (MEGA-10) detergent and dialyzed in the presence of lecithin and cholesterol (Huebschle *et al.*, 2003). An IgG_2 response was generated in mice while in cattle, predominant Th_1 pro-inflammatory response to *Mmm* showed IL-12 and IL-18 cytokine profiles (Abusugra *et al.,* 1997). Though, no protection was offered in vaccinated cattle (Huebschle *et al.,* 2003).

Vaccination experiments with a saponin-inactivated Mycoplasma were not successful (Nicholas *et al.* 2004). However, inoculation of animals twice but with very large quantities of inactivated mycoplasma mixed with Freund's complete adjuvant, provoked immune responses against the disease (Gray *et al.*, 1986; Garba and Terry, 1986). A recent study showed that dead *Mmm* may have to be present in large doses to induce protection in live *Mmm* inactivated vaccines (Mwirigi *et al.*, 2016).

1.2.5.2. Development of CBPP-DNA based vaccine

March *et al.,* (2006), tested the first *Mmm* DNA-based vaccines using bacteriophage as an expression vector. The vaccines were inoculated in mice, followed by infection with *Mmm*. Significant antibody responses to *Mmm* were observed following vaccination and a reduced mycoplasmaemia was observed for a clone expressing the MSC_0397 proteins. However, studies were not done in cattle to confirm the results observed in mice.

1.2.5.3. Lipoprotein Q subunit vaccine for CBPP

Abdo *et al.,* (2000) characterized LppQ after which the T1LppQ−MT1 derivative of the T1/44 live attenuated vaccine was obtained (Dedieu *et al.,* 2009; Janis *et al.,* 2008). It was suggested that this protein could be used in a DIVA assay for discriminating animals vaccinated with the depleted T1LppQ-MT1 from those infected with wild form strains (Bruderer *et al.,* 2002; Vilei and Frey, 2010). However, permission for the use of the depleted strain has not been sought from the national biosafety commission, as the process was deemed too expensive, so the protective capacity of the depleted vaccine strain has not been tested. Furthermore, it was still a live vaccine, with all its disadvantages. A study by Mulongo *et al.,* 2015, showed that cattle immunized with the recombinant peptide comprising LppQ antigen formulated in adjuvants elicited strong antibody responses. Unfortunately, these animals were more susceptible to infection with a virulent *Mmm* strain (Mulongo *et al.,* 2015).

1.2.5.4. Capsular polysaccharide subunit vaccine for CBPP

The CPS on bacteria's membrane possesses virulence factors that trigger the inflammatory process in cattle (Plackett and Buttery, 1964; Pilo *et al.,* 2007). Inoculation of CPS in calves through the intravenous route produced transient apnea, pulmonary arterial pressure, and oedema (Lloyd and Titchen,1976). Nonetheless, conjugation of carbohydrates and vaccination with capsule derived carbohydrates coupled to an immunogenic protein offers protection to encapsulated bacteria (Peeters *et al.,* 2003). The cellular and molecular mechanisms for adaptive immune responses mediated by glycoconjugate inoculation have been established (Avci1 *et al.,* 2011). The study revealed that upon uptake by antigen-presenting cells (APCs), glycoconjugate vaccines are depolymerized yielding glycan-peptide. The glycan-peptide is then displayed by an MHC-class II molecule on the surface of APCs

(figure 1.3). A recent study showed a reduction of 57% in the pathology in cattle vaccinated with CPS and challenged with *Mmm*, this in comparison to non-immunized controls (Mwirigi *et al.*, 2016). However, Mwirigi *et al.*, (2016) experiment was not 100% effective and it would constitute a very expensive vaccine as it would be difficult to make. The experiment was done to prove that antibodies to CPS are protective, and the reason why is only hypothetical, prevention of adhesion to bovine lung cells and is yet to be confirmed.

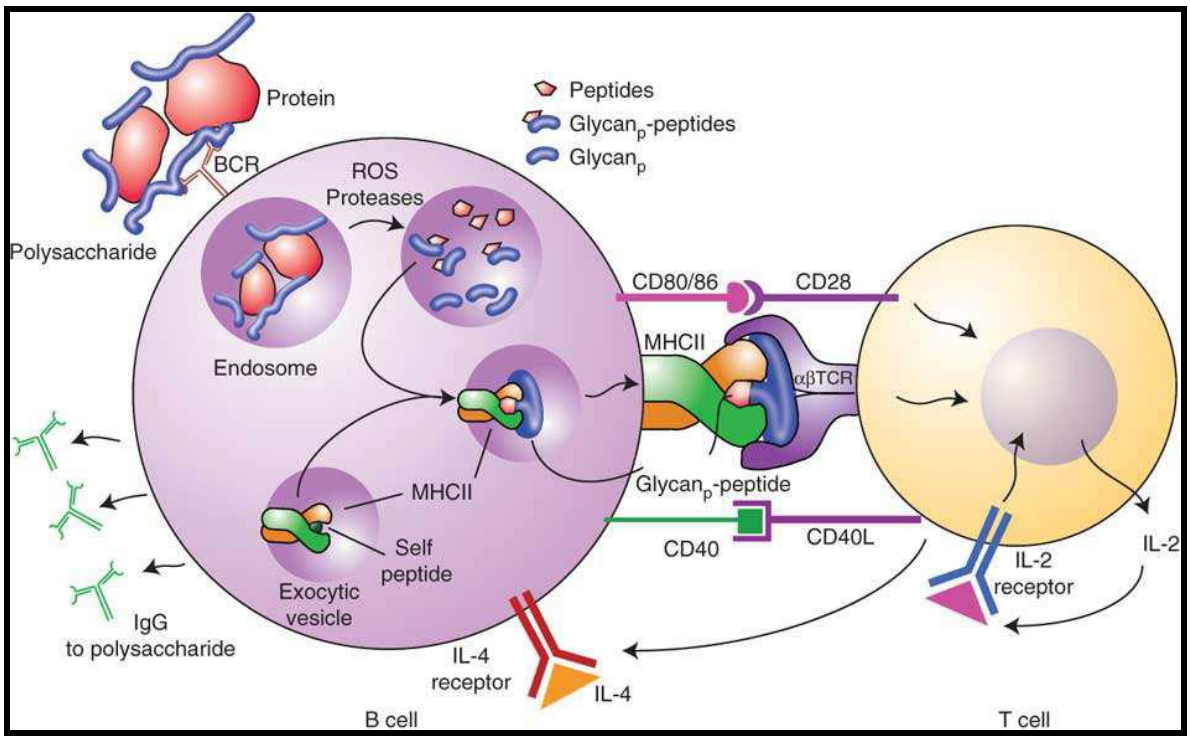

Figure 1.3: Schematic representation of mechanism of T-cell activation by glyco-conjugate vaccine; showing antigen processing and presentation of the vaccine resulting in CD4+ T_H-cell induction of B-cell production of IgG antibodies to the polysaccharide (Adapted from Avci1 *et al.*, 2011).

1.2.5.5. Recent studies of *Mmm* surface proteins as prospective subunit vaccines

In more recent laboratory work, data mining followed by computational analyses were used to identify 66 candidate *Mmm* proteins; selected based on exposure on the surface membrane or by secretion (Perez-Casal *et al.,* 2015). The selected antigens were then ranked in order of strong antibody responses of sera from CBPP-infected cattle. These *Mmm* proteins were then pooled in groups of five, male Boran cattle vaccinated, followed by a challenge with the virulent Afade *Mmm* strain (Nkando *et al.,* 2016). Two of the groups of vaccinated cattle showed protection after the *Mmm* challenge (Nkando *et al.,* 2016) and these subunit vaccines are being developed.

1.2.6. Diagnosis of CBPP infected cattle

Diagnosis of CBPP relies on a combination of clinical examination, post-mortem findings, and laboratory examination which is based on culturing methods and serological tests (FAO, 2002). Protein-based and nucleic acid molecular techniques are also available (Bashiruddin *et al.,* 1999; Gonçalves, 1994).

1.2.6.1. Clinical examination of post-mortem lesions and *Mmm* culture

Pathological post-mortem lesions in the acute stage include straw-colored pleural fluid, fibrinous deposits on the parietal surfaces of lungs, and distension of the interlobular spaces (Trichard *et al.,* 1981). Gross pathological lesions are typically unilateral, localized in the diaphragmatic section, and exist as characteristic grey and red marbling form (Masiga *et al.,* 1996; Provost *et al.,* 1987). Acute stage lesions progress into subacute cases, characterized by necrosis in lobules and early sequestra formation. The subacute lesions eventually develop into chronic, characterized by well-defined sequestrae. Chronic lesions also show adhesions connecting thickened viscera and parietal pleura (Trichard e*t al.,* 1981; Amanfu *et al.,* 2000).

Unfortunately, culturing of infected lung samples and isolation of mycoplasma organisms takes long and requires well-trained staff. A diagrammatic representation of lesions following post-mortem is described in chapter 2, figure 2.3.

1.2.6.2. Molecular detection of CBPP using polymerase chain reaction assay

Polymerase chain reaction (PCR) and molecular-based methods are mainly used for final confirmation of *Mmm* when the bacteria have been isolated from a sample grown in culture. The first molecular detection by PCR was reported in 1994 (Bashiruddin *et al.,* 1994). Even though PCR is a reliable method of detection of CBPP, it nevertheless has problems with the high risk of carryover-contamination (Persson *et al.,* 1999, Lorenzon *et al.,* 2008). Earlier reports suggested that single PCR is inadequate for diagnosis of CBPP (Le Grand *et al.,* 2004; Bashiruddin *et al.,* 2005). The development of real-time PCR assays has been shown to reduce the risk of contamination displayed by single PCR (Gorton *et al.,* 2005; Fitzmaurice *et al.,* 2008). However, the real-time PCR is less specific than real-time PCR assays that include specific probes. Currently, probe-based multiplex real-time PCR is the most advanced tool for reliable identification and sensitive detection of *Mmm* (Vilei and Frey, 2010), but is only available in fewer specialized laboratories and requires well-trained staff to carry out the assay.

1.2.6.3. LppQ-ELISA, Latex Agglutination and Immuno-Blotting Tests for *Mmm*

An iELISA based on the LppQ antigen that is specific to an early immune response to *Mmm* in infected cattle has been described (Abdo *et al.,* 2000; Bruderer *et al.,* 2002). This iELISA was pre-validated for sensitivity and specificity using 221 cattle sera from ten herds in Portugal. These cattle sera had previously been confirmed positive on post-mortem examinations, *Mmm* culture, and CFT titers (Bruderer *et al.,* 2002). The LppQ-ELISA was less sensitive in the acute clinical stage but gave positive results during the chronic period

post-inoculation with *Mmm* (Le Goff and Thiaucourt, 1998; Bruderer *et al.*, 2002). The assay was evaluated by the IAEA in 2008 (OIE, 2008) but the company licensed to produce the kit stopped its production.

Alternatively, a LAT that produces results in a few minutes was reported in 1999 (Ayling *et al.*, 1999). The LAT that uses a specific *Mmm* polysaccharide, had previously been described but were not recommended due to false-positive reactions to other Mycoplasmas (Nicholas *et al.,* 2000; March *et al.,* 2003). Another LAT based on a polyclonal antibody (pAb) coated with latex beads was reported (March *et al.*, (2003). However, adverse cross-reactions were also observed to *M. mycoides capri* when tested against field sera of cattle from Africa (March *et al.,* 2003).

An immuno-blotting test (IBT) is considered a sensitive and specific serological test for the analysis of immune responses in cattle (OIE, 2008). However, IBT is not suitable for mass screening but is only recommended as an option for other unreliable diagnostic tests (OIE, 2008).

1.2.6.4. OIE prescribed serological tests for detection of *Mmm* in cattle

The CFT by Campbell and Turner (1953) is the most well-known assay. Although the CFT has been harmonized in the European Union (EU), it is still difficult to standardize and perform (OIE, 2008, Le Goff and Thiaucourt, 1998). CFT is specific but less sensitive. A c-ELISA based on a monoclonal antibody (mAb) targeting a 3F3 site, the variably expressed PtsG surface protein was developed (Le Goff and Thiaucourt, 1998; Gaurivaud *et al.*, 2004). The c-ELISA test is simpler to carry out but validation studies are still insufficient to make good conclusions about its use in field surveillance studies while its specificity is uncertain (Sidibé *et al.,* 2012). Despite the shortcomings of current serological assays, serology is

better suited for large scale disease monitoring, especially in Africa where PCR-based and other molecular methods are not always practical (Nicholas *et al.,* 2000).

1.2.7. Problem statement

In Africa, CBPP is a contagious disease with a mortality rate of up to 50%. The economic losses as a result of the disease are estimated at 44.8 million euros per annum (Tambi *et al.,* 2006). The impact of the disease in Kenya alone is estimated at 7.6 million US dollars per year (Onono *et al.,* 2014). The studies describing the intubation and contact model of challenging cattle with *Mmm* during vaccine efficacy studies are limited. Vaccines in current use are live-attenuated, employing two strains, namely T1/44 and T1/SR making it difficult to differentiate between vaccinated from infected animals using OIE prescribed serological assays (c-ELISA or CFT). The prescribed tests have shortcomings of low specificity (c-ELISA) and low sensitivity for CFT (Rurangirwa, 1995; Sidibé *et al.,* 2012). Control of CBPP will not only be attained by the use of efficient vaccines but also with reliable diagnostics that can differentiate infected from vaccinated animals (Thiaucourt *et al.,* 2000, Bischof *et al.,* 2009). The introduction of a subunit vaccine will allow the development of diagnostic tests that could differentiate infected from vaccinated cattle (DIVAs).

1.2.7.1. *Mmm* surface proteins as DIVA assays for serological diagnosis of CBPP

In recent times, studies have been carried out to introduce identified *Mmm* proteins in the development of alternative tests (Perez-Casal *et al.,* 2015; Heller *et al.,* 2016). These components of the mycoplasma are variable surface proteins that are crucial in studies regarding the development of diagnostics for CBPP (Murray *et al.,* 2009). The 17 purified *Mmm* recombinant proteins were evaluated in a study by Heller *et al.,* (2016). These proteins had previously been identified as immunogenic with diagnostic potential and displayed strong antibody responses and high disease specificity in earlier collaborative studies

(Hamsten *et al.,* 2008; Jores *et al.,* 2009; Hamsten *et al.,* 2009; Neiman *et al.,* 2009; Naseem *et al.,* 2010). Genes were retrieved from *Mmm* PG1 (GenBank accession no. NC_005364.2) and compared to the African *Mmm* Afadé strain and B237 (Fischer *et al.,* 2015). These chosen sequences were codon-optimized, synthesized, expressed in *Escherichia coli*, and purified by GenScript Corp. (USA). The 17 purified *Mmm* recombinant proteins were used to screen more than 100 well-characterized bovine sera using a cocktail iELISA. Out of the 17 *Mmm* antigens tested, two of them (MSC_0136 and MSC_0636) showed the highest diagnostic potential (Heller *et al.*, 2016).

In another study, one mycoplasma protein previously identified as being a potential diagnostic antigen, the LppB (Miltiadou *et al.,* 2009) was tested for its usefulness to detect infected cattle in an iELISA. Samples obtained from naïve and cattle experimentally infected with *Mmm,* were compared using CFT and an iELISA based on LppB. The iELISA developed with the LppB antigen detected more positive samples especially those from chronically infected cattle compared to the CFT (Lutta *et al.,* 2018). However, studies by Heller *et al,* (2016) and Lutta *et al.,* (2018) did not confirm whether the selected *Mmm* antigens could be used to discriminate animals inoculated with *Mmm* from those vaccinated with recently developed subunit vaccines.

1.2.8. Justification

CBPP causes high morbidity and mortality in cattle in Africa (Tambi *et al.,* 2006) and limits international trade in cattle and cattle products. The CBPP vaccine efficacy studies require a model of inoculating cattle that provide an exact infection dose and precise time of disease manifestation to allow for a better comparison of clinical and pathological signs in individual animals (Nkando *et al.,* 2010). Control of CBPP in Africa can be possible through massive vaccination and diagnostic testing (Thiaucourt *et al.,* 2000, Bischof *et al.,* 2009). Such control

would tremendously improve the living standards of pastoral communities who rely on livestock as a main source of income. Improving trade would also increase their earnings in terms of foreign income from the sale of extra meat and milk. The Nkando *et al.*, (2016) experiment using pools of recombinant antigens conferred protection in vaccinated cattle. A DIVA diagnostic test, in conjunction with an effective vaccine, will contribute to the elimination of the disease in endemic regions in Africa, and help trade in cattle products. Sera from cattle immunized with a live attenuated vaccine could test positive for many *Mmm* antigens. Detection of antibodies against proteins not present in the subunit vaccine would discriminate between cattle immunized with a live-attenuated vaccine or exposed to *Mmm* in the field, and those vaccinated with a subunit vaccine containing known *Mmm* antigens.

The aim of this study was to: i) determine a suitable model of infecting cattle during vaccine efficacy studies, and ii) identify candidate *Mmm* antigens that could differentiate cattle infected with CBPP from those vaccinated with *Mmm* subunit based vaccines.

1.2.9. General objective

To develop sensitive and specific diagnostic assays that differentiate infected from vaccinated cattle.

1.2.9.1. Specific objectives

1. To establish a suitable model of infecting cattle during vaccination trials.
2. To evaluate the specificity and sensitivity of an indirect ELISA based on the LPPB antigen.
3. To select the best *Mmm* antigens for identifying CBPP-infected animals.
4. To characterize potential *Mmm* antigens as prospective tests differentiating CBPP infected cattle from those vaccinated with the subunit vaccine.

CHAPTER 2: DETERMINATION OF A SUITABLE MODEL OF INOCULATING CATTLE WITH *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* DURING VACCINES SAFETY AND EFFICACY TRIALS

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2.1. Abstract

Cattle infected by endotracheal intubation with *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) have been used in trials of contagious bovine pleuropneumonia (CBPP) pathogenesis. The advantage of this mode of infection is that the precise moment of infection is known, unlike with the in-contact transmission when the exact time of inoculation in each animal is not known. In this study, the two methods of inoculation were directly compared, by analyzing clinical signs and pathological outcomes in three controlled trials. Intubation produced smaller lung lesions and a much milder disease, mainly the chronic form of CBPP, than contact transmission. The mode of inoculation may influence disease outcome and this should be taken into consideration when studying the pathogenesis of CBPP and vaccine efficacy.

2.2. Introduction

There is no established laboratory animal model to conduct studies on CBPP. Previously, the challenge of vaccinated cattle in experimental vaccination trials used the subcutaneous route of inoculation (Piercy and Knight, 1957) but because this model of infection was unreliable, i*n vivo* studies on the disease since then have been conducted on cattle by infecting them by contact transmission and/or endotracheal intubation.

The contact transmission method, in which CBPP-infected cattle are allowed to graze and be in close contact with naïve animals at a ratio of one to two respectively, has the advantage of simulating a natural infection. However, it is costly because it utilizes an extra group of donor cattle during trials. Also, contact transmission has unpredictable transmission rates (Dedieu *et al.,* 2005; Provost, 1987) and transmission of mycoplasma occurs at different times in different individual animals. In contrast, with the endotracheal intubation method, the experimenter knows the exact infection dose and the precise time of inoculation in all animals can be determined (Hudson and Turner, 1963; Nkando *et al.,* 2010) thus allowing for a better comparison of clinical and pathological signs in individual animals. The only shortcoming of endotracheal intubation is in the use of sedatives reported to cause death in some cattle due to the inspiration of ruminal contents while recuperating from anesthesia. A few studies have shown that clinical and pathological outcome of CBPP was higher in animals exposed by contact than by intubation (Huebschle *et al.,* 2003; Scacchia *et al.,* 2011), however, these studies compared few animals. The objective of this study was to test and establish a suitable method of infecting cattle to be used in experimental trials involving vaccines and antimicrobials studies. This chapter compares disease severity, clinical and pathological events between endotracheal intubation and the in-contact method, in a total of 72 intubates and 61 contact cattle from several different experiments during controlled field

trials conducted at Kenya Agricultural and Livestock Research Organization (KALRO), formerly Kenya Agricultural Research Institute (KARI)-Veterinary Science Research Institute (VSRI), formerly Veterinary Research Centre (VRC).

2.3. Materials and methods

2.3.1. Experimental cattle

The study reports on three experiments performed at different times of which two are retrospective studies. All the cattle used in the studies were males of the zebu breed aged 2 to 3 years, purchased from Kakamega, a CBPP free zone. The animals were initially tested for CBPP using slide agglutination serum test (SAST) before purchase. They were then transported to the KALRO-Kakamega station where they were ear-tagged, drenched, and castrated for ease of management. Serum collected from each of the animals was tested by complement fixation test (CFT) seronegative animals were transported from Kakamega to KALRO-VSRI-Muguga.

On arrival at Veterinary Science Research Institute-Muguga, the cattle were grazed for one month before the commencement of the experiment. During this period, they were vaccinated against foot and mouth disease, a black quarter (blackleg), anthrax, and lumpy skin disease. The cattle were randomly assigned into groups for intubation and contact transmission. The endpoint of the experiments was either 10 days of fever or an animal recumbent for 48 hours and not eating or at the end of the experiment after a minimum of 8 weeks post-challenge. All cattle experiments from which the samples were derived were carried out in accordance with KALRO-VSRI, Institutional Animal Care and Use Committee (IACUC): VSRI/IACUC/1/29092009 and VSRI/IACUC/2/00122010.

