EXPERIMENTAL STUDY ON VACCINES: *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* SMALL COLONY TYPE BIOFILM. DIAGNOSTIC TECHNIQUES AND HUMORAL IMMUNE RESPONSE IN CONTAGIOUS BOVINE PLEUROPNEUMONIA CATTLE IN NAMIBIA

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DEDICATION

I dedicate this thesis

To my husband Daniel Ndjai Zaire, our children Rakotoka, Undjee and Uetuesa

To my parents, late dad Samuel Tjipura and mom Godfriedine Tjipura

In memory of

Otto Hübschle

2008

ABSTRACT

The control of contagious bovine pleuropneumonia (CBPP) has been identified as a priority by the Organisation of African Union/Inter-African Bureau of Animal Resources.

This project aimed to address some of the issues associated with the diagnosis and control of CBPP infections in cattle. Experimental studies tested current and novel vaccines which were used to provide more information about the importance of humoral immune response to CBPP and assess current and new diagnostic tests. Differences between infections caused by planktonic and biofilm grown isolates were also studied to assess if biofilm grown cells remain infective or are more virulent.

Two novel vaccine formulations, one consisting of five purified proteins and one using tween 20 washed cells, both formulations were combined with a commercial adjuvant designed to help stimulate the potentially prectective Th2 response. These novel vaccines efficacy was compared with the current T1/44 vaccine given subcutaneously as recommended, or intranasally to determine if that inoculation route stimulates a protective mucosal immune response. Six weeks after vaccination, the cattle were "in-contact" challenged using experimentally infected cattle.

A total of 45 cattle of Sanga and Africaner breeds were randomly selected from a CBPP free area and ear tagged for identification. Five cattle per vaccine (T1/44 sub-cutaneously, T1/44 intranasally, Purified Protein and Tween 20) were vaccinated six weeks prior to placing them with 5 non-infected control cattle and 10 cattle endobronchially intubated with a local Mmm field strain 40F05. After 122 days the cattle were sacrificed and gross pathology compared at post mortem.

The T1/44 vaccinated cattle had some small CBPP lesions but much less than the two novel vaccines. Both the Purified Protein and Tween 20 vaccines elicited low protection and in some cattle they appeared to have exacerbated the disease as their pathological lesions were comparable to the non-infected in-contact CBPP challenged control cattle.

At the same time a study was carried out to determine if a biofilm prepared isolate would be more pathogenic than one grown conventionally. Five cattle were endobronchially intubated with Mmm biofilm and housed together with 5 non-treated control cattle for the same duration as mentioned above. The biofilm intubated group appeared less pathogenic than the comparative planktonic intubated group although some of the biofilm in-contact group succumbed to CBPP.

Serological results obtained during the experiment were evaluated in four different tests, Lateral Flow Device (LFD), Competitive Enzyme Linked Immunosorbent Essay (cELISA), Lipoprotein Q Enzyme Linked Immunosorbent Essay (LPPQ ELISA) and Latex agglutination test (LAT). The results demonstrated that LFD is a promising field test at a cut-off point of 30%, but current serological tests are ineffective at detecting all stages of CBPP infection.

Furthermore, serological results were used to establish the importance of humoral immune response in protection. A high response to IgA (Immunoglobulin subclass A) in an indirect ELISA was observed in sera from chronically infected animals. This suggests that a high IgA immune response is an indication of disease progression. Additionally, proteins with a molecular mass of 110, 95 and 48 kDa may be important part of the protevtive immune response since these proteins were present in T1/44 sub-cutaneously vaccinated cattle that had less CBPP lesions than the other groups.

Further work is required to improve serological detection of CBPP and to develop more effective vaccines to help control the disease.

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LIST OF ABBREVEATIONS

APHA	Animal and Plant Health Agency
BIC	Bayesian Information Criteria
BSA	Bovine serum albumin
C3c	Complement component 3
CBPP	Contagious bovine pleuropneumonia
CCPP	Contagious caprine pleuropneumonia
cELISA	Competitive Enzyme Linked Immunosorbent Essay
CFT	Complement Fixation Test
cfu	Colony forming unit
CIRAD-EMTV	French Agricultural Research Centre for International Development: Animal Production and Veterinary Medicine Department
CO_2	Carbon dioxide
CsA	Cyclosporine A
CVL	Central Veterinary Laboratory
ELISA	Enzyme Linked Immunosorbent Essay
EMPRES	Emergency and Prevention System for transboundary animal and plants pests and diseases
FAO	Food and Agriculture Organisation
IBT	Immunoblotting
IgA	Immunoglobulin subclass A
IgG	Immunoglobulin subclass G
IgM	Immunoglobulin subclass M
IHC	Immunohistichemistry
ISCOM	Immunostimulating complex
Kb	Kilobases
KDa	Kilodalton
LAT	Latex agglutination test
LC	Large colony
LFD	Lateral Flow Device
LPPQ	Lipoprotein Q

MHM	Modified hayflick's medium
nm	Nanometer
OD	Optical density
OIE	Office International des Epizooties
PBS	Phosphate buffered saline
PPLO	Pleuropneumonia-like organism
RBC	Red Blood Cells
SC	Small colony
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCVF	Trans veterinary cordon fence
Th 1	T helper cells
µl	Microliter

CHAPTER ONE
GENERAL INTRODUCTION

1 INTRODUCTION

1.1 Classification and general characterization of Mycoplasmas

The class *Mollicutes* includes eight known genera of bacteria (Table 1.1), namely, *Entomoplasma, Mesoplasma, Spiroplasma, Acholeplasma, Asteroleplasma, Anaeroplasma, Ureaplasma* and *Mycoplasma* (Razin *et al.,* 1998; Messick, 2004).

Mycoplasmas are self-replicating prokaryotic microorganisms distinguished from other bacteria by their unique properties: a total lack of a cell wall, their small size, and minute genome (Razin *et al.,* 1998). In addition to their simple structure, mycoplasmas have a double stranded circular DNA chromosome ranging between 580 and 2,220 kilobase (kb) with a guanine plus cytosine content that varies between 23 and 40 mol% (Nicholas *et al.,* 1995) (Table 1.2). The absence of a cell wall makes the treatment of mycoplasma diseases difficult, therefore, rendering them to be refractory to some commonly used antibiotics, such as penicillin (Ayling, 2002).

Mycoplasmas are thought to have evolved from Gram-positive bacteria and through evolution; they have mainly retained the chromosomes that are essential for life (Ayling, 2002). This resulted in them having a small genome which lacks the ability to synthesize amino acids, nucleic acid precursors and lipids (Ayling, 2002). Therefore, these organisms lead a parasitic mode of life for their survival (Pilo *et al.,* 2005).

Mycoplasma mycoides subsp. *mycoides* (Mmm) is a member of the *Mycoplasma mycoides* cluster that is associated with diseases of ruminants (Thiaucourt *et al.,* 2005;

Egwu *et al.,* 1996). The cluster previously consisted of six species that share biochemical, immunological and genetic characteristics (Anon, 2001). However, Manso-Silvan *et al.,* (2009) revised the cluster and suggested that *M. mycoides* subsp. *mycoides* large-colony (MmmLC) and *M. mycoides* subsp. *capri* be classified as the same sub-species since they are only distinguished through serological tests. Furthermore, they suggested that Bovine Group 7 (BG7) be reclassified in its own group called *M. leachii* (Manso-Silvan *et al.,* 2009). Currently, the *Mycoplasma mycoides* cluster is composed of five taxa with three subclusters which correspond to *M. mycoides* subspecies, *M. capricolum* subspecies and species *M. leachii* (Table 1.3). Of these mycoplasmas, Mmm and *Mycoplasma capricolum* subspecies *capricolum* (Mccp) are the most significant and they are the causative agent of contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP) respectively (Kusiluka *et al.,* 2000). With this taxonomic change Mmm no longer requires the “small colony” (SC) designation. These two taxa are restricted to Africa, Asia and the Middle East (Tardy *et al.,* 2009). The species of the *Mycoplasma mycoides* cluster may cause respiratory, arthritis, genitourinary, or mammary disease with severity of the disease depending upon the virulence of the strain (Tardy *et al.,* 2009).

Table 1.1. The major characteristics and taxonomy of the class *Mollicutes*

Classification	Number of Recognized Species	Genome size (kb)	Mol% G+C	Cholesterol requirement	Distinctive properties	Habitat
Order I: *Mycoplasmatales*						
Family I: *Mycoplasmataceae*						
Genus I: *Mycoplasma*	129[b]	580-1,140	23-40	Yes	Optimum growth at 37°C	Humans, animals
Genus III: *Ureaplasma*	6	760-1,170	27-30	Yes	Urea hydrolysis	Humans, animals
Genus IV: *Eperythrozoon*[d]	5	730-770[c]	?	?	Parasitize RBC	Animals
Order II: *Entomoplasmatales*						
Family I: *Entomoplasmataceae*						
Genus I: *Entomoplasma*	5	790-1,140	27-29	Yes	Optimum growth at 30 °C	Insects, plants
GenusII: *Mesoplasma*	12	870-1,100	27-30	No	Optimum growth at 30 °C	Insects, plants
Family II: *Spiroplasmataceae*						
Genus: *Spiroplasma*	45[e]	770-2,220 [e]	24-31	Yes	Helical motile filaments Optimum growth at 30-37 °C	Insects, plants
Order III: *Acholeplasmatales*						
Family I *Acholeplasmataceae*						
Genus I: *Acholeplasma*	13	1,500-1,650	26-36	No	Optimum growth at 30-37 °C	Animals, also insects plants for some species
Order IV: *Anaeroplasmatales*						
Family I: *Anaeroplasmataceae*						
Genus I: *Anaeroplasma*	4	1,500-1,600	29-34	Yes	Oxygen sensitive anaerobes	Bovine, ovine rumen
Genus II: *Asteroleplasma*	1	1,500	40	No	Oxygen-sensitive anaerobes	Bovine, ovine rumen
Undefined taxonomic status						
Phytoplasma	ND[a]	640-1, 1185	23-29	not determined	uncultured *in vitro*	Insects, plants

[a]Uncultured phytoplama in not taxonomically defined; two candidates phytoplasma species have been published (Davies *et al.,* 1997; Zreik *et al.,* 1995). A modified Table adapted from Razin *et al.* (1998); [b]http://www.bacterio.cict.fr/m/mycoplasma.html ; [c]Messick *et al.* (2000)
[d]http://www.bacterio.cict.fr/e/eperythrozoon.html; [e]Williamson *et al.* (2011)
[?]not yet known

TABLE 1.2. Properties distinguishing *Mollicutes* from other eubacteria

Property	***Mollicutes***	**Other eubacteria**
Cell wall	Absent	Present
Plasma Membrane	Cholesterol present in most species	Cholesterol absent
Genome size	580-2,220 kb	1,050-10,000kb
G+C genome content	23-40 mol%	25-75 mol%
rRNA operons numbers	1 or 2[a]	1-10
5S rRNA length	104-113 nucleotides	Over 114 nucleotides
tRNA genes numbers	30 (*M. capricolum*) 33 (*M. pneumoniae*)	84 (*B. subtilis*) 86 (*E. coli*)
UGA codon usage	Tryptophan codon in *Mycoplasma, Ureaplasma, Spiroplasma* *Mesoplasma*	Stop codon
RNA polymerase	Rifampin resistant	Rifampin sensitive

[a]Three rRNA operons in *Mesoplasma lactucaea*

Table adapted from Razin *et al.* (1998).

TABLE 1.3. Members of the *Mycoplasma mycoides subspecies mycoides*cluster

Species	Disease	Main host (and others)
M. mycoides subspecies *mycoides* SC	Pleuropneumonia (CBPP)	Cattle, (goats, sheep, yak, reindeer, bison)
M. mycoides subsp. *capri*	MAKePs*	Goat, (sheep)
M. capricolum subsp. *capricolum*	MAKePs*	Goat, (sheep)
M. capricolum subsp. c*apripneumonia*e	Pleuropneumonia (CCPP)	Goat
M. leachii (formely BG7)	Arthritis, mastitis,	Cattle

Manso-Silvan *et al.,* (2009)

*MAKeps = mastitis, arthritis, keratitis, pneumonia and septicaemia syndrome

1.2 Contagious Bovine Pleuropneumonia

Contagious bovine pleuropneumonia (CBPP) is a bacterial disease caused by *Mycoplasma mycoides subspecies mycoides* (Mmm). It results in severe fibrinous exudative pleuropneumonia and mainly affects cattle. CBPP is an Office International des Epizooties (OIE) listed and notifiable disease. Endemic countries are restricted from international cattle trading (March, 2004; Dedieu *et al.,* 2005a). Since the control of rinderpest, CBPP is the most significant disease of animal health in Africa; where it is still present (Niang *et al.,* 2006a).

1.3 Causative agent of CBPP

Mmm is one of the smallest prokaryotic bacteria with a genome of 1,211,703 bp (Westberg *et al.,* 2004). It is fastidious but can be cultured in growth media described by Thiaucourt *et al.,* (2005) or in an extremely productive medium, PRM, developed by Rice and Miles at King's College, London (Nicholas *et al.,* 2000) and named after the developers. It was not until the end of the 19th Century that the causative agent (Mmm) was isolated by Nocard and Roux, (1898). However, it was more than 50 years before the organism was named as *Mycoplasma mycoides subspecies mycoides* by Edward and Freundt (Edward and Freundt, 1956).

1.4 Mode of transmission of *Mycoplasma mycoides*

CBPP mainly affects cattle although some Buffalo (*Bubalus bubalis*) that were in proximity of the infected cattle in Italy were affected albeit to a lesser extent (Santini *et al.,* 1992). The disease in Water buffalo, Yak, Reindeer and Bison has been reported (Provost *et al.,* 1987) but the African buffalo (*Syncerus caffer*) seems to be resistant to CBPP (Windsor and Wood, 1998). Mmm has been isolated from goats and sheep (Brandão, 1995, cited by Nicholas, *et al.,* 2008) and their role as reservoir of infection and in the epidemiology of the disease is not yet known.

CBPP is transmitted by direct or very close contact between a coughing infected animal and susceptible animals (Masinga and Domenech, 1995; Windsor and Wood, 1998); indirect spread has also been verified (Windsor and Masinga, 1977). Mmm may be found in urine of infected animals, thus alerting a possibility that transmission can occur from urinary tract to nose (Thiaucourt *et al.,* 2005). Similarly, Mmm was isolated from

semen of infected bull, suggesting that sexual contact and artificial insemination with infected semen may play a role in the spread of the disease (Thiaucourt *et al.,* 2005). Cattle movement has been implicated to be the major source of spreading the disease (Masinga *et al.,* 1996) and this remains a challenge for the control of CBPP in Africa (Thomson, 2005) where transhumance and nomadism are widely practiced.

1.5 CBPP: Disease manifestation

CBPP is a disease of the respiratory tract characterized by pneumonia and serofibrinous pleurisy (Masinga *et al.,* 1996) with an incubation period of 9 to 28 weeks in naturally infected cattle (Dedieu *et al.,* 2005a), however in most experimental cases, the disease manifests within 40 days of infection (Egwu *et al.,* 1996). The clinical signs depending on the severity of the disease or the virulence factors of the infectious agent are: 1) Hyperacute form: the animal does not show obvious clinical signs as it dies quickly; 2) Acute form: the animal has fever followed by respiratory complications (painful breathing, coughing, standing with an extended head and neck, nasal discharge and foamy saliva). Abortions as well as diarrhea have been reported and 3) Sub-acute form: the animal has similar symptoms as the acute form but to a lesser degree and fever being irregular (Anon, 2002). This form occurs in about 40-50% of affected animals (Anon, 2002). The animals that survive the acute form of the disease may become chronic. The chronically infected animals are usually emaciated and may present no apparent respiratory signs (Thiaucourt *et al.,* 2005). These chronic animals retain sequestra in their lungs in which the infection remains for more than 12 months and could be a major source of infection should the sequestra break (Thiaucourt *et al.,* 2005). CBPP affected calves may not present any signs of respiratory distress but

swollen joints are typical clinical signs (Anon, 2002). However, Trichard *et al.,* 1989, revealed typical CBPP pneumonic lesions in 4-9 months old calves, during the 1982 outbreaks in Namibia.

1.6 Geographical distribution of CBPP

A previous review indicates that CBPP existed in the ancient world (Provost *et al.,* 1987). Although, it was difficult then to be certain of the history of CBPP prior to the eighteenth century (Salmon*,* 1896), Dupuy *et a*l (2012) with the Bayesian evolutionary analysis estimated that CBPP may have emerged around 1700 AD in Europe. However, cattle movement due to Napoleonic military campaigns in the 19th Century has spread the disease rapidly throughout Europe. Other continents were at risk, and consequently, infection was observed beyond the European borders in the mid-1850s (Egwu *et al.,* 1996; Dupuy *et al.,* 2012). Egwu *et al* (1996) narrated the history of CBPP infections over the world. Even though CBPP may have been present in Britain during the 17th and 18th centuries, Britain was re-infected in 1839 from Holland through Island (cited in Nicholas *et al.,* 2008). Since there were no serious control measures in Britain, 1500 and 3000 outbreaks were reported in 1870 and 1875 respectively. However, with payment of full compensation to owners for slaughtered animals, the number of oubreaks dropped down to about 2000 in 1890 (cited in Nicholas *et al.,* 2008). CBPP was officially eradicated from Brittain in 1898 (Fisher, 2003). The disease was eradicated in many countries during the 20th Century by a combination of measures mentioned in 1.9 (Dedieu *et al.,* 2005a). The United States of America eradicated CBPP in 1892 after the Bureau of Animal Industry to fight the disease was set up by the Federal Government in 1887 (Amanfu 2009). CBPP persisted in Germany until 1923

and Poland up to 1936. By 1972, CBPP was eliminated from Australia (Anon, 2002). Sporadic outbreaks occurred on the French/Spanish border in 1956 and in Portugal in 1967. Subsequently, CBPP outbreaks were officially recognized in the eastern Pyrenees on both the French/Spanish border in 1980's. France eradicated the disease in 1984 and remained free ever since. A sudden reappearance of the disease between 1983 and 1990 was observed in southern Europe: Spain, Portugal and Italy (Scacchia *el al.*, 2007). It was effectively controlled and since 1999, no CBPP outbreaks have been reported in Europe (Nicholas *et al.,* 2000).

Besides reported cases of the disease in India, Bangladesh, Myanmar and the Middle East (Anon, 2002), the intensity of it in Asia and the Middle East remains unclear (Thiaucourt *et al.,* 2005). A detailed history of CBPP in China was not known until recently. The pathogen seems to have entered the country in 1919 and through a massive increase in cattle production in 1949, the disease spread to many regions in the country. As a result, China has initiated the production of vaccine from a virulent isolate (Ben-1) that was attenuated in rabbits and for a larger production, Tiben sheep were used (Xin *et al.,* 2012). About 74.5 million cattle in the affected regions were vaccinated with Ben-1 vaccine that has a high protection rate and duration of immunity of 28 months. CBPP has not been seen in China since 1989 (Xin *et al.,* 2012).

The status of CBPP in Africa differs greatly with that in Europe. The OIE, 2005 reports highlighted at least 26 countries in West, Central, East and Southern Africa that were infected. A total of 1289 deaths of cattle from 272 outbreaks with 7510 cases were reported from sub-Saharan countries in 2003 (Thomson 2005; Amanfu, 2009). The Central African Republic reported a recurrence of CBPP with 102 cases and 100 deaths (Anon, 2005 & 2007a). However, this number may have increased to at least 30

countries in Sub Sahara (Nicholas *et al.,* 2009). Jores *et al.* (2013) reported 28 countries in Sub Sahara to be infected with CBPP.

The introduction of CBPP into Africa was either via South Africa in 1854 by a bull originating from Holland or through Ethiopia and Sudan by infected Indian cattle belonging to the British Expeditionary Force in the late 19th Century (Egwu *et al.,* 1996). Subsequent to its introduction most of African countries were infected by CBPP (Egwu *et al.,* 1996). Within two years of CBPP introduction into South Africa in 1954 it had killed more than 100,000 head of cattle (Schneider *et al.,* 1994a; Trichard *et al.,* 1989). Through movement control, slaughtering and vaccination, CBPP was eradicated and South Africa has remained free of the disease since 1921 (Schneider *et al.,* 1994a; Trichard *et al.,* 1989). Zimbabwe was infected in 1861 and the disease was eradicated by 1904 (Thiaucourt *et al.,* 2005; Thomson, 2005). The disease entered Angola in 1888 and it has been endemic since 1914 (Thiaucourt *et al.,* 2005; Thomson, 2005). Zambia had managed to control the disease in 1946 after it was infected by Angola in 1915. However, outbreaks occurred in several years with a recent one reported in 2004 (Kabilika *et al.,* 2007).

The incidence of CBPP decreased in the 1970s, when research in some African countries (Kenya, Chad and Nigeria) was internationally sustained via “Joint Project 16”. However, in the late 1980s and early 1990s, possibly in the wake of the cessation of the combination vaccine and the interest of veterinary authorities moving to other concerns, the disease came back in the two fronts, the east and the south (Amanfu, 2009). According to Amanfu (2009) the outbreaks resulted in the reappearance of the disease in the endemic areas and the re-infection of the free countries. The re-infections in Tanzania: 1990, 1991 and 1995; Democratic Republic of Congo: 1991; Rwanda: 1994; Botswana: 1995; Northwestern-Zambia: 1997-present and Burundi: 1997 are due to

uncontrolled movement of cattle from infected areas, which may have happened because of the breakdown in the surveillance system, emergency preparedness and prompt reaction to the outbreaks (Amanfu, 2009).

The 1995 re-introduction of CBPP into Botswana (Amanfu *et al.,* 1998) after a period of over 50 years of freedom demonstrated that countries that are next to infected countries are constantly at risk. Botswana eradicated the disease by slaughter and compensation in 1996 and is currently free of CBPP. A recent increase in numbers of outbreaks was observed in West, East and Central Africa. Since the last occurrence of CBPP in Egypt in 1991, the countries in Northern Africa are free of the disease (Thiaucourt *et al.,* 2005; Thomson, 2005).