2.3.2. Preparation of *Mmm* inoculum for a challenge

Preparations for inoculum commenced one and a half months before intubation. During this period, a culture of pathogenic *Mmm* (B237 strain) from Thika, Kenya (Nkando *et al.,* 2010) stored at passage 3 was revived from the freeze-dried material. The *Mmm* culture for infection (inoculum) was prepared using modified Newings tryptose broth containing tryptose 20 g; (Sigma-Aldrich, UK), glucose (5 g), sodium chloride (5 g), thallous acetate (0.5 g; Sigma), disodium hydrogen phosphate (2.5 g), glycerol (5 ml), with the following additives: penicillin (0.03%), 0.5% phenol red (4 ml), fresh yeast extract (10 ml; Sigma), 25% sodium pyruvate (2 g) and pig serum (150 ml; obtained from the local slaughterhouse). Five ml of broth was added to a 5 ml aliquot of freeze-dried culture shaken and allowed to stand on the bench for 30 min before dispensing 0.3 ml into bijou bottles containing 2.7 ml of broth to make 1 in 10 dilutions. This was placed in the incubator at 37^0 C and upon growth, was upscaled to make quantities sufficient for animals at 60 ml per animal. Confirmation of growth was through observation of change of phenol red in the medium and filaments in the broth. At every stage of upscaling, the cultures were incubated for 48 hrs at 37^0 C. Growth was monitored daily based on colour change and appearance of filaments. Before use and after use, a sample of the culture was collected for titration to estimate the number of mycoplasmas in the culture used for inoculation. The titration was carried out in a 10-fold dilution series from 10^{-1} to 10^{-10} liquid medium.

2.3.3. Experimental infection of cattle by endotracheal intubation

Intubation was carried out by introducing a tube through the nostril to the larynx and down to the trachea of cattle. Each animal was inoculated with 60 ml of culture with an *Mmm* concentration of 10^8 cfu/ml, followed by 30 ml of 1.5% agar boiled in distilled water at 37^0 C and 30 ml of phosphate-buffered saline (1x PBS) to flush down the inoculum material. The

addition of agar was suggested to improve the infection rate (Nkando *et al.*, 2010).

2.3.4. Infection of cattle by contact transmission

For contact transmission, animals infected by intubation were initially observed for clinical signs, including fever before introducing them to naive ones in a ratio of one to two respectively, from day 14 until the end of the trial that lasted 6-12 months. An animal was considered to have a fever when it showed a rectal temperature of 39.5 ^{0}C and above. An animal was considered to show clinical signs of CBPP if it showed fever for three or more consecutive days. The animals were examined daily for the development of CBPP clinical signs such as fever, cough, nasal discharge, and labored breathing.

2.3.5. Clinical examination of cattle following infection

Rectal temperatures of each animal were recorded daily between 8.00 am and 10.00 am for the period of study following intubation and contact transmission. Clinical responses that were observed in both groups included nasal discharge, cough, and labored breathing.

2.3.6. Post-mortem analysis of cattle

A post-mortem was carried out for a selection of reasons including not eating for 48 hours, end of the trial, weight loss, and 10 days of continuous fever. The criteria for killing animals were the same in all three experiments and for intubated and in-contact animals; not eating for 72 hrs, loss of weight, or end of the experiment. Animals for necropsy were killed by captive bolt and exsanguinations. A record of gross pathological changes in all organs was made, with particular attention paid to the lungs. Lesion scoring as described by Hudson and Turner, (1963) was carried out to determine the severity of the disease in diseased animals using the pathology score. Briefly, using this method, the pathology score is calculated based on the size and duration of the lesion (whether adhesions are fibrous or fibrinous). Using the

diameter, the presence of encapsulated, resolving, or fibrous lesions or the presence of pleural fibrous adhesions only were allocated a score of 1 regardless of the size. Other types of lesions including consolidation (hepatization) due to fibrinous pneumonia or sequestration of necrotic mass were scored 2. Besides, if *Mmm* was isolated value of 2 was added to the lesion score. An arbitrarily selected factor was used to multiply the lesion score with the average diameter of the lesion. A lesion size under 5 cm was rated 1; that over 5 cm and under 20 cm was rated 2 while a lesion over 20 cm was rated 3. Hence, the maximum pathology score was (2+2) *3=12.

2.3.7. Data analysis

Data were entered into Microsoft excel 2010 and exported to SPSS 21.0 for analysis. Chi-square and ANOVA were used to measure association between clinical signs, deaths, lesions and analysis of in-group variations between intubates and in-contacts in different trials. General linear model was used to test for the differences in pathology scores. R-statistical was used to plot medians for pathology scores between intubates and in-contacts in all trials.

2.4. Results

Tables 2.1 and 2.2 show details of findings in different groups of experiments, including the cattle that showed fever, those that were killed in extremis after showing severe clinical signs, and pathology of CBPP in cattle infected by either intubation or contact transmission. More in-contacts developed lesions than intubates in all trials as shown in figure 2.1. Although the median of intubates and contacts in the trial I were comparable, there were significant variations in medians between intubates and in-contacts in trials II and III as shown in figure 2.2.

Table 2.1: Details of cattle showing clinical signs and average scores in all trials

	Trial I		Trial II		Trial III		Total of 3 trials	
	Intubates	Contacts	Intubates	Contacts	Intubates	Contacts	Intubates	Contacts
Total cattle used	40	41	15	10	17	10	72	61
Number that showed fever	22	30	10	8	12	8	44	46
Average scores:								
• Coughing	1	2	2	2	2	2	2	2
• Nasal discharge	2	2	1	2	2	2	2	2
• Respiratory effort	1	2	2	2	2	2	2	2
• General appearance	1	2	1	2	2	2	1	2
Total of all scores	1	2	2	2	2	2	2	2

The scores in table 2.1 were rounded off to the nearest whole number. The clinical scores were assigned based on averages of the number of animals showing clinical signs, time of appearance, and severity as follows: 0: Coughing, nasal discharge, a respiratory effort were absent and general appearance was normal; 1: Cough and nasal discharge were mild; the respiratory effort was hyperpnoea; general appearance was subdued; 2: Severe coughing; significant nasal discharge; respiratory effort-obvious dyspnoea; appearance-reluctant to rise; 3: Distressed respiratory effort; general appearance-unresponsive to external stimuli.

Table 2.2: Number of cattle killed in extremis, developed lesions and pathology scores

	Trial I			Trial II			Trial III			Total of all trials		
	intub.	cont.	p	intub.	cont.	p	intub.	cont.	p	intub.	cont.	p
Total cattle used	40	41		15	10		17	10		72	61	
Number killed in extremis	10	13	0.337	1	5	0.023	0	5	0.003	11	23	0.003
Number with lung lesions	36	40	0.172	12	10	0.198	12	8	0.475	60	58	0.029
Average pathology scores	7.3	8.9	0.025	6.2	9.1	<0.001	3.3	8.8	<0.001	6.1	8.9	<0.001

Data used in these experiments in table 2.2 was as described by (Wesonga and Thiaucourt, 2000). Whereas in table above, intub. ≈ intubates; cont. ≈ contacts.

2.4.1. On-station trial one on the model of inoculating cattle with *Mmm*

In this trial, a total of 40 intubates and 41 in-contacts were used. No unwanted reactions were observed in cattle following manipulation related to intubation. The 22 (55%) intubates showed fever with 10 (25%) cattle being killed in extremis after showing severe clinical CBPP and 36 (90%) intubates showing chronic lesions of pleural fibrosis and encapsulated parenchymal necrosis (sequestration) at post-mortem. Similarly, 30 (73%) in-contact cattle showed fever with 13 (31%) of them being killed in extremis after showing severe clinical CBPP and 40 (97.6%) with pathology characteristic of CBPP at post-mortem. The first acute infections occurred on day 7 after intubation, while the first acute cases occurred on day 45 in the in-contact animals, suggesting faster manifestation of clinical disease in intubates than in-contacts. The last post mortem was carried out 133 days after intubation, or 127 days after contact. There was significant difference ($p<0.05$) in pathology scores between contacts and intubates, although medians are comparable (figure 2.2). However, in this experiment, there was no significant association ($p>0.05$) between the mode of transmission and the number of animals with clinical signs, killed in extremis, or the number of animals with lesions.

2.4.2. On-station trial two on the model of inoculating cattle with *Mmm*

In this study, a total of 15 intubates and 10 in-contacts were used. No unwanted reactions were witnessed in cattle following manipulation related to intubation. Ten (66.7%) and eight (80%) animals showed fever in intubates and in-contacts respectively. The first acute infections occurred on day 16 after intubation, while the first acute cases occurred on day 42 in the in-contact animals, suggesting faster manifestation of clinical disease in intubates than in-contacts. One intubate (6.7%) was killed in extremis after showing severe clinical CBPP and 12 (80%) intubates showed lesions at post-mortem with 3 developing acute lesions of fibrinous pneumonia and 9 showing chronic lesions of pleural fibrosis and encapsulated

parenchymal necrosis (sequestration). In the group of in-contact cattle, 5 (50%) cattle were killed in extremis after showing severe clinical CBPP and all others developed lesions, 5 with acute and 5 with chronic lesions. Three (3) of the in-contact cattle had pleural fluid in their thoracic cavity. There was no significant association ($p>0.05$) between mode of transmission to the development of clinical signs and lesions, although the magnitude of the lesions was higher in the in-contact animals (figure 2.2). There was significant difference between contacts and intubates in pathology scores ($p<0.05$) and in the number of animals killed in extremis ($p<0.05$).

2.4.3. On-station trial three on the model of inoculating cattle with *Mmm*

In this trial, a total of 17 intubates and 10 in-contact cattle were used. No unwanted reactions were witnessed in cattle following manipulation related to intubation. None of the animals was killed in extremis in all the 17 intubates within the observation period. The last animals were killed on day 144 after intubation or on day 128 days in-contact. In the in-contact infection trial, 5 (50%) of the cattle survived until the end of the trial. Two (20%) animals were killed in extremis while three (30%) were killed for not eating for at least three days due to CBPP. There was no significant association ($p>0.05$) between the mode of transmission to the development of clinical signs and lesions. However, there was significant association ($p<0.05$) between the mode of transmission, mortality, and pathology scores between contacts and intubates.

2.4.3.1. Clinical response and necropsy in intubated cattle

The cattle started showing clinical CBPP signs on day 2 post-intubation, including fever, cough, and mucus secretion. In the 17 intubates, 12 (66.7%) exhibited fever for at least two days and up to 10 days in the experimental period. One of the animals showed fever intermittently for ten days, starting from the second-day post-inoculation. Three animals

showed fever for only two days. Fever was generally intermittent in all animals and ranged from 39.5 ^{0}C to 41.1 ^{0}C. Five (29.4%) animals did not show fever. Chronic lesions were observed in the twelve (70.6%) cattle that experienced fever at slaughter at the end of the trial while 5 (29.4%) cattle did not develop lesions. A culture for isolation of *Mmm* from the lung tissues was made in all intubates and was found positive.

2.4.3.2. Clinical response and necropsy in contact cattle

Eight out of ten animals (80%) showed a temperature rise above 39.5^{0} C for a period between 2 and 10 days. Temperature above 39.5 ^{0}C was first recorded on day 54 post-exposure. One animal showed fever for 10 days. Seven animals showed fever for at least 3 days. Fever was not recorded in two animals. On post-mortem examination, the pleural cavity in 5 animals contained copious amounts of yellowish coloured clear fluid. Other lesions included consolidation of the lung tissue, marbling with heavy deposits of fibrin flocculates. In 3 of cattle, the lesions were well-developed sequestra. Two animals did not develop lesions. *Mmm* was isolated in all the 10 cattle. Figure 2.3 shows representative post-mortem findings of cattle challenged by intubation or contact.

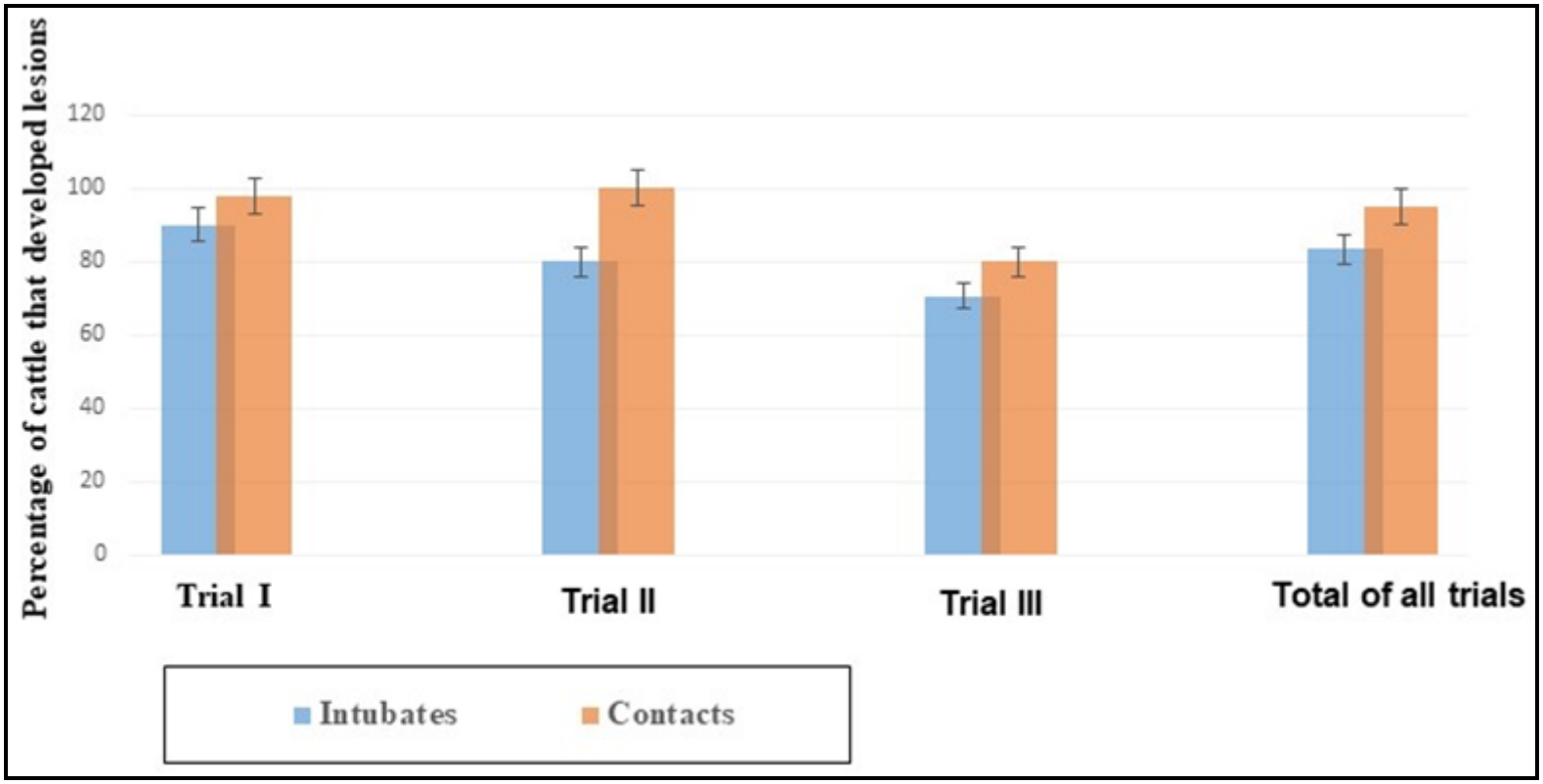

Figure 2.1: Proportion (%) of animals with lesions from the different trials

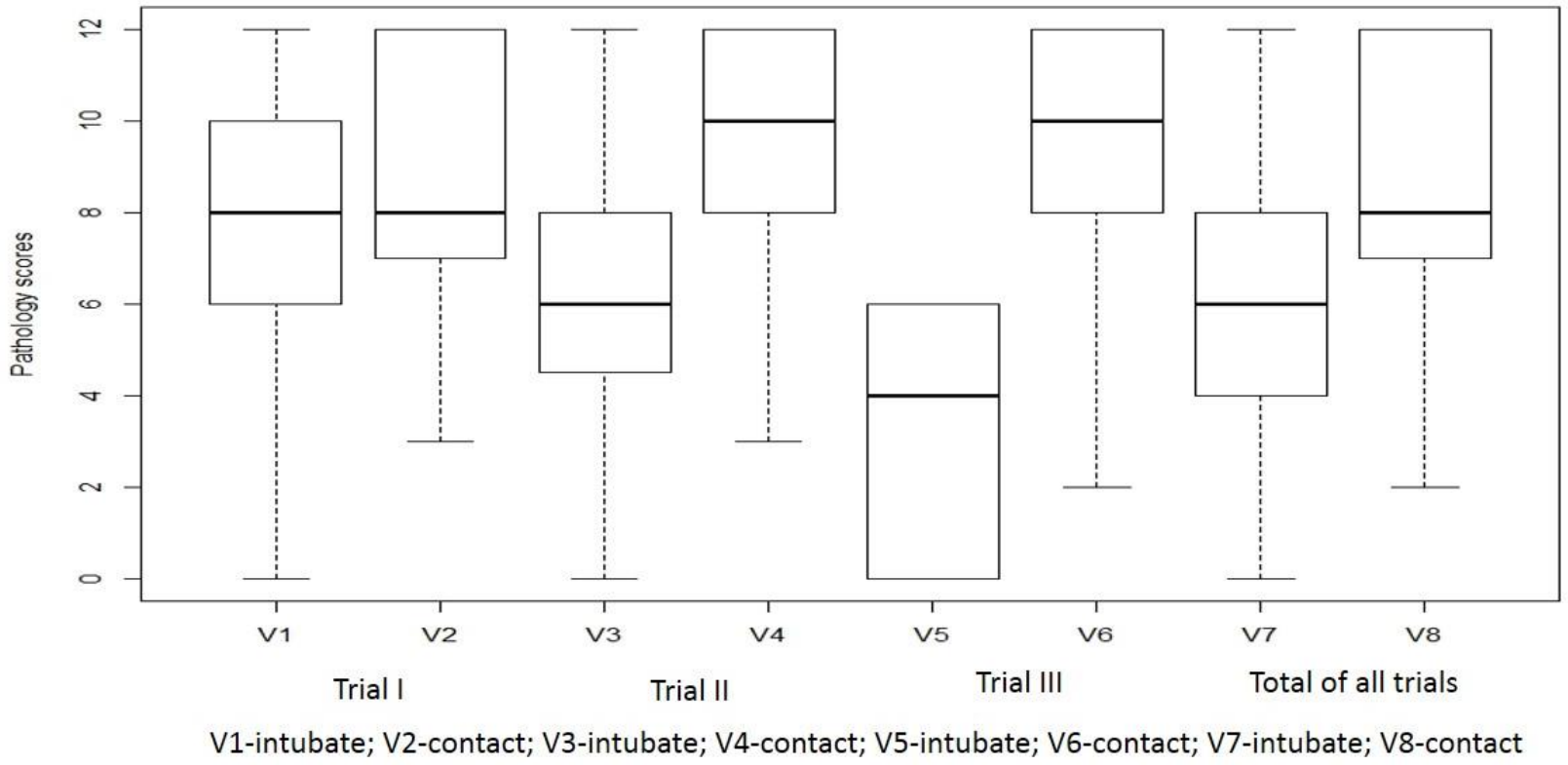

Figure 2.2: Pathology scores (with median shown) between intubates and in-contacts from different trials

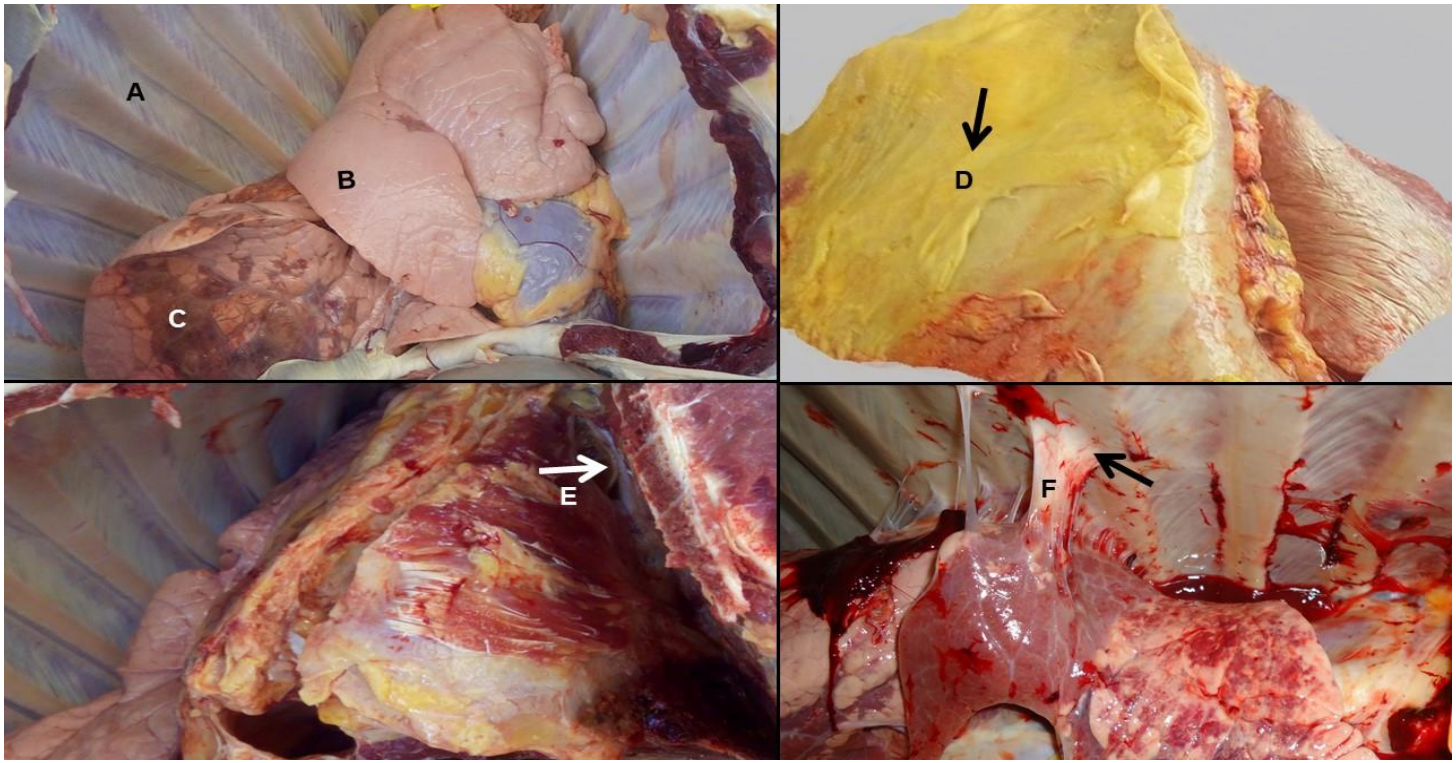

Figure 2.3: Representative post-mortem findings of cattle challenged by intubation or contact: A) Normal thoracic cavity of an animal killed on day 28 post-intubation; B) Typical normal lung of an animal killed on day 28 post-intubation; C) Less severe lesion of an animal killed on day 28 post-intubation; D) Contrast of enlarged lung lobes on the left with marbling (black arrow) and less severe reaction on the right in an animal killed in extremis on day 42 post-contact; E) Fibrous adhesions on thoracic cavity (white arrow) of an animal killed in extremis on day 42 post-contact and F) Thoracic adhesions, less severe lesion of intercostal adhesions between parietal and visceral pleurae (black arrow) in an animal killed at the termination of trial post-intubation.

2.4.4. Combined results of three experiments on the model of infecting cattle with *Mmm*

Overall, combined results for the three experiments indicated that there was no significant association ($p>0.05$) between mode of transmission to development of clinical signs such as fever but there was significant association ($p<0.05$) between mode of transmission to development of lesions. In addition, there was significant association ($p<0.05$) between the mode of transmission and mortality. Similarly, there was significant difference ($p<0.05$) in pathology scores between contacts and intubates. Tables 2.1 and 2.2 show the total cattle used in each group, details of clinical signs, the proportion of animals euthanized in extremis, the number of animals with lesions, and the average pathology scores of cattle infected by intubation or contact transmission in different trials.

2.5. Discussion

The results of the study suggest that infection by intubation produced a milder form of the disease than transmission by contact. Significantly more animals presented with clinical signs and more animals had to be killed in extremis in the in-contact groups. There were no significant differences in the number of animals with lesions, but that is likely to be because those percentages were high in all experiments. But the severity of lesions was significantly higher in the contact groups. Other reports also suggested differences between the two methods (Huebschle *et al.*, 2003; Scacchia *et al.*, 2011), however, the suggestions were based on a few animals.

Although it is impossible to compare the infective doses between the two groups, largely because it is not possible to correctly estimate the number of mycoplasma that is transmitted between animals by aerosol, it seems unlikely to be the cause of the difference. Cultures contain a high mycoplasma, easily 10^8 organisms per ml, and animals get 60 ml of such suspension poured straight into the lungs. It is difficult to see how an animal could inhale such numbers in a natural situation. The reason for the difference must lie elsewhere, such as the precise location in the lungs where the pathogens establish colonies or the lack of optimal conditions for adhering to the lung epithelial cells. A large suspension of mycoplasma may not provide the ideal environment for colonization and growth. Alternatively, a large mycoplasma in one location may provide a stimulus for innate host responses that may rapidly prevent pathogen expansion, while smaller numbers of pathogens in different locations may delay the initiation of inflammatory responses until the pathogens have multiplied to a sizeable colony.

When comparing the three experiments, it was noticed that in-contact animals develop a similar degree of pathology, unlike intubates which differ between the three experiments. This could be due to various factors, such as the passage of mycoplasma culture, culture

conditions, or the intubation procedure. Conditions of in-contact infections are more homogeneous because in these cases two naïve animals are put in contact with an infected animal showing clinical signs. It is likely that repeated intubations would also produce higher infection rates and more reproducible results, however, that has never been tested.

Finally, a potential major contributory factor is differential gene expression between mycoplasma originating from the lung and mycoplasma cultivated *in vitro*. A number of such differences have been described recently (Weldearegay *et al.,* 2015), as well as differences between *in vitro* and biofilm-grown mycoplasma, the latter possibly being the natural infective state of mycoplasma and expressing proteins that may be involved in adhesion (McAuliffe *et al.,* 2008). Because of this, cultured mycoplasma may be much less effective in establishing a lung infection than mycoplasma originating *in vivo*. A lower expression of adhesion molecules or other virulence factors in cultured mycoplasma may make them less infectious after transmission. In this scenario, it is important that data obtained from intubated animals are confirmed by the in-contact method.

2.5.1. Conclusion

The mode of infection appears to have an effect on the outcome of the disease with contact transmission producing a more severe disease compared to intubation. This should be taken into consideration when planning for studies on the pathogenesis of CBPP. In addition to reproducing a more severe disease, contact transmission is a suitable method in trials because it simulates a natural infection, and is therefore recommended, especially where finances are not a limiting factor. However, intubation assures that animals are infected at the same time, while the exact time of infection of each animal after contact is not known and can vary widely (Dedieu *et al.,* 2005).

CHAPTER 3: PRELIMINARY FINDINGS OF LIPOPROTEIN B IN DETECTING CATTLE CHRONICALLY INFECTED WITH CONTAGIOUS BOVINE PLEUROPNEUMONIA

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3.1. Abstract

Complement Fixation Test (CFT) and competitive Enzyme-Linked Immunosorbent Assay (cELISA) are the only serological tests recognized for the diagnosis of CBPP. The performance of CFT depends on the quality of the antigen and the combination of reagents used and only detects CBPP during the acute phase of the disease. There is a need to develop a more sensitive and specific test. Therefore, one mycoplasma protein previously identified as being a potential diagnostic antigen, lipoprotein B (LppB), was tested for its usefulness to detect infected cattle in an indirect ELISA (iELISA). Samples obtained from pre-challenged and challenged cattle were compared using CFT and an i-ELISA based on lipoprotein B. The iELISA developed with the new antigen detected more positive samples than the CFT, which is considered a gold standard. The LppB recombinant protein could be explored further as a target for screening cattle infected chronically with CBPP.

3.2. Introduction

Serological tests recommended by the Office of International Epizootics (OIE) for serological diagnosis of CBPP employs either a complement fixation test (CFT) and/or competitive enzyme-linked immunosorbent assay (cELISA). These tests have drawbacks and no single serological test can detect all CBPP clinical stages necessitating the need for the development of more robust tests. The CFT has high specificity but takes long to perform and requires more elaborate training of staff. The CFT is also less effective at diagnosing animals with chronic lesions (Rurangirwa, 1995). The cELISA test, on the other hand, is simpler to carry out. However, validation studies are still insufficient to make good conclusions about its use in surveillance studies where vaccinations are undertaken in enzootic areas of infected zones. Also, the test is sensitive but its specificity is uncertain (Sidibé *et al.*, 2012). There is a need for the development of another test as sensitive as cELISA and as specific as CFT.

Preliminary studies have been carried out to introduce surface proteins of the mycoplasma *Mmm* strain to develop sensitive and specific diagnostics and efficacious vaccines (Miltiadou *et al.,* 2009; Perez-Casal *et al.,* 2015; Nkando *et al.,* 2016). In this study, a few serum samples that had been tested and confirmed positive by CFT were re-tested against LppB recombinant protein to determine if there are differences in antibody responses, sensitivities, and specificities between the two tests.

3.3. Materials and methods

The cattle used in the study were purchased from Kakamega in western Kenya, a CBPP-free zone. The samples were obtained following a challenge by contact transmission using a field isolate of *Mmm* referred to as strain B237 from Thika, Kenya (Nkando *et al.*, 2010). All cattle experiments from which the samples were derived were carried out in accordance with the KALRO-VSRI, IACUC: VSRI/IACUC/1/29092009 and VSRI/IACUC/2/00122010.

3.3.1. Expression and purification of lipoprotein B

The 5 ml Luria-Broth (LB) medium containing 30 µg/mL kanamycin was inoculated with a single colony of BL21DE3 STAR cells containing a pETite expression construct of LppB. The culture was shaken overnight at 220 rpm and 37^0 C in a shaker incubator. The following morning, 0.5% glucose was added before the addition of 5 ml to 250 ml LB media plus kanamycin. The cultures were grown in a shaker incubator until the optical density (OD at 600nm) was between 0.5-0.7, before inducing with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hours. Cells were centrifuged at 14,000 x g for 30 minutes at 15^0 C, then purified under denaturing conditions as described by Miltiadou *et al.*, 2009.

3.3.2. Antibody responses as determined by CFT and LppB-ELISA

Serum samples were selected based CFT results and the presence or absence of lesions. They were tested using an iELISA based on the LppB antigen. An analysis was then carried out to compare antibody responses, sensitivities, and specificities of the two tests. The CFT was carried out according to Campbell and Turner, (1953).

The LppB assay was performed as follows: Wells of polysorp 96-micro-well plates (Nalge International, Roskilde Denmark) were pre-coated with 150 µl of 1.2 µg/µl LppB recombinant antigen in phosphate-buffered saline (PBS) pH 7.4 for 2 hrs at 37^0 C. The wells

were blocked with 300 µl of blocking buffer (0.2% casein in PBS) for 20 min at 37^0 C and washed 3 times in washing buffer (PBS + 0.1% Tween 20). Serum test samples were diluted 1:400 in 1% skimmed milk in PBS, 150 µl added in duplicate on the plate, and incubated at 37^0 C for 40 min before washing 5 times in washing buffer. One hundred and fifty microlitres of horse reddish peroxidase (HRP)-conjugated anti-bovine IgG (Svanova, Sweden) were added at a dilution of 1:10, 000 in PBS, and the plate incubated at 37^0 C for 30 min. The diammonium salt 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) was used as chromogen and H_2O_2 as a substrate for peroxidase to detect bovine anti-LppB antibodies. The plate was incubated in the dark with gentle shaking for 30 min before OD readings were obtained using an Immunoskan ELISA plate reader at 405 nm.

3.3.3. Determination of sensitivities and specificities between CFT and LppB-ELISA

The sensitivities and specificities between tests were determined using a 2×2 contingency table as shown in table 3.1 (adapted from Amanfu *et al.*, 2000).

Table 3.1: Calculation of sensitivities and specificities of CFT and iELISA based on LppB

Type of test	**Tested (+)**	**Tested (-)**
Test (+)	T+	F-
Test (-)	F+	T-
Sensitivity (*Se*)= $\left(\frac{T+}{(T+)+(F-)}\right)\times 100\%$	Specificity (*Sp*)= $\left(\frac{T-}{(T-)+(F+)}\right)\times 100\%$	

T+: true positive; T-: true negative; F-: false negative; F+: false positive

3.3.4. Data analysis

Data were entered into Microsoft excel 2010 and SPSS 22.0 where Cohen's Kappa test was used to determine if there was an agreement between CFT and LppB serological tests in detecting positive and negative serum samples.

3.4. Results

3.4.1. Antibody responses as determined by CFT and LppB-ELISA

Table 3.2 shows the results of pre and post-challenge sera, tested with the two assays. Post-challenge sera were further split into sera from animals without lesions, with sequestrae or from early acute cases. These post-challenge sera were selected from a total of 36 cattle, where 7/36 had no lesions, 20/36 had sequestrae and 7/36 had an acute infection. The eight pre-challenge samples were samples collected 21 days before the challenge. Acute sera were collected within 1 month after challenge while sera from animals with sequestrae and no visible lesions were collected between 3 to 12 months after challenge. The negative and positive sera in various disease stages were collected at the same time points. LppB-ELISA was very efficient in detecting serum samples from infected animals; in particular, from animals with sequestrae (100%), and just over half of sera from animals without lesions. However, it did not identify any sample from acutely infected cattle. It also detected one of the pre-vaccination serum samples.

3.4.2. Determination of sensitivities and specificities between CFT and LppB-ELISA

Table 3.2 shows CFT and LppB-ELISA results on post-challenge serum samples for animals without lesions, with sequestrae and early acute cases. *Mmm*SC was isolated from the lungs of all challenged cattle. Upon challenge, fever was first recorded on the 40^{th} day post-contact. Eight out of nine animals with sequestrae presented with fever. The longest period with fever recorded was 14 days in one animal while the shortest fever period recorded lasted 2 days in two animals. All 7 animals with early acute lesions presented with fever. The highest fever recorded lasted 13 days in one animal while the least fever recorded lasted one day in one cattle.

Table 3.2: Number of serum samples testing positive pre-challenge and at various disease stages post-challenge

	Number of serum samples showing seroconversion			
	Pre-challenge (n = 8)	Post-challenge (n = 36)		
Type of assay		Without lesions	With sequestrae	With acute infection
CFT (KALRO)	0/8 (0%)	2/20 (10%)	3/9 (33.3%)	1/7 (14.3%)
LppB (i-ELISA)	1/8 (12.5%)	14/20 (70%)	9/9 (100%)	0/7 (0%)

3.4.2.1. Sensitivities and specificities of CFT and iELISA based on LppB

The sensitivities of the assays were calculated as follows: it was assumed that all infected animals were positive, including those that had no lesions since *Mmm*SC was isolated from lung tissues of all cattle. Specificity was calculated using pre-challenge samples only since these were assumed to be true negatives. In table 3.3, sensitivity of LppB-ELISA was found to be higher than that of CFT while specificity of CFT was higher.

Table 3.3: Results of sensitivities and specificities of CFT and iELISA based on LppB

Type of assay	**CFT**	**LppB**
Se (%)	17	64
Sp (%)	100	87.5

3.4.3. Determination of agreement between CFT and iELISA based on LppB

Table 3.4 presents the frequency counts for positive and negative serum samples as assessed using CFT and the indirect ELISAs based on the LppB recombinant antigen. Although there was no agreement between CFT and LppB in the detection of antibodies in no lesions and sequestrae cases respectively, there was significant agreement between CFT and LppB in the detection of antibodies in acute cases (p = 0.659).

Table 3.4: A comparison of similarities and differences between CFT and iELISA based on LppB

Categories		*Kappa*	*p*
Acute lesions (N=7)	CFT and LppB-ELISA	-0.167	0.659
Sequestrae (N=9)	CFT and LppB-ELISA	0.000[a]	0.000
No lesions (N=20)	CFT and LppB-ELISA	-0.212	0.023

a. No statistics are computed because the test is a constant.

3.5. Discussion

The observed differences between the sensitivities obtained in this study and previous studies (Amanfu *et al.,* 2000) and (Muuka *et al.,* 2011), are probably due to the disease status of the animals after challenge and the time point at which sera were collected. Animals in this study were infected by an in-contact challenge, and there is always uncertainty on the time point at which a particular animal gets infected (Lutta *et al.,* 2017). In this study, CFT had significantly lower sensitivity, 17%. In this study the test was performed on serum samples at the end of 3rd week for acute cases, end of 4th month for cases with sequestrate, and end of also 4th month for cases with no lesion. Besides, the immune status of the individual animal seems to play a role in the time course and level of specific antibody production (Schubert *et al.,* 2011). During the acute phases of the disease, CFT detects both IgM and IgG1 that bind complement but not IgG2 (Le Goff and Thiaucourt, 1998). Antibodies detected by CFT decrease early and the number of positive results decreases dramatically when a blood sampling has occurred 3 months later in an outbreak. This possibly explains the low sensitivity of the CFT assay, which depends on complement binding antibodies, mainly IgM (Niang *et al.,* 2006). In contrast, c-ELISA strongly detects IgG2 and therefore will identify positive animals in later stages of disease compared to CFT (Le Goff and Thiaucourt, 1998; Niang *et al.,* 2010).

The sensitivities of the i-ELISA with LppB, detecting mainly specific IgG, was 64%. This figure is comparable to the sensitivity of an assay based on another recombinant protein, LppQ (Muuka *et al.* 2011), at 69% but with a specificity of 100%. It is known that LppQ induces a very high antibody response (Abdo *et al.,* 2000), and is also specific for *Mmm*. However, the i-ELISA assays detected the highest number (100%) of positive serum samples in cases with sequestrae, while 70% of animals without lesions (Table 3.2). Progress of CBPP in animals with

the acute form of the disease is rapid and such animals might have not had time to produce high enough IgG antibody titers to the proteins. High levels of mycoplasma antigens in the acute form will mop up any specific antibodies generated early in the infection, resulting in negative diagnosis, as suggested before (March *et al.*, 2003). In contrast, animals with sequestrae have been infected for a longer time and therefore IgG antibody titers may have had time to build up in the blood. In animals without lesions, the antigen is relatively low compared to animals with sequestrae, so the number of positive samples detected is intermediate.

3.5.1. Conclusion

Though the iELISA based on LppB antigen detected more positive serum samples than CFT, additional work is required to optimize the assay conditions. Since the LppB-based diagnostic test detected all animals with sequestrae, it holds the possibility that a diagnostic test could be developed to identify animals with carrier status.

CHAPTER 4: IDENTIFICATION OF THE BEST *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* ANTIGENS FOR DETECTING ALL CLINICAL STAGES OF CONTAGIOUS BOVINE PLEUROPNEUMONIA

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4.1. Abstract

CBPP is mainly a disease of cattle in Africa that is controlled by early diagnosis and vaccination using live attenuated vaccines. The prescribed serological diagnostic tests are either a CFT or cELISA. However, none of these prescribed diagnostic tests are capable of detecting all clinical stages of CBPP. This study undertook research to identify *Mmm* antigens that could be used to detect both acute and chronic stages of CBPP in cattle experimentally infected with *Mmm*. Serum samples from well-characterized cattle infected with the Afade strain of *Mmm* were screened using i-ELISA. The antigens tested were MSC_0397, MSC_0636, MSC_0653, and LppB. These antigens were selected following extensive literature review and previous laboratory studies in which they had been predicted or shown to have higher sensitivities and specificities in detecting cattle infected with *Mmm*. All antigens tested negative on sera from naïve animals. However, the performances of MSC_0636 and LppB showed significance differences ($P < 0.05$) by detecting both acute and chronic clinical stages of CBPP compared to other antigens post-inoculation. Therefore, MSC_0636 and LppB would be the most suitable antigens recommended for the development of pen-side assays for detecting CBPP infected cattle.

4.2. Introduction

Investigations to understand pathogenicity factors associated with *Mmm* have been undertaken to introduce bacterial antigens in the development of alternative tests and vaccines (Westberg *et al.,* 2004; Perez-Casal *et al.,* 2015). A virulent factor that injures cattle cells, the H_2O_2 formed by L-ά-glycerophosphate oxidase (GlpO) has since been identified during *in-vivo* studies (Miles *et al.,* 1991; Pilo *et al.,* 2005). A comparison of Australian-African-cluster strains of *Mmm* and European cluster strains revealed that the latter is less virulent. The virulence traits of Australian-African-cluster strains of *Mmm* were due to their ability to introduce glycerol, a precursor of H_2O_2 while the European strains lack this function, due to erasure of the operon coding the glycerol uptake structure (Vilei and Frey, 2001). It has also been revealed that cytotoxic effects occur in nasal epithelial cells of hosts inoculated by either membrane-bound glycerol-phosphate oxidase (GlpO), capsular polysaccharides (CPS), or thick galactan layer surrounding *Mmm* (Buttery *et al.,* 1976; Bischof *et al.,* 2009).

Lipoproteins and variable surface proteins of *Mmm* contain virulence factors that severely affect the host (Brenner *et al.,* 1997). Lipoproteins are extremely antigenic membrane proteins and are involved in Mycoplasma's survival links such as adhesion and stimulation of pro-inflammatory cytokine release (Herbelin *et al.,* 1994; Calcutt *et al.,* 1999; Marie *et al.,* 1999). To date, several immunogenic proteins of *Mmm* have been described including; MSC_397, MSC_636, MSC_653, LppB, and LppQ (Abdo *et al.,* 2000; Miltiadou *et al.,* 2009; Perez-Casal *et al.,* 2015). By varying the antigenic range of surface proteins, mycoplasma might survive better in the mammalian host (Rosengarten and Wise, (1990); Razin *et al.,* 1998; Denison *et al.,* 2005). The variations are achieved at a genomic level using "cases" of repetitive sequences that

are switched by addition, erasure to form varied protein modifications in mycoplasmas (Lysnyansky *et al.,* 1996; Persson *et al*., 2002).

Since *Mmm* is a pathogen affecting the respiratory system, the local and mucosal antibody response plays the main part by preventing the growth and colonization of bacteria on animal cells (Niang *et al*., 2006). The defensive role of antibodies in humoral responses during infection with *Mmm* has been established and shown that transmission of sera from cattle that had recuperated from CBPP, was able to confer protective immunity to beneficiary calves (Masiga *et al*., 1975). Strong serological responses from immunoglobulin isotypes IgM, IgG_1, and IgG_2 antibody profiles were obtained in infected cattle but only the levels of mycoplasma-specific IgA correlated with the acute clinical stage of CBPP (Niang *et al.,* 2006). Earlier studies proposed that both humoral and T-cell immunity played a role in immune responses that correlated with protection against *Mmm* (Masiga *et al*., 1975; Dedieu *et al*., 2005). Several studies have explored surface-exposed proteins of *Mmm* as likely targets of antibody responses (Kiarie *et al*., 1996; Abdo *et al*., 1998).

Diagnosis of CBPP mainly employs a combination of any of the following: clinical signs, serological tests, post-mortem findings, and PCR (Bashiruddin *et al.,* 1994; Wesonga and Thiaucourt, 2000). The CFT and c-ELISA are the only prescribed serological tests by the OIE for the diagnosis of CBPP infected cattle. These prescribed serological tests have limitations necessitating the need for the development of user-friendly, sensitive, and specific assays. Earlier studies described the use of iELISA based on the LppQ antigen (Bruderer *et al.*, 2002) and also several *Mmm* proteins in a cocktail ELISA setup (Neiman *et al.,* 2009). However, it is expensive to produce various antigens for cocktail ELISA and also limited data was available to confirm that antigens used in previous studies can detect all clinical stages of CBPP. The goal of this trial

was to: i) optimize protein-encoding genes for *Escherichia coli* expression and purification, ii) characterize humoral immune responses in infected cattle specific for purified antigens of *Mmm* that can detect all clinical stages of CBPP.

4.3. Materials and methods

All protocols of the studies in chapters 4 and 5 were designed and performed in strict accordance as per applicable animal welfare regulations with the approval of KALRO-VSRI, IACUC: VSRI/IACUC009/15072016.