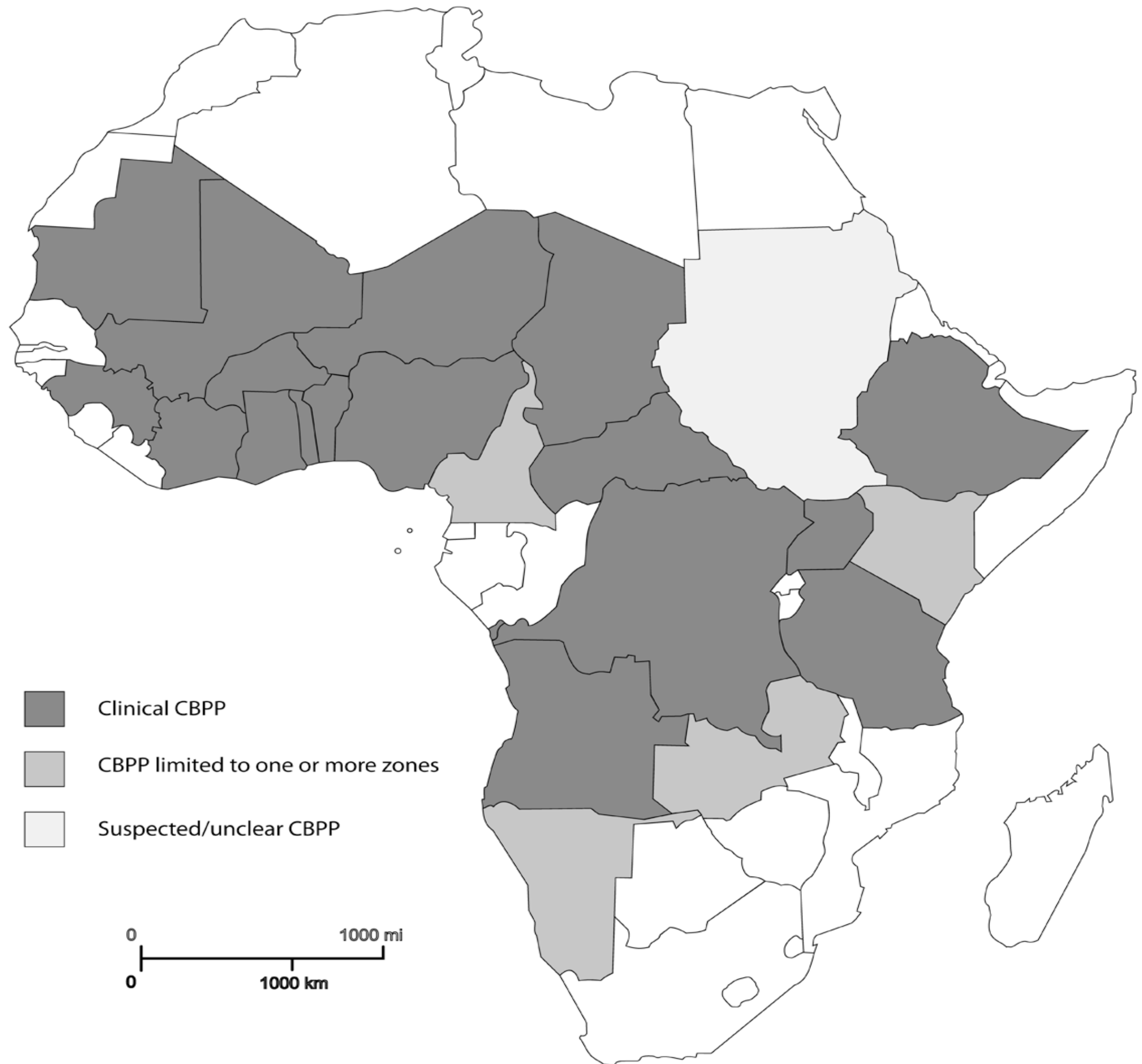


Fig.1.1. Occurrence of CBPP in Africa: 2006-2008 (Hamsten, 2009)

The history of CBPP in Namibia is traced back to 1856 when the disease was introduced through Cape Province by infected oxen (Schneider *et al.,* 1994a). Through animal quarantine measures adopted then, the disease was confined to a localized outbreak at Warmbad in the south, and subsequently eradicated (Hübschle *et al.,* 2003). However, it was re-introduced in 1859 by cattle coming either from Botswana or the Cape Province and spread throughout the country. As a result, a large number of cattle died and 1860 was called the year of “Otjipunga” (lung) by the Herero people (Schneider *et al.,* 1994a).

CBPP was eradicated in 1919 from central commercial farming areas of Namibia by control measures outlined in 1.9.1 and 1.9.2 (Schneider *et al.,* 1994a, Trichard *et al.,* 1989). Despite being eradicated from these areas, CBPP remains endemic today in the northern communal areas (Hübschle *et al.,* 2004; Schneider, 1994b) where half of the Namibian population resides and it is separated from the rest of the country by a Trans Veterinary Cordon Fence (TVCF). Animals in this area are quarantined for 21 days before they are slaughtered at local abattoirs in that area (Anon, 1956), creating a discrepancy in marketing prices between farmers in quarantine zones and that outside quarantine (Windsor and Wood*,* 1998).

The CBPP situation in northern communal areas has improved since 1997 after the implementation of yearly T1/44 vaccination. However, a threat from unvaccinated Angolan cattle to resident cattle remains a challenge due to animal movement to Angola in search of grazing as well as from Angola into Namibia for marketing (Anon, 2003).

In August 2003, after a respite of over 60 years, Linyanti constituency in the Caprivi region, northeast of Namibia experienced a CBPP outbreak (Fig 1.2). The infection is believed to have originated from a neighbouring country. By December 2003, a total of 206 cattle died of the disease. By the end of the outbreak in early 2005, nearly 380 cattle at three crush pens in the Linyanti constituency succumbed to CBPP. In order to halt the spread of the disease, a total of 37,181 cattle were vaccinated with 96% coverage. Table 1.4 below depicts the trend of the disease between 2005 and 2015. The measures applied to control CBPP in Europe and elsewhere cannot be emulated in many African countries because of inadequate financial resources and transhumance way of living. Therefore, CBPP remains endemic in many African countries (Anon, 2002).

In view of the disease situation in Africa and the paucity of data from Asia and the Middle East as well as the high demand in international trade of live cattle, a re-introduction of the disease to free countries is probable (Heller *et al.,* 2007). Hence, a concerted effort from infected countries together with international communities is vital in the fight against CBPP.

Fig.1.2. The map of Namibia. This shows the Linyanti village in the Maunga constituency (red star) in the Zambezi region (formerly Caprivi) where CBPP outbreaks occurred in 2003 (Anon, 2003).

Table1.4. The trend of CBPP infection in the Northern Communal Areas between 2005 and 2015 (Anon, 2016)

	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
No of Outbreaks	19	6	5	1	6	12	6	7	9	1	1
No. of Clinical Cases	150	26	10	3	30	28	40	47	68	3	3
No. of Deaths	39	22	3	0	2	12	9	14	23	2	0

1.7 The economic impact of CBPP

CBPP poses a major threat to cattle farming worldwide and especially in Africa (Dedieu *et al.,* 2005a; Nicholas *et al.,* 2000). In 1995 the OIE, reported that CBPP was causing heavy losses in cattle and had a more economic impact than any other cattle disease in Africa (Egwu *et al.,* 1996).

The economic as well as the social cost accrued from this devastating disease in the world is significant (March, 2004). High mortality rate, stamping-out, vaccinations, loss of weight and reduced working ability among others are factors associated with economic and social cost. (March, 2004). In Africa, losses accrued from CBPP are estimated to be in the region of US$2 billion per annum (Waite and March, 2001; March *et al.,* 2000). The economic loss accrued by Britain in 1860, when 187,000 cattle died of CBPP was

estimated at £2 million per annum (Egwu *et al.,* 1996). In Italy during the 1990 to 1993 outbreaks, more than 24,000 cattle were slaughtered in three infected areas (Regalla *et al.,* 1996). The Botswana veterinary authority in 1996 slaughtered 320,000 head of cattle at an estimated cost of US$100 million, with indirect cost in over US$400 million during the outbreak (Geering *et al.,* 1999; Windsor and Wood, 1998). Tambi *et al.* (2006) investigated the cost accrued from controlling CBPP in twelve sub-Saharan African countries and arrived at an estimate cost of 30 million euros (2.5 million euros per country) per annum due to morbidity and mortality while the total ecomonic cost (direct and indirect production losses as well as disease control costs) was estimated at 44.8 million euros. However, an investment of 14.7 million euros to control CBPP would prevent a loss of 30 million euros (Tambi *et al.*, 2006). The CBPP outbreaks in China between 1949 and 1989 have resulted in the death of 178,570 cattle at an estimated loss of 356 million RMB (Xin *et al.,* 2012).

CBPP restricts cattle production and has grave implications for food security and people's livelihoods in Africa (Amanfu, 2009). The eradication of the disease in Botswana has resulted in the increase of malnutrition in children due to the massive slaughter of cattle; therefore, fewer animals were available for food (Boonstra *et al.,* 2001).

1.8 Diagnosis of CBPP

The diagnosis of CBPP is based on clinical signs, necropsy findings augmented by serological, molecular and cultural tests (Thiaucourt *et al.,* 2005).

1.8.1 Clinical diagnosis of CBPP

CBPP may be diagnosed through clinical symptoms during acute stage. Usually, the animal is depressed, has modest fever and lacks desire to eat. These clinical signs are succeeded by coughing and heavy painful breathing due to pleuropneumonia, which progresses as the intensity of the disease advances. The infection in calves tends to be restricted to arthritis with swelling of the joints (Anon, 2002), although it was reported differently by Trichard *et al.* (1989) (section 1.5).

1.8.2 Necropsy findings

The pathological lesions of CBPP are typically unilateral and restricted to the thoracic cavity and lungs (Egwu *et al.,* 1996). During the acute stage, the gross pathological lesions are characterized by fibrinous deposits on the parietal and visceral surface of the lungs (Provost *et al.,* 1987). The different stages of the disease among other factors are observed through, red, gray and yellow hepatisation of the lung lobules. Furthermore, marble appearance of the lung which sometimes accompanied by adhesion of the parietal and visceral surface is evident. A sequestrum surrounded by fibrous capsules is obvious of chronic stage of CBPP. The thoracic cavity of diseased animal may contain litres of pleural fluid (Egwu *et al.,* 1996). Figures 1.3 and 1.4 represent typical CBPP lesions seen at post mortem.

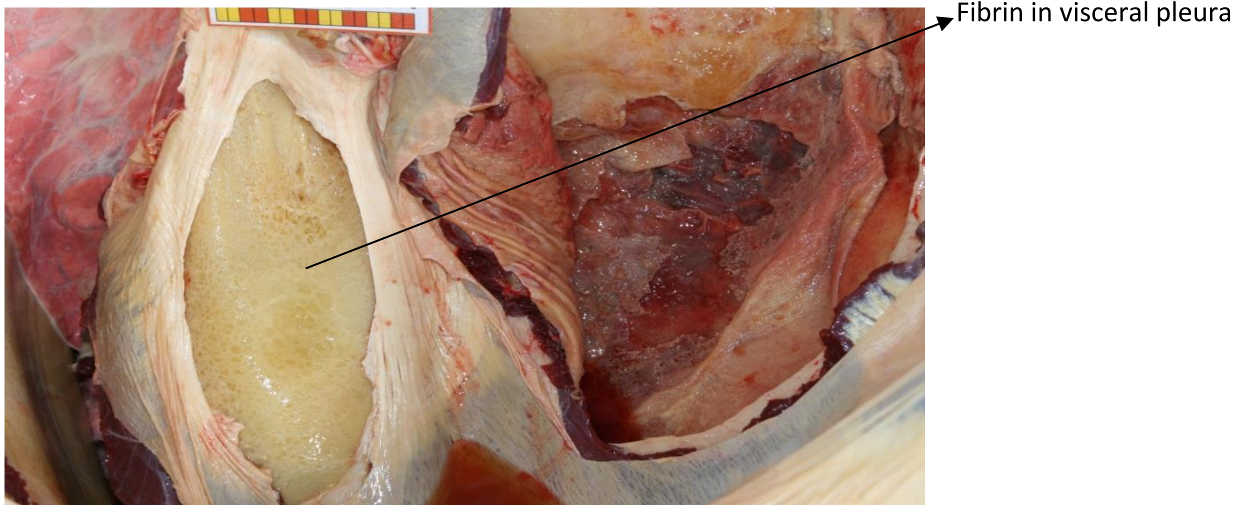


Fig. 1.3. CBPP lesions in animal experimentally infected with Mmm.

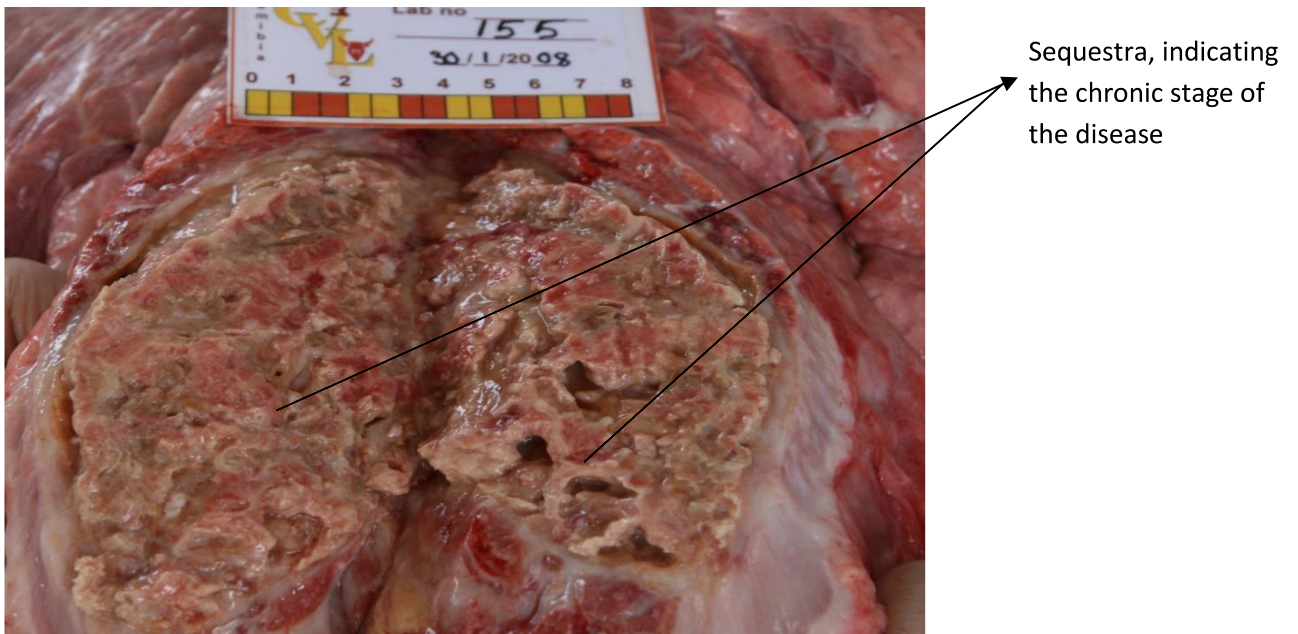


Fig.1.4. Sectioning of the lung revealing CBPP lesions in animal experimentally infected with Mmm by contact (images derived from the current study).

1.8.3 Laboratory diagnosis of CBPP

The laboratory techniques involved in the diagnosis of CBPP are outlined in OIE manual (Anon, 2008) and are described in chapter 2 of this thesis. The presence of the disease can be confirmed by:

1) The detection of the causative agent through either culture, or polymerase chain reaction (PCR).
2) Detection of serum antibodies through different serological tests such as: Complement Fixation Test (CFT), Competitive enzyme-linked immunosorbent assay (cELISA) and Immunoblotting (IBT).

Mmm is also detected by immunohistochemistry in tissues fixed in 10% formalin (Bashiruddin *et al.,* 1999). Many different tests such as ELISAs using recombinant antigens (Thiaucourt *at al*., 2005) and rapid diagnostic test using polysaccharide-sensitized latex beads have been developed (March *et al*., 2003; Ayling, 2002).

In Namibia CBPP is diagnosed through clinical signs and necropsy findings in the field and at abattoirs. The laboratory diagnosis comprised of determination of causative agent through culture, PCR and antibody detection by CFT and cELISA.

1.9 Control of CBPP

CBPP is controlled through various control measures such as; vaccinations, strict cattle movement control and stamping-out policy. The implementation of these measures varies according to the epidemiological situations, animal husbandry and the effectiveness of the veterinary service in a particular country (Thiaucourt *et al.,* 2005).

1.9.1 CBPP vaccinations

CBPP in endemically infected areas is mainly controlled by vaccinations (Thiaucourt *et al.,* 1998). The control of CBPP using vaccination in Europe dated back to 1852 with a possibility of being tried earlier than that in Africa. In an early attempt to build immunity in susceptible animals a piece of lung or pleural fluids from infected animal were inoculated at the tip of the tail and on the bridge of the nose of animals in Europe and Africa respectively (Thiaucourt *et al.,* 2003; Blancou, 1996). Although, immunity was conferred, a high mortality was observed. As a result, attenuated vaccines were formulated and used in Australia and Africa in the 1950s. These include KH3J, T1/44 and its streptomycin resistant derivative, T1/SR (Thiaucourt *et al.,* 2003).

These vaccines have been passaged several times in broth culture or embryonated egg to reduce the pathogenicity and are affected by factors such as a drop in titer, viability, media, storage and transport (March, 2004; Thiaucourt *et al.,* 2000). In addition, observations from the field together with experimental studies have highlighted the low efficacy of the current vaccines which results in not effectively protecting cattle from outbreaks of disease (Waite *et al.,* 2001; Rweyemamu *et al.,* 1995; Thiaucourt *et al.,* 2000) and reversion to virulence among others (Thiaucourt *et al.,* 2000; Mbulu, *et al.,* 2004; Totte *et al.,* 2008). The T1/44, the OIE recommended vaccine for use in endemic areas of Africa, confers immunity up to one year (Thomson, 2005; Rweyemamu *et al.,* 1995), therefore, requiring yearly vaccination, which is a burden to poorly financed veterinary services (Musisi *et al.,* 2003). Thomson (2005) estimated a five-year economic cost accrued from CBPP vaccination in nine endemically infected countries in Africa (Table 1.5).

A streptomycin resistant derivative, T1/SR vaccine that was used during the 1990s CBPP outbreaks frustrated both the farmers and livestock experts in Botswana

during the 1995 outbreaks because it failed to stop the spread of the infection; hence, its use was discouraged (Tulasne *et al.,* 1996).

Therefore, the development of an improved vaccine with long lasting immunity is a prerequisite for the eradication of CBPP in Africa. Many attempts to formulate an inactivated vaccine have proved futile. The lipoprotein Q (LppQ) immunostimulating complex (ISCOM) vaccine that has explored the role of a Th1 response in the pathogenesis of Mmm and in protection and a whole cell mycoplasma vaccine inactivated with saponin exacerbated the disease as the vaccinated animals were affected to the same extent or even more than the in-contact control animals (Nicholas *et al.,* 2004). The more recently held experiment with purified subunit vaccine consisting of five recombinant putative variable surface proteins of Mmm did not confer protection and the animals were similarly affected as the in-contact non-immunized animals (Hamsten *et al.,* 2010).

Therefore, no breakthrough has been seen in the development of a better protective vaccine for CBPP.

Table1.5. Annual cost of CBPP vaccination in nine endemically infected countries (1998-2003)[1]

Country	Average no. of cattle vaccinated annually (million)	Range (million)	Cattle population estimate for 2000[2] (approx. average annual vaccine coverage)	Estimated annual expenditure in US$ million (range)
Angola	0.83	0.53-0.91	4.15 (20)	0.42 (0.27-0.46)
Burkina Faso	1.53	1.34-2.14	5.10 (30)	0.77 (0.76-1.07)
Cameroon	2.88	1.80-3.70	5.95 (48)	1.44 (0.91-1.85)
Ethiopia	0.30	0.06-0.41	35.50 (0.8)	0.15 (0.03-0.21)
Ghana	0.14	0.05-0.34	1.34 (10)	0.29 (0.03-0.17)
Kenya	1.18	0.26-1.96	11.50 (10)	0.59 (1.30-1.71)
Mali	3.02	2.59-3.41	7.30 (41)	1.51 (1.30-1.71)
Nigeria	1.43	0.19-3.20	15.20 (9.4)	0.72 (0.09-1.60)
Tanzania	1.77	0.07-5.10	17.70 (10)	0.89 (0.03-2.60)

[1] Based on average cost of US$ 0.50 per dose

Table adapted from Thomson (2005)

1.9.2 Strict cattle movement control

Implementation of strict animal movement is the ideal method of reducing the spread of CBPP and this coupled with other control measures mentioned earlier proved to be a success in eradication of CBPP (Anon, 2002). However, in Africa where pastoralism and transhumance are widely practiced, the method is far from being successful. Furthermore, porous borders, financial constraint and political instabilities faced by some African Governments results in veterinary authorities not being able to impose regulatory measures pertaining to animal control (Thomson, 2005).

1.9.3 Eradication strategies for CBPP

As demonstrated in Europe and USA in the 1860s, the control of CBPP is better achieved by slaughtering the whole herd population when it is found in a disease-free country or area. CBPP was eradicated from Europe through culling combined with quarantine and strict cattle movement (Sylla *et al.,* 1995). In 1995, Botswana successfully eradicated the disease through culling and cattle owners were compensated (Amanfu *et al.,* 1998).

1.9.4 Factors hindering the control of CBPP

The long disease incubation period, cattle movement due to various factors such as differences in availability of pasture and water as a result of drought are a major obstacle to the control of CBPP (Masinga *et al.,* 1995; Windsor and Wood, 1998). In addition, the short lasting immunity conferred by currently used CBPP vaccines; result in the failure to control the disease (Masinga and Domenech, 1995). The detection of affected animals in CBPP endemic countries might be hampered by the illegal or misuse of antibiotics which reduces the clinical signs (Musisi *et al*., 2003).

Although, the use of antibiotics has been officially discouraged on the grounds that they may mask disease symptoms and create carriers (Provost *et al.,* 1987), it is still widely used in Africa (Thiaucourt *et al.,* 2003; Ayling *et al.,* 2000). In the absence of a better vaccine, antibiotics continue to be employed as an applicable control-measure by farmers to reduce CBPP mortality (Lesnoff *et al.,* 2004). An Office International des Epizooties (OIE) meeting held in Rome in 2006 under the theme “CBPP control: Antibiotics to the rescue?” was mainly to assess the efficacy of antibiotics in treating CBPP infected animals. Various experimental studies were carried out *in vitro* and *in*

vivo. An *in vitro* study (Ayling *et al.,* 2000), investigated the effectiveness of five antimicrobials against CBPP in an attempt to assist the Veterinary authorities. The study suggested that tilmicosin and danofloxacin are useful in the control of CBPP. Hence, an experimental study using danofloxacin in treating CBPP in naturally infected cattle in 2004 outbreak in Caprivi Region of Namibia proved that antibiotics could reduce the spread of the disease to healthy animals (Hübschle *et al.,* 2006). The use of Long Acting (LA) oxytetracycline in the treatment of CBPP infected animals had a positive effect as 12 out of 14 animals recovered although complete elimination of the mycoplasma was not achieved. Furthermore, the treatment stopped the spread of the pathogen to healthy in-contact animals (Niang *et al.,* 2006a). This might pave a way to treating CBBP infected animals in endemic areas whilst waiting for a more efficacious vaccine candidate. The lack of sensitivity and specificity of the tests applied in Sub-Saharan Africa is also a limitation to effectively control the infection (Naseem *et al.,* 2010). To this end, the only practical means to contain CBPP in Africa is therefore by prophylactic methods (Lesnoff *et al.,* 2004). However, Aligaz and Munganga (2019) suggested three possibilities of controlling the disease by (1) treating 85.7% of infectious cattle with antibiotics, without vaccinating healthy cattle, (2) vaccinating 80% of susceptible cattle within a period of 49 days, without treating the infectious cattle and (3) assuming that 50% of susceptible cattle are vaccinated within 73 days and 50% of infectious are treated with antibiotics. The option of using the combination of vaccination with antibiotics is the one likely to be successful (Aligaz and Munganga, 2019). However, for this option to be a success, vaccines and antibiotics should be readily available to farmers of which cattle are at risk of exposure to infection. One of many problems associated with failure to control CBPP in sub-Saharan Africa is a failure to deliver control services to farmers whose cattle are at high risk of exposure to infection (Onono *et al.,* 2017). In order to assist public sectors to deliver control services (vaccinating and

antibiotics treatment), a contractual agreement between public and private sectors should be adopted (Onono *et al.,* 2017).