4.3.1. Tagging and transformation of *Mmm* genome sequences

Genome sequences encoding MSC-397, MSC_636, and MSC-653 genes were submitted to a commercial company (GenScript USA Inc) for tagging into the PQE60 expression vector. Frozen competent *Escherichia coli* cells (BL 21 DE3 STAR) were thawed on ice for 30 min. 5 µl of 0.05 µg/µl recombinant plasmid DNA was dispensed in a microcentrifuge tube, 50 µl *E. coli* cells added and the contents gently vortexed to mix. Tubes were heat-shocked at 42^0 C in a water bath for 45 secs then placed on ice for 2 min. 950 µl of super optimal broth media (S.O.C from Invitrogen life technologies, USA) without antibiotic was added to the contents and grown at 37^0 C in a shaker incubator (220 rpm) for 45 min. 50 µl of transformed cells were plated on an LB broth with agar (Sigma Life Sciences, USA) containing 100 µg/ml ampicillin (Sigma Aldrich, USA). Agar plates were incubated at 37^0 C overnight (O/N). Glycerol stocks were made by placing a recombinant colony of each plasmid into 10 ml LB broth with 100µg/ml ampicillin. The contents were grown at 37^0 C in Innova 4335 New Brunswick Scientific USA incubator shaker until the absorbance at 600 nm was between 0.5 to 0.6 on a spectrophotometer. 1 ml

culture of each plasmid was put in 2 ml sterile cryovials, 0.5 ml of sterile 100% glycerol (VWR Prolabo chemicals, Belgium) added, mixed and kept at -80^0 C for future use.

4.3.2. Extraction of DNA from recombinant *Mmm* plasmids and running of agarose gel

Plasmid DNA was harvested from transformed bacteria using the QIAprep miniprep kit Inc. A colony of bacteria was grown under ampicillin selection (100 µg/ml) in 3 ml LB culture media O/N at 37^0 C with vigorous shaking (220 rpm). Two milliliters of O/N cultures were dispensed in Eppendorf tubes and span at 14,000 rpm for 3 min at 15^0 C to harvest cells. The supernatant was completely discarded and the pellet resuspended in 250 µl P1 buffer (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose), containing RNase-A. Two hundred and fifty µl P2 buffer (1% Sodium Dodecyl Sulfate (SDS) was added and mixed by gently inverting tubes 6X within 5 min. Three hundred and fifty µl of neutralizing N3 buffer (4.2 M guanidine hydrochloride, 0.9 M potassium acetate, pH 4.8) was added, contents mixed by inverting the tube 6X until the solution was cloudy. The contents were span for 10 min, 14, 000 rpm at 4^0 C, and supernatant applied to QIAprep spin columns. The columns were span for 1 min at 14,000 rpm and the flow-through discarded. The columns were washed by adding 500 µl PB buffer (5 M guanidine hydrochloride and 30% isopropanol) to remove traces of nuclease activity. After centrifugation for 1 min at 14, 000 rpm, the spin columns were washed with 750 µl PE buffer (10mM Tris-HCl pH 7.5 and 80% ethanol) and centrifuged for 1 min at 14,000 rpm. The filtrate was discarded and the columns subjected to additional centrifugation to remove any residual wash buffer. The columns were then placed in clean 1.5 microcentrifuge tubes and the bound plasmid DNA eluted in 50 µl of double-distilled water. The DNA was quantified using Thermo Scientific NanoDrop 1000 spectrophotometer and resolved in 1% agarose gel (appendix 1).

4.3.3. Western blotting using 6x-his antibody

This protocol was modified from the QIAexpress Ni-NTA Fast Start Handbook, June 2006). The 15 µg of purified antigen was loaded per well in 10% SDS-PAGE separating gel (appendix 4) along with 3 µg of Fisher Scientific molecular marker, USA. The gel was run in an SDS-PAGE electrophoresis tank for 1hr 50 min and thereafter placed in 1x transfer buffer (appendix 5) for 15 min. The transfer sandwich (ATTA electrophoresis tank AE-6675, Japan) was assembled, gel placed in the sandwich tank, a voltage of 25 V applied at maximum current, and transferred for 1 hr. Briefly, the blot was dipped in tap water then into the Ponseau S solution (Sigma Aldrich, USA) to visualize the protein bands. The Ponceau S solution was rinsed off the blot with three washes of Tris-buffered saline-tween (TBST), prepared as shown in appendix 5. 1% casein in TBST was used for blocking at room temperature for 1 hr. The primary antibody (6x-his monoclonal antibody from Sigma Aldrich, USA) was applied and incubated O/N at 4^0 C at a concentration of 1: 2000 for the 6x-his antibody and 1: 100 for sera in 50 ml of blocking buffer. the following day, the blot was rinsed 5 times with TBST. Thereafter incubated in the 0.2 µg/ml HRP-conjugated secondary antibody (Sigma Aldrich, USA) in 50 ml blocking buffer for 1 hr at 24^0 C. The blot was rinsed 5 times in TBST and developed by adding the substrate {50 ml PBS; 50 µl 30% H_2O_2; 0.05g 3,3´-diaminobenzidine (DAB from Sigma Aldrich, USA)}. The blot was then washed after 5 min and visualized by Bioanalytical Imaging System (Azure Biosystems, USA).

4.3.4. Expression of recombinant *Mmm* proteins and determination of protein solubility

Firstly, LB agar plates containing 100µg/ml ampicillin were streaked with recombinant plasmids in glycerol stocks and incubated overnight in Thermo Scientific HERACELL 150i CO_2

incubator, Germany. The following morning, the desired isolate was inoculated with 5ml culture of Luria broth (LB from Sigma Life Sciences, USA) with 100µg/ml ampicillin, incubated at 37^0 C and shaken at 220 rpm. On day 3, 250 ml of broth plus ampicillin was inoculated with the 5 ml of O/N culture in 1 liter flask, incubated at 37^0 C until spectrophotometer readings at 600 nm were within 0.5 to 0.6 before adding 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) from Promega Corporation, USA, incubated for 6 hours at 37^0 C by shaking at 220 rpm. The culture was then centrifuged in Beckman coulter Avanti J-301 at 8000 rpm for 15 min. The supernatant was discarded and pellets suspended in 1 ml of 25% sucrose/50 mM tris pH 8.0, transferred to 50 ml corning tubes and kept at -80^0 C until purification.

Before the purification of proteins, the solubility of each protein was determined. The cells were removed from -80^0 C and thawed on ice for 15 min. The lysis buffer (formulation is shown in appendix 3) at pH 8.0 (10 ml of lysis buffer per gram wet weight of pellet) was added then placed on the rotor (Denley Spirax, England) for 1 hour to break open the cells and sonicated (Ultrasonic Homogenizer Model 150VT, Biologicals, Inc., USA) at 50% amplitude for 2 min to further break the remaining cells**.** The lysate was span at 10, 000 x g for 30 min at 15^0 C, supernatant collected, and pellet resuspended in 10 ml 1 x PBS. Five microliters of supernatant and/or pellet were mixed with 5x SDS sample buffer (appendix 4) at a ratio of 1 : 3 and placed in a water bath at 95^0 C for 4 min, thereafter loaded on wells of 10% SDS-PAGE gel and run for 1 hr 50 min. The gel was stained with coomassie blue, destained (appendix 4), and visualized by Bioanalytical Imaging System (Azure Biosystems, USA) to confirm the presence of proteins.

4.3.5. Purification of *Mmm* proteins

The protein purification procedure was adapted from QIA expressionist, June 2003 with modifications. Briefly, the expressed cell pellets were removed from -80^0 C freezer, thawed for 15 min, 8M urea lysis buffer added at 10 ml per gram wet weight. 0.1 mM Phenyl methyl sulfonyl fluoride (PMSF)-Sigma Aldrich, USA was added to inhibit protease activity. The corning tubes with cells were put on the rotor and allowed to mix for 60 min at 24^0 C taking care to avoid foaming. The lysate was span at 10, 000x g for 30 min at 15^0 C to pellet the cellular debris and supernatant saved. Five µl of 2x SDS-PAGE sample buffer was added to 5µl supernatant and stored at -20^0 C for SDS-PAGE analysis. One ml of the 50% Ni-NTA slurry (Sigma Aldrich, USA) was added to 10 ml lysate and mixed gently by shaking on the rotor for 30 min at 24^0 C. The lysate-resin mixture was loaded carefully into an empty column (BioRad, USA) and flow-through collected for SDS-PAGE analysis. The columns were washed 3x with 4 ml of wash buffer (appendix 3) at pH 6.3 and wash fractions kept for SDS-PAGE analysis. Recombinant antigens were eluted 3x with 1ml elution buffer (appendix 3) at pH 7.4. The purified proteins were mixed with 2x SDS sample buffer (appendix 4) and ran in SDS-PAGE gel. The LppB *Mmm* gene was tagged, transformed, and sent as glycerol stocks to BecA-LRI from Agricultural Research Council (ARC)-Onderstepoort Veterinary Institute (OVI), South Africa for expression and purification of proteins (Miltiadou *et al.,* 2009).

4.3.6. Dialysis and estimation of protein concentration

The purified proteins were extensively dialyzed in 4M urea for $1^1/_2$ hour then 1x PBS (appendix 2) for either 1hour (MSC_0499, MSC_0636, MSC_0653 & MSC_0775) or O/N at 4^0 C (MSC_0136, MSC_0397, MSC_0431 & LppB) and concentrated using polyethylene glycol from

Sigma Aldrich, Germany. Quantification was performed as described by BCA Protein Assay Kit (Thermo Scientific, USA). In this protocol, 25 µl of a sample was pipetted in a replicate in a microplate well (working range = 20-2000 µg/ml). 200 µl of the working reagent was added to each well and mixed for 30 seconds. The plate was then incubated at 37^0 C for 30 min, then absorbance measured at 562 nm in a plate reader (BioTek Synergy HT, USA). The calculated and standardized absorbance was plotted in a standard curve and used to determine unknown protein concentrations.

4.3.7. Serum samples from naïve and experimentally infected animals

Thirty-two well-characterized sera were used in this study. Twenty-four of the sera were collected from 8 CBPP-infected Zebu cattle. Twenty-four of those sera from infected cattle were collected at different clinical stages: Acute, subacute and chronic phases of the disease. Eight of the remaining sera were collected from 8 naïve cattle in an on-station experiment. Eight of the sera from the other 8 naïve cattle, were well-characterized sera and confirmed to be negative by both OIE recommended serological tests, the CFT, and c-ELISA. Animals were bled at an interval of 14 days.

4.3.7.1. Screening of serum samples from on-station trials using indirect ELISA

This protocol was adapted from Nkando *et al.,* (2016). The iELISAs were done in duplicate and repeated on three different occasions. The negative and positive control sera were placed in duplicates in the first and last row of plates coated with 1 µg/ml of each antigen. No serum sample was placed in the second row and an OD of this row was subtracted from the OD containing sera. The ELISA-plates were coated with 1 µg/ml antigen in coating buffer (100 µl/well) of Carbonate: bicarbonate buffer and kept at 4^0 C overnight. The following day, plates

were washed 5x in phosphate-buffered saline-tween (PBS-T). 200 µl blocking buffer (1x PBS-T and 0.5% Horse serum from Gibco Life Technologies^{TM}, New Zealand) was added and then test serum on dilution plates to each well. For anti-IgG, the initial concentration was obtained at 1/100 then diluted 4-fold, plates incubated with gentle shaking at 37^0 C for 1 hr, and washed 5x with PBS-T (appendix 2). 100 µl of secondary antibody conjugated to alkaline phosphatase (KPL151-12-06, USA) at a dilution of 1/5000 in blocking buffer was added in each well and kept for 1 hr with gentle shaking at 37^0 C. The plates were again washed 5x in PBS-T and 100µl/well of para-Nitrophenylphosphate (pNPP) substrate from Sigma Aldrich, USA (appendix 2) added. The plates were placed in the dark for 45 min and read at a wavelength of 405 nm in BioTek Synergy HT, USA plate reader.

4.3.8. Determination of cut-off point, diagnostic sensitivity, and specificity of *Mmm* antigens

To analyze iELISA data based on *Mmm* antigens, the change-point method was determined as the cut-off point formula of a mean + 3 standard deviation of negative controls (Lardeux *et al.*, 2016). Sensitivity (Se), specificity (Sp), positive predictive value (PPV), and negative predictive value (NPV) were calculated as follows; Se: **{**TP/(TP + FN**)}** ×100%, where TP = true positive, FN = false negative; Sp: **{**TN/(TN + FP)**}**×100%, where TN = true negative, FP = false positive; PPV: **{**TP/(TP + FP)**}** × 100%; NPV: **{**TN/(TN + FN)**}** × 100% (Amanfu *et al.*, 2000; Heller *at al.,* 2016). In this study, the number of animals per group was based on previous immunization trials (Nkando *et al.*, 2016; Prysliak *et al.*, 2016; Mwirigi *et al.*, 2016).

4.3.9. Data Analysis

Data for iELISA with different antigens in chapters 4 and 5 were entered into MS Excel (Microsoft® Excel, Washington, 2016) and transferred into SPSS version 22, analyzed by two-

way Analysis of Variance (ANOVA) and least significance difference used to separate the means. Data were imported into R software, version 3.6.0 (R Core Team, 2019) for analysis and drawing of the box and scatter plots. Cohen's Kappa measurement assessed the level of agreement between the *Mmm* antigens.

4.4. Results

Sera from naïve and CBPP-infected cattle were tested against the antigens. None of the animals seroconverted before infection with *Mmm* (Table 4.1). When antigens were used to detect antibodies in sera from CBPP-infected, MSC_0636 and LppB detected more animals as CBPP positive than MSC_0397 and MSC_0653. The iELISA results showed that MSC_0636 detected 7/8, 7/8, and 8/8 animals in acute, subacute, and chronic stages of the disease, respectively, while LppB detected 6/8, 6/8, and 8/8 animals. However, MSC_0397 detected 4/8, 3/8, and 6/8 animals in acute, subacute, and the chronic stages of the disease, respectively, while MSC_0653 detected 4/8, 4/8, and 8/8 animals. The cut-off point for the infected cattle was obtained at a titer of 1: 400, an OD of ≤0.2 at a wavelength of 405 nm. Thus, these two antigens (MSC_636 and LppB) appear suitable for detecting antibodies in sera from acute and chronic clinical stages of CBPP as they yielded specificities of 100% and sensitivities of between 83.3%-91.7% (Table 4.2).

Table 4.1: ELISA results based on MSC_0397, MSC_0636, MSC_0653 and LppB *Mmm* antigens showing true negative or positive serum samples

Test (Antigen)	*Naïve cattle*	*Infected cattle*		
		Acute	Sub-acute	Chronic
MSC_0397	8/8	4/8	3/8	6/8
MSC_0636	8/8	7/8	7/8	8/8
MSC_0653	8/8	4/8	4/8	8/8
LppB	8/8	6/8	6/8	8/8

Table 4.2: Sensitivities, specificities and predictive values of iELISA based on MSC_0397, MSC_0636, MSC_0653 and LppB *Mmm* antigens

Test (Antigen)	*Sensitivity (se)*	*Specificity (sp)*	*PPV (%)*	*NPV (%)*
MSC_0397	54.2%	100%	100	42
MSC_0636	91.7%	100%	100	80
MSC_0653	50%	100%	100	57
LppB	83.3%	100%	100	66.7

Figure 4.1 shows sizes of different plasmid DNA on agarose gel; control (uncut plasmid DNA), and those linearized with restriction enzymes (BamHI/Hind III). Identities of specific proteins are shown on a western blot using anti-HIS antibody (Figure 4.2). The proteins were then expressed and purified (Figure 4.3).

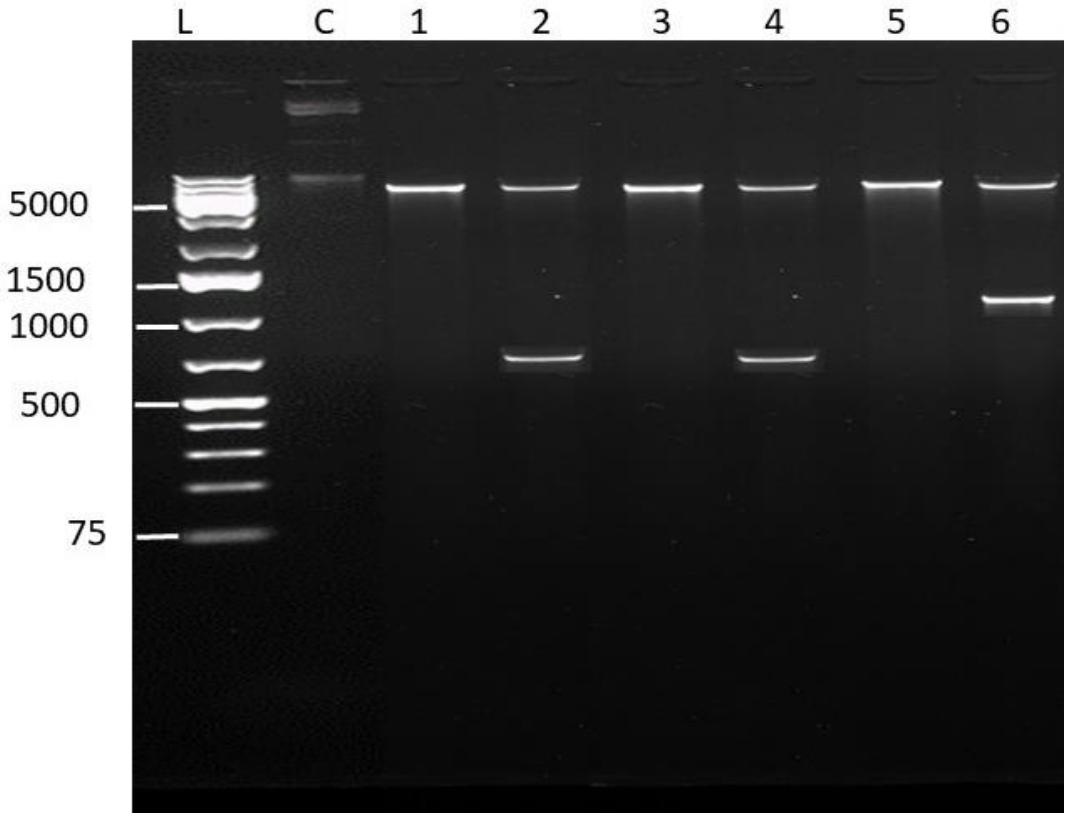

Figure 4.1: Agarose gel showing sizes of *Mmm* recombinant plasmid DNAs: Restriction digestion was done using BamHI/ Hind III. 5 µl of BamHI (single digest) was removed before adding Hind III for the double digest. 1% of agarose gel was used to run the samples. L = 1 kb plus DNA Ladder (Thermo Scientific); C= uncut pDNA control; 1= MSC_0397 cut by BamHI, 2= MSC_0397 cut by both BamHI/HindIII (642 bp); 3= MSC_0636 cut by BamHI, 4= MSC_0636 cut by both BamHI/HindIII (600 bp); 5= MSC-0653 cut by BamHI, 6= MSC_0653 cut by both BamHI/HindIII (1086 bp).

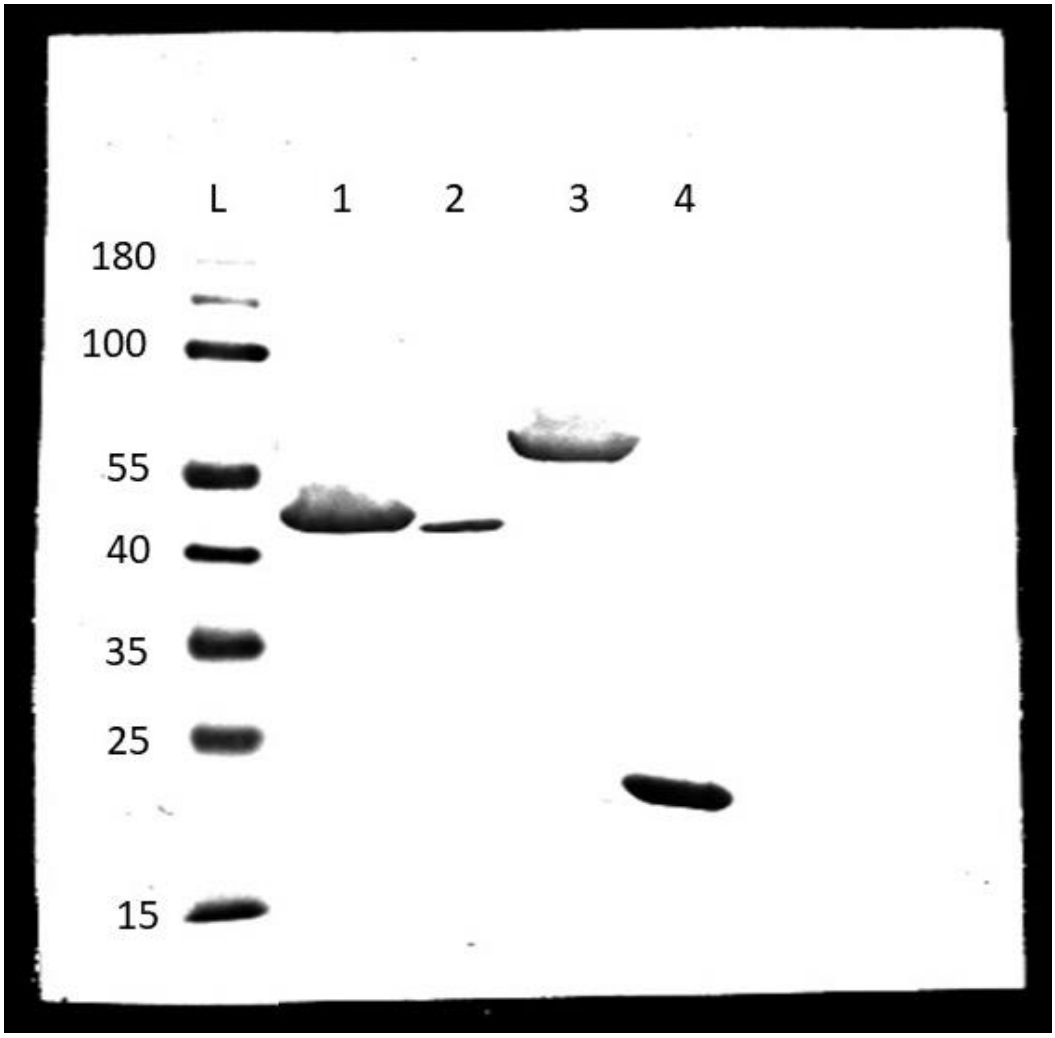

Figure 4.2: Western blot identifying sizes of specific *Mmm* proteins. An anti-HIS antibody was used to detect the proteins: L= Protein ladder (Thermo Scientific), 1=MSC_0397 (45 kDa), 2= MSC_0636 (43 kDa), 3= MSC_0653 (72 kDa), 4=LppB (27 kDa).

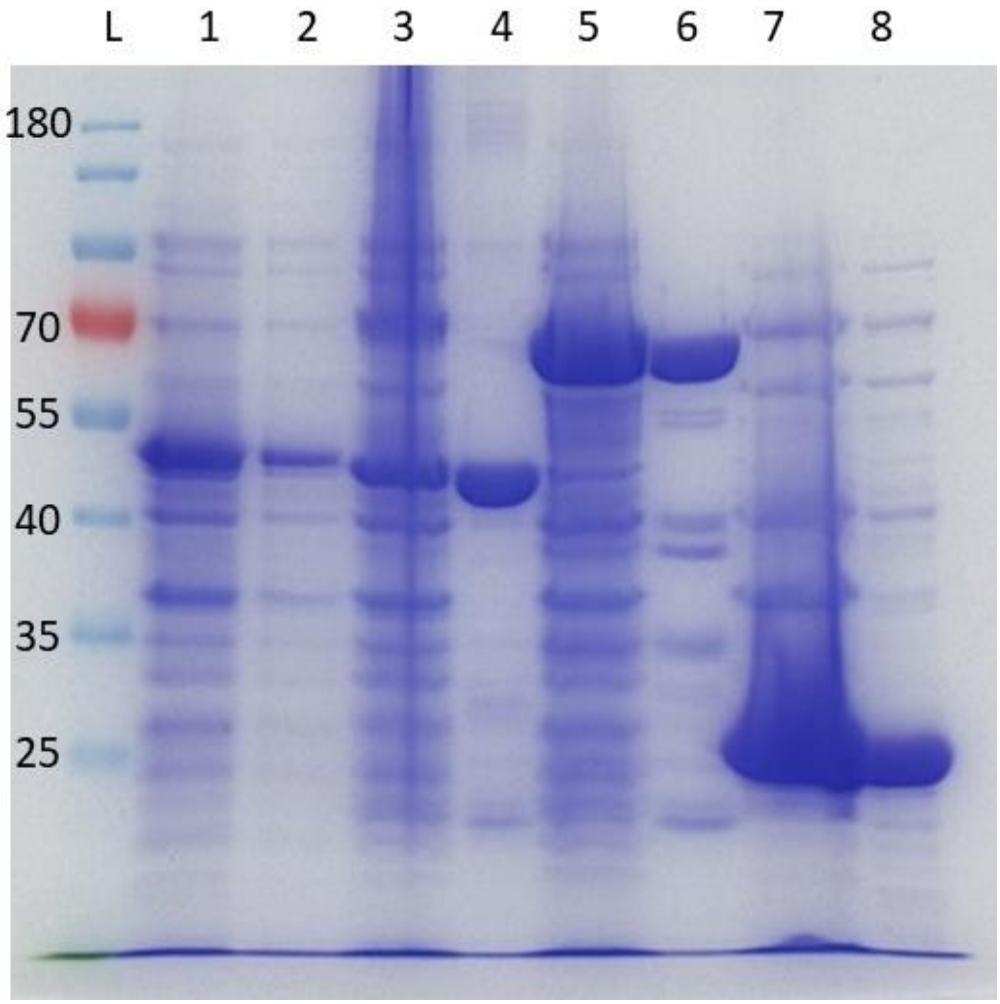

Figure 4.3: SDS-PAGE (10%) of four expressed and purified *Mmm* recombinant proteins. Lanes: L = Protein ladder (Thermo Scientific); 1= MSC-0397-expressed, 2= MSC_0397-purified (45 kDa); 3= MSC_0636-expressed, 4= MSC_0636-purified (43 kDa); 5= MSC_0653-expressed, 6= MSC_0653-purified (72 kDa); 7=LppB-expressed, 8=LppB-purified (27 kDa).

4.4.1. Determination of the best *Mmm* antigens detecting all CBPP clinical stages

In figure 4.4, there were no significance differences ($P > 0.05$) in the performance of antigens on sera from naïve animals. MSC_0636 showed a significance difference ($P < 0.05$) in detecting CBPP-infected cattle, as did LppB (figure 4.5).

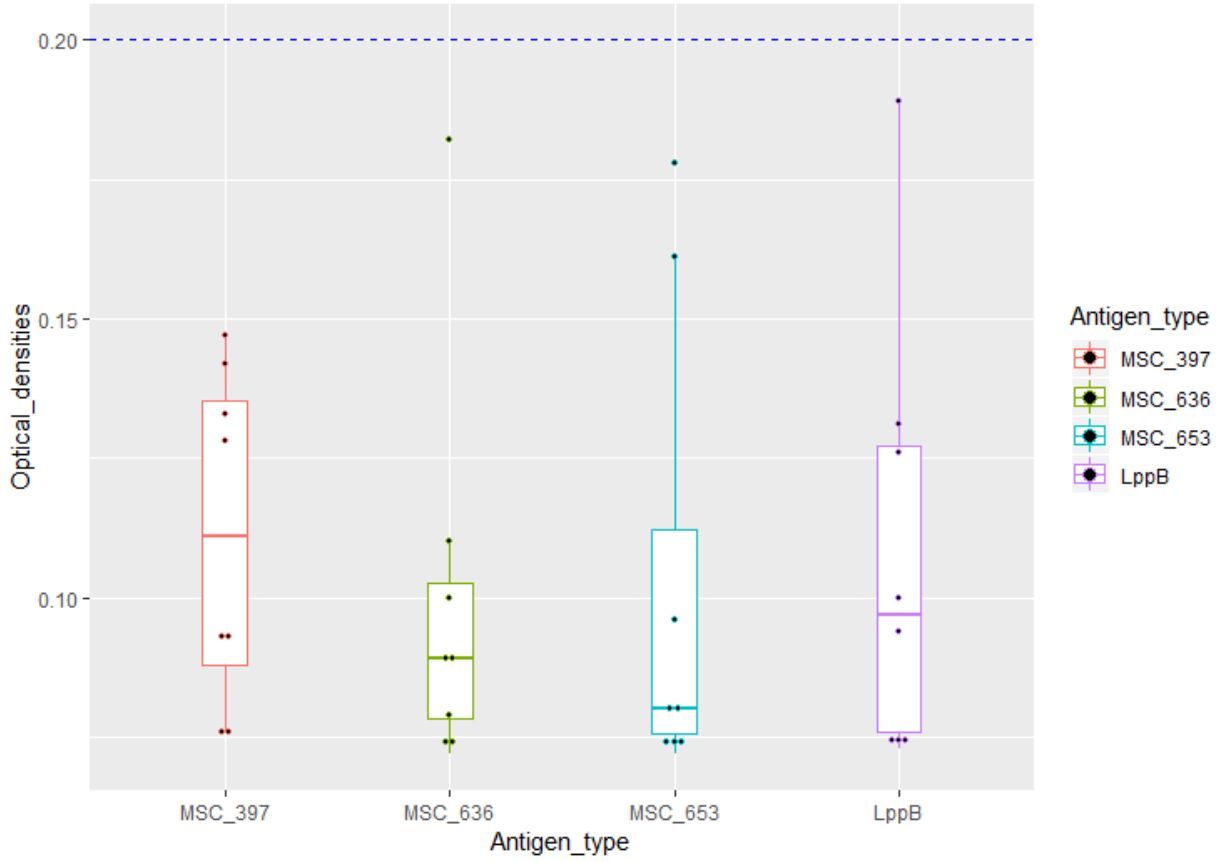

Figure 4.4: Box and scatter plots showing ODs of *Mmm* antigens screened against sera from naïve cattle. None of the antigens showed activity with sera from naïve, pre-vaccinated animals at an OD cut-off of 0.2.

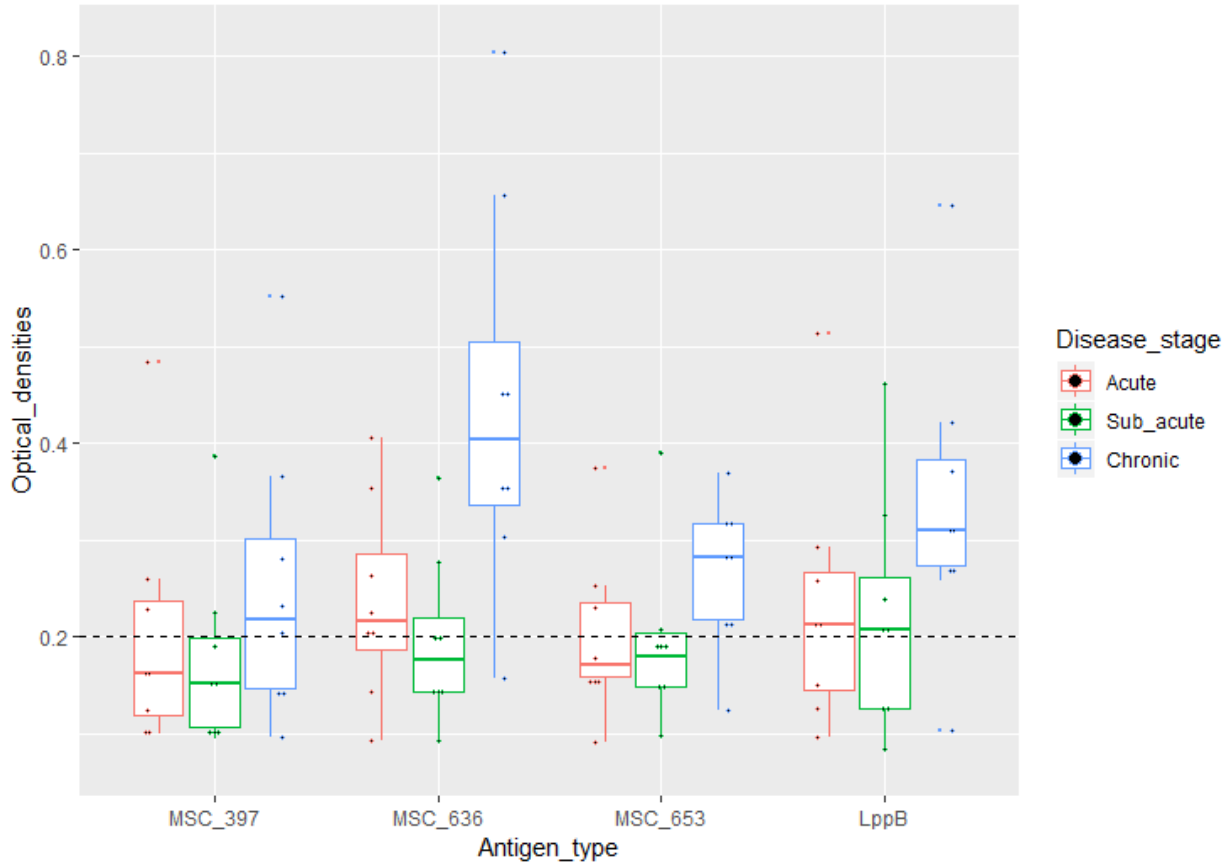

Figure 4.5: Box and scatter plots showing ODs of *Mmm* antigens screened against sera from CBPP-infected cattle. MSC_0636 significantly detected antibodies in sera from infected cattle as did LppB. As expected, the highest seroconversion of animals was observed in the chronic disease stage (blue boxes) at an OD cut-off 0.2.

4.5. Discussion

The OIE-prescribed tests for serological diagnosis of CBPP are cELISA (Le Goff and Thiaucourt, 1998) and CFT (Etheridge and Buttery, 1976). Nevertheless, these assays require well-trained staff, are mechanized, less sensitive particularly during chronic stages of the disease and none of them can detect all clinical stages of CBPP (Muuka *et al.,* 2011; Schubert *et al.,* 2011). Numerous research works have used genomic approaches to identify immunogenic *Mmm* antigens to develop vaccines and diagnostics for CBPP (Hamsten *et al.,* 2009; Perez-Casal *et al.,* 2015). The genes were tagged into plasmid vectors, expressed, and antigens purified. The purified antigens were used for setting indirect ELISA (iELISA) essays to screen sera from on-station trials from naïve and animals experimentally inoculated with *Mmm*. To enhance the accuracy of iELISA, sera were selected at different CBPP clinical stages; 3 weeks for acute, 7 weeks for subacute, and between 8 to 20 weeks for chronic disease stages. Four antigens (MSC_0397 MSC_0636, MSC_0653, and LppB) were selected for iELISA based on literature assessments and earlier laboratory conclusions that predicted or reported their higher sensitivity and specificity to detect cattle infected with *Mmm* (Miltiadou *et al.,* 2009; Naseem *et al.,* 2010; Heller *et al.,* 2016).

Previously, Naseem *et al.,* (2010) obtained both 100% sensitivity and 100% specificity in an ELISA based on hypothetical antigen, MSC_0636, and MSC_0108. The findings by Naseem *et al.,* (2010) are comparable to those obtained in this study, sensitivities of MSC_0636 and LppB being 91.7% and 83.3% with both antigens showing specificities of 100%. The results of MSC_0636 by Naseem *et al.,* (2010) and those obtained in this study seem to reach the same level with recent results by Heller *et al.,* 2016; they identified MSC_0136, MSC_0397, and MSC_0636, as the best performing proteins in a cocktail iELISA. However, the performance of

MSC_0136 was not tested in this study because it was a vaccine candidate in a parallel vaccine efficacy study. Heller *at al.,* (2016) obtained sensitivity and specificity of 85.6% and 96.4% respectively in a cocktail ELISA using MSC_0136 and MSC_0636, which was found to be in the same range with those obtained in this study. Although Naseem *et al.,* (2010) reported sensitivity and specificity of 100% using MSC_0108, the performance of this antigen was lower than MSC_0397 in a study by Heller *et al.,* (2016) using a large number of sera. Therefore, MSC_0108 was not included in this study.

The percentages of sensitivity and specificity of an ELISA depend on the sera from infected cattle, if they have high antibody titers or low. Cattle sera used in this study was not the same with previous authors and therefore, before the validation of simple diagnostic tests, more cattle sera from a lot more time points after infection should be tested. The sensitivities and specificities obtained in this study are comparable to those of other assays such as CFT and cELISA prescribed by OIE for serological diagnosis of CBPP also LppQ (Bruderer *et al.,* 2002; Sidibe *et al.,* 2012). The outcome of the best two antigens determined in this study provides proof of the concept of developing a simple pen-side test to detect CBPP infected cattle. It was also noted that all antigens used for assays in this study detected all positive sera collected at chronic stages of the disease, findings that correlate with earlier studies using LppB (Lutta *et al.,* 2018). Early diagnosis and treatment of CBPP including the ability to detect chronic stages of the disease are important since it could accelerate CBPP control and/or eradication programs by testing, isolating, and/or treating infected cattle. Treatment prevents transmission of disease from carriers to naïve animals. Macrolides such as danofloxacin and more recently tulathromycin have been reported to reduce the spread of CBPP to healthy in-contact cattle that are treated at an appropriate time (Huebschle et al., 2006; Muuka *et al.,* 2018).

Miltiadou *et al.,* (2009) characterized LppB and predicted that it could be a possible antigen for setting up a serological test. Our study confirms the prediction by Miltiadou *et al.,* (2009), based on a few well-characterized serum samples selected at different disease stages. The sensitivities and specificities of LppB were higher than MSC_0397, reported earlier by Heller *et al.,* (2016). Although Lutta *et al.,* (2018) showed that LppB detects chronically infected cattle, limited data was available to show that LppB could as well detect acute stages of CBPP. The results of this experiment show that MSC_0636 and LppB detect both acute and chronic stages of CBPP. Though iELISA in our trial was given a relatively high cut-off (OD_{405} at ≤ 0.2; titer of 1: 400) to make the most of specificity, the sensitivity was not affected as earlier reported (Dedieu *et al.,* 1996). It was also noted that all antigens showed seroconversion on sera collected on day 142 (Table 4.1). Since Heller *et al.,* (2016) indicated that MSC_0636 provoke strong antibody responses in immunized cattle, future studies should focus on investigating the humoral responses using the two selected antigens (MSC_0636 & LppB) on sera from cattle, especially those vaccinated with the recently developed subunit vaccines (Nkando *et al.,* 2016). The outcome will guide whether MSC_0636 and LppB could be used to develop a test that differentiates infected from vaccinated animals. At this point, it was not necessary to examine the cross-reactivity of our assay to antibodies from other mycoplasmas, since such cross-reactivity have never been pronounced in Africa and cannot disturb the diagnosis of an iELISA based on recombinant proteins (Gonçalves *et al.,*2008; Heller *et al.,* 2016). However, future studies should explore the possibility of cross-reactivity and if the proteins can distinguish cattle vaccinated with live attenuated vaccines before the development and validation of such assays.

4.5.1. Conclusion

Lipoprotein B and hypothetical antigen MSC_0636 were used as antigens to detect all clinical stages of cattle infected with contagious bovine pleuropneumonia. Future studies should explore the possibility of embedding these two antigens in lateral flow strip or latex agglutination test to detect naturally infected cattle in the field. Development of lateral flow devices will solve the limitation of current serological tests since the lateral test will offer results within 10 minutes, is easy to store, and transport and does not require highly trained staff and automation to perform.

CHAPTER 5: CHARACTERIZATION OF *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* ANTIGENS AS TARGETS FOR A DIVA ASSAY FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA

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5.1. Abstract

A subunit vaccine for CBPP is under development. Nevertheless, a diagnostic test for the differentiation of infected from vaccinated animals (DIVA) is still missing. This study characterized *Mmm* proteins to classify potential antigens for DIVA assays. A total of ten proteins encoding genes were codon optimized, expressed and purified for indirect ELISA (iELISA) on experimental sera from animals vaccinated with subunit vaccines and those infected with CBPP. Two-way ANOVA and least significance difference was used to separate the means of iELISA based on each of ten antigens. The results demonstrated that two vaccine antigens (MSC_0499 and MSC_0776) were the best in identifying animals vaccinated with the subunit vaccines while two non-vaccine antigens (MSC_0636 and LppB) detected all clinical stages of infected animals. The iELISA based on MSC_0499, MSC_0776, MSC_0636 and LppB *Mmm* proteins are sensitive and specific for DIVA diagnostic tests. For vaccinated serum samples, MSC_0499 iELISA provided sensitivity and specificity of 100% while MSC_0636 showed sensitivity of 87.5% and specificity of 100% on sera from infected animals. Further testing using sera from vaccinated and infected animals at different time intervals should establish how useful a cocktail of an iELISA based on MSC_0499, MSC_0776, MSC_0636 and LppB *Mmm* antigens will be.

5.2. Introduction

Mycoplasma mycoides subsp. *mycoides* (*Mmm*), the causative pathogen of contagious bovine pleuropneumonia (CBPP) in cattle, belongs to the classical '*Mycoplasma mycoides* cluster' (Manso-Silvan *et al.,* 2009). Approaches to the control of CBPP rely on vaccination with live attenuated strains, diagnosis of the sick and treatment with antibiotics, and quarantining of the infected herds (Wanyoike *et al.,* 2004). Therefore, effective control of the disease not only needs more efficient vaccines, but also dependable diagnostics (Thiaucourt *et al.,* 2003; Wesonga *et al.,* 2003; Mbulu *et al.,* 2004). Numerous live attenuated vaccines have been produced by serially culturing the bacteria in broth or embryonated eggs (Thiaucourt *et al.*, 2004). The V5 and KH3J were the first vaccines but had poor efficacy in vaccinated cattle and were therefore replaced by freeze-dried T1 strain that include T1/44 and T1/SR. Though T1/44 and T1/SR live attenuated vaccines elicit some protection, regular immunizations are essential to obtain suitable protection. Noticeable reactions at site of inoculation have been witnessed with the T1/44 strain, signifying substantial virulence, established when cattle were inoculated with the vaccine through the endobronchial way (Mbulu *et al.*, 2004).

The *Mmm* genome encodes various proteins that play a role in a host (Westberg *et al.*, 2004). Formerly, several of these proteins were characterized, including LppB (Vilei & Frey, 2001) and LppQ (Abdo *et al.,* 2000). Currently, reverse vaccinology has been used to identify more Mycoplasma proteins (Perez-Casal *et al.,* 2015). The identified antigens have since been used to formulate subunit vaccines that confer protection and do not manifest residual pathogenicity (Nkando *et al.*, 2016).

Diagnosis of CBPP employs the following: clinical signs, post-mortem findings with lungs showing grossly a fibrinous broncho-pneumonia accompanied with pleuritis and isolation

of *Mmm* from lungs (Wesonga and Thiaucourt, 2000). Also, polymerase chain reaction (Dedieu *et al.,* 1994; Miserez *et al.,* 1997; Bashiruddin *et al.,* 1999; Vilei *et al.,* 2000; Miles *et al.,* 2006), real time PCR (Gorton *et al.,* 2005; Bischof *et al.,* 2006; Lorenzon *et al.,* 2008) and serological tests: the c-ELISA of Le Goff and Thiaucourt, (1998) and CFT of Etheridge and Buttery, (1976) are used in specialized laboratories. A LppQ-ELISA was developed to differentiate infected from animals vaccinated with the current live attenuated vaccine (Bruderer *et al.,* 2002). However, this LppQ iELISA is no longer produced.