1.10 Future prospects for the control of CBPP

The requirements set out by OIE to be adopted by countries that wish to acquire a disease free status, such as slaughtering of infected as well as in-contact herds (Anon, 2002) may not be fiscally realistic for some of the African governments, which are already burdened by wars and famine (Lesnoff *et al.,* 2004; Windsor, 2000). Therefore, the control of CBPP in endemically affected areas requires a mass vaccination for not less than five years augmented by strict cattle movement control where it is possible (Masinga and Domenech, 1995). Based on the economic implications of the disease in the world, and the threat for re-introduction into countries that are free of the disease (Lorenzon *et al.,* 2003), a concerted effort, involving regional and international coordination in controlling international cattle movement as well as harmonizing control strategies is needed for the intended eradication to be a success (Masinga *et al.,* 1995; Sylla *et al.,* 1995). In addition, veterinarians and community health workers should be trained in detecting clinical symptoms in the cattle as early as possible (Heller *et al.,* 2007; Sylla *et al.,* 1995).

For CBPP to be eradicated a joint effort should work towards the understanding of pathogenesis of Mmm as well as research to formulate a better vaccine. The Pan African Rinderpest Campaign (PARC) has financially invested in CBPP vaccine research aimed at ISCOM with a hope to come up with improved vaccines. Until then, continuous vaccinations should remain the method of choice to contain CBPP in Africa (Thiaucourt *et al.,* 2003). Abusugra *et al.* (1997) envisaged the future eradication of CBPP to be through the development and use of an improved vaccine.

In an attempt to set up control measures for CBPP, the Southern African Developing Communities (SADC) Member States convened at a workshop in Dar Es Salaam, Tanzania in September 2007 and decided that considering the porous borders, CBPP will be better managed when countries develop robust early warning systems, harmonized surveillance and disease control strategies. The trading of livestock and livestock products should be encouraged to follow a formal regime either within the country or in the region. There must be mutual agreements that the activities taken to control the disease in all Member States are similar and equally effective (Anon, 2007b). These resolutions look promising on papers; however, their implementation is likely to prove more difficult.

The measures that have to be implemented to control CBPP are not feasible in many countries in Africa. Therefore, the only option is the use of T1/44 live attenuated vaccine, even though it has some limitations mentioned in 1.9.1. The efforts to formulate new vaccines have so far not given desirable outcomes. Therefore, it is against this background, that an understanding of the humoral immune responses of the host, in relation to pathogenicity or protection may pave the way towards the formulation of better vaccines against CBPP. Only after successful intervention through prophylactic measures, the disease that has affected the continent for more than 150 years will be finally eliminated.

1.11 Pathogenicity of CBPP

The close interaction between the mycoplasma and the host cells is complex (Howard and Taylor, 1985). However, the ability of an infectious organism to attach to particular host receptors is important for establishing colonization (Howard and Taylor, 1985) and may lead to the development and severity of the disease as well

as the spread of the infection in the body (Razin *et al.,* 1998). Mycoplasmas elicit specific and non-specific immune responses upon entering the host immune system (Razin *et al.,* 1998; Howard and Taylor 1985). The specific immune response involves the host defense mechanisms to produce different antibodies, stimulation of cell-mediated immunity, and opsonisation and phagocytosis of organisms (Razin *et al.,* 1998).

The pathogenicity of Mmm is not fully understood; however, Mmm being a mucosal disease enters the host via the respiratory route and attaches itself to the epithelial cells (Thiaucourt *et al.,* 2005). It colonizes the mucous membrane and this may subsequently lead to its pathogenicity (Thiaucourt *et al.,* 2005). The membrane lipoproteins (LppA, LppB, LppC, and LppQ) are alleged to be involved in the virulence of Mmm (Hamsten *et al.,* 2008). Persson *et al.* (2002) described the role of variable surface proteins (Vmm) of mycoplasmas in augmenting colonization and adapting to host cells at different stages during infection leading to it to evade the host immune system. Dedieu *et al.* (2005b) investigated the virulence factors leading to apoptotic cell death and demonstrated that Mmm secretes toxic components *in vitro* which cause cell death. This might suggest that when Mmm invades the immune system it causes apoptotic cell death to the major cells of the immune system, the CD4 T-cells. The CD4 T-cells are essential for the host defense mechanism at the onset of infection (Dedieu *et al.*, 2005b). The hydrogen peroxide (H_2O_2) formed by L-alpha-glycerophosphate oxidase (GlpO), that is secreted by Mmm plays a role in the oxidation of glycerol and it is responsible for cell death (Pilo *et al.,* 2005, 2007; Dedieu *et al.,* 2005b). The H_2O_2 has been demonstrated as one differentiating factor between European and African strains. The African strain is more virulent possibly due to the production of H_2O_2 (Houshaymi *et al.,* 1997).

In addition to the virulence factors of Mmm, the capsular galactan attaches to lung tissue and trigger the formation of self-antibody to pneumogalactan which is

associated with autoimmune reaction and may induce pathological lesions seen in the lung of CBPP affected animal (Anon, 2001). It is however, unclear how pathological lesions observed in diseased animals are produced (Thiaucourt *et al.,* 2005; Anon, 2001).

In addition, although mycoplasmas have a very limited genome, little is known about their methods of persistence in the host (McAuliffe *et al.,* 2006). Mycoplasma species vary in their abilities to produce prolific biofilm. Biofilms are bacteria that attached to a substratum or each other and surrounded by an extracellular polysaccharide matrix. Bacteria are believed to persist in the host by a formation of an adherent biofilm (McAuliffe *et al.,* 2006). Biofilms are stubornly resistance to host defences and stress than planktonic cells. Furthermore, biofilms are resistant to antibiotics, antibodies as well as phagocytes and may cause host damage as phogotytosis is not taking place and the released phagocytic enzymes damage surrounding tissues and exacerbate infection (McAuliffe *et al.,* 2006).

Although previous studies have shown that cattle recovered from CBPP are normally immune or resistant to re-infection (Dedieu *et al.,* 2006; Lanzavecchia and Sallustro, 2005; London *et al.,* 1999; Windsor and Masinga, 1977; Gourlay, 1975), the immune response that is essential for protection is unknown. It has been demonstrated that humoral and cellular immune responses are important for protection (Dedieu *et al.,* 2005a; Dyson and Smith, 1975), to understand their involvement during the progression of the disease remains a challenge. There is insufficient information in this area because the use of cattle for experiment is too costly (Dyson and Smith, 1975; Dedieu *et al.,* 2005a; Masinga *et al.,* 1975; Gourlay, 1975; March, 2004).

1.12 The immune responses

Mycoplasmas cause respiratory diseases in animals and humans and have a huge economic and health impact over the world (Blanchard and Browning, 2005). Due to the severity of the infection brought by them, it is possible that both innate and adaptive immune systems are employed to combat the disease (Blanchard and Browning, 2005). Vaccines elicit the immune responses to resist infection and or the immune responses to stop the infection from disseminating to other tissues. However, in many Mycoplasma diseases, the pathogen persists in the host and result in the establishment of the immunopathologic lesions merely because the immune responses are unable to resist the infection. Immune responses are considered to be important factors in the pathogenesis of disease induced by mycoplasma (Blanchard and Browning, 2005). The innate immunity is needed to clear the infection as soon as the pathogen enters whereas the adaptive immune responses take time to develop and they are pathogen specific (Blanchard and Browning, 2005). Both B and T lymphocytes are the uniqueness of adaptive immune responses and are accountable for the progression of antibody (humoral) and cell-mediated immune responses during infection (Blanchard and Browning, 2005).

Mmm is an extracellular organism (Dedieu *et al.,* 2005b) that elicits both cellular and humoral immune response upon entering the host (Rana and Srivastava, 2001). The role of cell mediated immunity; whether it is important in protection or actively contributes to pathology had remained a mystery for a long time (Gourlay and Palmer*,* 1965; Dyson and Smith, 1975; March, 2004) because most of the earlier studies focussed on antibody responses. However, the existence of Mmm-specific cellular immunity has been suggested albeit, not characterized. Dedieu *et al.* (2005a) investigated the cell-mediated immune response elicited in cattle by Mmm. The authors concentrated on the Mmm immunity in acute and recovered CBPP animals.

The study revealed among other factors that Mmm–specific IFNy-CD4 T-cell immune response was observed in blood from all recovered cattle. Although a Mmm-specific CD4 Th1-like T-cell response persisted in the peripheral blood mononuclear cells (PBMC) of acute cattle, it did not produce IFN-γ. A similar study that investigated the immune response in lymph nodes revealed similar findings (Dedieu *et al.,* 2006). Mmm–specific IFN-γ secreting $CD4^{+}$ T-cells were observed in lymph nodes of recovering animals and this persisted for the duration of the experiment (Dedieu *et al.,* 2006). Furthermore, IFN-γ secreting $CD4^{+}$ T-cells were higher in magnitude in completely recovered animals compared to recovered animals with lung sequestra. Both studies revealed the importance of IFN-γ in protection against Mmm (Dedieu *et al.,* 2005a; 2006). The latter study also revealed that the Mmm recovered animals are immune from further infection. Totte *et al.* (2008) unravelled the importance of lymphocyte memory T cells in CBPP chronic animals. In that study, lymphocytes were analysed *in vivo* for proliferation, production of cytokine as well as the expression of activation and memory markers in reaction to Mmm. Proliferation of CD4+ T and B cells was observed in cultures from CBPP chronic animals when stimulated with heat-killed Mmm. This phenomenon was not observed in cultures from CBPP acute animals. Therefore, TH1 Memory lymphocytes are thought to be essential in the control of CBPP (Totte *et al.,* 2008).

Other researchers failed to prove the importance of IFN-γ in protection. Jores *et al.* (2008) could not establish a correlation between IFN-γ and the absence or presence of pathological lesions in CBPP experimental animals. The immunohistochemistry analysis revealed the presence of other cells and no increase of IFN-γ cells were seen (Jores *et al.,* 2008). Also, Scacchia *et al.* (2007) reported suppression in the cell- mediated immune response with Cyclosporine A (CsA) to influence the pathogenesis of CBPP and did not find a correlation between IFN-γ and clinical

lesions or severity of the disease. Therefore, the role of $CD4^{+}$ secreting IFN-γ in the control of CBPP needs further research due to this contradicting information.

Earlier immunological research was mainly concentrated on serological responses which yielded contradictory outcomes (Dedieu *et al.,* 2005a). The literature contains conflicting reports on the importance of humoral antibody in the protection of cattle against Mmm. Several experiments that investigated the passive antibody transfer insinuated that the humoral immunity is important in protection (Smith 1967, 1971; Dyson and Smith, 1975), whilst others implied differently, due to the lack of correlation observed between antibody response and subsequent immunity (Lloyd 1967; Gourlay 1975; March, 2004). There may not be a correlation between the antibody titers measured by either CFT or ELISA and the severity of the lesions found at post mortem in naturally or experimentally infected cattle. Cattle with high antibody titers may have no visible lesions and *vice versa* (Nicholas *et al.,* 1996). The disparities might be due to differences in the age, the breed, mode of infection, the strain used as a source of infection and the health status of the cattle used for the experiment (Nicholas *et al.,* 1996).

Dedieu *et al.* (2005a) failed to establish the correlation between the onset of the disease, CFT results (antibody response) and the different clinical forms of CBPP. It was shown that the onset of the humoral response in acute cases developed later whereas it appeared early in recovered animals (Dedieu *et al.,* 2005a). However, a correlation between the immune response measured and CBPP lesions was observed by Hübschle *et al.* (2003) during the CBPP outbreak in Northern Namibia.

An experiment with immunostimulating complexes (ISCOM) vaccine postulated further the possibility of Th-1 immune response to be responsible for CBPP lesions (Hübshcle *et al.,* 2003). The immuno dominant proteins of humoral immune response have been studied and characterized in experimentally infected animals with the

African and European strains (Abdo *et al.,* 1998). This study showed that the African and the European strains have similar immunogenic proteins (110, 98, 95, 85, 80, 72, 62, 48 and 39 kDa) except the 98 kDa which is missing in the European strains. Samples from bronchial lavage revealed 6 immunogenic proteins (110, 95, 85, 80, 72 and 48 kDa) of IgA with Afade strain. A strong IgA reaction to lipoprotein P72 was observed (Abdo *et al.,* 1998). No correlation between antibody titres, clinical lesions and lung lesions could be established when humoral immune responses were monitored for one year in animals naturally exposed to CBPP (Niang *et al.,* 2006b). However, specific IgA immune response was found at both local and systematic levels in acute, sub-acute and chronic animals. Animals in sub-acute to chronic lesions had high level of IgA immune response (Niang *et al.,* 2006b). The results of the IgM, IgG1 and IgG2 did not seem to be different in all groups and no correlation of them with the severity of the disease was established (Niang *et al.,* 2006b). Therefore, the locally produced IgA may be important in protection against CBPP.

Studies that investigated the immunity in other mycoplamas species postulated the importance of local immunity in host defense mechanism. A correlation was established between IgA antibody in respiratory secretions in man and immunity to *M. pneumoniae* (Krause and Taylor-Robinson, 1992). Furthermore, resistance to *M. bovis* in cattle was related to specific IgG in lung washings (Gourlay and Howard, 1982). Therefore, poor correlation between serum antibody concentrations and resistance to infection for Mmm might be because Mmm is located on the mucous membrane of the respiratory tract (Ayling, 2002). In serum, immunoglobulin M (IgM) responses are usually detected first after Mmm infection followed by immunoglobulin G (IgG) (Barber *et al.,* 1970), however the role of these immunoglobulins in immunopathology of CBPP is yet to be investigated.

It appears that both antibody and cell mediated immune responses are essential for prevention and resolution of CBPP (Nicholas *et al.,* 1995) and further studies are needed.

1.13 Aim and objective of the study

1.13.1 Background

Contagious bovine pleuropneumonia (CBPP) caused by Mmm has been in existence since time immemorial. It has been eradicated in many parts of the world during the 19th century through a combination of control measures.

The only practical control measure for Africa is through the use of vaccines. Current vaccines have limitations as they elicit short lived immunity and repeated coverage of all animals is impractical. Hence, development of an improved vaccine providing longer immunity and increased protection is vital. An improved understanding of the protective immune response is essential to facilitate the development of a new vaccine.

1.13.2 Aim

The aim of the study is to investigate the issues associated with CBPP by providing information on:

1. The effectiveness of the current T1/44 vaccine given sub-cutaneously as recommended by OIE.
2. The effectiveness of the current T1/44 vaccine given intranasally to see if it stimulates a protective mucosal immune response.

3. Two novel vaccine formulations using a commercial adjuvant designed to help stimulate the Th2 response, which may stimulate protection.
4. The importance of humoral immune response
5. Differences between infections caused by planktonic and biofilm grown isolates.
6. The comparative performance of old and new diagnostic tests.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental cattle

The experimental protocol and justification was reviewed and approved by the Chief Veterinary Officer of the Directorate of Veterinary Services of Namibia and his team. A total of 45 cattle of Sanga and Afrikaner breeds which derived from a cross breed of *Bos taurus* and *Bos indicus* reared on a Government research farm in Windhoek district, an area free of CBPP for more than 85 years, were randomly selected and ear tagged for identification. The age of cattle was between 31 and 132 months old. Prior to the experiment all cattle were clinically screened and their sera tested negative for antibodies to Mmm by Complement Fixation Test (CFT) and Competitive Enzyme-Linked Immunosorbent Assay (cELISA), brucellosis and bovine viral diarrhoea (results not included). The cattle were transported to an experimental research farm, Omashare that is situated in the Okavango region, which is approximately 700 km north of Windhoek. The study had to be carried out in the Okavango region which is situated in the CBPP endemic area because of trade implications.

Upon arrival at Omashare experimental research farm, cattle were divided into two groups, A and B. The older cattle were used for intubation to cause infection and serve as the challenge animals for the other groups that were all of similar ages circa 3 years. The two groups were kept at two different paddocks about 500 meters far from each other for the duration of the experiment.

Group A consisted of thirty-five cattle: ten intubated, five non-treated in-contact control, twenty (5 per group) vaccinated and referred to as vaccine group, whilst

group B had 10 cattle of which five were intubated with biofilm cells and 5 served as non-treated in-contact control (ratio of 1:1) and is referred to as biofilm group. Table 2.1 below shows the details of the experimental animals of Afrikaner and Sanga breed.

Table 2.1. Animal number, age in months and the type of treatment.

Animal number	Age	Type	Group
2	132	Intubated	
3	132	Intubated	
4	132	Intubated	
6	131	Intubated	
7	131	Intubated	A
8	131	Intubated	
9	131	Intubated	
10	131	Intubated	
41	92	Intubated	
66	69	Intubated	
146	34	T1/44 sub-cutaneous	
178	33	T1/44 sub-cutaneous	A
179	33	T1/44 sub-cutaneous	
181	33	T1/44 sub-cutaneous	
192	32	T1/44 sub-cutaneous	
147	34	T1/44 intranasal	
152	33	T1/44 intranasal	
176	33	T1/44 intranasal	A
186	32	T1/44 intranasal	
195	30	T1/44 intranasal	
150	33	Purified Protein	
155	33	Purified Protein	
161	33	Purified Protein	A
162	33	Purified Protein	
189	32	Purified Protein	
139	32	Tween 20 cells	
158	33	Tween 20 cells	
170	33	Tween 20 cells	A
182	32	Tween 20 cells	
194	31	Tween 20 cells	
145	34	In-contact	
165	33	In-contact	
175	33	In-contact	A
177	33	In-contact	
188	32	In-contact	
11	127	Biofilm intubated	
20	116	Biofilm intubated	
62	69	Biofilm intubated	B
67	69	Biofilm intubated	
78	69	Biofilm intubated	
138	34	Biofilm in-contact	
154	33	Biofilm in-contact	
159	33	Biofilm in-contact	B
180	33	Biofilm in-contact	
183	32	Biofilm in-contact	

All animals were a breed of Afrikaner and Sanga which derived from a cross of Bos *inducus* and Bos *taurus*. Group A “Vaccine group and Group B “Biofilm group”.

2.2 Vaccine studies

Four groups of five cattle (see Table 2.1) were vaccinated on the 22nd of August 2007 with one of the vaccines T1/44 (2 different routes), Purified Proteins or Tween 20 as described in sections 2.2.1 to 2.2.3.

2.2.1 T1/44 Vaccine

The Panvac modified (PERIBOV) T1/44 freeze dried vaccine, with a minimum colony forming unit (cfu) of 10^7 as manufacturer's information, was obtained from the Botswana Vaccine Institute in Gaborone. It was reconstituted as per manufacturer's instructions immediately prior to use. Five cattle were injected sub-cutaneously behind the left shoulder with 1 ml of the vaccine as recommended by the manufacturer. A further five cattle were intranassally injected with the same dose of this vaccine.

2.2.2 Recombinant protein vaccine

A novel vaccine consisting of a cocktail of five purified proteins, herein referred to as purified protein vaccine, was evaluated. The recombinant putative variable surface proteins (MSC_0117, MSC_0364, MSC_0847, MSC_0816 and MSC_1033) were prepared as described previously (Hamsten *et al.,* 2008). Briefly, these proteins were expressed in *E. coli* BL21 (DE3) and purified by Immobilized Metal Affinity Chromatography (IMAC). The vaccine consisted of equal amounts of each protein to a total protein concentration of 1 mg/ml (0.2 mg/ml of each protein) with the same adjuvant as 2.2.3 (Hamsten *et al.,* 2010). The vaccine was prepared at Department of Proteomics, School of Biotechnology, Royal Institute of Technology, Stockholm, Sweden.

Five cattle (Table 2.1), age between 32 and 33 months were sub-cutaneously injected with 1 ml of the purified protein vaccine behind the left shoulder.

2.2.3 Tween 20 vaccine

A culture of the Mmm isolate designated “Matapi”, previously isolated in 2004 from the Okavango region of Namibia was grown overnight at 37°C in 100ml of Eaton’s broth media (Table 2.3) (Nicholas and Baker, 1998). The culture was harvested by centrifugation at 10,000 *g* for 40 minutes at 4°C. The pellet was washed in 0.1M PBS pH7.2 by centrifuging three times as above. After the last centrifugation, the sediment was suspended at 1 mg/ml in 0.0125M PBS containing 1% Tween 20. The concentration of 1 mg/ml (measured by using a Pierce BCA protein assay kit) was selected based on the concentration used for *M. bovis* vaccine (Nicholas *et al.,* 2002). The culture was incubated for two hours at 37°C then centrifuged and the sediment suspended in 0.1M PBS pH7.2 with Emulsigen® (MVP Laboratories Inc., Omaha, USA) and AIOH, Rehydragel-LV® (Reheis Inc. New Jersey, USA) as per manufacturer’s instructions. Five cattle (Table 2.1) were injected sub-cutaneously behind the left shoulder with 1 ml of the Tween 20 washed cells vaccine.

2.3 Experimental infection of cattle

2.3.1 Mycoplasma strain and growth conditions

A culture of the second passage of Mmm field strain 40F05 from a previous outbreak in Okavango which has been frozen at -80 ^{o}C was grown in a sterile Erlenmeyer flask. A four point five millilitre of Modified Hayflick’s Medium (MHM) (Table 2.2) (Freundt, 1993) was inoculated with 0.5 ml of 40F05 Mmm strain and incubated

overnight at 37°C under gentle agitation. A 5 ml culture was added to 245 ml of MHM and incubated as above. To measure the titer of the culture, a serial dilution was prepared into 10 test tubes containing 10 ml of MHM. Briefly, a 1 ml of the broth culture was added to the first tube, and then a serial dilution was performed by transferring 1 ml to the next tubes until the last tube. Two hundred microliter of the culture was placed on MHM and blood agar media in order to observe and count the Mmm colonies and to ensure that the culture was not contaminated. A titre of 10^9 colony forming units (cfu) per ml at a second passage was used for the experimental infection of cattle. The culture was placed into 10 x 20 ml Falcon tubes and stored at +4°C until intubation. Meanwhile, MHM agar was prepared and 30 ml was poured into each 15 x Falcon tubes. The Falcon tubes were stored at +4°C until the cattle were intubated which was within 72 hours.

Table 2.2. Composition of MHM (pH 7.8)

Components	Concentration
Heart infusion broth (Difco)	28.5g
Deionized water	900 ml
Fresh yeast extract (25%, w/v)	100 ml
Horse serum	200 ml
Calf thymus DNA (0, 2%, w/v)	2 ml
Thallium acetate (1%, w/v)	10 ml
Penicillin G (200, 000 units/ml)	2.5 ml

Table 2.3. Composition of Eaton medium (pH 7.6- 7.8)

Components	Concentration
Difco PPLO broth	21g
Deionized water	700 ml
Fresh yeast extract	100 ml
Glucose	10g
Horse serum	200 ml
DNA	0.02g
Phenol red (0.2%)	12.5 ml
Penicillin (200, 000 IU/ml)	0.5ml

2.3.2 Infection of cattle

Ten cattle, age ranging between 69-132 months old were infected on the 1st of October 2007. For each cow intubation was carried out by inserting a horse stomach tube (Hübschle *et al.,* 2003) into the trachea of the animal down to the site where the lungs split (bifurcation). After it was ascertained that the horse stomach tube was at the right place, a 20 ml of the Mmm culture that was prepared as described in section 2.3.1 was used for the inoculum followed by 30 ml of MHM agar (crushed and mixed by using an ultra-turrax machine). Lastly a fifty millilitre of MHM was added to flush down all materials to the target site.

2.3.3 Growth of biofilm

A one millilitre aliquot of a second passage of the strain 40F05 described in section 2.3.1 was added to 9 ml of MHM and incubated overnight at 37°C. The overnight culture was diluted at 1/40 into fresh pre-warmed MHM. Using sterile forceps, 0.1 µm pore size 48 mm diameter cellulose acetate filter membrane (Sartorius) was placed on dry MHM agar and 1 ml of the diluted culture was placed on the membrane. The plates were left under a laminar flow hood for one hour at room temperature for the culture to soak into the filter membrane. Then, the plates were incubated at 37°C with 5% CO_2 for 72 hours. Five millilitre of MHM was placed into 10 x 50 ml Falcon tubes. Two biofilm membranes at a time were removed from the plates and placed face inwards into the Falcon tube containing 5 ml MHM. The adherent cells were removed from the membrane by vortexing for 1 minute. Each Falcon tube had 10 membranes re-suspended in it. The 5 ml culture from each Falcon tubes was pooled (50 ml) and centrifuged at 10,000 *g* for 20 minutes at 4°C. The supernatant was removed and the sediment re-suspended in 50 ml MHM. It was aliquoted into 5 x 10 ml tubes and stored at 4°C, ready for intubation.