The CFT of Etheridge and Buttery, (1976) and c-ELISA of Le Goff and Thiaucourt, (1998), the only OIE prescribed serological tests have drawbacks. CFT has a high specificity but takes long to execute, requires rigorous training of staff and is less sensitive at detecting animals with chronic clinical stage of disease (Rurangirwa, 1995). On the other hand, c-ELISA is sensitive but its specificity is uncertain (Sidibe *et al.,* 2012) and both tests cannot differentiate infected from vaccinated animals. Control of CBPP will not only be attained by the use of efficient vaccines, but also reliable diagnostic strategies that will be able to differentiate infected from vaccinated animals (Thiaucourt *et al.,* 2000, Bischof *et al.,* 2009).

DIVA assay will be an important tool in control of CBPP because it will enhance discrimination of naturally infected cattle from those vaccinated with the recently develop subunit vaccines. The infected animals could then be stamped out or treated with recommended macrolides while the naïve animals will be vaccinated. This trial aimed at identifying the most potent *Mmm* antigens that can differentiate cattle infected with CBPP from those vaccinated with the subunit vaccine.

5.3. Materials and methods

5.3.1. Experimental design

The vaccines used in this study were formulated in Vaccines and Infectious Disease Organization (VIDO)-University of Saskatchewan, Canada. Vaccine C (Table 5.1) was as formulated earlier by Nkando *et al.,* (2016). Table 5.1 below shows details of trial vaccines and controls.

Table 5.1: Details of trial vaccines and control

Group	*Antigen (50µg/dose)*	*Adjuvant*	*No. of cattle (n)*
A	None (Control group)	Montanide ISA61 VG	8
B	MSC_0136, MSC_0431, MSC_0499 and MSC_0775	Montanide ISA61 VG	8
C	MSC_0136, MSC_0431, MSC_0499, MSC_0776 and MSC_0957	Montanide ISA61 VG	8

5.3.2. Sera from naive and CBPP infected cattle

Sera were obtained from the experiments described previously (Lutta *et al.*, 2017). Thirty-two serum samples were used in this study. Twenty-four of the sera were collected from eight male Zebu cattle (two to four years old, weighing 105-214 kg) that had been infected with Mmm Afade and collected at different clinical stages: acute (3 weeks), subacute (7 weeks), and chronic (above 8 weeks) of the disease. From these eight infected animals at necropsy time, three had chronic sequestrae and the other five had consolidating acute lesions. *Mmm* was isolated from lung specimens of all the 8 CBPP-infected animals. The remaining serum samples were collected from eight male naïve zebu cattle (two to four years old, weighing 105-214 kg). The sera from naïve cattle (sourced from Kakamega county, a CBPP negative area in Kenya) were well characterized and confirmed to be negative by both OIE prescribed serological tests, a CFT, and a c-ELISA.

5.3.3. Sera from control group and subunit vaccinated cattle

Male Boran cattle (one to two years old, weighing 249-390 kg) purchased from a CBPP free ranch, Kapiti Plains Estate Kenya were used in this study as previously described (Nkando *et al.*, 2016). Briefly, using Microsoft Excel's function, cattle were randomly assigned into three groups comprising 24 animals (Table 5.1). Eight of the animals from the unvaccinated group, were used as controls, while sixteen cattle were vaccinated with two test vaccines, each group consisting of eight animals. All experimenters did not know the correspondence between the groups and treatments before the end of the trial and the blinding integrity was maintained throughout the study period. The vaccines used in this study were formulated at the VIDO-InterVac. Animals in each group were restrained in a cage, vaccinated twice subcutaneously

using an 18-gauge needle with 2 ml of the vaccine formulation, first on the left side of the neck on day 0, and the booster vaccine on the right side of the neck on day 28. Sampling was done on the following days: 0, 28, 49, 60, 81, 102, and 123. The blood samples were obtained from the jugular vein and collected into labeled BD Vacutainer® tubes (Becton, Dickson & Company, USA), then allowed to coagulate at room temperature for 2 hrs. The coagulated blood was centrifuged to separate the serum which was then aliquoted into Nunc® CryoTubes® (Sigma-Aldrich®, Germany). The serum samples were transported from the site of the trial (KALRO-VSRI) to Biosciences eastern and central Africa (BecA-ILRI) and stored at -20^0 C until further serological analysis to develop a DIVA test. At sixth week post-challenge, cattle were euthanized by stunning with a captive bolt pistol and exsanguination. The carcasses were opened and lungs examined for CBPP lesions. No study animal entered the human food chain. The carcasses of all animals were disposed of in deep lime pits.

5.3.4. Expression and purification of proteins for serology

Plasmids in agar slants were sent from VIDO to BecA-ILRI and *E. coli* containing the plasmids were immediately plated on LB agar plates with 100 µg/ml ampicillin. A colony was scooped from agar plate and grown in LB broth (Sigma Life Sciences, USA) until OD_{600} was between 0.5-0.6. Western blotting, expression and purification of proteins, dialysis and estimation of proteins concentrations, iELISA, cut-off point, sensitivity, specificity, predictive values, and data analysis of antigens were performed as described in chapter 4, sub-sections 4.3.3, 4.3.4, 4.3.5, 4.3.6, 4.3.7.1, 4.3.8, and 4.3.9.

5.4. Results

Six *Mmm* proteins were evaluated as vaccine antigens in the prototype subunit vaccines, and four non-vaccine antigens selected based on their potential to detect infected cattle, as reported in previous studies were tested against sera from control groups, CBPP-infected and subunit vaccinated animals (Table 5.2).

5.4.1. Characterization of sera from control group and subunit vaccinated cattle

To define vaccine antigens that were targeted by antibody responses in naive and subunit vaccinated cattle, we determined the antibody responses against antigens in an experimental subunit vaccine. Antibodies were detected against four out of five antigens (MSC_0136, MSC_0431, MSC_0499, and MSC_0776) in the group C vaccine (Table 5.1) at or above the titer of 1:1600. None of the animals seroconverted before vaccination (Table 5.3). Figure 5.1 shows representative iELISA results using MSC_0431 from sera from the control group and the group vaccinated with the test antigens. The trend shown in figure 5.1 with MSC_0431 was also observed using other vaccine antigens, MSC_0136, MSC_0499, and MSC_0776 in the subunit vaccine group C (data not shown). However, when vaccine antigens were tested on sera from CBPP-infected cattle to establish whether they could discriminate these from subunit-vaccinated cattle, MSC_0431 detected antibodies in sera from infected cattle, rendering it unsuitable for a DIVA diagnostic test (figure 5.2). The results with MSC_0499 when used to test sera from vaccinated cattle, showed a significance difference ($P < 0.05$) to those from infected animals, as did those using MSC_0776, thus making the two antigens suitable for a DIVA test.

5.4.2. Characterization of sera from naive and CBPP infected cattle

Sera from naïve and CBPP infected cattle were tested against the non-vaccine antigens. None of the animals seroconverted before infection with *Mmm* (Table 5.3; Figure 5.3). When non-vaccine antigens were used to detect antibodies in sera from animals challenged with *Mmm*, MSC_0636 and LppB detected more animals as *Mmm* positive than MSC_0397 and MSC_0653. The iELISA results showed that MSC_0636 detected 6/8, 4/8, and 7/8 animals in acute, subacute, and chronic stages of the disease, respectively, while LppB detected 4/8, 5/8, and 7/8 animals (figure 5.4). However, MSC_0397 detected 3/8, 2/8, and 5/8 animals in acute, subacute, and the chronic stages of the disease, respectively, while MSC_0653 detected 3/8, 4/8 and 7/8 animals (figure 5.4). When non-vaccine antigens were tested on sera from subunit-vaccinated cattle to establish whether they could discriminate these from the CBPP-infected cattle, MSC_0653 detected antibodies in sera from vaccinated cattle, rendering it unsuitable for a DIVA diagnostic test (figure 5.5). MSC_0636 showed a significance difference ($P < 0.05$) in discriminating infected from vaccinated animals, as did LppB. Thus, these two antigens (MSC_636 and LppB) appear suitable for a DIVA diagnostic test.

Table 5.2: Name, type and predicted molecular weight of various proteins

Name	*Description*	*Size (KDAL)*
Vaccine antigens		
MSC_0136	Hypothetical lipoprotein	66
MSC_0431	Prolipoprotein	70
MSC_0499	Prolipoprotein	111
MSC_0775	Prolipoprotein	81
MSC_0776	Prolipoprotein	120
MSC_0957	Prolipoprotein	79
Non-vaccine antigens		
MSC_0397	Prolipoprotein	45
MSC_0636	Hypothetical lipoprotein	50
MSC_0653	Prolipoprotein	75
LppB	Lipoprotein	27

Vaccine antigens refer to proteins used to formulate prototype subunit vaccines. Non-vaccine antigens refer to proteins selected based on their potential to detect CBPP-infected cattle as found in the literature.

Table 5.3: ELISA results showing animals that seroconverted, sensitivities, specificities and predictive values

Test (Antigen)	Control		Sera from vaccinated		Se (%)	Sp (%)	PPV (%)	NPV (%)
	+Ve	-Ve	+Ve	-Ve				
MSC_0136	0/8	8/8	7/8	1/8	87.5	100	100	88.9
MSC_0431	0/8	8/8	8/8	0/8	100	100	100	100
MSC_0499	0/8	8/8	8/8	0/8	100	100	100	100
MSC_0775	0/8	8/8	7/8	1/8	87.5	100	100	88.9
MSC_0776	0/8	8/8	5/8	3/8	62.5	100	100	72.7
MSC_0957	0/8	8/8	0/8	8/8	0	100	0	50
			Sera from infected					
MSC_0397	0/8	8/8	5/8	3/8	62.5	100	100	72.7
MSC_0636	0/8	8/8	7/8	1/8	87.5	100	100	88.9
MSC_0653	0/8	8/8	7/8	1/8	87.5	100	100	88.9
LppB	0/8	8/8	7/8	1/8	87.5	100	100	88.9

This table represents results through the use of sera from naïve, CBPP-infected, and subunit-vaccinated cattle only. +Ve., positive; -Ve., negative. Cohen's kappa index value, classified as very good (1-0.76), good (0.75-0.61), acceptable (0.6-0.4), and poor (<0.4). All CBPP-negative serum samples obtained from a group of 8 cattle (n = 8) were correctly identified as negative.

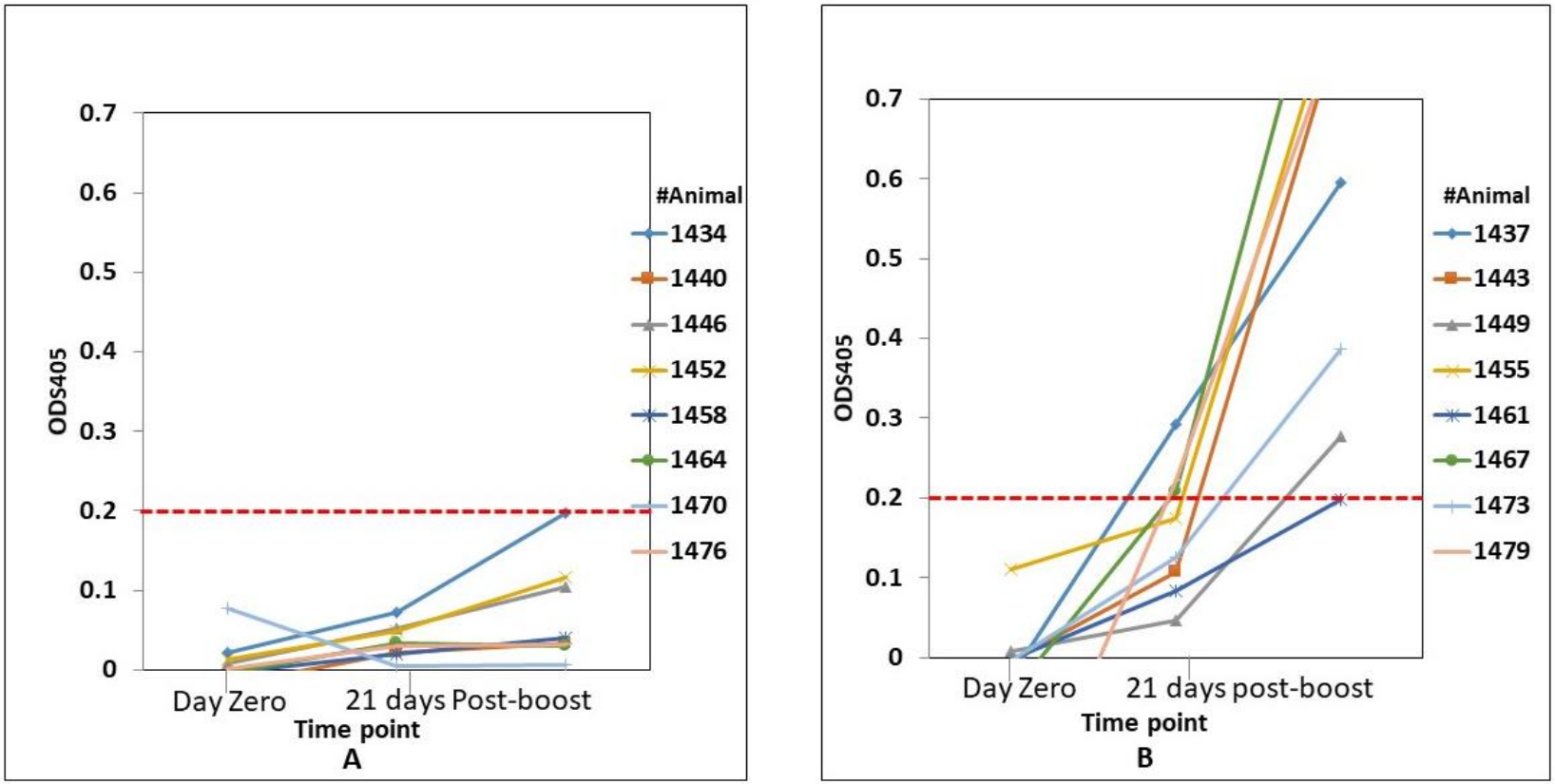

Figure 5.1: Representative iELISA results of the control group (A), and those formulated with test antigens (B). The iELISA results were based on MSC_0431 Mmm antigen screened on sera post-boost (time point at second immunization). Seroconversion was observed in the group formulated with the test antigens but not the control group.

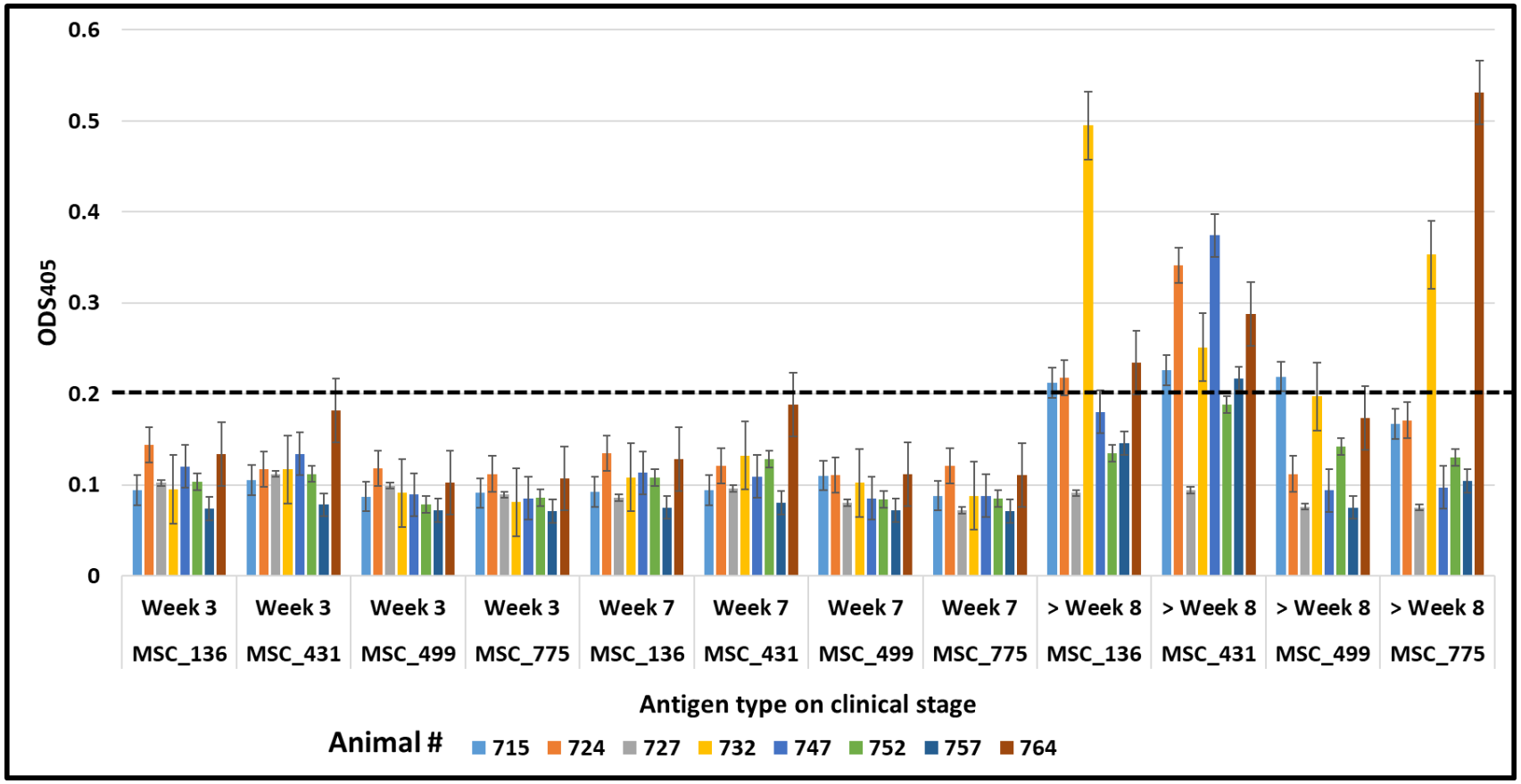

Figure 5.2: ELISA results using vaccine antigens on sera from animals infected with *Mmm*. None of the antigen showed seroconversion in acute and subacute disease stages. MSC_0431 had the highest number of animals (6/8) seroconverting in chronic stage of disease followed by MSC_0136 (4/8).

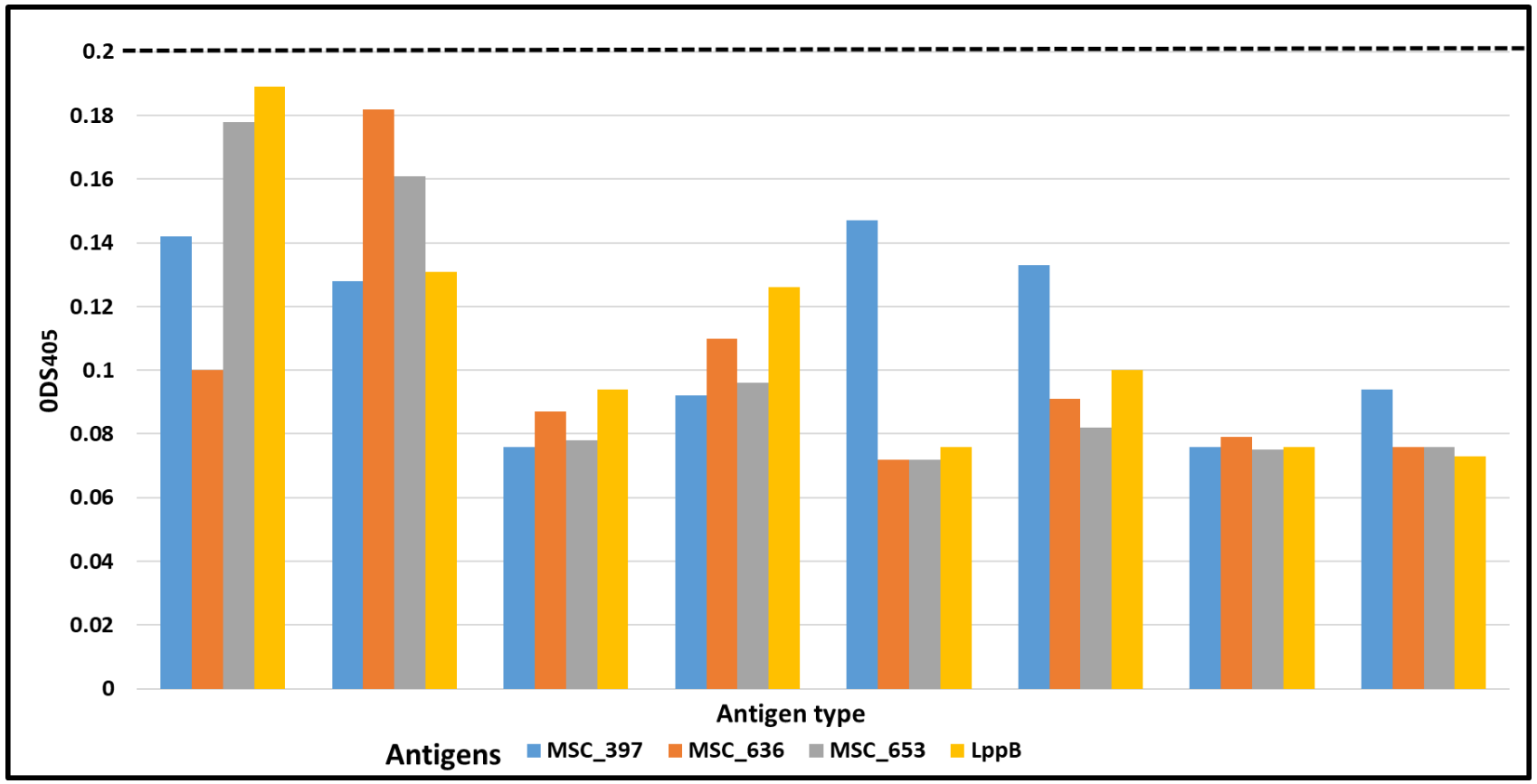

Figure 5.3: Performance of non-vaccine antigens on sera from experimental control cattle. None of the antigen showed seroconversion against the control group.