2.3.4 Infection of cattle with biofilm culture

Five cattle 69-127 months old were infected on the same day and in the same manner as described in section 2.3.2 with a 10 ml of the biofilm culture prepared in section 2.3.3 with the cell count of 10^{9} cfu per ml.

2.3.5 Exposure of vaccinated and control cattle to intubated cattle

To challenge controls and vaccinated cattle, the intubated cattle were placed in their respective groups (group A and B) in two different paddoks , 500 meters away from each other on the day the intubation was carried out. At each camp, the cattle had access to clean water and were supplied daily with adequate quantities of pellets of Lucerne/hay. Subsequently, daily temperature and clinical signs were recorded by a trained veterinarian. Also, blood was collected once a week from each animal through the jugular vein using sterile 21 gauge needles and 10ml vacutainer tubes that were labelled with the animal number and date of collection. The clotted blood was centrifuged at 800*g* for 10 minutes and sera were collected into 15 ml Falcon tubes (BD) which had the same label as the vacutainer tubes. The sera were stored at -20°C prior to overnight shipping to the Central Veterinary Laboratory via a courier. The experiment continued for 122 days following the initial intubation of the cattle.

2.4 Serological tests

2.4.1 Complement Fixation Test (CFT)

The CFT is a method for demonstrating the presence or absence of antibody in the serum. The test is based on the property of antigen-antibody complexes to fix complement that is demonstrated by testing for free complement using a haemolytic indicator system. The CFT was carried out as described in the OIE Manual of Standards for Diagnostic Tests and Vaccines (Anon, 2004) with minor modification. Sera, including positive and negative reference were pre-diluted 1/10 in calcium-magnesium veronal buffer (VB) (IDVET innovative diagnostics) before inactivation at

56°C for 30 minutes. The antigen and reference sera used were obtained from Instituto Zooprofillatico Esperimentale, dell Abruzzo, an OIE Reference Laboratory for CBPP. An antigen dilution of 1/70 was used at a dose of 2 complement fixing units. The complement (Dade Behring) at a dilution required for 2 units in 25 µl was used. A haemolysin (Dade Behring Diagnostic) was used at a dilution of 1/700 in VB (14 µl into 10 ml VB). An equal volume of sheep red blood cells (SRBC) was collected into a graduated bottle containing Alsever's solution from a research farm and stored at 4°C for at least five days prior to use. On the day of the test, the cells were washed by centrifugation at 1500*g* for 10 minutes which was repeated three times in VB. Then the SRBC fraction was reconstituted in VB to make a 6% suspension. An equal amount of the diluted haemolytic serum was added to the 10 ml of 6% SRBC suspension to make a 3% SRBC suspension (haemolytic system). A 25 µl aliquot of VB was added to the appropriate wells of a round bottom microtiter plate. A 25 µl aliquot of the diluted sera were added to the appropriate wells and serial dilutions of 25 µl were performed and the last 25 µl was discarded followed by the addition of 25 µl aliquot of diluted antigen to the appropriate wells. A 25 µl aliquot of the 1/20 complement was added to all the wells. The mixture was incubated at 37°C for 30 minutes together with the haemolytic system. After 30 minutes, a 25 µl aliquot of the haemolytic system was added to the plate and incubated for 30 minutes. The plate was centrifuged at 2000*g* at 4°C for 5 min. The results were read visually and interpreted as described in the OIE Manual for Diagnostic Tests and Vaccine for Terrestrial Animal with a positive samples having 100% inhibition of haemolysis at a 1/10 dilution.

2.4.2 Competitive Enzyme Linked Immunosorbent Assay (cELISA)

The cELISA developed by CIRAD and described in the OIE Manual for Diagnostic Tests and Vaccine for Terrestrial Animal was used. This ELISA was designed to use a specific monoclonal antibody (117/5) which does not cross react with other mycoplasmas. The test was performed following the manufacturer's instructions. Briefly, 100 µl aliquot of dilution buffer (DB) was added to all the wells of a pre-test plate (dilution plate). Then 11 µl of the test sera and control sera were added to the appropriate wells. A 110 µl aliquot of the monoclonal (diluted 1/120 in DB) was added to all the wells, leaving wells A1 and A2 as conjugate controls. After that, a 100 µl of this mixture was transferred to the corresponding wells of the antigen coated plate and incubated for sixty minutes at 37°C under constant gentle shaking. The plate was washed three times with washing buffer and a 100 µl aliquot of 1/100 diluted conjugate was added and incubated as described previously for 30 min. After a further wash, a 100 µl aliquot of 3,3',5,5' Tetramethylbenzidine (TMB) substrate was added and incubated at 37°C for 20 min. A 100 µl aliquot of stop solution (0.5 M sulphuric acid) was added and the plate was read using the microtiter plate reader (Multiskan EX, Thermo Electron Corporation) with 450nm filter. The percentage inhibition (PI) value for each serum was calculated using the following formula:

$$PI = \{(OD_{mab}-OD_{test\ serum})/\ (OD_{mab}-OD_{conjugate})\} \times 100\%$$

OD_{mab} = optical density for the monoclonal antibody; $OD_{test\ serum}$ = optical density for the test serum and $OD_{conjugate}$ = optical density for the conjugate.

2.4.3 Procedure for LPPQ ELISA

An indirect ELISA based on the antigenic recombinant peptide N'-terminal half of the lipoprotein LPPQ of the Mmm developed by CHEKIT (Bommeli Diagnostics, Switzerland) (Bruderer *et al*., 2002) was used to test only some sera because the production of the kits was discontinued during the course of the experiment. The sera were diluted at 1/10 in dilution buffer. A 100 µl aliquot was added to the appropriate wells of the coated plate and incubated for 90 minutes at 23^oC. The wells were washed three times using the washing buffer. Subsequently, 100 µl aliquot of anti-ruminant IgG-conjugate horseradish peroxidase was added and incubated for 60 minutes as previously described. After a further wash, 100 µl aliquot of the chromogen solution was added. The optical density was measured with a microtiter plate reader at 405 nm and the value for the sample was expressed as below:

$(OD_{sample}-OD_{mean\ of\ negative\ control}/ OD_{mean\ of\ positive\ control})$ x 100%

2.4.4 Procedures for the indirect ELISA

2.4.4.1 Preparation of Antigen for the indirect ELISA

A 0.5 ml freeze dried stock culture of Mmm from APHA was reconstituted and sub cultured in 4.5 ml Eaton medium. After overnight incubation at 37^oC with 5% CO_2, the culture was poured into 90 ml pre-warmed medium in order to increase the volume of the culture and was incubated as previously on the shaker for 48 hours. Then the culture was centrifuged at 10,000*g* for 40 minutes. The supernatant was discarded and the sediment re-suspended in 20 ml of 0.1M PBS. A Thermo 2mg/ml

concentration of antigen was determined with a bicinchoninic acid (BCA) protein Assay (Scientific Pierce).

2.4.4.2 Chequerboard titration for the indirect ELISA

A chequerboard titration was performed to determine the specific dilutions for both antigen and immunoglobulins. Different dilutions of antigens (1/100; 1/200; 1/400; 1/800; 1/1600 and 1/3200) were prepared in carbonate/bicarbonate buffer (one Tablet 0.05M (Sigma) was dissolved in 100 ml of distilled water). Two columns (row A to H) of the flat bottom shape microtiter plates were coated with 100 µl aliquot of the appropriate dilution of antigen. The plates were incubated at 37°C for 2 hours. The plates were washed 4 times with PBS using the microtiter plate washer and after the last wash the plates were tapped on absorbent paper to remove any excess fluid. A 100 µl aliquot of the positive and negative control sera at a dilution of 1/100 in serum/conjugate diluent buffer (0.1M PBS, 0.025% Tween 20 and 1% powdered milk) were added to the appropriate wells. The plates were incubated for 30 minutes before being washed as previously described.

The dilutions of conjugate (1/1000; 1/2000; 1/3000 and 1/4000) were performed in conjugate diluent buffer and a 100 µl aliquot of 1/1000 conjugate was added to wells of rows A and B, a 100 µl aliquot of 1/2000 conjugate to wells of rows C and D, a 100 µl aliquot of 1/3000 to wells of rows E and F, and a 100 µl aliquot of 1/4000 conjugate was added to wells of rows G and H. The plates were incubated at 37°C for 30 minutes and washed as above. A 100 µl aliquot of freshly prepared enzyme substrate (mix substrate buffer 1:1 with substrate chomogen) (Vetequinol Cat no 0701360 & 0701349) was added to all the wells. When the OD of positive control was between

0.28 and 0.32 when measured at 405nm, a 50 µl aliquot of stop solution (1M Citric acid) was added and the final reading was measured at 450nm. The optimum dilution of the antigen and the conjugate were taken as the highest dilutions that gave an OD_{450} value of approximately 1 for the positive serum and 0.20 for the negative serum. The chequerboard titration was carried out for each immunoglobulin (IgG1, IgG2, IgM and IgA).

2.4.4.3 Test procedure for indirect ELISA for *Mycoplasma mycoides subspecies mycoides*antibodies

An indirect ELISA was performed to detect the antibodies to Mmm using different immunoglobulins. The antigen was diluted in carbonate/bicarbonate buffer at 1/400, 1/100, 1/200 and 1/100 for IgG1, IgG2, IgM and IgA respectively as previously determined by chequerboard titration described in section 2.4.4.2 and incubated for 2 hours at 37°C. After two hours of incubation the plates were washed 4 times with PBS and a 100 µl aliquot of 1/100 diluted sera (including controls) in dilution serum/conjugate diluent buffer were added to appropriate wells. A 100 µl aliquot of the serum/conjugate diluent buffer was added to G11 and H to serve as blank control wells. After 30 minutes of incubation at 37°C, the plates were washed as above and a 100 µl aliquot per well of secondary antibody (IgA, IgG1, IgG2 and IgM) anti-cow horseradish conjugate (Sigma) was added. The secondary antibodies were diluted in the same buffer as for serum at a dilution of 1/10,000; 1/500; 1/10,000 and 1/2000 for IgG1, IgG2, IgM and IgA respectively as previously determined in chequerboard titration as described under section 2.4.4.2. The plates were incubated for a further 30 minutes at the same temperature as described previously. After 4 washes, substrate

was added, followed by stop solution when the OD of the positive control was between 0.30 and 0.40 nm, determined by Multiplate reader at 405nm. The final optical density reading was measured with a photometer at a wavelength of 450nm. The calculation for each serum was automatically done using the generic Newbovis program with this formula.

$OD_{\text{mean sample}} - OD_{\text{mean of blank}} / OD_{\text{mean of positive control}} - OD_{\text{mean of blank}}$

2.4.5 Test procedures for Latex Agglutination Test (LAT) to detect the antibodies against the *Mycoplasma mycoides*

The BoviLAT from Animal and Plant Health Agency (APHA), Weybridge, UK, was used as per manufacturer's instructions. The test utilizes a polysaccharide antigen extracted from the Mmm capsule that is bound to latex beads. Mmm was prepared as described in 2.4.4.1. The supernatant was used as it contains more carbohydrate than cells. After the pH of the supernatant was adjusted to 5.0 it was boiled for one hour before filtered through a grade 1 Whatman paper, (Sigma-Aldrich). Ethyl alcohol was added to the filtrate. After an overnight incubation of the mixture at 4°C, the precipitate was collected by centrifugation for 15 minutes at 1000*g*. The precipitate was suspended in distilled water and agitated for 2 hours at room temperature before the mixture was centrifuged for 30 minutes at 3000*g*. The sediment was discarded and carbohydrate was extracted by adding an equal volume of aqueous phenol. The mixture was incubated at 68°C for one hour and left overnight at 4°C followed by another centrifugation for 30 minutes at 3000*g*. After that the aqueous layer was separated through a PD10 column with distilled water. After several steps of centrifugation, the precipitate was lyophilized. The lyophilized precipitate was

suspended in PBS (pH 7.2) and carbohydrates were separated according to size by filtration through a column with BioSep Sec 3000, 60 x 2.1 cm (Phenomerex, UK) using an M45 HPLC/low pressure system (Waters, UK). The extracted polysaccharide antigen was used to bind the latex beads. Briefly, the latex beads were mixed with polysaccharide extract in PBS containing sodium ethylene diamine tetra-acetate and sodium azide (Ayling, 2002). After properly shaking the mixture, it was incubated at 37°C for one hour.

For the execution of the test, 10 µl of the sera collected during the course of the experiment was added to an equal volume of the latex beads with bound polysaccharide antigen on microscope slide. After two minutes of mixing the presence or absence of the agglutination was observed against a dark background (Ayling, 2002).

2.4.6 Test Procedures for the Lateral Flow Device (LFD) to detect the antibodies against the *Mycoplasma mycoides*.

The performance of the newly constructed LFD by Foresite Diagnostics (York, UK) in conjunction with APHA, Weybridge (Churchward *et al.,* 2007) was evaluated. Essentially, the test uses the same Mmm carbohydrate extract antigen that is used in the LAT. However, it includes within one slide, the test and control. Briefly; a 9 µl of serum or whole blood was added to the dilution buffer and mixed well and 75 µl of the mixture was placed on the LFD and left for 5 minutes to incubate. Although a clear single band or double bands is visible when the test is negative or positive respectively, a battery operated portable reader was used to obtain a clear-cut result of a percentage value relating the colour of the test band to the positive control band.

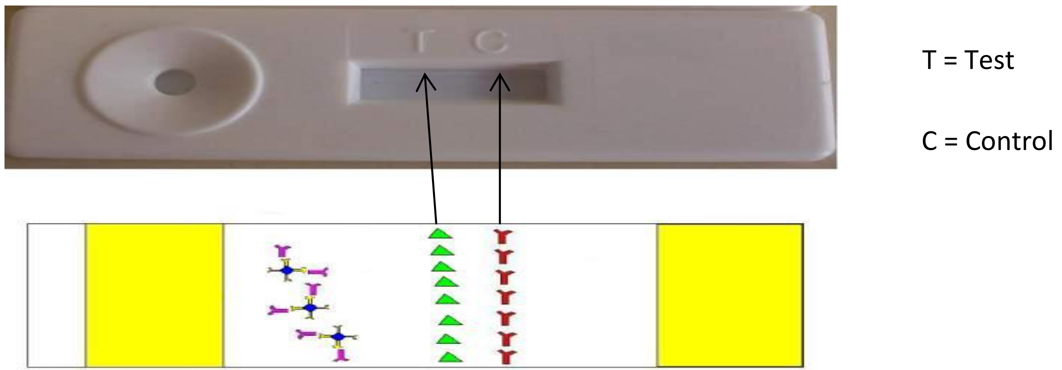


Fig. 2.1. Lateral Flow Device

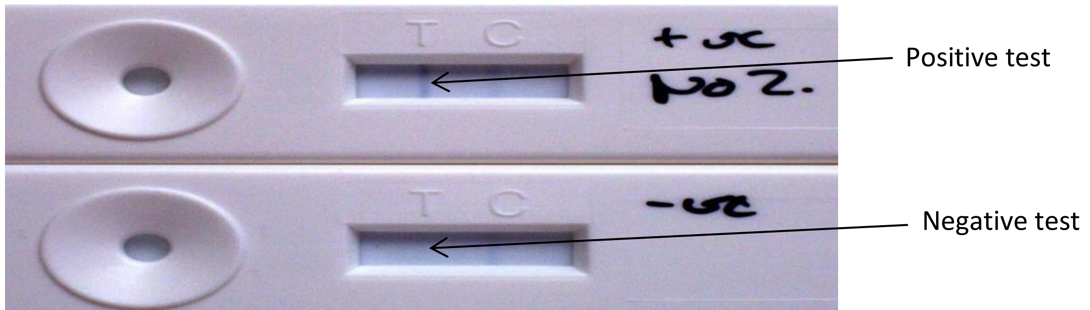


Fig 2.2. Reading of Test

Fig. 2.3. Portable Lateral Flow Reading Device

2.4.7 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting for the determination of antibodies against the *Mycoplasma mycoides subspecies mycoides* in sera collected during this experiment

2.4.7.1 Preparation of CBPP antigen for SDS PAGE

The antigen was prepared as described in section 2.4.4.1 and 5.2 µg/ml concentration of the protein was used. Seventy-one microliter of antigen was added to 29 µl of PBS and 100 µl Laemmli buffer (Laemmli (1970). The mixture was boiled for 10 minutes and cooled on ice before it was briefly vortexed.

2.4.7.2 SDS PAGE Gel Preparation

Mini-Protean III gels plates with 1mm spacers were used. The procedure was carried out according to Laemmli (1970). Briefly, a 12% resolving gel was prepared using 30% Acrylamide, HPLC water, 0.5M Tris-HCL (pH 6.8), 10% Sodium dodecyl sulphate (SDS), 10% Ammonium Persulphate (APS) and Tetramethylethylenediamine (TEMED). A five millilitre of the prepared gel was dispensed between the assembled glass plates to about 4 cm below the top and one millilitre of isobutyl alcohol was immediately added to the gel to allow the gel to settle evenly. After the gel polymerised, the isobutyl was removed and the gel was washed with running tap water. The residual water was removed by inserting a thin blotting paper. A four percent stacking gel (30% Acrylamide, HPLC water, 1.5M Tris-HCL (pH 8.8), 10% SDS, 10% APS and TEMED) was prepared and layered over the solidified resolving gel. The comb was inserted into the gel before the gel was allowed to set for 30 minutes. The casting was dissembled, comb removed and the glass plates containing the gels were placed into the central core of the electrophoresis tank. A

concentrated electrode buffer (75g Tris (hydroxymethyl) aminomethane, 25g Sodium Dodecyl Sulphate (10% w/v), 360g Glycine, 5 liters of H_2O) was diluted at one to five in deionised water and enough volume to cover the lower part of the gel was added to the tank. A 195 µl aliquot of the antigen prepared as described in section 2.4.7.1 was added to each gel and a 15 µl aliquot of the molecular weight marker (Dual color plus, Bio-Rad) was loaded into the well. The tank was connected to the power pack 200 (Bio-Rad) and electrophoresed for 45 minutes at 200 volts.

2.4.7.3 Transfer of the gel from the electrophoresis apparatus

The electrophoresis apparatus was dismantled and the gels were transferred to a container containing Bjerrun and Schafer-Nielsen transfer buffer (Tris NH_2C $(CH_2OH)_3$) 5,82g, Glycine 2,93g, SDS 0,0375g, Methanol 200ml and Water 1L). The gels were placed on top of pre-soaked 0.45 µm nitrocellulose membranes that were cut to the same size as the size of the thick blotting paper and the gel. The apparatus was connected to the power source at 15 volts for 30 minutes. The membranes were removed and stained with 0.2% (w/v) Ponceau S (2 g Ponceau S, 30 ml acetic acid (3% v/v), to make up 1L in ddH_2O) in order to visualize that the protein was successfully transferred from the gels to the membranes. The membranes were rinsed with deionised water followed by PBS and placed in blocking buffer (1M Glycine, 1% Ovalbumin, 5% Skimmed milk in 0.1M PBS) at either room temperature for one hour under slow shaking or overnight at 4^oC. Subsequently, they were washed twice with PBS + 0.1% Tween 20 and rinsed once in PBS.

2.4.7.4 Western Blotting for the detection of antibodies against *Mycoplasma mycoides*

The Western blotting was carried as described by Nicholas *et al.* (2008b). The membranes were cut into 4mm wide strips. A single strip was used for each serum including negative and positive controls. Each strip was placed on a channel of the incubation tray and soaked in 1 ml of serum diluted at 1/50 in serum/conjugate diluent (0.1% skimmed milk, 0.1% chicken egg albumin, 0.1M PBS) and incubated at 37°C for 2 hours under gentle shaking. The strips were washed thrice in washing buffer (0.1M PBS + 0.1% Tween 20) and rinsed once with PBS. One millilitre of the anti-species conjugate diluted to the correct concentration as previously determined (IgM =1/10,000; IgG1 = 1/1000; IgG2 = 1/1000) was added and incubated for 1 hour as before. After washing as above, the strips were soaked in 4-chloro-1-naphthol (4CN) (Thermo Scientific, Pierce Biotechnology) at room temperature until the band developed to a desired intensity and then the reaction was stopped by rinsing the strips with deionised water.

2.5 Detection of CBPP antigen in lungs and lymph nodes of experimental animals by histological and Immunohistochemistry (IHC) analysis

Pieces of lungs and lymphnodal tissue were cut and fixed in neutral buffered formalin. The fixed tissues were passed through baths of concentrated ethanol in order to remove the excess water. The alcohol was removed from the tissues with a hydrophobic clearing agent (xylene) before they were infiltrated in paraffin wax. The infiltrated tissues were then embedded in wax blocks (Scanziani *et al.,* 1997). Sections of 5µm were stained with standard haematoxylin and eosin. Sections were also used for immuonohistochemical staining to assess the localization of the Mmm

antigen in the lung and lymph node. The Streptavidin Biotin Complex Peroxides (DAKO) method was used. All samples were tested with rabbit hyper-immune serum anti-Mmm (IZS A&M) diluted 1:1560 in tris-buffered saline solution, pH 7.6 containing 5% skimmed milk powder. The rabbit serum was used for negative control.

2.6 Pathology and clinical scores of experimental animals

At the end of the experiment, cattle were euthanized and examined for gross pathological changes, including adhesions, the presence of pleural and pericardic fluids. The clinical scoring system (Chapter 3, Table 3.1) for CBPP as described by Hübschle *et al.* (2006) was used. The aim was to quantify the serological response, clinical signs and post mortem findings weighting the score in favour of the size of CBPP lesion. The maximum scoring number is 18. The cattle that were sacrificed early due to CBPP during the course of the experiment or that died of CBPP were given a maximum clinical score of 18 by a veterinarian.

2.7 Statistical analysis of data of indirect ELISA and CFT

A Proc Mixed statistical tool (SAS, 2003) was used for immunological study on data analysed with indirect ELISA because it gives flexibility in covariance structures for repeated measurements data and accounts for within animal, time-dependent correlations (Littell *et al.*, 1998). The predictor variables considered in the models were type of vaccine (A = T1/44 sub-cutaneous; B = T1/44 intranasal; C = Purified Protein; D = Tween 20 and E = Control), time (week = to 18) and treatment X time interaction. The dependent variables were IgG1, IgG2, IgM and IgA.

CHAPTER THREE

EXPERIMENTAL STUDIES ON THE EFFECT OF T1/44 VACCINE, PURIFIED PROTEIN VACCINE, TWEEN 20 VACCINE, IN THE CONTROL OF CBPP AND THE INTERACTIONS BETWEEN Mmm BIOFILMS AND THE HOST *IN VIVO*

3.1 Introduction

Although vaccines are available to control CBPP, they lack the required efficacy with limited duration of immunity, therefore requiring repeated annual vaccinations (Thiaucourt *et al.,* 2000). Furthermore, the T1/44 strain maintains a level of pathogenicity when administered to cattle through the intra-tracheal route (Hübschle *et al.,* 2002). T1/44 is nonetheless used in the absence of a better product (Scacchia *et al.,* 2007). To date, the development of a new generation of vaccines has not given satisfactory results (Abusugra *et al.,* 1997; Hübschle *et al.,* 2004). Although the use of antibiotics is officially discouraged due to the possibility that it may mask the disease and create chronic carriers (Provost *et al.,* 1987), they are still widely used.

In order for an improved vaccine to be formulated, the understanding of the humoral immune response, clinical signs and pathological lesions caused by Mmm is needed. Formation of biofilms by mycoplasma species was reported by Simmons and Dybvig, (2009) and McAuliffe *et al.* (2006). This phenomenon is alleged to lead to the survival of mycoplasmas in harsh environment. It is further alleged to be responsible for mycoplasmas resistance to antibiotics as well as persistance in the host. When different strains of 11 mycoplasmas species were tested for their abilities to form biofilms on 11 mm^2 glass coverslips, Mmm did not show biofilm formation (McAuliffe *et al.,* 2007). However, Mmm formed biofilm when it was grown on cellulose acetate filter membrane (McAuliffe *et al.,* 2008). The role of Mmm biofilm in the establishment of the disease, its pathogenicity, evasion of the immune system or possible re-

emergence has not yet been evaluated. Therefore, the interactions between Mmm grown as a biofilm and the host *in vivo* were evaluated.

This study was therefore in two folds. First, to assess the clinical signs and pathological lesions in experimentally vaccinated, intubated and control animals. Secondly, to assess the same parameters as above in animals intubated with a biofilm grown mycoplasma.

3.2 Materials and Methods

3.2.1 Experimental cattle

The experimental protocol and justification was reviewed and approved by the Chief Veterinary Officer of the Directorate of Veterinary Services of Namibia and his team. A total of 45 cattle of Sanga and Afrikaner breeds (age 31 and 132 months old) which derived from a cross breed of *Bos taurus* and *Bos indicus* reared on a Government research farm in Windhoek district, an area free of CBPP for more than 85 years, were randomly selected and ear tagged for identification. Prior to the experiment all cattle were clinically screened and their sera tested negative for antibodies to Mmm by Complement Fixation Test (CFT) and Competitive Enzyme-Linked Immunosorbent Assay (cELISA), brucellosis and bovine viral diarrhoea (results not included). Animals were examined for the presence of endo- and ecto-parasites and no treatment was considered necessary.

Upon arrival at Omashare experimental research farm (approximately 700 km north of Windhoek, in the Okavango region) cattle were divided into two groups, A and B. Group A consisted of thirty-five cattle: ten intubated, five non-treated in-contact

control, twenty (Four groups of five cattle) vaccinated and referred to as vaccine group, whilst group B had 10 cattle of which five were intubated with biofilm cells and 5 served as in-contact control (ratio of 1:1) and is referred to as biofilm group. See Chapter 2, table 2.1 which shows the details of the experimental animals of Afrikaner and Sanga breed. The two groups were kept at two different paddoks which were about 500 meters far from each other for 122 days.

3.2.2 Infection of experimental cattle with *Mycoplasma mycoides* subspecies *mycoides*

3.2.2.1 Infection of experimental cattle through endobronchial intubation

3.2.2.1.1 Intubation of experimental cattle with conventional Mmm culture

The mycoplasma culture was prepared as described in Chapter 2, section 2.3.1. Briefly, an endo-tracheal intubation was performed by inserting a horse stomach tube into the trachea until bifurcation (the site where the lungs split). Briefly, each of the 10 cattle in the intubated group (group A) (see Chapter 2, Table 2.1) was infected with 20 ml of mycoplasma culture with 10^9 colony forming units (cfu) per ml at a second passage as described in Chapter 2, section 2.3.2.

3.2.2.1.2 Intubation of experimental cattle with biofilm Mmm culture

The procedure for the preparation of biofilm mycoplasma culture is described in Chapter 2 under section 2.3.3. Briefly, five cattle 69-127 months old were infected on the same day and in the same manner with a 10 ml of the biofilm culture with the cell count of 10^9 cfu per ml (see sections 2.3.2 and 2.33

3.2.3 Infection of experimental animals by contact with experimentally infected animals

In-contact non-treated animals and animals vaccinated with various vaccines (see Chapter 2, sections 2.2.1, 2.2.2 and 2.2.3) five weeks prior to infection were placed together with the intubated cattle on the day the intubation was performed. The biofilm intubated animals were also placed together with the in-contact non-treated animals on the day the intubation was carried out. The animals in the vaccine group (Group A) and the biofilm group (Group B) were kept at 2 different paddocks which were 500 meters apart from each other for 122 days (17 weeks).

3.2.4 Clinical examinations of experimental animals

A Veterinary Officer was stationed at the site of the experiment. All animals were observed daily and clinical findings were recordedy for the duration of the experiment. These included rectal temperatures, cough, nasal discharge and dyspnea. Animal with a temperature exceeding 39.0^{o}C was considered febrile.

3.2.5 Serological examination of experimental animals

Blood samples were collected once a week from each animal via jugular venepuncture using sterile 21 gauge needles into 10ml vacutainer tubes that were labelled with the animal number and date of collection. The clotted blood was centrifuged at 800*g* for 10 minutes and sera were collected into 15 ml Falcon tubes (BD) which had the same label as the vacutainer tubes. The sera were stored at -20°C prior to overnight shipping to the Central Veterinary Laboratory via a courier.

Samples from each animal were tested for the presence of Mmm antibodies using CFT. The CFT was carried out as described in the OIE Manual of Standards for Diagnostic Tests and Vaccines (Anon, 2004) with minor modification (see Chapter 2 section 2.4.1).

3.2.6 Postmortem examination of experimental animals

At hundred and twenty-two days' post infection, cattle were euthanized by stunning with a captive bolt pistol followed by exsanguination. The nature of lung lesions and the percentage of parenchyma involved in the pathological process were recorded. The lesions recorded in the lungs of affected animals were characterized as:

- Acute lesions – hepatisation at different stages, thickening of interlobular septa, presence of serous pleural exudates in the thoracic cavity and of fibrinous pleuritis
- Sub-acute lesions – fibrinous adhesions between visceral and parietal pleura, necrotic areas in lung parenchyma
- Chronic lesions – encapsulated sequestra at different stages of organisation or liquefaction.

The clinical scoring system for CBPP (Table 3.1) as described by Hübschle *et al.* (2006) was used. The cattle that were sacrificed early during the course of the experiment due to CBPP were given a maximum clinical score of 18. Samples of lung tissues, pleural fluids, lung sequestra, lymph nodes and kidneys were collected from all animals.

Lungs and lymphnodal tissue collected at post mortem were fixed in neutral buffered formalin and embedded in paraffin (see Chapter 2, section 2.5).

Table 3.1. The pathological scoring system for CBPP of Hudson and Turner (1963) was adapted as follows:

Indicators of infection	Pathological scores
Serological response (CFT) to challenge	
<10	0
10	1
20-80	2
≥160	3
Clinical response to challenge	
Pyrexia at least 39.5 ^{0}C for >3 but <7 days	1
Pyrexia for >7 days	2
Animal succumbed/euthanized	3
PM findings	
Chronic lesions (sequestra)	1
Acute/sub-acute (marbling etc.)	2
Mmm isolated or detected	2
Multiply the sum by a factor depending on lesion size	
<5cm	x 1
>5cm but <20	x 2
>20cm	x 3
Maximum score is	
Serology	3
Clinical	3
PM (2 +2) 3	12
Total	18

Hübschle *et al.* (2006)

3.3 Results

3.3.1 Clinical and serological findings of animals within the vaccine group

3.3.1.1 Clinical and serological findings of conventionally Intubated animals

Elevated body temperatures were first recorded between days 6 and 106 post intubation. The average time of elevated temperatures among animals was 11 days. Persistence of pyrexia varied from 1 to 16 days. Respiratory distress of variable intensity was recorded in 8 animals. Serological response measured by CFT was first detected at day 6 post intubation while the last animals sero-converted at day 107 post intubation. The CF titres varied between 640 and 10240. Nine animals were CFT positive at post mortem. One animal remained CFT negative with no apparent clinical signs for the duration of the experiment. None of the animals succumbed to CBPP; they were only necropsied at the end of the experiment, at week 17 (122 days).

3.3.1.2 Clinical and serological findings of animals vaccinated sub-cutaneously with T1/44

The elevated body temperature was recorded in one animal at day 100 post infection. Three animals did not show any respiratory distress while two presented respiratory distress of variable mild intensity. Two animals (#146 and 178) were CFT positive 2 weeks post vaccination and all five animals became positive in CFT 4 weeks post vaccination. However, the CF titres in four animals (#146, 179, 181 and 178) declined to zero between days 74 and 114 post infection and remained negative up to the end of the experiment. The fifth animal that was febrile for one day was CFT positive at post mortem. None of the animals died during the course of the experiment.

3.3.1.3 Clinical and serological findings of animals vaccinated intranasally with T1/44

Pyrexia was recorded in two animals between days 61 and 86 post infection with an average time of two days. Respiratory distress of variable intensity was recorded in four animals. The average time for detecting serological response was 7 days' post infection with the earliest reaction recorded on day 44 and the latest on day 93 post infection. Peak CF titres varied between 10 and 5120. One animal remained sero-negative and one became sero-negative 3 weeks prior to post mortem examination. All 5 cattle were culled at the end of the experiment.

3.3.1.4 Clinical and serological findings of animals vaccinated with Purified Protein

Elevated body temperatures were recorded between days 44 and 84 post infection with an average time of 9 days. Two animals presented respiratory distress of variable intensity. The average time for detecting serological response in all animals was 11 days; the earliest reactions were recorded on day 30 post infection and the latest on day 86. One animal (#150) was CFT positive 4 weeks post vaccination and the titer increased in intensity at day 72 post infection. All animals were CFT positive at post mortem. None of the animal succumbed to the disease during the course of the experiment.

3.3.1.5 Clinical and serological findings of animals vaccinated with Tween 20 cells

Pyrexia was first recorded between days 44 and 76 post infection in three out of five animals. Persistence of high fever varied between 9 and 12 days. Respiratory distress was recorded in all animals. Three animals had vaccine titers prior to infection. However, the intensity of the CF titers increased between days 37 and 51 post infection at which time the animals were febrile. Two animals (#182 and 194) succumbed to CBPP during the course of the experiment on week 16 and 12 with CF titers of 2560 and 1280 respectively. Two of the three surviving animals had CF titers at post mortem while the third animal that had low CF titers varied between 10 and 40 for 8 weeks was negative for additional 8 weeks prior to post mortem examination.

3.3.1.6 Clinical and serological findings of non-treated in-contact control animals

Raised body temperatures were first recorded between days 44 and 93 post infection with an average time of 5 days. Respiratory distress of variable intensity was recorded in three animals. In three animals (#165, 188 and 177), sero-conversion preceded pyrexia whereas in two, the contrary was observed. The first serological response was detected at day 23 post infection while the last animal sero-converted at day 100. Peak CF titres varied between 40 and 5120. All animals were CFT positive at slaughter.

3.3.2 Clinical and serological findings of animals within biofilm group

3.3.2.1 Clinical and serological findings of biofilm Intubated animals

Pyrexia was recorded in one animal (#78) on days 112 and 113 post intubation. The same animal had low CF titres of 20 for two days. Sero-conversion preceded pyrexia in this animal. Respiratory distress was recorded in two animals. Animal #67 was the only animal with high CF titres from day 31 post intubation until at post mortem examination. Sero-conversion was recorded in the total absence of pyrexia (Table 3.3). All cattle were sacrificed at the end of the experiment.

3.3.2.2 Clinical and serological findings of non-treated in-contact control animals

One animal (#138) was sacrificed on day 79 post infection, due to an acute form of CBPP. The body temperatures were recorded in 2 animals (#138 and 159) on days 37 and 96 post infection with an average time of 3 days. Persistence of pyrexia varied from 2 to 3 days. These two animals sero-converted on days 73 and 93 post infection. In animal #138, sero-conversion was observed before pyrexia while in animal #159, the contrary was observed.

The clinical findings of the animals within the vaccine and the biofilm groups are summarised in Tables 3.2 and 3.3 respectively.

3.3.3 Post mortem findings of animals within both vaccine and biofilm groups

The post mortem findings of animals in vaccine and biofilm groups are summarised in Tables 3.2 and 3.3 respectively. Necropsy was carried out on all animals at the end of

the experiment. The types of lesions in the thoracic cavity were recorded on a specifically designed form (see appendix 1) and classified as: acute lesions: hepatisation at different stages, thickening of interlobular septa, presence of pleural fluid in the thoracic cavity, fibrinous pleurisy- “omelette”; sub-acute lesions: adhesion between visceral and parietal pleura, necrotic area in lung parenchyma; chronic lesions: encapsulated sequestra at different stages of liquefaction (Scacchia *et al.,* 2011). Three cattle out of 45 were necropsied on welfare ground at different times during the cause of the experiment. The typical lesions observed at post mortem were characterized by straw coloured fluid and the affected portion of the lung was large and solid. Increased interlobular septa; red and gray areas of hepatisation; fibrinous deposits on parietal surface of lungs; liquefaction necrotic areas and sequestra were observed.

3.3.3.1 Post mortem findings of animals intubated with conventional Mmm culture

Chronic lesions were observed in seven animals. One animal (#8) had three stages of the lesions: acute, sub-acute and chronic while animal #10 had sub-acute and chronic lesions. Figure 3.3 shows fibrin in visceral pleura of animal number 8. Animal #9 did not have CBPP lesions but the lung was hypertrophic. CBPP was confirmed by Real Time PCR (carried out at IZS, Teramo) in all animals excluding #9. The total integrated score for the intubated animals was 94.

3.3.3.2 Post mortem findings of animals vaccinated sub-cutaneously with T1/44

Pathological lesions found during post mortem examinations in these cattle were less severe compared to the cattle vaccinated with the same vaccine but intranasally and those vaccinated with Tween 20 and Purified Protein. Two animals (#179 and 192) had chronic lesions with small sequestra of 1 cm and 5 cm (Table 3.2). No CBPP lesions were observed in the other three animals. The cattle in this category had less clinical symptoms with a total integrated score of 12.

3.3.3.3 Post mortem findings of animals vaccinated intranasally with T1/44

Chronic lesions were observed in all the animals. However, two animals (#152 and 176) had small sequestra of 1.2 and 2 cm. The total integrated score was 39.

3.3.3.4 Post mortem findings of animals vaccinated with Purified Protein

Animals in this group had chronic pathological lesions with large sequestra varied between 8 and 25 centimeter in diameter (Table 3.2). The entire lung of one animal was sequestrated. Figure 3.1 depicts the encapsulated sequestrum with necrotic core from an animal vaccinated with purified protein vaccine. The lesions in the animals of this group are comparable to the lesions of the in-contact control animals. The total integrated score was recorded at 59.

3.3.3.5 Post mortem findings of animals vaccinated with Tween 20 cells

Two animals (#194 and 182) succumbed to CBPP at weeks 12 and 16 post infection. Chronic pathological lesions were recorded in four animals including the animals which died during the course of the experiment. Animals # 94 and 182 had large sequestra that invaded the left epical lobes. The total integrated score among all animals was 44.

3.3.3.6 Post mortem findings of none treated in-contact control animals

Chronic lesions with various sizes of sequestra were recorded in all animals at post mortem examination. Apart from chronic lesions, animal #145 had sub-acute lesions with lung hepatisation and marbling. Figure 3.2 shows lung adhesion of animal number 177. The total integrated score was 58.

3.3.3.7 Post mortem findings of animals within Biofilm Intubated and none treated in-contact control cattle

Chronic pathological lesions were observed in one animal (#159) of the control group. Animal #138 in the control group died of acute form of CBPP. There were no CBPP lesions observed in the rest of the animals (Table 3.3). Biofilm mycoplasma seemed to have established a mild form of the disease when compared to the counterparts in the vaccine group. The total integrated score was 11 for intubated animals and 30 for in contact challenged animals.

Table 3. 2. Summary of clinical, pathological and serological results for cattle within vaccine group

Cattle No	Age in Month	Group Type	No of days Temp ≥39 ^{0}C	Cough	Clinical score	CFT at PM	CFT + Duration (weeks	CBPP mortality	Type of lesions	Size of sequestrum
2	132	Intubated	9	No	14	1280	6	No	S, PF	>40 cm
3	132	Intubated	7	Yes	13	320	10	No	S, PF	30 cm
4	132	Intubated	1	Yes	10	160	6	No	S, PF	12 cm
6	132	Intubated	15	Yes	10	80	16	No	S	6 cm
7	132	Intubated	16	Yes	8	320	17	No	S, PF	3.5 cm
8	132	Intubated	11	Yes	17	2560	3	No	S, PF	
9	132	Intubated	0	Yes	2	0	0	No	N	
10	132	Intubated	11	No	9	640	3	No	S, PF	
41	92	Intubated	15	Yes	7	40	17	No	S, PF	2.5 cm
66	69	Intubated	14	Yes	14	2560	7	No	S	40 cm
146	34	T1/44 s/c	0	Yes	2	0	19	No	PF, PF*	
178	33	T1/44 s/c	0	No	2	0	16	No	PF	
179	33	T1/44 s/c	0	No	2	0	16	No	S, PF*	1 cm
181	33	T1/44 s/c	0	Yes	2	0	13	No	PF, PF*	
192	32	T1/44 s/c	1	No	4	160	20	No	S, PF	4 cm
147	34	T1/44 i/s	4	Yes	12	5120	1	No	S, PF	10, 20, 15 cm
152	33	T1/44 i/s	0	No	3	0	7	No	S, PF*	1.2 cm
176	33	T1/44 i/s	0	Yes	3	0	0	No	S, PF	2 cm, 2 cm
186	32	T1/44 i/s	0	Yes	12	1280	5	No	S	>40 cm
195	30	T1/44 i/s	2	Yes	9	640	14	No	S, PF	10 cm
150	33	PP	1	No	9	2560	15	No	S, PF	10 cm
155	33	PP	12	Yes	11	1280	9	No	S, PF, PF*	15 cm
161	33	PP	13	Yes	14	2560	6	No	S, PF	>40 cm
162	33	PP	9	No	11	320	13	No	S	8 cm
189	32	PP	10	No	14	320	12	No	A, S, PF	25 cm
139	32	Tween 20	12	Yes	8	320	12	No	S, PF	5 cm
158	32	Tween 20	0	Yes	2	0	8	No	H, PF	
170	32	Tween 20	0	Yes	4	80	18	No	PF	
182	32	Tween 20	13	Yes	15	2560	*	Yes	S, PF	>40 cm
194	31	Tween 20	12	Yes	15	1280	*	Yes	S	>40 cm
145	34	Contact	2	No	15	640	5	No	H, M, S, PF	28 cm
165	33	Contact	1	Yes	6	160	15	No	S, PF, PF*	<2 cm
175	33	Contact	1	Yes	9	160	9	No	S, PF	5 cm; 3 cm
177	33	Contact	8	Yes	14	5120	7	No	S, PF	>40 cm
188	32	Contact	11	No	14	1280	10	No	S	35 cm

A (Lung adhesion); S (sequestrum); PF (Pleural Fluid); H (Lung hepatisation); PF* (Pericardic fluid); N (Negative); i/s (Intranasal) s/c (Sub-cutaneous)

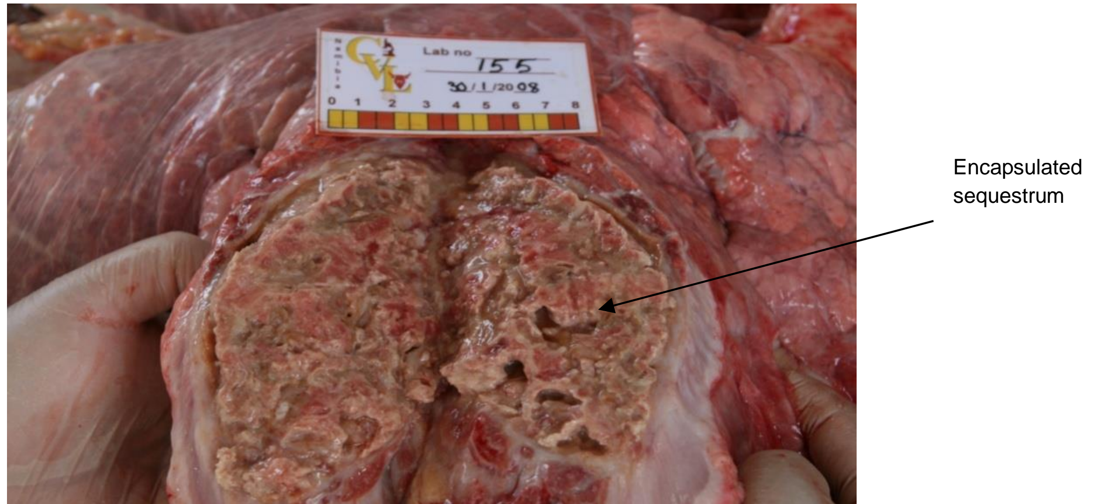


Fig.3.1. Encapsulated sequestrum with a necrotic core from an animal vaccinated with a purified protein vaccine

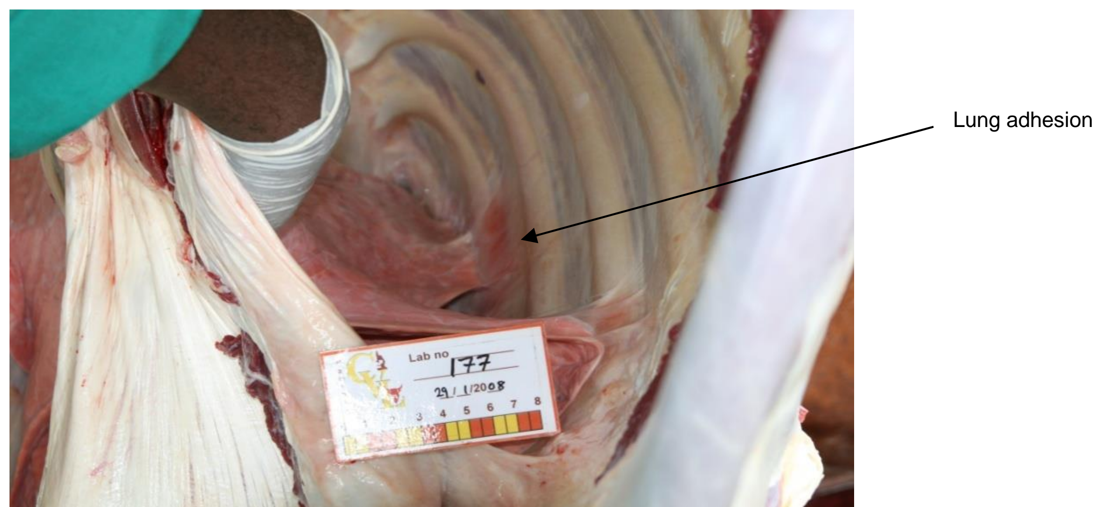


Fig.3.2. Lung adhering to the chest wall of an in-contact control animal from the vaccine group (images from this study)

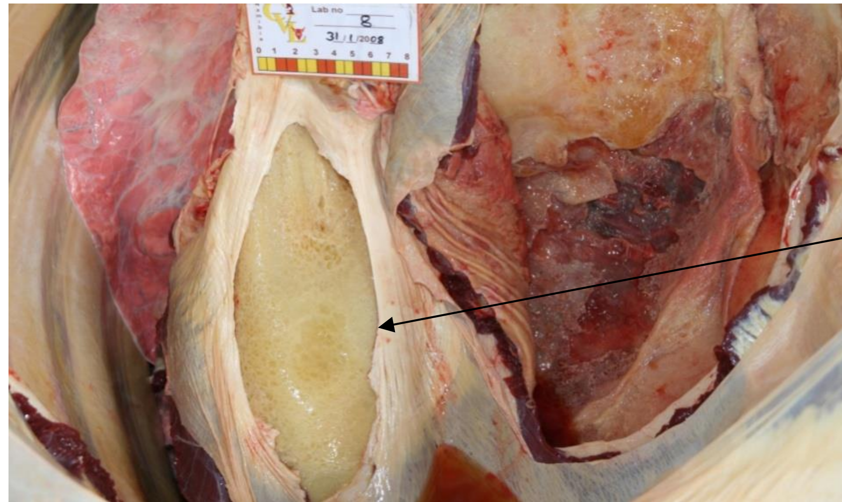


Fig.3.3. Fibrin in visceral pleura of an Mmm intubated animal

Fig.3.4. Marbled lung with thickened interlobular septa from an Mmm intubated animal (images from this study)

Table 3. 3. Summary of clinical, pathological and serological results for cattle within biofilm group

Cattle No	Age in Month	Group Type	No of days Temp ≥39 ^{0}C	Cough	Clinical score	CFT at PM	CFT + Duration (weeks	CBPP mortality	Type of lesions	Size of sequestrum
11	127	Intubated	0	Yes	2	0	0	No	H, PF*	
20	116	Intubated	0	No	2	0	0	No	PF*	
62	69	Intubated	0	No	2	0	0	No	N	
67	69	Intubated	0	Yes	3	160	14	No	PF*	
78	69	Intubated	2	No	2	0	2	No	N	
138	34	Control	3	No	13	5120	*	Yes	S	
154	33	Control	0	No	2	0	0	No		
159	33	Control	3	No	10	1280	5	No	S, PF	15-17 cm
180	33	Control	3	No	3	0	0	No	PF, PF*	
183	32	Control	1	Yes	2	0	1	No		

A (Lung adhesion); S (sequestrum); PF (Pleural Fluid); H (Lung hepatisation); PF* (Pericardic fluid); N (Negative)

3.3.4 Histopathological findings on animals within the vaccine and biofilm groups

Histopathological findings were similar in most animals and they were characterized as following:

- The observations on the lung tissues were: catarral fibrinous pneumonia and pleuropneumonia; fibrosis of septa with infiltration of inflammatory neutrophils, eosinophils and macrophages. Also, edema, emphysema, atelectasis, necrosis and congestion were observed on the lungs.
- The lymph nodes displayed the lymphoid infiltration around the lymphatic vessels. The lymphatic vessels were dilated and lymphoid hyperplasia with marked enlarged follicle was visible.
- No major findings were seen in kidney tissues, however interstitial nephritis and glomerulonephrosis were observed in one animal.