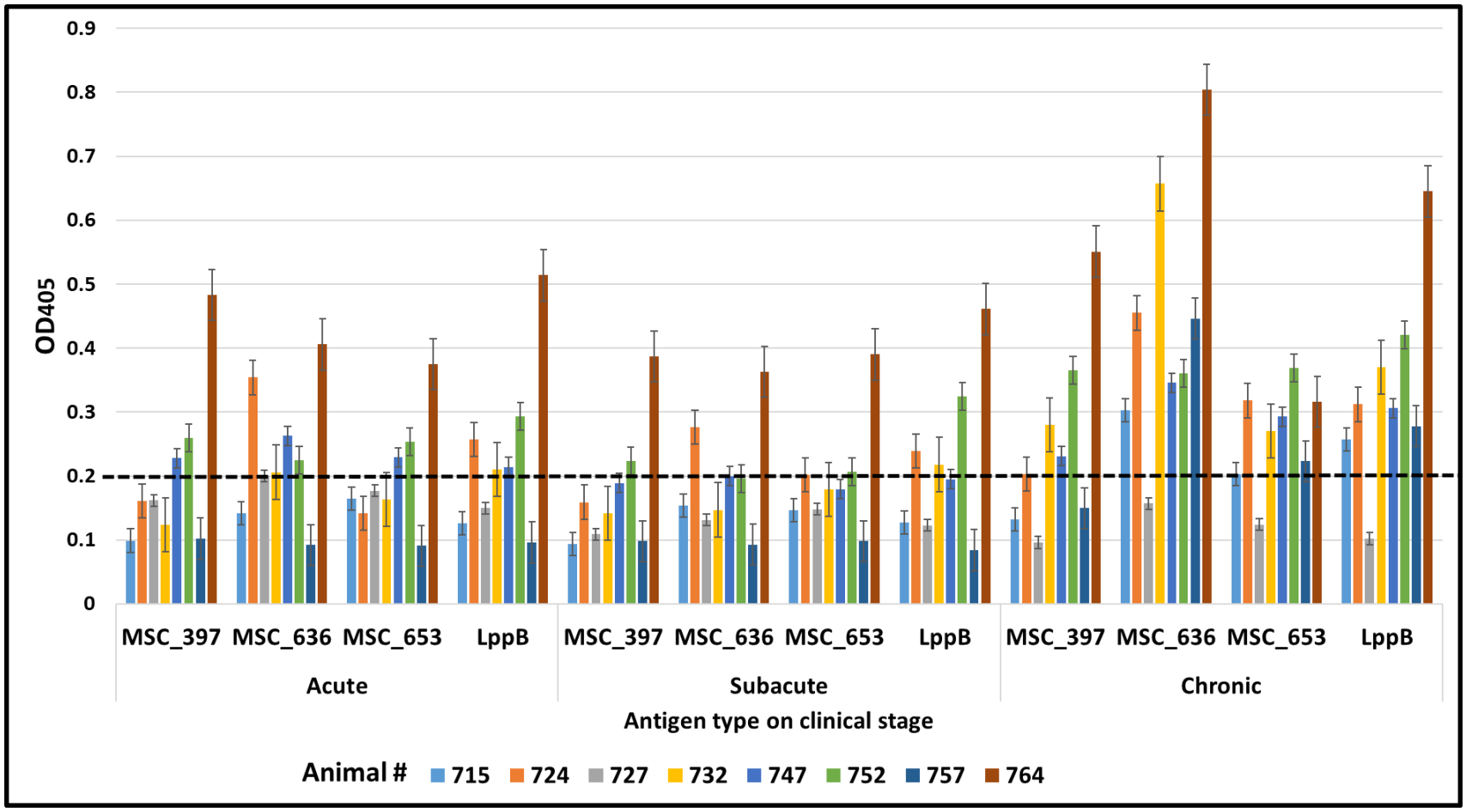

Figure 5.4: Performance of non-vaccine antigens on sera of cattle post-inoculation with *Mmm*. MSC_0636 detected more CBPP infected cattle in all disease stages followed by LppB.

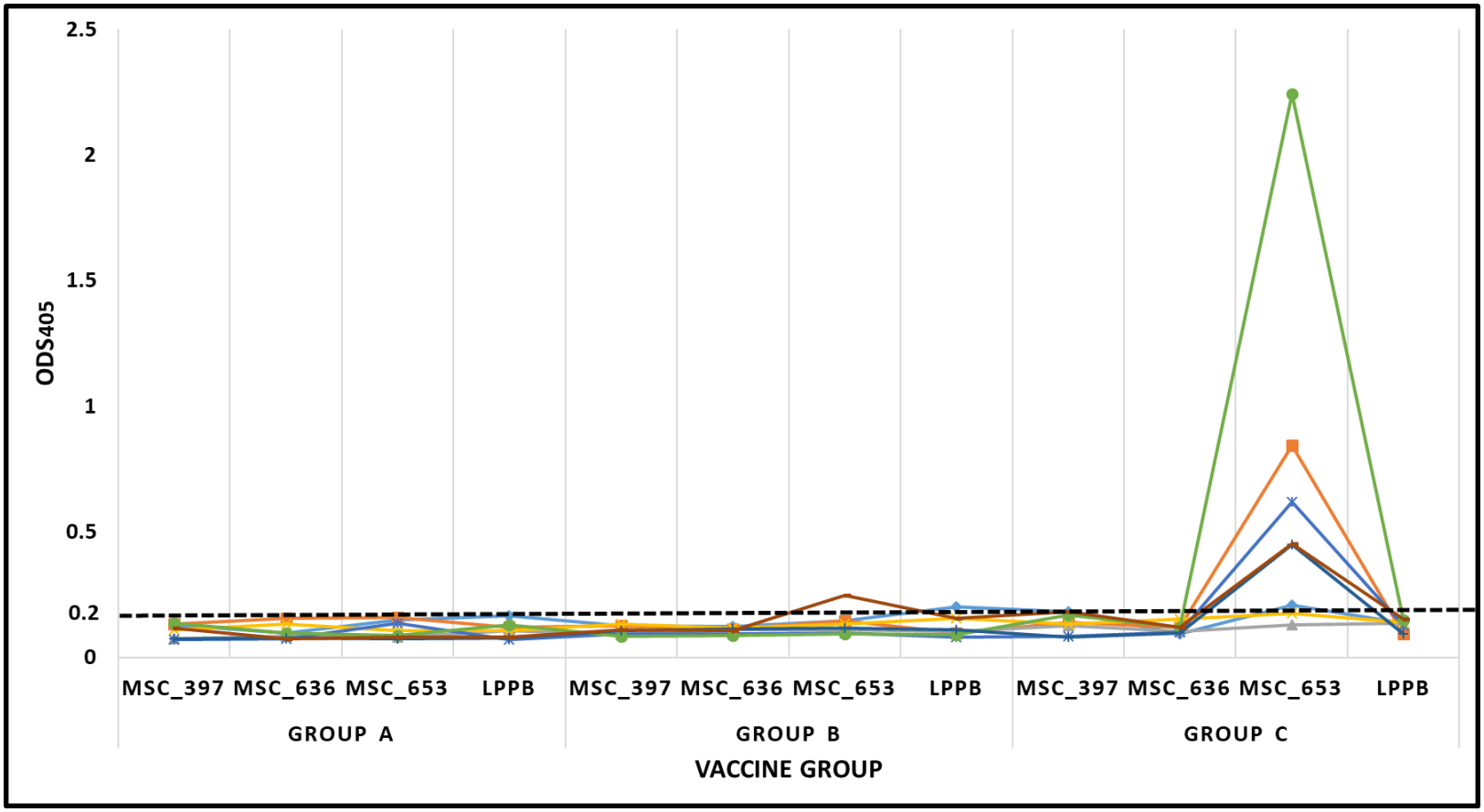

Figure 5.5: Performance of non-vaccine antigens on sera from animals vaccinated with subunit vaccines. One animal in group B vaccine and six animals in group C vaccine seroconverted against i-ELISA with MSC_0653.

5.4.3. Determination of cut-off point, diagnostic sensitivity, and specificity of *Mmm* antigens

Serum samples from naïve, CBPP-infected and vaccinated cattle returned a mean ± standard deviation OD values of 0.10 ±0.03 (OD range of 0.07-0.19), 0.23 ±0.14 (OD range of 0.15-0.66), and 0.24 ±0.20 (OD range of 0.15-0.85), respectively. The change point for negative controls was as good as the cut-off point at an OD of ≤0.2 at a wavelength of 405 nm, at a titer of 1:1600 for vaccinated animals and 1:400 for infected cattle. There was a poor index value among the iELISA based on 10 *Mmm* antigens on sera from CBPP-infected, and subunit-vaccinated cattle, respectively (Cohen's Kappa statistic k = 0.398; 0.239). The Cochran's Q test showed that the diagnostic sensitivity of MSC_0636, LppB, MSC_0499, and the MSC_0776 differed significantly from the rest of the antigens on sera from CBPP-infected and subunit-vaccinated cattle, respectively ($Q = 41.013$; $df = 9$, $p < 0.001$; $Q = 54.419$, $df = 9$, $p < 0.001$).

For the vaccine antigens, there were no significant variations in medians of ODs between *Mmm* antigens on sera from the control/naive group (figure 5.6). However, there were significant variations in medians of ODs between *Mmm* antigens post-vaccination with the prototype recombinant vaccines. MSC_0499 showed a statistically significant ability to differentiate vaccinated from infected animals as did MSC_0776 (figures 5.7 and 5.8).

When the non-vaccine antigens were assessed, MSC_0636 showed a statistically significant ability to differentiate infected from vaccinated animals, especially in the chronic clinical phase as did LppB (figures 5.7 and 5.9). MSC_0499, MSC_0776, MSC_0636, and LppB were therefore, determined to be the best antigens for a DIVA diagnostic, as they yielded specificities of 100% and sensitivities of between 62.5-100% (Table 5.3).

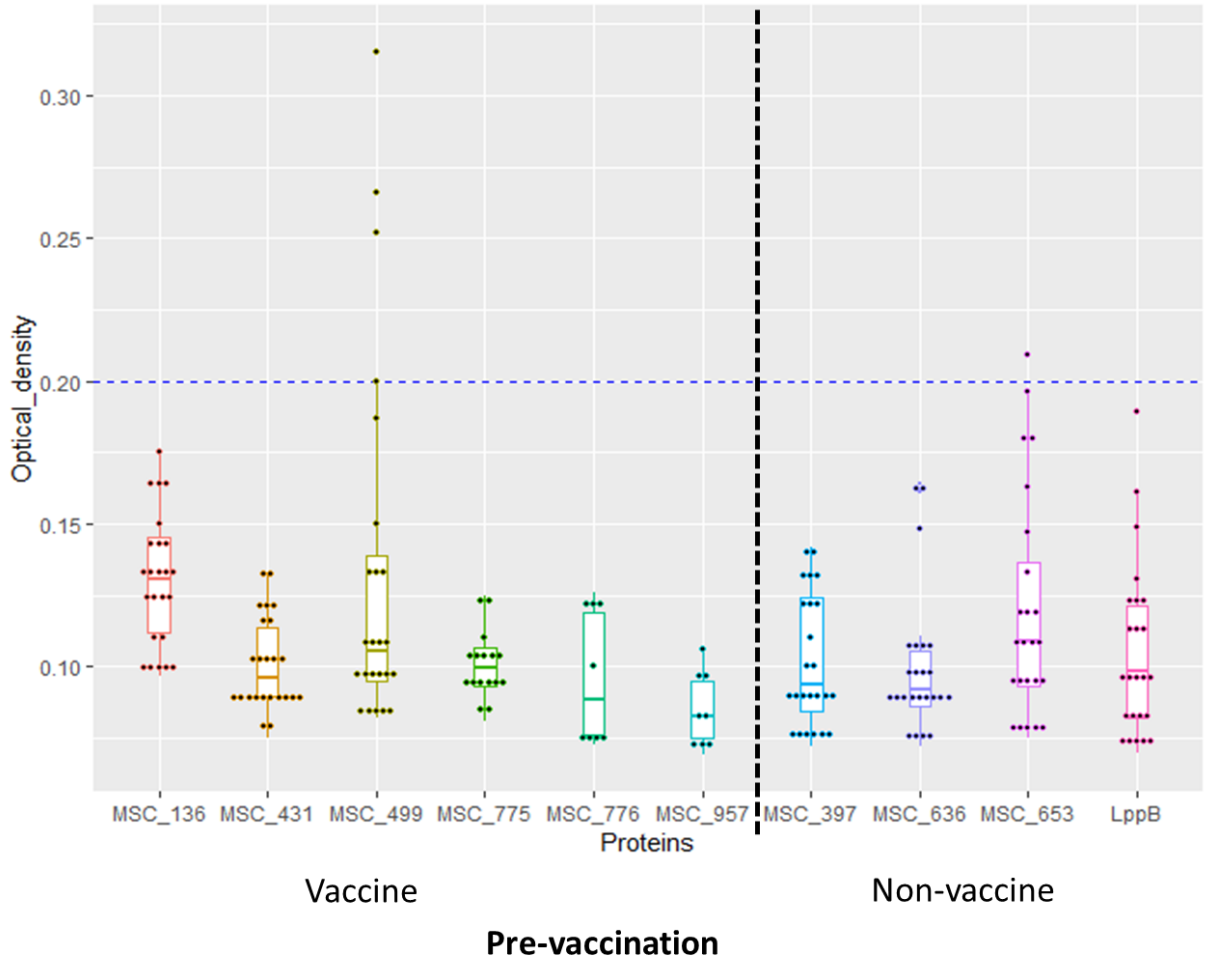

Figure 5.6: Box and scatter plots showing ODs for different *Mmm* antigens screened against sera pre-vaccination. None of the antigens showed activity with sera from naïve, pre-vaccinated animals at an OD cut-off of 0.2.

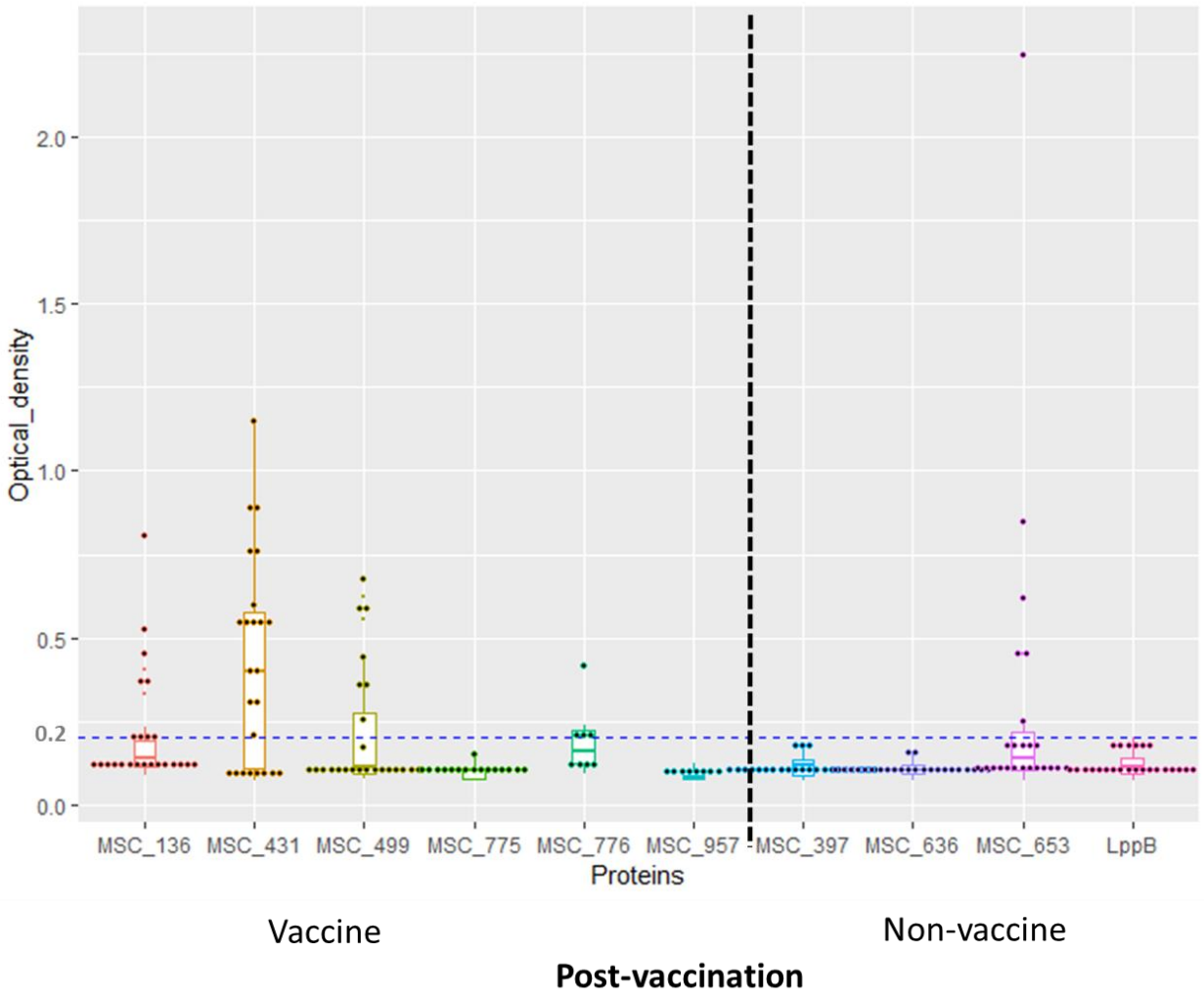

Figure 5.7: Box and scatter plots representing ODs for *Mmm* antigens screened against sera from vaccinated cattle. The horizontal blue dotted line is at OD = 0.2. Although MSC_0653 was a non-vaccine antigen and not part of the prototype vaccines, the antigen detected antibodies in sera from vaccinated animals.

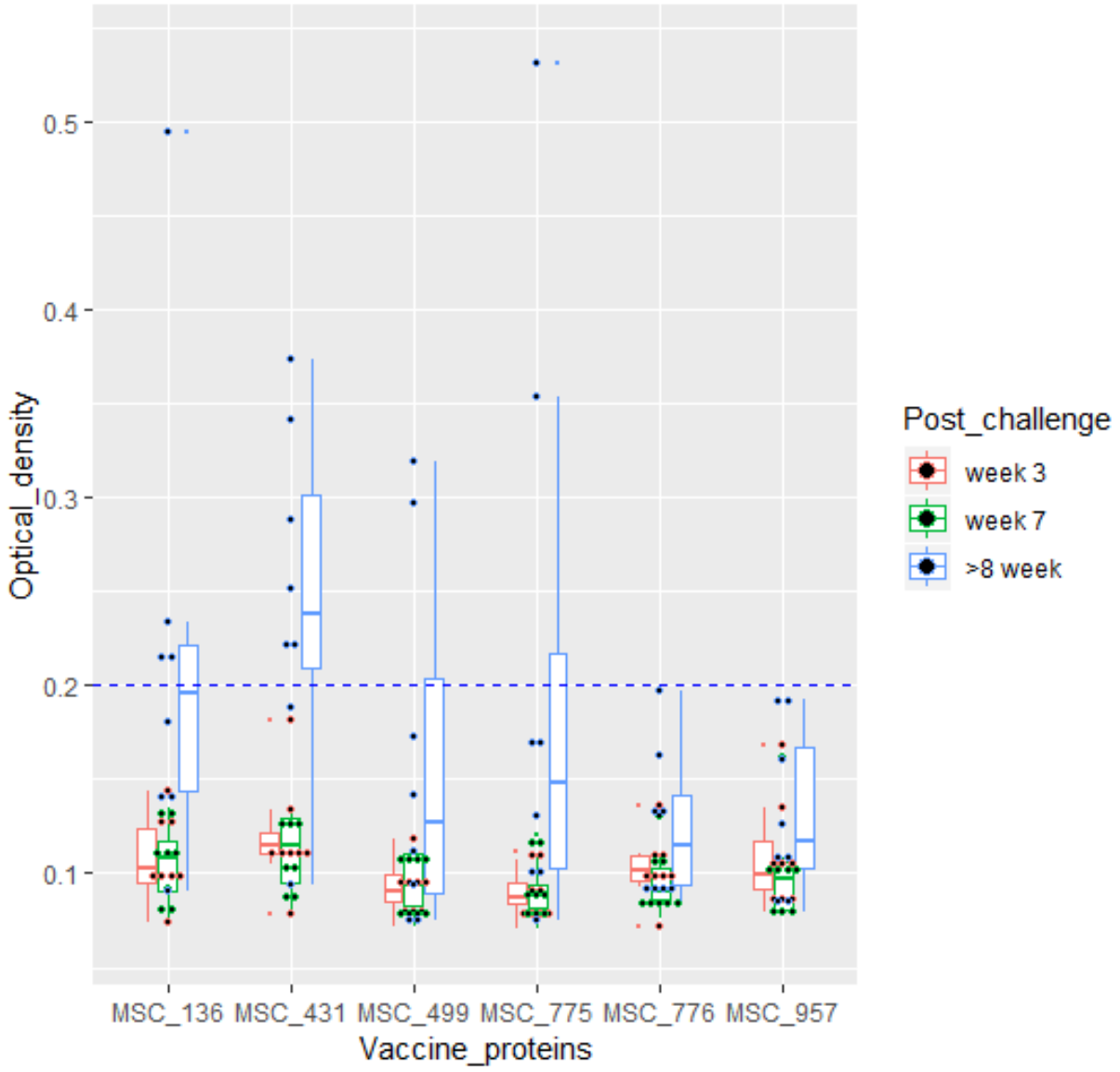

Figure 5.8: Box and scatter plots showing ODs of vaccine antigens screened against sera from CBPP-infected cattle. Although MSC_0431 and MSC_0136 were part of the prototype vaccines, the antigens detected more antibodies in sera from chronically infected animals at an OD cut-off 0.2.