3.3.5 Immunohistochemistry findings of animals within both vaccine and biofilm groups

The result of the immunohistochemistry (IHC) is detailed in Table 3.4. A distinctive immunostaining of macrophages, alveoli, fibrin, bronchial lumen, epithelium and BALT was recorded in lung tissues of most animals. The follicle germinal centers and medulla of the lymph nodes stained positive for the presence of Mmm.

Mmm antigen was detected by IHC in the lungs and/or lymph nodes of most of the animals. The biofilm intubated animals did not present detectable stained Mmm antigen in their lungs, however, Mmm antigen was detected in the lymph node of cow number 154.

The immunoglobulin A was detected in the majority of the vaccine non-treated in-contact and Purified Protein vaccinated cattle. The antibody responses in the Purified Protein cattle were comparable to the responses of cattle in the vaccine non-treated in-contact. The Complement component 3 (C3c) responses were detected in the majority of animals with high CF titers at post mortem.

Table 3. 4. Immunohistochemistry activities of animals in vaccine and biofilm groups

An.	Type	IHC CBPP		IHC IgA		IHC IgM		IHC IgG1		IHC IgG2		IHC C3c	
No		Lung	Lymph node	Lung	Lymph node	Lung	Lymph node	Lung	Lymph node	Lung	Lymph node	Lung	Lymph node
2	IN	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
3	IN	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
4	IN	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
6	IN	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	POS
7	IN	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	NEG	POS
8	IN	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG
9	IN	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
10	IN	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	POS	NEG
41	IN	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	POS	POS
66	IN	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
146	T1/44 s/c	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
178	T1/44 s/c	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
179	T1/44 s/c	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
181	T1/44 s/c	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
192	T1/44 s/c	POS	POS	NEG	NEG	NEG	NEG	POS	POS	NEG	NEG	POS	POS
147	T1/44 i/n	POS	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	POS	POS
152	T1/44 i/n	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
176	T1/44 i/n	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
186	T1/44 i/n	POS	POS	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	POS	NEG
195	T1/44 i/n	POS	POS	NEG	NEG	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG
150	PP	POS	POS	POS	POS	POS	POS	POS	POS	POS	NEG	NEG	POS
155	PP	POS	POS	POS	POS	NEG	POS	NEG	POS	NEG	NEG	NEG	POS
161	PP	POS	POS	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
162	PP	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
189	PP	NEG	POS	NEG	POS	NEG	POS	NEG	POS	NEG	NEG	NEG	POS
139	T20	POS	POS	NEG	POS	NEG	POS	NEG	POS	NEG	NEG	NEG	POS
158	T20	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
170	T20	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
182	T20	POS	NEG	POS	NEG	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG

194	T20	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG
145	VC	POS	POS	POS	NEG	POS	POS	POS	POS	NEG	NEG	POS	POS
165	VC	NEG	POS	NEG	NEG	NEG	POS	NEG	POS	NEG	NEG	NEG	POS
175	VC	POS	POS	POS	NEG	POS	POS	POS	POS	NEG	NEG	POS	POS
177	VC	POS	POS	POS	NEG	POS	POS	POS	POS	NEG	NEG	POS	POS
188	VC	POS	POS	POS	NEG	POS	POS	POS	POS	NEG	NEG	POS	POS
11	BFI	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
20	BFI	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
62	BFI	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
67	BFI	NEG	POS	POS	ND	POS	POS	ND	POS	NEG	NEG	NEG	NEG
78	BFI	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
138	BFC	POS	POS	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	POS	POS
154	BFC	NEG	NEG	NEG	ND	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
159	BFC	POS	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG	POS	NEG
180	BFC	POS	POS	NEG	ND	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
183	BFC	POS	NEG	NEG	NEG	NEG	NEG	ND	NEG	NEG	NEG	NEG	NEG

NEG = Negative; POS = Positive and ND = Not done

3.4 Discussion

Mycoplasma mycoides subsp. *mycoides* (Mmm) is the causative agent of contagious bovine pleuropneumonia (CBPP) (Mulongo *et al.,* 2013). CBPP is still a priority disease for FAO-Emergency Prevention System for Transboundary Animal Diseases (FAO-EMPRES) (Niang *et al.,* 2010). It is a serious respiratory disease of cattle that causes major economic losses, specifically in Sub-Saharan Africa (Egwu *et al.,* 1996; Manso-Silva *et al.,* 2009; Jores *et al.,* 2013). In most countries in Africa, where the disease is present, vaccination with live attenuated strain T1/44 is the preferred control measure (Amanfu, 2009). However, it elicits short-lived immunity (Niang *et al.,* 2010). Therefore, an improved vaccine is urgently needed. An animal experiment was conducted with the aim of assessing the capacities of the two novel vaccines (Tween 20 and Purified Protein) to protect animals from CBPP. Their protection capacities were compared to the current T1/44 vaccine.

Animals that were vaccinated with Purified Protein, Tween 20 cells and conventional T1/44, 5 weeks earlier were placed together with artificially infected animals and in-contact control animals. The purpose of the artificially infected animals was to transmit the infection to other animals (Niang *et al.,* 2006b). Furthermore, the artificially infected animals give an approximate evaluation of the quantity of organism and incubation period (Gull *et al.,* 2013). Similarly, animals that were endobronchially infected with biofilm prepared mycoplasma cultures were placed with in-contact healthy control animals, also to simulate natural infection of the disease.

At necropsy, the intubated cattle had chronic pathological lesions. This observation was in accordance with Scacchia and colleagues (2011). The one animal that did not have CBPP lesions was serologically negative for the duration of the experiment. This could be that the circulating antibodies bound to soluble antigen and prevented the antibodies to be detected by serological tests (Provost *et al.,* 1987). However, no CBPP lesions were seen in this animal at post mortem examination, which make this phenomenon unlikely. The same situation, although that animal had acute lesions, was observed by others (Nicholas *et al.,* 1996) where one animal was serologically negative. The author postulated that the lesions may have been caused by other respiratory pathogens (Nicholas *et al.,* 1996). The sequestra observed in the lung of the majority of animals in the vaccine group were well formed and the affected lungs had acute and/or sub-acute and chronic lesions. The lesions in all animals were unilateral. Nunes Petisca *et al.* (1990), observed unilateral lesions in 95% of the 566 affected lungs (cited by Nicholas *et al.,* 2008). The lesions observed in this study are in accordance with lesions described by Bygrave *et al.* (1968).

Judging from the lesions found at post mortem, the Tween 20 and Purified Protein vaccines appeared to have exacerbated the disease. There were no clear differences in terms of pathological lesions and clinical signs between the animals vaccinated with Purified Protein and the non-vaccinated control animals. Mulongo *et al.* (2013) observed a similar situation between immunized and control animals, although in their experiment Freund's adjuvant formulations were used. The Purified Protein contained the variable surface protein (Vmm). Variable surface proteins (Vmm) are alleged to enhance colonization of the host tissue (Hamsten *et al.,* 2008; Persson *et al.,* 2002). The rationale

for the formulation of the Purified Protein was to elicit the immune responses which avert the action of the Vmm (Hamsten *et al.,* 2010). The failure of the Purified Protein to mount protection may be due to the protein concentration, the adjuvant used and the administration conditions (Hamsten *et al.,* 2010). The vaccines formulated so far have failed to elicit protective immune responses in all animals. The ISCOM and Saponin vaccines experiment held in Namibia exacerbated the disease as the vaccinated animals were as equally affected as the in-contact control animals (Hübschle *et al.,* 2003; Nicholas *et al.,* 2004).

The T1/44 vaccine in sub-cutaneously administered animals elicited a better protection. Cattle in this sub-group had less clinical score. A strong humoral immune response to MSC_1046 (LppQ) in the T1/44 sub-cutaneously vaccinated animals was reported. It is assumed that this protein plays an important role in protection provided it induces immune response prior to the onset of the disease (Hamsten *et al.,* 2010). However, this assumption needs to be determined. In order to evoke local immune response, a mucosal route of vaccine administration needs to be explored (Abusugra and Morein, 1999). Although T1/44 vaccine is not recommended to be administered in mucosal tissues, its performance was nonetheless evaluated. The majority of animals in this sub-group developed chronic lesions. This discrepancy might be attributable to the dose given. It is possible that a higher dose might have elicited protection. Mice immunized intranasally with a dose three times higher exhibited higher IgA and IgG subclass responses (Abusugra and Morein, 1999).

A second part of the experiment was to evaluate the interaction of the biofilm grown Mmm in the establishment of the infection. The term 'Biofilm' was discovered and

described in 1978 (Costerton *et al.,* 1978). Biofilm is formed by a group of bacteria that aggregate together and/or attached to a biotic or abiotic substratum and are protected by a polysaccharide matrix (McAuliffe *et al.,* 2008). This formation enables them to survive in harsh environmental conditions such as heat (McAuliffe *et al.,* 2007). A biofilm is alleged to persist in the host and cause damage to the host tissues as well as to exacerbate the infection (McAuliffe *et al.,* 2008). Although the ability of Mmm to form biofilm has been evaluated (McAuliffe *et al.,* 2008), the evolution of the disease in the host caused by biofilm mycoplasma has not yet been evaluated. The intubated cattle in the biofilm group had fewer clinical scores than anticipated. However, judging from clinical scores and the pathological lesions found in in-contact control animals, the intubation was a success. The differences in the evolution of the disease in the biofilm intubated animals and control animals could possibly be that when the planktonic cells are released from biofilm in the non-treated cattle, they mutate and become more virulent.

The immunohistochemistry examination revealed the concentration of Mmm/antigen in the macrophages cells, bronchial lumen as well as in the germinal centers of the lymphoid follicles. Scanziani *et al.* (1997), observed large concentration of Mmm antigen in the germinal centers of the follicles. This could be due to the activation of the B-cell that takes place in most chronic microbial diseases (Cotran *et al.,* 1994 cited by Scanziani *et al.,* 1997). The majority of the animals were positive for IgG1 and C3c. Chronically infected animals in the in-contact non-treated control animals (Group A) and animals vaccinated with Purified Protein exhibited strong IgA immune response. It seems that IgA is involved in the progression of the disease in this study. This is

contrary to the observation made by Niang *et al* (2006b). The histopathology results showed no noteworthy difference on the lesions observed with-in and between groups of animals. The microscopic lesions of the lungs included fibrinous pleuropneumonia, fibrosis septa and lymphangiectases in most of the animals. The lesions of the tracheobronchial lymph nodes were lymphoid hyperplasia with marked enlarged follicle germinal centre in all animals but three animals that were vaccinated with T1/44 sub-cutaneous and two animals in biofilm intubated group did not have lesions. These lymph node lesions are in accordance with lesions found in animals that were infected with various strains of Mmm (Gull *et al.,* 2013).

Animal experiments are important in simulating the disease. However, the high cost involved in setting up animal experiment results in the use of few cattle that makes it difficult to arrive at meaningful conclusions from pilot experiments, however they give a good indication of whether novel vaccines or antibiotic treatments are worth pursuing. Moreover, the use of different breeds of cattle and different inoculum dose make difficult to compare data from different experimental infections. Therefore, as suggested by Jores *et al.* (2013), a uniform challenge model for study of CBPP infection would be ideal. Nonetheless, this study concluded that the two novel vaccines did not provide protection and the T1/44 sub-cutaneously vaccinated animals were better protected. However, it may be that changes in the concentration of the antigens in the vaccines, or a different adjuvant may have had different outcomes. Therefore, these vaccine formulations should not be totally disregarded in future, but the immunological data obtained should be used for future vaccine design. Similarly, the biofilm animals did not derive meaningful conclusion but it was interesting to note that intubated animals had

fewer lesions, although they were evidently infected and passed the infection to in-contact control animals. May be some form of biofilm based vaccine could be useful in future, therefore, more research is needed.

In conclusion, the author's results indicate that induction of humoral immune response to Purified Protein and Tween 20 vaccines failed to protect animals against challenge with the organisms, with vaccinated animals showing exacerbated pathology. Therefore, in the absence of better vaccines, improved diagnostic tests should be one of the options for CBPP control in Africa (Ssematimba *et al.,* 2015). Sera collected during this experiment were used to evaluate the performance of Lateral Flow Device (LFD) as a potential field test (Chapter 4).

CHAPTER FOUR

COMPARATIVE EVALUATION OF COMPETITIVE ELISA, LIPOPROTEIN Q ELISA, LATEX AGGLUTINATION TEST AND LATERAL FLOW DEVICE FOR THE DETECTION OF Mmm ANTIBODIES IN EXPERIMENTAL CATTLE

4.1 Introduction

In the absence of efficient CBPP vaccines, an improved and easy to use diagnostic test that is able to identify animals in all stages of diseases will assist in the control of CBPP in Africa (Heller *et al.,* 2016; Ssematimba *et al.,* 2016). A quick and reliable diagnosis is a prerequisite for any disease treatment and more so for the diseases that have trade implications. Conventional Polymerase Chain Reaction (PCR) and real-time PCR are available for the quick diagnosis for early response as well as for disease monitoring (Schnee *et al.,* 2011; Vilei and Frey, 2010; Bashiruddin *et al.,* 2005). However, these tests require sophisticated equipment and skilled technicians (Mair *et al.,* 2013, Ayling *et al.,* 2007). Moreover, not all laboratories in developing countries where the disease is present have enough resources to carry out these tests.

At present, the ideal method to detect CBPP is through the detection of antibodies using serological diagnostics techniques (Amanfu *et al.,* 2002). Different serological tests such as Complement Fixation Test, Tube Aggutination Test, Tube Precipitation Test, Agar Plates, Enzyme Linked Immunosorbant Assay and Slide Agglutination Test were used in the diagnosis of CBPP. These tests are sensitive but the specificity is questionable (Onsa *et al.,* 2018). Therefore, modified and or new tests: Latex agglutination (BoviLAT),

Competitive ELISA (CIRAD EMVT, Montpeller) and Immunoblotting were developed for the diagnosis of Mmm (Nicholas *et al.,* (1996), Le Goff *et al.,* (1998), Gonclaves *et al.,* (1998). However, these serological tests have some advantages and limitations as well.

The immunoblot is highly sensitive and specific. It was used in Portugal during the outbreak. It is a useful technique in case of uncertain results from CFT and/or cELISA (Gonçalves *et al.,* 2008). However, some report that the test is not reproducible and would not be useful during mass campaign (Schubert *et al.,* 2011). On the other hand, the CFT, an OIE recommended test, is the preferred serological diagnostic test (Nicholas *et al.,* 1996). It has low sensitivity and specificity and can only detect about 70% of the chronically infected animals (Ayling, 2002). CFT is good during acute stages and alleged not to detect antibodies from asymptomatic animals during the early stage of the infection (Provost *et al.,* 1987). The test is difficult to standardize due to different antigens and fresh red blood cells used (Le Goff and Thiaucourt, 1998). The cELISA, also an OIE recommended test has almost the same sensitivity and specificity as the CFT. Its sensitivity was found to be 71% when compared to CFT as a gold standard (Enyaru *et al.,* 2003). Although cELISA, CFT, LAT and Immunoblot are available, there is still no "gold standard" serological test for this disease (Ayling, 2002) and they are expensive (Onsa *et al.,* 2018).

A diagnostic test that is capable of detecting all infected animals and be able to differentiate the vaccinated from infected animals would be the ideal test in controlling CBPP. It should be able to fulfil most if not all the criteria for a test. These criteria are: Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment free and

Deliverable to the user (ASSURED) (Mabey *et al.,* 2004). The Lateral Flow Device (LFD) was developed with the aim to meet these criteria as a field test.

The LFD constructed by Foresite Diagnostics (York, UK) in conjunction with APHA, Weybridge uses the same Mmm antigen that was successfully used in Latex Agglutination Test. Additionally, an anti-bovine IgG2 conjugate is imbedded in. Previously, the performance of LFD was evaluated against the CFT. The LFD detected less positive samples than CFT and this might be due to the difference in the type of immunoglobulins LFD (IgG) and CFT (IgM) recognized (Tjipura-Zaire *et al.,* 2008). Therefore, due to differences in immunoglobulins recognized by CFT and LFD, the aim here was to compare the performance of the LFD with Competitive Enzyme Linked Immunosorbent Essay (cELISA), Lipoprotein Q Enzyme Linked Immunosorbent Essay (LppQ ELISA) and Latex Agglutination Test (LAT).

4.2 Materials and Methods

4.2.1 Sera from experimental animals

A total of 1,495 sera collected from experimental animals described in Chapter 2 under section 2.1 were used. One thousand three hundred and five sera were from animals in the vaccine group whilst the remaining 190 were from animals in the biofilm group as described in Chapter 2, section 2.1. The collection of blood and centrifugation was done as described in Chapter 2 under section 2.3.5.

The majority of sera were subjected to 3 tests (cELISA, LAT and LFD). It was not possible to analyse all the sera using the LppQ ELISA as the production of the ELISA

kits ceased during the course of the experiment. The test procedures for cELISA, LppQ ELISA, LAT and LFD are described in Chapter 2 Materials and Methods under sections 2.4.2, 2.4.3, 2.4.5 and 2.4.6 respectively.

4.3 Results of the LppQ ELISA, LAT and LFD

Serological results are summarized in Tables 4.1 to 4.3.

4.3.1 Measurement of *Mycoplasma mycoides* subspecies *mycoides* antibody by cELISA

A total of five hundred and nineteen sera were analysed in cELISA and 204 (39%) were positive. Most of the positive samples were from Tween 20 vaccinated animals followed by sera collected from animals vaccinated sub-cutaneously with T1/44.

4.3.2 Measurement of *Mycoplasma mycoides* subspecies *mycoides* antibody by LppQ ELISA

Two hundred and sixty-four sera were analysed with LppQ ELISA. Twenty sera reacted positive for Mmm. It is worth to note that the LppQ ELISA was used in the analysis of sera collected in the early stage of the experiment. This could be the reason why the majority of the sera tested negative. The majority of the sera tested positive were from animals #146, #181 (both T1/44 sub-cutaneously vaccinated) and #194 (Tween 20).

4.3.3 Measurement of *Mycoplasma mycoides* subspecies *mycoides* antibody by LAT

Not all sera were analysed with LAT, however a total of three hundred and ninety-nine sera were tested with LAT and 194 (49%) were positive. The LAT had the most positive samples compared to the cELISA and LFD.

4.3.4 Measurement of *Mycoplasma mycoides* subspecies *mycoides* antibody by LFD

A total of three hundred and ninety-five sera were analysed and 151 (38%) were positive at a 30% cut-off. The LFD had the least positive samples in comparison to cELISA and LAT.

4.3.5 Comparison of the the results of cELISA, LppQ ELISA, LAT and LFD in detecting *Mycoplasma mycoides* subspecies *mycoides* antibodies for experimental animals

Table 4.1 shows a summary of 770 sera tested with cELISA, LAT and LFD. All the tests detected almost equal numbers of positive and negative samples. The agreement was 38% and 45% between cELISA and LFD in positive and negative sera respectively. For cELISA and LAT, the agreement was 37% and 42% for positive and negative sera respectively. Table 4.2 shows rhe comparion of the performance of LppQ, LAT and LFD with cELISA an OIE recommended test for the diagnosis of CBPP. A total of 353 sera were analysed in all four tests. LppQ ELISA had less sensitivity compared to the other three tests (table 4.2). Most of the positive sera detected by LppQ ELISA are from

animal #194 vaccinated withTween 20 which succumbed to the disease during the course of the experiment. The results of the comparisons of cELISA, LFD and LAT for intubated animals in the vaccine group for specified weeks are depicted in tables 4.3. Out of 40 sera tested in three tests from intubated animals in the vaccine group (group A) 20 (50%) were positive in all 3 tests while 15 (37.5%) were negative in all tests (table 4.3). The results of the comparisons of 4 serological tests (cELISA, LppQ, LFD and LAT) are shown in table 4.4 to 4.9 in appendix 2. The cELISA and LppQ ELISA are measured in percentage inhibition at a cut-off point of 50. The LFD is measured in percentage related to the positive control and the cut-off here was set at 30%. The LAT is measured by the degree of clumping as expressed + to +++ depending on the degree of clumping to be positive while a negative sample is expressed as -.

Table 4.1. Comparison of tests on 770 sera (381 sera between cELISA and LAT) and (389 sera between cELISA and LFD) from experimental animals

	LAT +VE	LAT –VE	LFD +VE	LFD -VE
cELISA +VE	144	67	*140	43
cELISA –VE	35	*135	*44	*162

*5 samples were tested in cELISA and LFD only while 8 samples were tested in cELISA and LAT only

Table 4.2. Comparison of tests on 353 sera from experimental animals

	LppQ +VE	LppQ -VE	LAT +VE	LAT -VE	LFD +VE	LFD -VE
cELISA +VE	11	47	20	30	12	38
cELISA -VE	2	63	4	61	9	56

Table 4.3. Comparisons of the results of the three serological tests in sera collected from intubated cattle in the vaccine group.

Cattle No	2			3			4			6			7		
Date in weeks	**cELISA %**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**
8	45	11.0	+	68	34.5	+++	19	19.1	_	92	38.7	+++	96	52.1	+++
9	21	17.6	_	66	103.1	+++	29	17.7	_	84	55.5	+++	89	46.1	++
10	35	19.9	_	67	101.4	+++	43	17.1	++	79	31.0	++	92	42.3	+++
17	79	71.9	++	77	43.2	+++	78	46.4	+++	71	27.1	++	73	43.4	+++

Cattle No	8			9			10			41			66		
Date in weeks	**cELISA %**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**	**cELISA%**	**LFD**	**LAT**
8	41	5.8	_	40	7.9	+	26	11.3	_	80	68.7	+++	18	14.5	_
9	41	10.0	_	12	14.0	+	35	10.7	_	91	43.5	+++	23	8.9	_
10	47	13.9	_	22	24.0	_	42	13.7	_	87	43.3	+++	8	11.7	_
17	70	173.0	+	36	25.5	+	73	104.9	++	70	29.3	+++	68	129.3	+++

cELISA is measured in percentage inhibition at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

4.4 Discussion

The diagnostic accuracy of a Lateral Flow Device that is embedded with Mmm carbohydrate extract antigen and anti-bovine IgG2 conjugate as a field test was compared to that of cELISA, LppQ ELISA and LAT. Using the combined results of the four tests, the possible cut-off point for LFD was determined.

Many serological diagnostics techniques are available for the diagnosis of CBPP. However, their inability to detect antibodies against Mmm at all stages due to disease outcomes (acute, subacute, or chronic disease) associated with different or a total absence of clinical signs hamper the diagnosis of CBPP requiring more than one serological test to be used in an eradication programme (Amanfu *et al.,* 2002, Muuka *et at.,* 2011; Schubert *et al.,* 2011; Enyaru *et al.,* 2012), which makes it more difficult in some African countries were resources are inadequate (Olabode *et al.,* 2013).

Diagnostic tests should be highly specific and sensitive as well as easy to execute. However, this is difficult to attain, as often an increase in sensitivity results in a decrease in specificity, which may lead to false results (Ayling, 2002). The Complement Fixation Test (CFT), an OIE recommended test, is cumbersome and requires a well-trained analyst to perform. Similarly, the cELISA, also an OIE recommended test requires specialized equipment (Ayling, 2002). Both tests have similar sensitivity (Marobela-Raborokwe *et al.,* 2003). The CFT performs well in detecting antibodies during the acute stage while the Immunoglobulin M (IgM) is dominant (Bashiruddin *et al.,* 1994). On the other hand, the cELISA detects Immunoglobulin G (IgG). It is, however, not yet known at which stage of the disease is the IgG dominant (Muuka *et al.,* 2011). Due to the risk of false positive or false negative results in individual animals, CFT and cELISA are recommended for diagnosis of CBPP at herd level only (Heller *et al.,* 2016). Since both

tests require a fully equipped laboratory the development of improved and easy to use diagnostic tests comprising antigens able to identify animals in all stages of disease is considered a research priority (Heller *et al.,* 2016). Therefore, pen-side tests, such as LAT and the newly tested LFD, would be the ideal diagnostic tests where a quick diagnosis is required.

In China, where 4000 sera were tested, LppQ ELISA had a good agreement with cELISA and CFT (Nicholas *et al.,* 2008). The LppQ ELISA performed well in the T1/44 vaccinated cattle. This could be further substantiated by the fact that LppQ is a marker for T1/44 vaccine and the MSC_1046 (LppQ) was the major component of the T1/44 induced response in the bead-based Mmm recombinant surface protein array (Hamsten *et al.,* 2010).

The LAT, as it was previously reported by Ayling (2002), performed well under this study, and hence remained a valid screening test for CBPP. The advantage of the LAT is that, it is not expensive, does not require specialized equipment and can be fairly performed by any personnel (Ayling, 2002). In general, LAT scored well in sera collected at post mortem. Although, LAT uses carbohydrate extract from a European strain, it can detect antibody responses elicited by cattle infected by contact or through bronchial intubation with African strain. The same observation was obtained when LAT was used to test immune response in sera from Uganda (Ayling, 2002).

The LFD was tested for the first time under an experimental set-up. The test was developed by Foresite Diagnostics (York, UK) in conjunction with APHA (Weybridge). It uses the same Mmm carbohydrate extract antigen that is used in the Latex Agglutination Test for the detection of antibodies against Mmm. The LFD uses an anti-bovine IgG2 conjugate**.** Under this study, the LFD displayed a low sensitivity. However, at a cut-off

point of 30% it compared well with the cELISA and the LAT. The LFD demonstrated a low sensitivity in sera collected from T1/44 sub-cutaneously vaccinated cattle. This could be that the LFD is incapable of distinguishing the immune response elicited by T1/44 vaccine from the CBPP infection. However, this observation will be better tested in a real outbreak where vaccination as a control measure is practiced. The LFD as a field test is more advantageous than LAT because the result is displayed on a portable reading device. This would potentially rule out any ambiguous results usually seen in LAT which is a subjective test and low positive result might be interpreted as negative. All serological tests currently used have limitations.

Although the cELISA has a low sensitivity, it compared well with other diagnostics tests (Muuka *et al*, 2011). The cELISA detected more positive cattle in chronic stage of infection in Zambia (Muuka *et al*, 2011). Cattle in the conventionally intubated group were positive 9 days after infection. Provost *et al.* (1987), reported that antibodies can be detected from about 10 days after the onset of the clinical signs and remain detectable for a few months. This finding is in agreement with the observations made in this study. The cELISA is an established test with a defined cut-off point of 50%. The LAT gives either a positive or a negative result based on agglutination. Based on the correlation of the results of LFD with those of cELISA and LAT in 770 sera (105 positive and 141 negative) a cut-off value for LFD was set at 30%. At a cut-off point of 20%, LFD would have detected 235 samples positive out of 395. Work done earlier using positive and negative reference sera showed that LFD compared well with other serological tests (Ayling *et al.,* 2007).

It was noted that none of the three tests (cELISA, LAT and LFD) have the sensitivity to detect all infected cattle at all stages of the disease. Similar findings were reported (Enyaru *et al.,* 2012, Gonçalves *et al.,* 2008). Consequently, more than one test should

be used in order to have a better understanding of the extent of the infection. The data presented in this chapter suggested that the LFD could be a good alternative field test as it is not subjective. However, it needs to be refined using different conjugates. Therefore, continued research for a test that is capable of detecting CBPP during all stages of the disease is important. Furthermore, it is important during the infection of CBPP to establish which immunoglobulins (Ig) play role in the protection or progression of the disease. The next chapter (Chapter 5) assessed the role of humoral immune responses in experimental animals.

CHAPTER FIVE

HUMORAL IMMUNE RESPONSE IN EXPERIMENTAL CATTLE TO *MYCOPLASMA MYCOIDES* SUBSPECIES *MYCOIDES* (Mmm)

5.1 Introduction

There is little information available regarding the role of Mmm in regulating the host's immune response. Although previous studies have shown that cattle recovered from CBPP are normally immune to re-infection or have some resistance (Gourlay, 1975; Windsor and Masinga, 1977), it is still not known whether cell-mediated or humoral immune response is essential for protection. While both the humoral and cellular immune responses are important for protection, their involvement during the progression of the disease remains a challenge because the research in the immunopathology is hampered by the numbers of cattle used for experiment (Dyson and Smith, 1975; Dedieu *et al.,* 2005a; Masinga *et al.,* 1975; Gourlay, 1975; March, 2004).

Dedieu et al. (2005a) revealed the importance of Mmm–specific IFNγ -CD4 T-cell. The study postulated the importance of IFN-γ in protection against Mmm. However, studies from Jores *et al*. (2008) and Scacchia *et al*. (2007) revealed contradictory information. Neither study found correlation between IFN-γ and the absence or presence of pathological lesions in CBPP experimental animals (Jores *et al*., 2008; and Scacchia *et al*., 2007). The immunohistochemistry examination revealed the presence of other cells and no increase of IFN-γ cells was observed (Jores *et al*., 2008). The role of CD4+ secreting IFN-γ in the control of CBPP needs to be further investigated. The immunostimulating complexes (ISCOM) vaccines experiment postulated the possibility of Th-1 immune response in the development of CBPP lesions (Hübshcle *et al*., 2003).

Therefore, it is possible that antibodies against any non-protective antigens will not be neutral and might enhance pathological lesions (Nkando *et al*., 2016).

Earlier immunological research on serological responses yielded contradictory outcomes (Dedieu *et al.,* 2005a; March, 2004; Nicholas *et al.,* 1996; Smith, 1967, 1971; Dyson and Smith, 1975; Lloyd, 1967; Gourlay, 1975). Schieck *et al.* (2014) considered that if antibody response played a critical role in controlling the outcome of the disease, the animals with less or no pathological lesions would have higher antibody response than animals that have more pathological lesions. The severely affected animals had higher antibody response which suggested that antigens are being released from sequestra which results in the high antibody response (Schieck *et al.,* 2014). However, in the study done by Nkando *et al.* (2016) animals with high titres had lower pathology indexes, suggesting that protection was dependant on titres.

Although there are serological diagnostics techniques such as CFT and cELISA for CBPP diagnosis, they lack sensitivity (Ayling, 2002). A more sensitive and specific western immunoblot test is available. The immunoblotting test confirms the positive and negative samples. However, the analysis of the protein bands requires experience and the test is cumbersome, therefore not possible for mass screening (Nicholas *et al.,* 2008a). The immune dominant proteins of humoral immune response were studied and characterized in experimentally infected animals with the African and European strains of Mmm (Abdo *et al.,* 1998). They are 110, 98, 95, 85, 80, 72, 62, 48 and 39 kDa but the European strains do not have a 98 kDa protein. Samples from bronchial lavage revealed 6 immunogenic proteins (110, 95, 85, 80, 72 and 48 kDa) of IgA with Afade strain (Abdo *et al.,* 1998).

The role of humoral immune responses has so far not been determined in animals experimentally intubated with biofilm Mmm culture. Biofilms are bacteria that stick

together and are surrounded by a polysaccharide matrix and are believed to remain in the host through the formation of an adherent biofilm (McAuliffe *et al.,* 2006). Furthermore, biofilm is believed to enable bacteria to survive in harsh environmental conditions (McAuliffe *et al.,* 2007). It is further believed that biofilm is an important stage for bacteria in disease initiation (McAuliffe *et al.,* 2006). Studies of Mmm culture from biofilm cells were not yet undertaken *in vivo*. In this study, the role of humoral immune response elicited by biofilm cells in cattle was evaluated.

The main part of this study was to determine the role of humoral immunity in: vaccinated, non-treated control and intubated animals. Furthermore, antibody response as a result of biofilm culture was also investigated. This information is important in understanding the immunopathology and the role of the antibody response in protection or in the progression of the disease. The information derived from this study might be useful in the development of improved vaccines.

5.2 Materials and Methods

5.2.1 Experimental animals

A total of forty-five animals were divided into two groups: vaccine and biofilm as described in Chapter 2 under section 2.1. The animals were treated and kept as described in Chapter 2 sections 2. 2 and 2. 3.

5.2.2 Serological tests

The sera were collected weekly for 18 weeks and analysed using indirect ELISA, CFT and Western (immuno) blotting.

The CFT was executed as described in section 2.4.1 of Chapter 2. The indirect ELISA is an in-house developed diagnostic technique whereby the plate was coated overnight with antigen described in Chapter 2, section 2.4.4.1. After an overnight incubation, the plate was washed and blocked with PBS that contained 0.5% skim milk. The test was performed as described in Chapter 2, section 2.4.4.2.

The Western immunoblot has been used to test for antibody responses in *M. bovis* (Poumarat *et al*., 1994), Mmm (Gonçalves *et al.,* 1994; Nicholas *et al.,* 1996) and *M. capricolum* subsp. *capripneumoniae* (March *et al.,* 2002). In this study, specific immune responses to Mmm proteins were determined using IgG1, IgG2 and IgM anti-cow horseradish peroxidase conjugated secondary antibodies. The procedures are detailed in Chapter 2, section 2.4.7.

5.2.3 Statistical analysis of Indirect ELISA and CFT data

The observations on humoral immune responses of cattle under different treatments were made sequentially over an 18-week period and constitute repeated measures, hence the correlation amongst observations on the same animal needs to be taken into account. Proc Mixed (SAS, 2003) was used for the data analysis because it gives flexibility in covariance structures for repeated measurements data and accounts for within animal, time-dependent correlations (Littell *et al.*, 1998). The predictor variables

considered in the models were type of vaccine (A = T1/44 sub-cutaneous; B = T1/44 intranasal; C = Purified Protein; D = Tween 20) and E = Control), time (week = 1-18) and treatment X time interaction. The dependent variables were IgG1, IgG2, IgM and IgA.

The first step in using Proc Mixed is to select an appropriate covariance structure based on information criteria such as REML log likelihood (REML logL) and Bayesian Information Criterion (BIC); the covariance structure that gave the value of information criterion closest to zero indicated a model fit to the data for each immunoglobulin. The next step is to select an appropriate model.

Three types of comparisons employing estimate statements made: (i) differences among treatments averaged over weeks; (ii) differences among means for treatments at each week; and differences between means for each week and the average of means for subsequent weeks for each treatment.

5.3 Results of the indirect ELISA and CFT

The Mmm-specific antibody response was assessed for each animal using the indirect ELISA with different immunoglobulins as well as the CFT. However, for easier comparisons the averages OD per week for animals under the same treatment were used for Indirect ELISA whilst averaged titers per week for animals under the same treatment were used for CFT. All animals except one animal in conventional intubation seroconverted. Animal seroconverted at different periods post infection. Animals in the T1/44 vaccine (sub-cutaneous) group were used as a gold standard since this is the OIE recommended vaccine. They are compared to the animals in the other groups. Table 5.1 shows the selected covariance structures for the different immunoglobulins and the effects included in the models.

Table 5.1 Selected covariance structures for the different humoral responses and their fit criteria.

	Covariance structure	BIC	REML Logl	Dependent Variables
IgG1	Simple	75.8	72.6	Treatment, week, treatment*week
IgG2	AR(1)	193.1	186.6	Treatment, week, treatment*week
IgM	Simple	-68.9	-72.1	Treatment, week, treatment*week
IgA	ANTE (1)	509.7	397.0	Treatment, week

*AR(1) = Autoregressive order 1; ANTE(1) = Ante-dependence order 1

5.3.1 Measurement of Mmm-specific serological isotypes and antibody responses

5.3.1.1 Measurement of Mmm-specific Immunoglobulin G1 (IgG1) response

IgG1 response was affected ($P < .05$) by treatment, week and treatment x week interactions (Figure 5.1). Treatment mean for each week was compared separately due to the significant interaction in the IgG1 response. A significantly higher ELISA OD response was observed in animals vaccinated with Tween 20 compared with all other treatments; OD increased from week 1 to 9 before OD declined. The animals vaccinated with purified protein displayed similar kinetic distribution pattern as those in Tween 20 group although their average OD per week were low. The T1/44 sub-cutaneous group maintained a fairly steady antibody OD from week 1 to 8 where it reached a plateau, before declining from week 12 to 18. Although the T1/44 intranasal and the non-treated in-contact control groups had low serological response at the onset; the responses

increased steadily with time; there was no significant difference observed between T1/44 intranasal and the non-treated in-contact control over the 18-week test period. Both treatments with T1/44 sub-cutaneous and T1/44 intranasal steadily increased the IgG1 response, attaining a plateau at week 9.

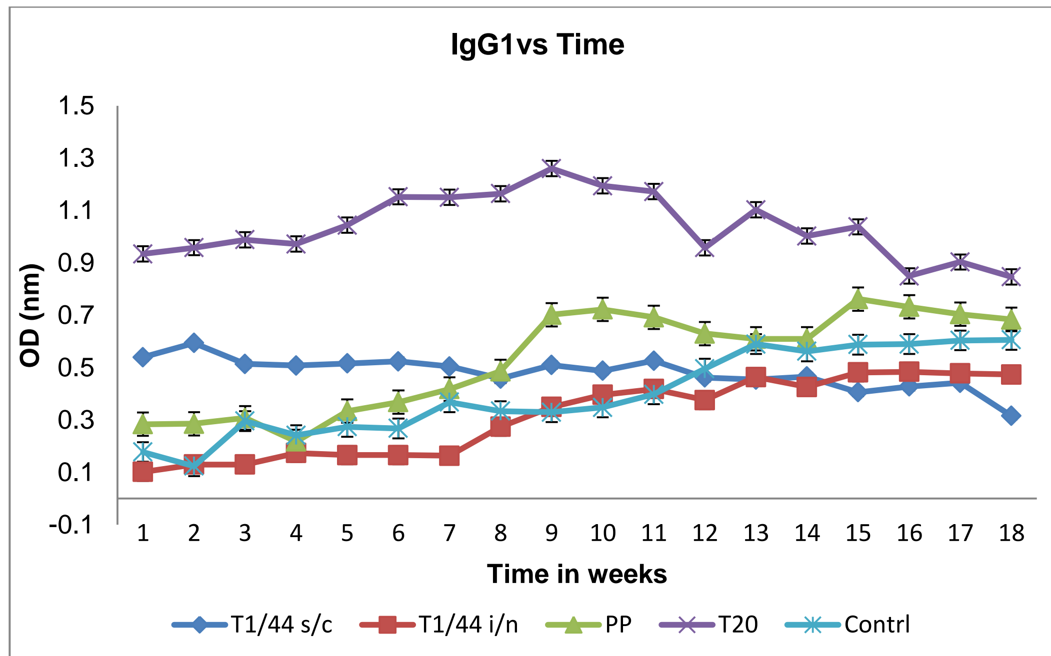


Fig.5.1 shows the average OD data for IgG1 as measured by indirect ELISA for animals in vaccine group. N= number of animals (5 animals per group).

5.3.1.2 Measurement of Mmm-specific Immunoglobulin G2 (IgG2) response

IgG2 response was significantly ($P < .05$) affected by treatment, week and treatment times week interactions. Although the interaction was marginally significantly ($P =.0483$) affected, the least square means for IgG2 are plotted in Figure 5.2. IgG2 responses for T1/44 sub-cutaneous were significantly ($P <.05$) higher than the animals in the non-treated in-contact control group from week 1 to 12. Tween 20 response was significantly ($P < .05$) higher than T1/44 sub-cutaneous from weeks 1 to 18. In other groups except

the T20, the IgG2 responses were lower than T1/44 sub-cutaneous and increased from week 8 for Purified Protein group and from week 13 onwards for non-treated in-contact control group as well as the animals in the T1/44 intranasally vaccinated group. The immune response for T1/44 sub-cutaneous was steadily maintained although it was slightly above 0.2 OD for the first four weeks and started declining towards the end of the experiment. Treatment with Tween 20 evoked a significantly ($P < .0001$) higher IgG2 response than Purified Protein.

T1/44 sub-cutaneous and Tween 20 attained a plateau in IgG2 production at week 10; other treatments failed to attain a plateau.

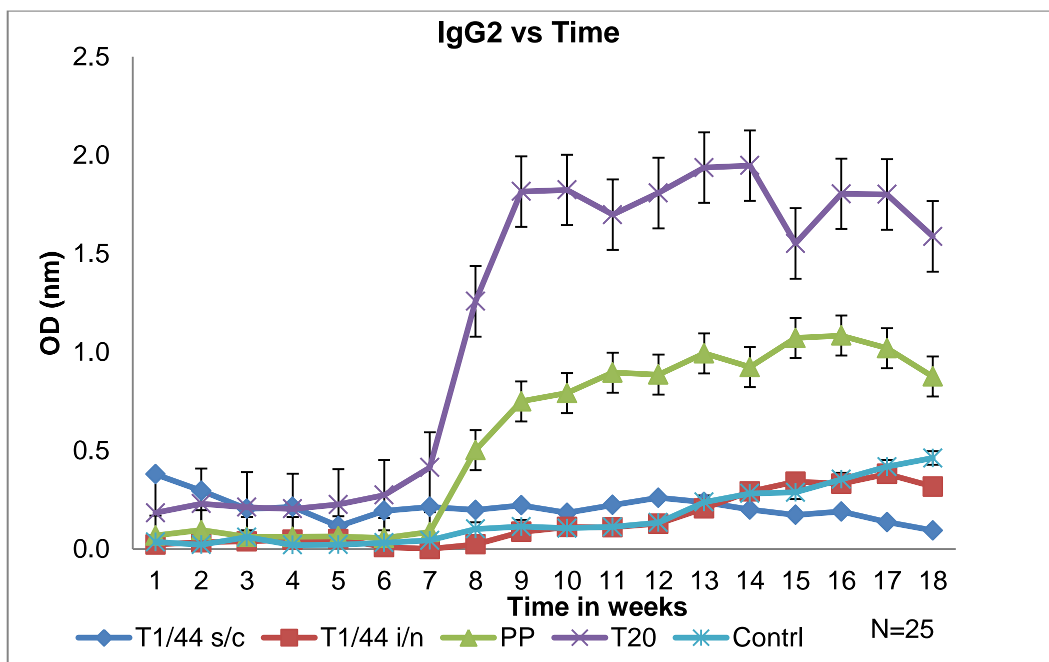


Fig.5.2. shows the average OD data for IgG2 as measured by indirect ELISA for animals in vaccine group. N= number of animals (5 animals per group).

5.3.1.3 Measurement of Mmm-specific Immunoglobulin M (IgM) response

IgM response was significantly ($P < .05$) affected by treatment, week and treatment times week interactions. Figure 5.3 shows the least square means for serological Mmm-specific IgM response. The figure shows that the Mmm infection elicited a strong but maintained response in all groups. The first high peak was observed at around week 8 in four groups except the T1/44 sub-cutaneous. As with IgG1 & IgG2, Tween 20 group maintained the highest optical density values. The response in T1/44 group was more sustained but with intermittence for the duration of the experiment.

Throughout the 18-week period, there was no significant difference in mean response on IgM between treatments T1/44 sub-cutaneous and Tween 20. From around week 6 onwards, Tween 20 generally evoked a significantly ($P < .05$) greater response than Purified Protein. Furthermore, Tween 20 evoked a significantly ($P < .05$) higher response of IgM than the non-treated in-contact control group between week 8-13. Similar to what was observed for IgG1 and IgG2, treatment with T1/44 sub-cutaneous reached a plateau at week 9 possibly reflecting the onset of the pathological lesions. Treatments with T1/44 intranasal and Tween 20 all gave rise to a plateau in IgM production at about weeks 8-9. Purified Protein gave no plateau in IgM production, similar to what was observed for IgG1 and IgG2. For the non-treated in-contact control, a plateau in IgM production was surprisingly observed much later at week 12.