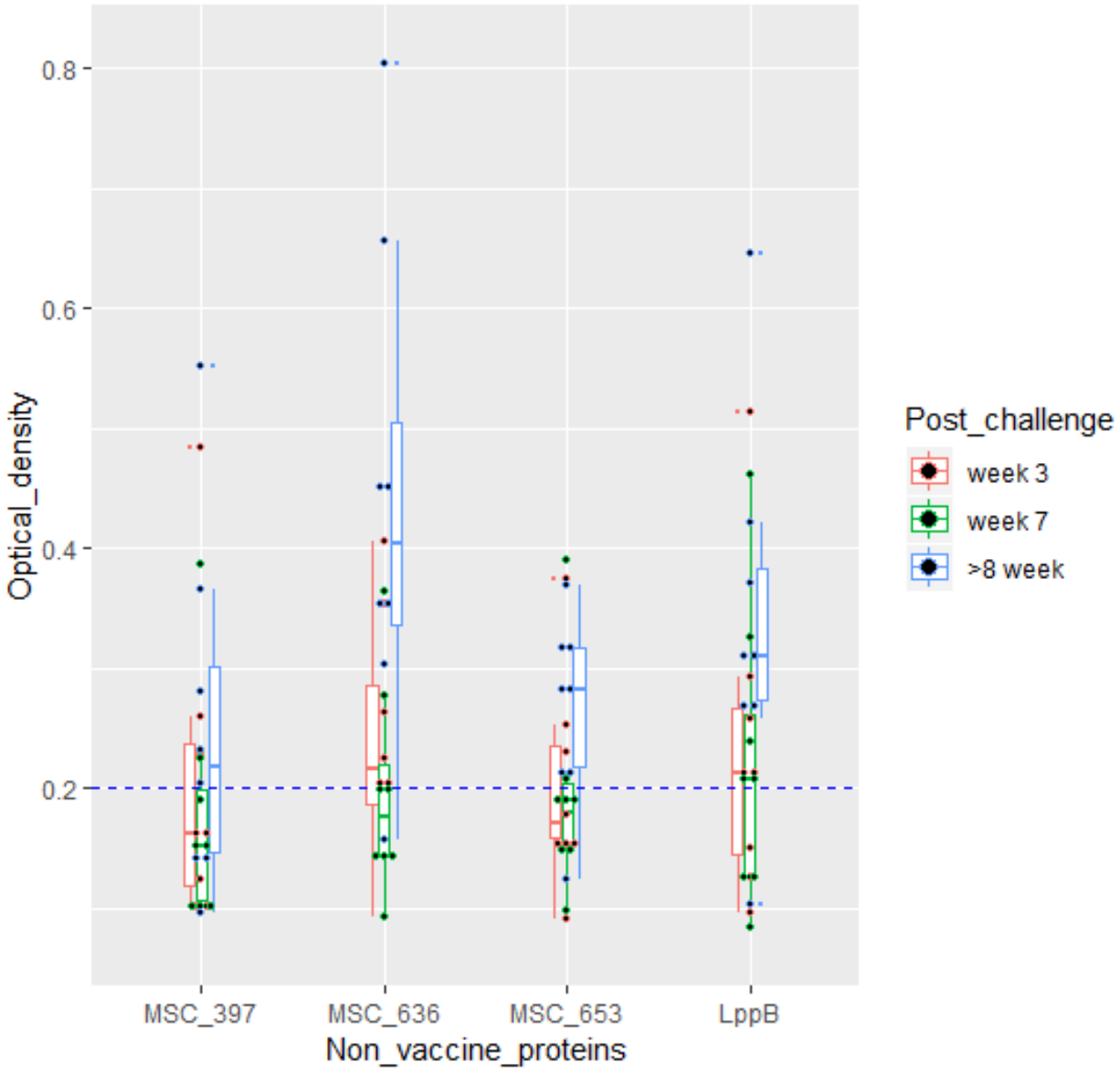

Figure 5.9: Box and scatter plots showing ODs of non-vaccine antigens screened against sera from CBPP-infected cattle. MSC_0636 significantly detected antibodies in sera from infected cattle as did LppB. As expected, the highest seroconversion of animals was observed in the chronic disease stage (blue boxes) at an OD cut-off 0.2.

5.5. Discussion

Recently, KALRO, VIDO and ILRI laboratories developed subunit vaccines using *Mmm* recombinant antigens and affirmed that they protect cattle against experimental endotracheal challenge with *Mmm* Afade strain (Nkando *et al.,* 2016). In Phase 1, the study tested the protective effect of 14 pools of five recombinant antigens on a CBPP challenge, and three of these pools showed clear indications of a reduction in the pathological index (Nkando *et al.,* 2016). In Phase II, the formulations of phase I recombinant vaccines was evaluated by selecting those that showed the strongest immune responses as earlier described (Nkando *et al.,* 2016); 3 antigens from Phase1 group A and 1 antigen from Phase1 group C, plus one extra group vaccinated with the preparations for groups A as used in Phase 1. The newly formulated subunit vaccine based on a combination of four recombinant proteins offered an efficacy of 81% in the Zebu breed of cattle (Data not published). The vaccine formulations have since been patented (International Patent Application No. PCT/CA2016/050864) and are under development. In this study, the *Mmm* antigens were characterized then used to analyze humoral responses in cattle infected with *Mmm* Afade strain, those vaccinated with the subunit vaccines and the control groups.

Several studies describe the development of DIVA diagnostics/vaccines for *M. bovis* (Zhang *et al.,* 2014; Han *et al.,* 2015; Khan *et al.,* 2018). Zhang *et al.,* (2014) used the *M. bovis*-150 strain to develop the live attenuated vaccine that elicited protection against a challenge with the virulent strain of *M. bovis* HB0801. DIVA assays using sodium thiocyanate (NaSCN) or recombinant proteins assay for use alongside the *M. bovis*-150 live attenuated vaccine have been reported (Han *et al.,* 2015; Khan *et al.,* 2018). Han *et al*. (2015), used NaSCN in a competitive iELISA for the detection of IgG. Khan *et al.,* (2018), used proteomic techniques to identify and characterize membrane-associated proteins of *M. bovis* HB0801 and its attenuated strain (*M.*

bovis-150). The results identified a putative lipoprotein encoded by a functionally unknown gene MbovP730 that demonstrated high sensitivity and specificity in an IgG iELISA. A Western blot test confirmed that MbovP730 is absent in attenuated *M. bovis*-150, indicating that this antigen could be used to develop a DIVA assay.

In a study to establish a DIVA diagnostic test to be used in combination with the CBPP live-attenuated vaccine, a very immunogenic antigen, LppQ was characterized and an LppQ-knock-out derivative of the T1/44 live attenuated vaccine strain, the T1LppQ-MT1 was created (Abdo *et al.,* 2000; Janis *et al.,* 2008; Dedieu *et al.,* 2009). It was suggested that LppQ could be used in a DIVA assay for discriminating animals vaccinated with the depleted T1LppQ-MT1 strain from those infected with the strains from the field (Bruderer *et al.,* 2002; Vilei and Frey, 2010). However, a genetically modified live attenuated vaccine might not be easily adopted by regulatory agencies, and there remain the disadvantages associated with a live attenuated vaccine including, the need for cold chain and possible side effects at the site of inoculation (Dedieu-Engelmann, 2008).

In this trial, an amalgamation of extensive literature review, genomics, and immuno-proteomics was used to identify more specific and sensitive proteins for developing DIVA assays. A total of 6 *Mmm* vaccine genes (MSC_0136, MSC_0431, MSC_0499, MSC_0775, MSC_0776, and MSC_0957) and 4 *Mmm* non-vaccine genes (MSC_0397, MSC_0636, MSC_0653, and LppB) were codon-optimized, expressed and antigens purified for setting up iELISAs. Amongst the 10 antigens used in this study, MSC_0499, MSC_0636, MSC_0776, and LppB were determined as sensitive and specific antigens for DIVA in an iELISA platform. The MSC_0499 and MSC_0776 were selected as best performing vaccine antigens because they detected vaccinated animals and showed very little reactivity on sera from infected animals. The MSC_0636 and LppB were selected as best performing non-vaccine antigens because they

detected infected animals but showed very little reactivity on sera from vaccinated animals. However, it should be noted that in this study, a relatively low number of animals were available. But the data suggest that MSC_0499 and MSC_0636 serological assays might be currently the most sensitive and specific methods for diagnosis of CBPP

5.5.1. Conclusion

This study characterized ten *Mmm* antigens for use in a potential novel DIVA diagnostic for CBPP. The MSC_0499, MSC_0776, MSC_0636, and LppB proteins were able to differentiate cattle vaccinated with the subunit vaccine from those infected with a virulent *Mmm* Afade strain. This preliminary analysis shows that *Mmm* antigens are potential targets for developing a DIVA diagnostic assay, though further testing of field sera from vaccinated and infected animals collected at different time intervals, and a cocktail made of the four antigens, should be undertaken to establish how useful a diagnostic test based on *Mmm* antigens will be.

CHAPTER 6: GENERAL DISCUSSIONS AND CONCLUSIONS

This thesis outlines sequences of on-station and laboratory trials involving recombinant *Mmm* antigens to establish whether they can differentiate cattle infected with CBPP from those vaccinated with the recently developed subunit vaccines. The *Mmm* antigens were characterized then used to analyze humoral responses in cattle infected with *Mmm* Afade strain, those vaccinated with the subunit vaccine, and control groups.

Two procedures of inoculating cattle with *Mmm* were compared (Chapter 2), by analyzing clinical signs and pathological outcomes in three controlled trials. The two models of inoculating cattle compared were endotracheal intubation and contact transmission. Although differences in severity have been mentioned before (Huebschle *et al.,* 2003; Scacchia *et al.,* 2011), it was never carried out in a large number of cattle in a statistical way. In this study, data were pooled from three different experiments during controlled on-station trials conducted at KALRO-VSRI. This trial aimed at identifying a suitable model of inoculation of cattle with *Mmm* that would transmit the disease at the same time to all animals, so that disease development between groups of animals can directly be compared. Infection by intubation produced a milder form of the disease, but its advantage was that the precise moment of infection was known, the day of intubation, and symptoms occurred in all animals around the same time, even between experiments. In contrast, with contact transmission, the exact time of inoculation in each animal is not known, as it depends on close contact between animals (Dedieu *et al.,* 2005), so the disease could occur at very different times after infection. There were also differences in pathology between the three experiments. In subsequent CBPP trials in our laboratories, cattle have been infected by endotracheal intubation.

Chapters 3 and 4 describe the best antigens that detected both acute and chronic stages of CBPP. In chapter 3, the LppB *Mmm* antigen was tested for its ability to detect humoral responses

in cattle infected with *Mmm* in an indirect ELISA platform. This protein had been characterized before and predicted to be a potential diagnostic antigen (Miltiadou *et al.,* 2009). Therefore, the LppB antigen was tested against sera from cattle infected with *Mmm* and naïve control group. Sera obtained from naive and CBPP-infected cattle were compared using CFT and LppB-iELISA. The iELISA developed with LppB antigen detected more positive samples than the CFT, which is considered a gold standard. The observed differences between the sensitivities obtained in this study and previous studies (Muuka *et al.,* 2011) of 68.8% using LppQ antigen, are probably due to the disease status of the animals after challenge and the time point at which sera were collected. Sera in this study were obtained from animals infected by the contact method, and there is always uncertainty on the time point at which a particular animal gets infected (Lutta *et al.,* 2017). Although the specificities of both CFT and LppB tests were above 87.5% in this study, significantly lower sensitivity by CFT of 17% was observed. The low sensitivity of CFT could have been because of various factors: i) during the acute phases of the disease, CFT detects both IgM and IgG1 that bind complement but not IgG2 (Le Goff and Thiaucourt, 1998) and thus may miss chronic carriers ii) the immune status of the individual animal play a role in the level of exact antibody production (Schubert *et al.,* 2011). The higher sensitivity of cELISA (64%), was because it strongly detects IgG2 and therefore can identify positive animals in later stages of disease compared to CFT (Niang *et al.,* 2010). The iELISA with LppB detected all cattle with the chronic clinical stage of the disease. The LppB detected the highest number (100%) of positive serum samples in cases with sequestrae and 70% of animals without lesions.

In chapter 4, four well-characterized antigens were used to describe humoral immune responses on sera from cattle that had been inoculated with the Afade strain of *Mmm.* The four antigens (MSC_0397, MSC_0636, MSC_0653, and LppB) were selected based on extensive literature review and earlier laboratory conclusions (Hamsten *et al.,* 2009; Miltiadou *et al.,* 2009;

Naseem *et al.*, 2010; Perez-Casal *et al*., 2015; Heller *et al.*, 2016; Nkando *et al.*, 2016; Khan *et al.*, 2018). This chapter aimed to develop user-friendly assays using *Mmm* recombinant antigens that could detect both acute and chronic stages of the disease. The genes were tagged into plasmid vectors, expressed, and proteins purified. The purified proteins were used for setting iELISA to screen sera from on-station trials from naïve and animals experimentally inoculated with *Mmm*. To enhance the accuracy of iELISA, sera were selected at different CBPP clinical stages as follows; 3 weeks for acute, 7 weeks for subacute, and between 8 to 20 weeks for chronic disease stages. In this study, the performance of MSC_0636 in chronic clinical cases was the best compared to other antigens post-inoculation with *Mmm*, as did LppB. The MSC_0636 and LppB detected both the acute and chronic stages of CBPP. The findings observed in this study equals those reported by other authors (Naseem *et al*., 2010; Heller *et al*., 2016; Lutta *et al*., 2018). The outcome of the best two antigens (MSC_636 and LppB) used in this study, provided proof of the concept of developing a test to detect both acute and chronic clinical stages of CBPP.

In chapter 5, *Mmm* proteins were characterized for DIVA assays. An amalgamation of extensive literature review, genomics, and immuno-proteomics was used to identify more specific and sensitive proteins for developing DIVA assays. A total of 6 vaccine genes (MSC_0136, MSC_0431, MSC_0499, MSC_0775, MSC_0776, and MSC_0957) and 4 non-vaccine genes (MSC_0397, MSC_0636, MSC_0653, and LppB) were codon-optimized, expressed and proteins purified for iELISA on experimental sera from animals vaccinated with subunit vaccines, those inoculated with *Mmm* and control groups. The goal of chapter 5 was to identify the best antigens that could differentiate animals vaccinated with the novel subunit preparation from those infected with CBPP.

Amongst the 10 antigens used in this study, MSC_0499, MSC_0776, MSC_0636, and LppB were determined as sensitive and specific antigens for DIVA assays in an iELISA platform.

The MSC_0499 and MSC_0776 were selected as the best antigens for detecting antibodies in sera from subunit-vaccinated cattle because they detected vaccinated animals and showed very little reactivity on sera from CBPP-infected animals. The MSC_0636 and LppB were selected as the best antigens for detecting antibodies in sera from CBPP-infected cattle because they detected antibodies in sera from infected animals and showed very little reactivity on sera from subunit-vaccinated cattle. The data in this study suggest that iELISA based on MSC_0499 and MSC_0636 *Mmm* antigens were the most sensitive and specific methods for diagnosis of CBPP and DIVA assays. Although this is the first study to reported the MSC_0499 as the best vaccine antigen to discriminate infected from vaccinated animals, several studies recommended MSC_0636 in detecting animals infected with *Mmm.* Naseem *et al.*, (2010) and Heller *et al.*, (2016) reported sensitivity and specificity of (MSC_0636) to be above 96.4% respectively. These findings are comparable to those obtained in this study, sensitivity, and specificity of MSC_0636 being 87.5% and 100% respectively.

In conclusion, during the vaccine safety and efficacy studies, endotracheal intubation was used to inoculate animals with *Mmm* because, with this model, the time of infection of cattle was precisely the same. This study characterized ten *Mmm* antigens for use in a potential novel DIVA diagnostic for CBPP infected cattle. The MSC_0499, MSC_0776, MSC_0636, and LppB proteins were able to differentiate cattle vaccinated with the subunit vaccine from those infected with a virulent *Mmm* Afade strain. This preliminary analysis shows that *Mmm* antigens are potential targets for developing DIVA diagnostic assays. Future studies should explore the possibility of developing more friendly field applicable assays such as lateral flow or latex agglutination tests, using MSC_0499, MSC_0776, MSC_0636, and LppB *Mmm* antigens to discriminate CBPP infected from subunit vaccinated. This will solve the limitation of current serological tests since the lateral flow or latex agglutination tests offer results within 10 minutes, easy of transportation,

can differentiate CBPP infected from subunit vaccinated cattle, and no requirement for highly trained staff and automation.

6.1. Recommendations

1. Further testing of field sera from naïve, CBPP-infected, and subunit-vaccinated animals collected at different time intervals should be carried out before validation and commercialization of DIVA assays.
2. ELISAs based on cocktails made of the four selected *Mmm* antigens should be tested to establish how useful diagnostic assays will be.
3. Communication factors leading to the adoption of the developed CBPP DIVA diagnostics should be established during field validation studies.

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APPENDIX:

SOLUTION AND BUFFER COMPOSITIONS

1. **Preparation of 1% agarose gel:**

   Measure 1 g of agarose powder, mix with 100 ml of Tris-Borate-EDTA (TBE buffer), and boil for 2 min. Add 2.5 µl of gel red (for 2 gels) and boil for 30 secs. Stir using a magnetic stirrer for 2 min, boil for 30 secs, and allow to cool. Pour on the gel tank with combs and wait for 30 min. Remove combs and load the DNA samples as follows: 1 kb ladder (6 µl); 1.5 µl undigested sample + 1 µl dye (control); 5 µl of single digest (Bam HI); 10 µl of a double digest (Bam HI/Hind III).

2. **ELISA solutions and buffers:**

   - para-Nitrophenylphosphate (pNPP) substrate

   990 ml distilled water, 10 ml diethanolamine, 1 ml 500 mM $MgCl_2$, adjust pH to 9.8 with HCL and add 1 mg PNPP powder/ ml of the above buffer.

   - Phosphate buffered saline (1x)

   8g NaCl, 0.2g KCL, 1.44g Na_2HPO_4, 0.24g K_3PO_4. Top up to 1 liter of distilled water, mix and autoclave at 121^0 C for 15 min.

3. **Protein expression and purification solutions and buffers:**

   - Ampicillin

   100 mg/ml of powder in distilled water, pass solution through

   0.4 µm sterile filter, store aliquots at -20^0 C.

   - Elution buffer (1 liter)

   0.1M NaH_2PO_4 (MW 137.99 g/mol), 1.5 M NaCl (MW 58.4 g/mol) and 480.5 g urea (MW 60.06 g/mol), pH 7.4. Add 250 mM Imidazole (stock solution is 2 M) before use. Pass through 0.4 µm sterile filter.

- Isopropyl β-D-1-thiogalactopyranoside (IPTG):

238 mg/ml of powder in dH_2O, sterile filter using 0.4 µm filter, store in aliquots at -20°C.

- Lysis buffer (1 liter):

13.8 g NaH_2PO_4 (MW 137.99 g/mol), 1.2 g Tris base (MW 121.1 g/mol) and 480.5 g (MW 60.06 g/mol) urea. Adjust pH to 8.0 using NaOH. Pass through 0.4 µm sterile filter.

- Wash buffer (1 liter)

13.8 g NaH_2PO_4 (MW 137.99 g/mol), 1.2 g Tris base (MW 121.1 g/mol) and 480.5 g urea (MW 60.06 g/mol). Adjust pH to 6.3 using HCl. Pass through 0.4 µm sterile filter.

4. SDS-PAGE sample solutions and buffers:

- 30% Acrylamide/0.8% Bisacrylamide

Mix 30.0 g acrylamide and 0.8 g N, N'-methylene-bisacrylamide in a total volume of 100 ml dH_2O. Filter the solution through a 0.45 µm filter and store at 4^0 C in the dark.

- Coomasie blue staining buffer

1.25g Coomasie blue, 500 ml Methanol, 100 ml Acetic acid, and 400 ml dH_2O. Mix by stirring for 30 min and sterile filter using a 0.4 µm filter.

- Destaining buffer

600 ml Methanol, 140 ml Acetic acid, and 1260 ml dH_2O.

- Running buffer (1x)

3.0 g Tris base, 14.4 g Glycine, 1.0 g SDS. Top up with 1000 ml dH_2O.

- Sample buffer (2x)-16 ml

10.4 ml distilled water, 1.2 ml 0.5 M Tris pH 6.8, 1.9 ml Glycerol, 1.0 ml 20% SDS, 0.5 ml Mercaptoethanol, 1 ml Bromophenol blue.

- Sample buffer (5x)-16 ml

6.8 ml distilled water, 2.0 ml 0.5 M Tris pH 6.8, 3.2 ml Glycerol, 1.6 ml 20% SDS,

0.8 ml Mercaptoethanol, 1.6 ml 1% Bromophenol blue.

- 10% SDS-PAGE separating gel (for 2 gels)

6.25 ml dH_2O, 3.75 ml 1.5 M Tris/SDS, 5 ml Acrylamide/Bisacrylamide, 100 µl 10% Ammonium persulfate and 10 µl Temed.

- SDS-PAGE stacking gel (for 2 gels)

3.05 ml dH_2O, 1.25 ml 0.5 M Tris/SDS, 650 µl Acrylamide/Bisacrylamide, 50 µl 10% Ammonium persulfate and 10 µl Temed.

- 4x Tris-Cl/SDS, pH 6.8 (0.5 M Tris-Cl containing 0.4% SDS

Dissolve 6.05 g Tris-base in 40 ml dH_2O. Adjust to pH 6.8 with HCl. Add dH_2O to 100 ml in total volume. Filter the solution through a 0.45 µm filter. Add 0.4 g SDS, and store at 4^0 C.

- 4x Tris-Cl/SDS, pH 8.8 (1.5 M Tris-Cl containing 0.4% SDS

Dissolve 91 g Tris-base in 300 ml dH_2O. Adjust to pH 8.8 with HCl. Add dH_2O to 500 ml in total volume. Filter the solution through a 0.45 µm filter. Add 2 g SDS, and store at 4^0 C.

5. Western blotting buffers:

- Transfer buffer

25 mM Tris, 190 mM Glycine, 20% Methanol. Add 0.1% SDS for protein larger ≥80 kD.

- Tris-buffered saline with Tween 20 (TBST) buffer

20mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20.