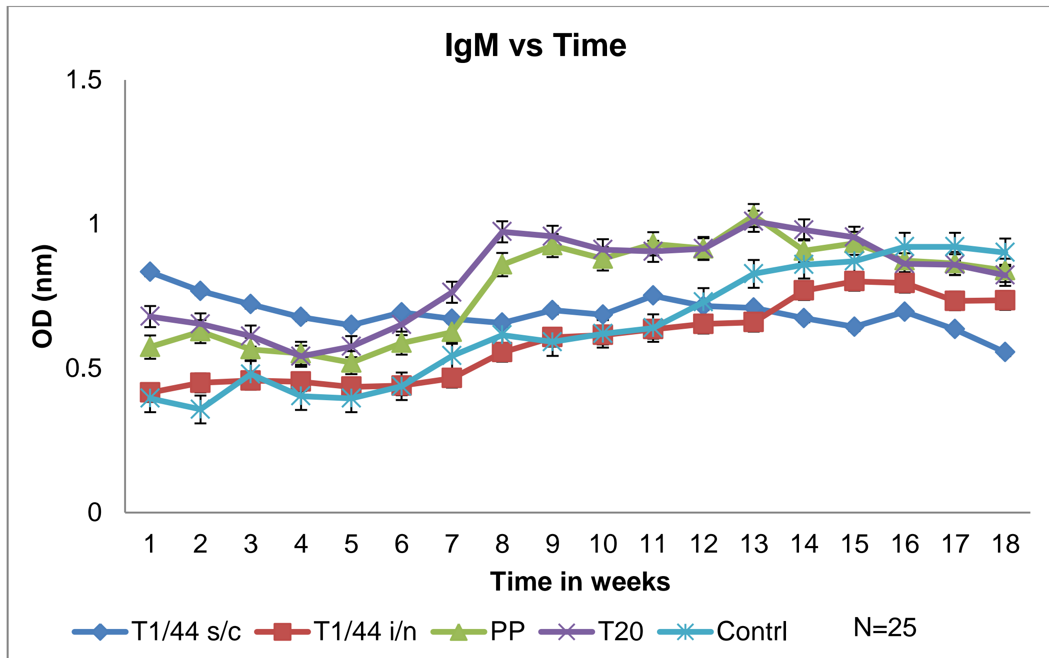


Fig.5.3. shows the average OD data for IgM as measured by indirect ELISA for animals in vaccine group. N= number of animals (5 animals per group).

5.3.1.4 Measurement of Mmm-specific Immunoglobulin A (IgA) response

IgA response was significantly (P < .05) affected by treatment and week. Figure 5.4 shows the least square means of IgA response. Since there was no significant difference in treatment X weeks, treatment means were averaged and compared over the 18-week trial period and these are given in Table 5. 2. The immune response of animals in the Purified Protein was significantly (P < .05) higher than the non-treated in-contact control. The difference in immune response between T1/44 sub-cutaneously vaccinated and the non-treated in-contact control animals neared significance (P =. 075). Interestingly, the Tween 20 presented lower but sustained IgA response than Purified Protein. The second and strong peaks were observed at week 14, 15 and 16 for non-treated in-contact control; T1/44 intranasal and Purified Protein respectively.

T1/44 sub-cutaneous brought about a peak in IgA production at week 7 and again at week 11 and 12 before declining (Table 5. 3).

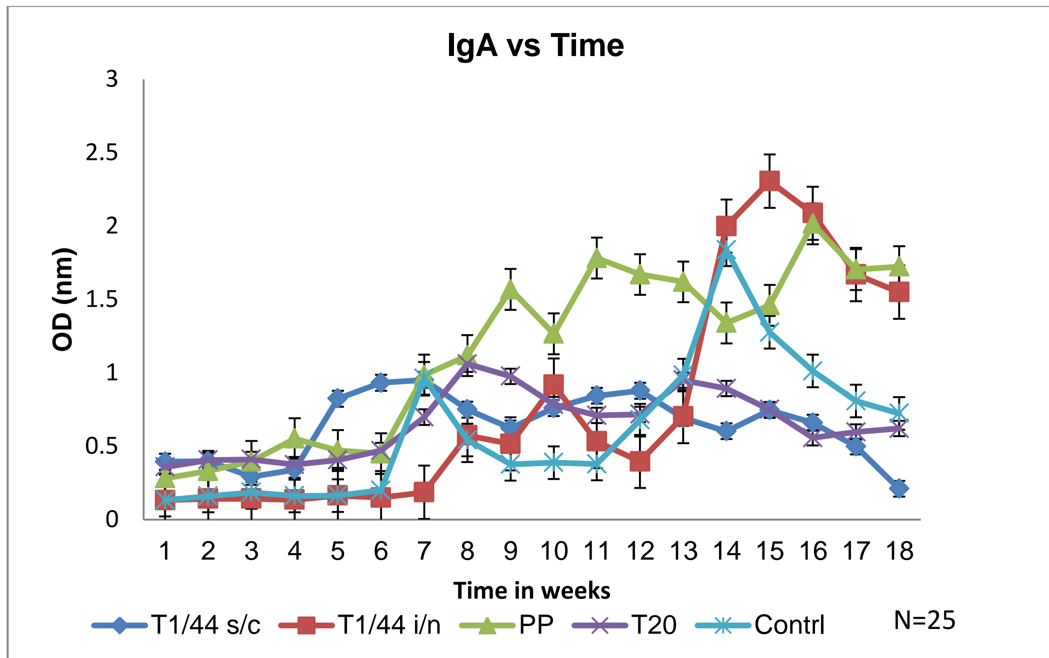


Fig.5.4. shows the average OD data for IgA as measured by indirect ELISA for animals in vaccine group. N= number of animals (5 animals per group).

Table 5.2 Estimates of treatment differences in IgA averaged over weeks, with standard errors*

Label	Estimate	Standard Error	Pr > |t|
trt A-B avg. over week 1 - 18	0.128	0.069	0.0823
trt A-C avg. over week 1 - 18	-0.075	0.069	0.2962
trt A-D avg. over week 1 - 18	-0.097	0.070	0.1775
trt A-E avg. over week 1 - 18	0.131	0.069	0.0751
trt B-C avg. over week 1 - 18	-0.203	0.069	0.0088
trt B-D avg. over week 1 - 18	-0.225	0.070	0.0043
trt B-E avg. over week 1 - 18	0.003	0.069	0.9618
trt C-D avg. over week 1 - 18	-0.022	0.070	0.7474
trt C-E avg. over week 1 - 18	0.206	0.069	0.0079
trt D-E avg. over week 1 - 18	0.229	0.070	0.0038

*A = T1/44 sub-cutaneous; B = T1/44 intranasal; C = Purified Protein; D = Tween 20; E = Control

Table 5.3 Differences between means for each week and subsequent weeks for treatment T1/44 sub-cutaneous, with standard errors*

Label	Estimate	Standard Error	Pr > |t|
week 1-weeks 2-18 in trt A	0.542	0.113	<0.0001
week 2-weeks 3-18 in trt A	0.546	0.120	<0.0001
week 3-weeks 4-18 in trt A	0.585	0.126	<0.0001
week 4-weeks 5-18 in trt A	0.597	0.138	<0.0001
week 5-weeks 6-18 in trt A	0.543	0.154	0.0004
week 6-weeks 7-18 in trt A	0.551	0.177	0.0021
week 7-weeks 8-18 in trt A	0.256	0.256	0.3183
week 8-weeks 9-18 in trt A	0.224	0.197	0.2569
week 9-weeks 10-18 in trt A	0.246	0.178	0.1680
week 10-weeks 11-18 in trt A	0.264	0.204	0.1981
week 11-weeks 12-18 in trt A	0.272	0.177	0.1272
week 12-weeks 13-18 in trt A	0.300	0.197	0.1286
Week.18 in trt A	-0.096	0.082	0.2416

*Treatment A = T1/44 sub-cutaneous

5.3.1.5 Measurement of CFT-specific antibody response

The systemic antibody response as measured by CFT (Fig. 5.5) revealed a first increase at week 10 p.i for T1/44 sub-cutaneous, T1/44 intranasal, PP and at week 9 p.i for Tween 20. The second increase appeared between week 12 and 14 post infection in all sub-groups in the vaccine group except T1/44 sub-cutaneous which appeared to be decreasing during that time. The humoral response in non-treated in-contact control animals increased steadily from week 9 and reached the first highest peak at week 15 with the second at week 17.

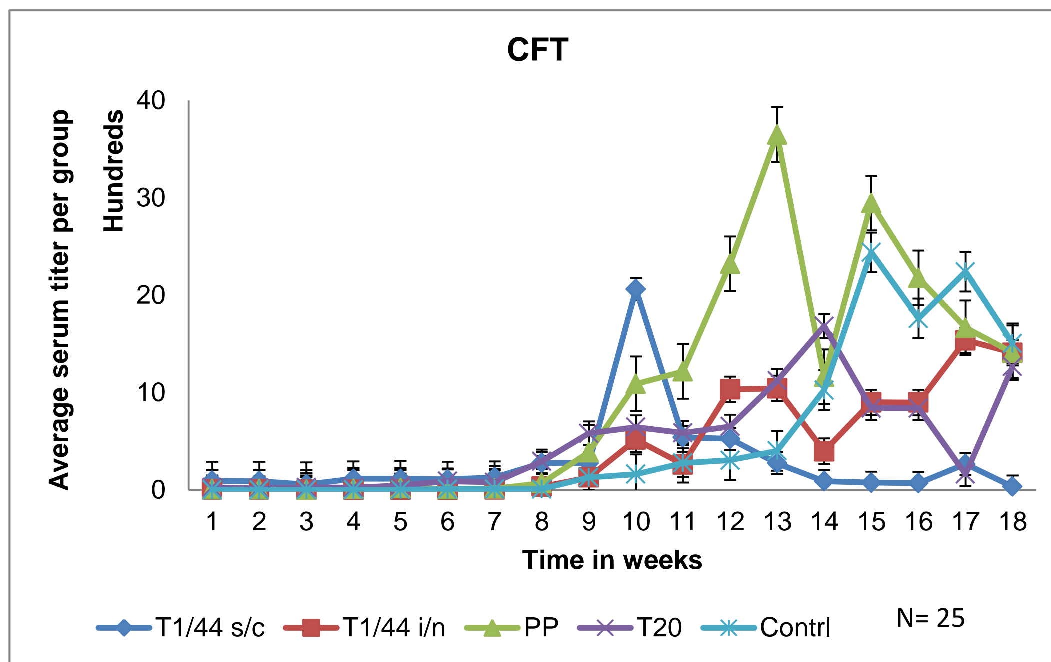


Fig. 5.5. Shows the results of the CFT during the course of the infection for all animals in the vaccine group. Cf titers of animals in the same group per week are averaged. N= number of animals (5 animals per group).

5.3.1.6 Measurement of Mmm-specific serological isotypes and antibody responses for artificially infected animals

The results of the indirect ELISA and the CFT are shown in figures 5.6 and 5.7 respectively. The immunoglobulin (M) appeared between weeks 2 and 4 post intubation. Intubated animals had high IgA response followed by IgM, then IgG2 responses. The CFT responses occurred from week 1 post intubation in animal #41 and animals #7 and

#41 sero-converted 2 weeks post intubation. The rest of the animals except #9 became positive in CFT by week 15. Animal #9 remained negative in CFT, IgG1 and IgG2; however, it had a low positive response in IgM and IgA at week 3 and 4 post intubation.

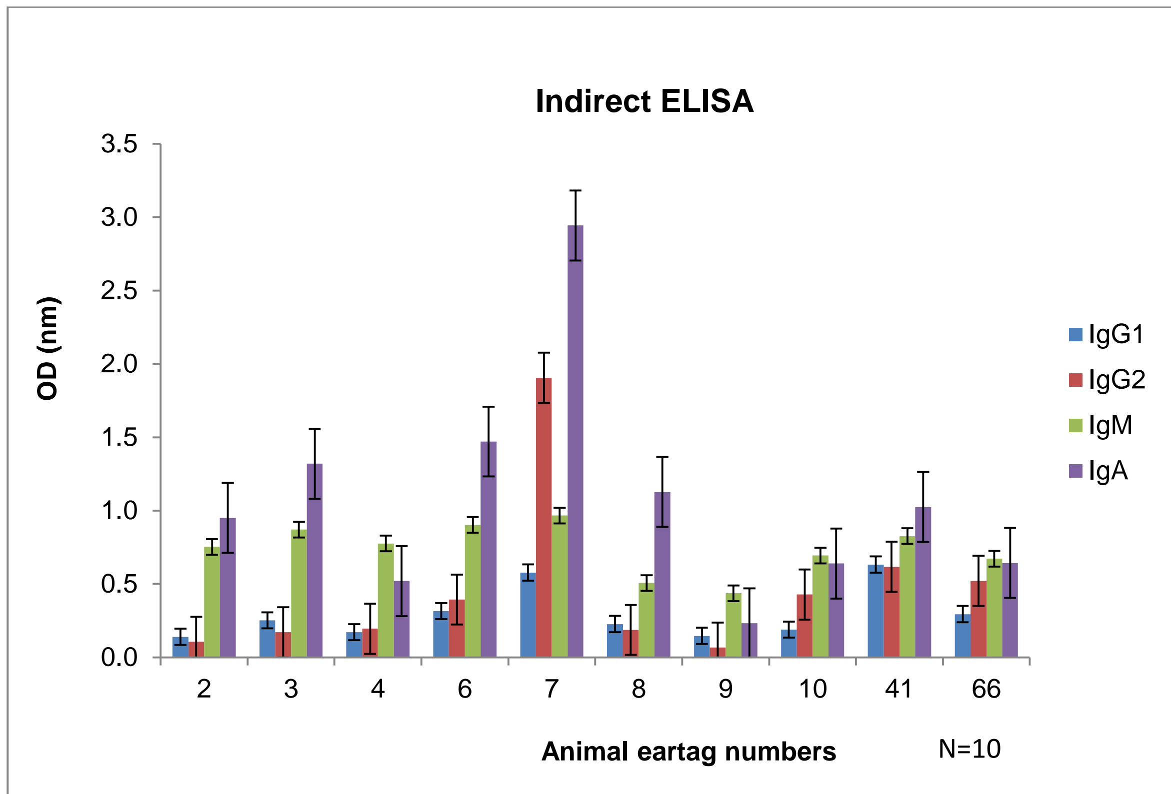


Fig.5.6. shows the average OD data for immunoglobulins as measured by indirect ELISA for artificially intubated animals. N= number of animals.

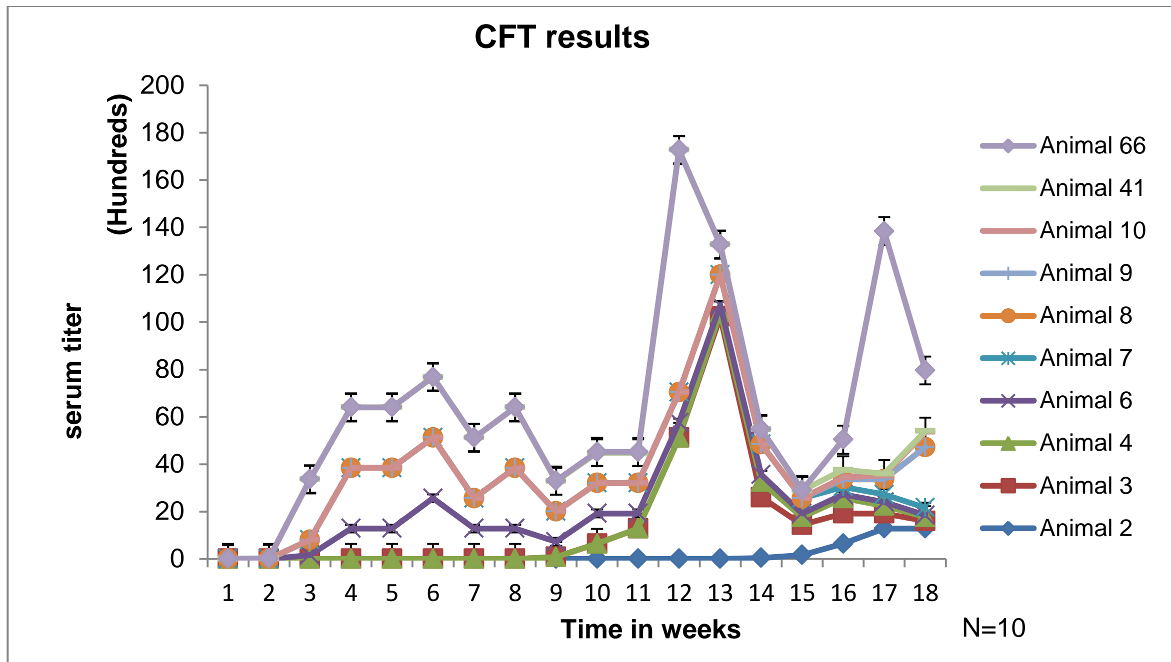


Fig. 5.7. shows the results of the CFT per animal during the course of the infection for artificially intubated animals. N= number of animals.

5.3.1.7 Measurements of Mmm-specific IgG1, IgG2, IgM, IgA and CFT-specific antibody responses for animals in Biofilm group

The comparison of intubated and control animals gave a significant effect (P < .05) for intubated group in IgG1. The best covariance structure for IgG1 based on the Bayesian Information Criterion (BIC) of -54.4 was Toeplitz (TOEP). Figure 5.8 shows the least square means of IgG1 in ELISA. The highest peak for non-treated in-contact control group was observed at week 11. The intubated group had slightly higher IgG2 and IgM responses than the control group. The optical density values of intubated animals were higher than the control group from day 1 to 6 (results not shown). Animal # 67 in the intubation group was the only one which seroconverted from week 7 onwards with a highest cf titer of 2560. In the non-treated in-contact control group, animal # 159 seroconverted for the last 5 weeks of the experiment with a highest cf titer of 1280 whilst animal # 139 seroconverted at weeks 11 and 12 before it succumbed to the disease (results not shown).

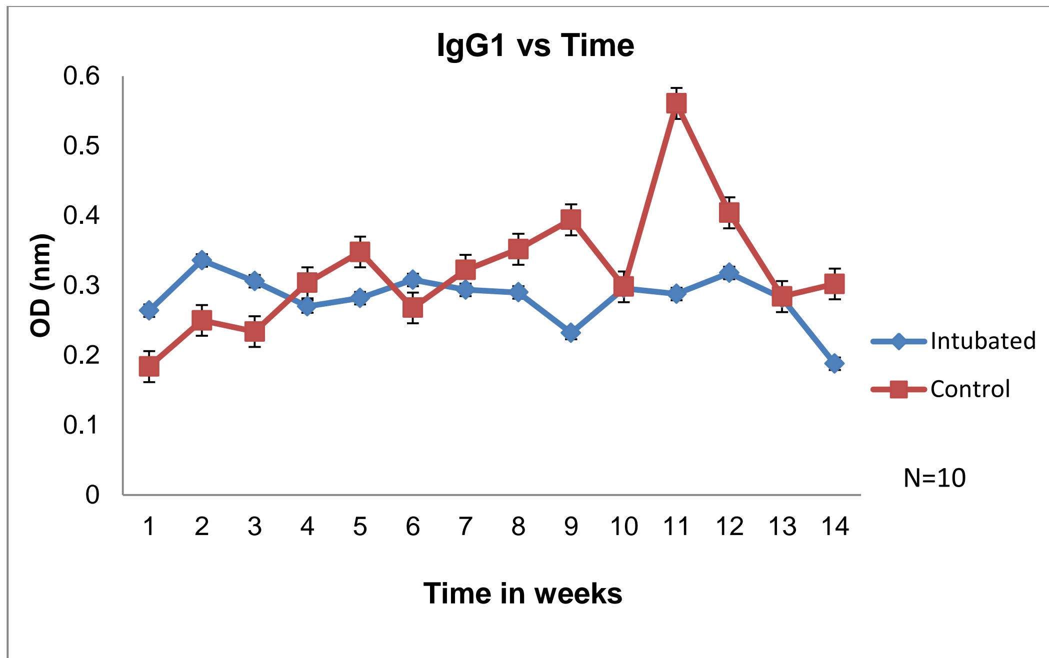


Fig.5.8. shows the average OD of IgG1 for animals (intubated and control) in the biofilm group as measured by indirect ELISA.

5.3.2 Determination of the immunogenic antigens by western immunoblot

5.3.2.1 Determination of the immune responses in experimental animals in the vaccine group

The sera collected during the course of the experiment were used to determine the humoral immune response. There was no significant difference in the western-blot profiles from the sera of animals either within the same group or between groups. Furthermore, the responses from different immunoglobulins were almost the same except that IgM immunoblot had more insignificant protein bands (appendix 3). None of the animals in this study revealed all the 7 Mmm specific protein bands. The responses for the three immunoglobulins appeared at different time periods for all animals. However, where it was apparent it intensified from week 14 onwards. Antibody response to a 60 kDa protein seemed to be dominant and was present in 5 animals in

the vaccine group for IgG1 and in 3 animals for IgG2. The antibody responses typical for CBPP produced bands: 110 kDa; 98 kDa; 95 kDa; 74 kDa; 48 kDa and 30 kDa proteins in IgG1 and IgG2. Since the Immunoblot appeared almost the same, only the most significant ones are shown here below in figures 5.9, 5.10 and 5.11 whilst others are in appendix 3

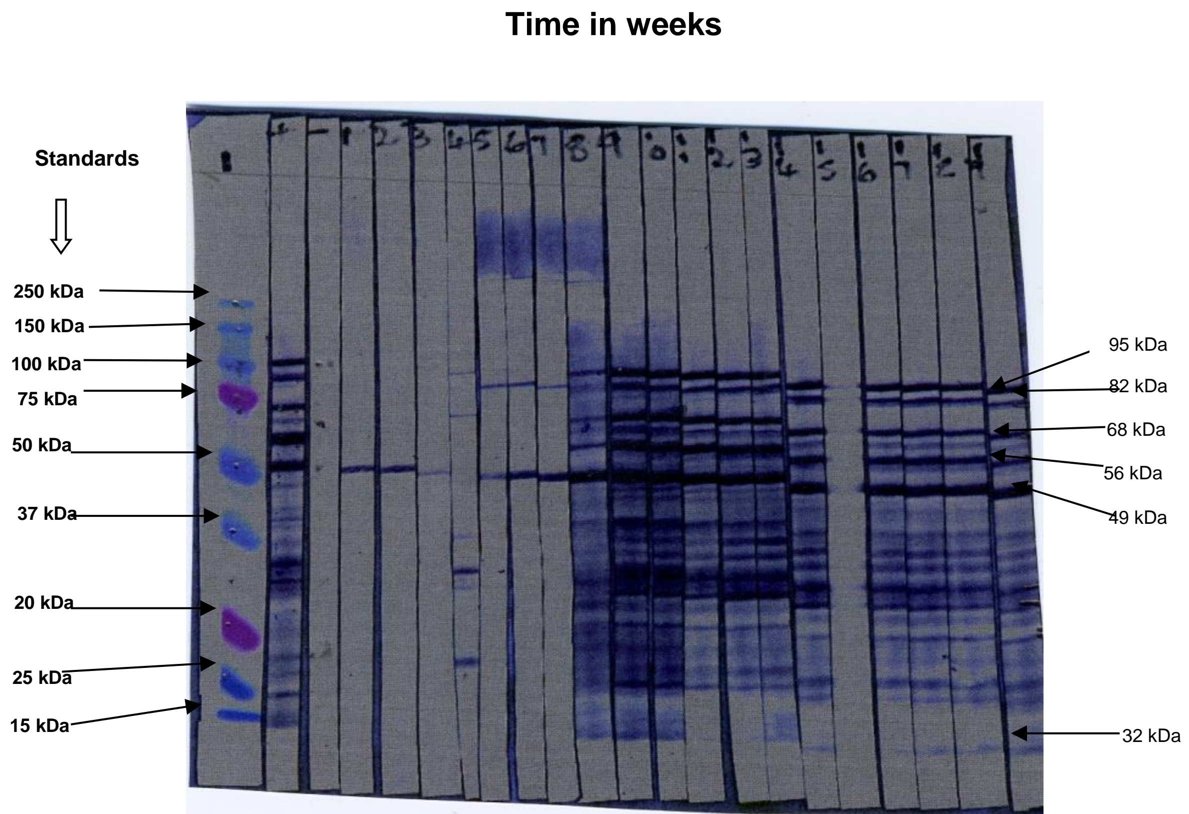


Fig.5.9. Humoral immune response to a local strain “Matapi”. Western-blot based on gradient SDS-PAGE illustrates the response of IgG1. The reactions of the sera taken sequential every week from the control untreated animal (#165) and infected by contact are shown. The major bands shown on the right appeared prominently between weeks 9 and (except week 15) 19 post infection.

Time in weeks

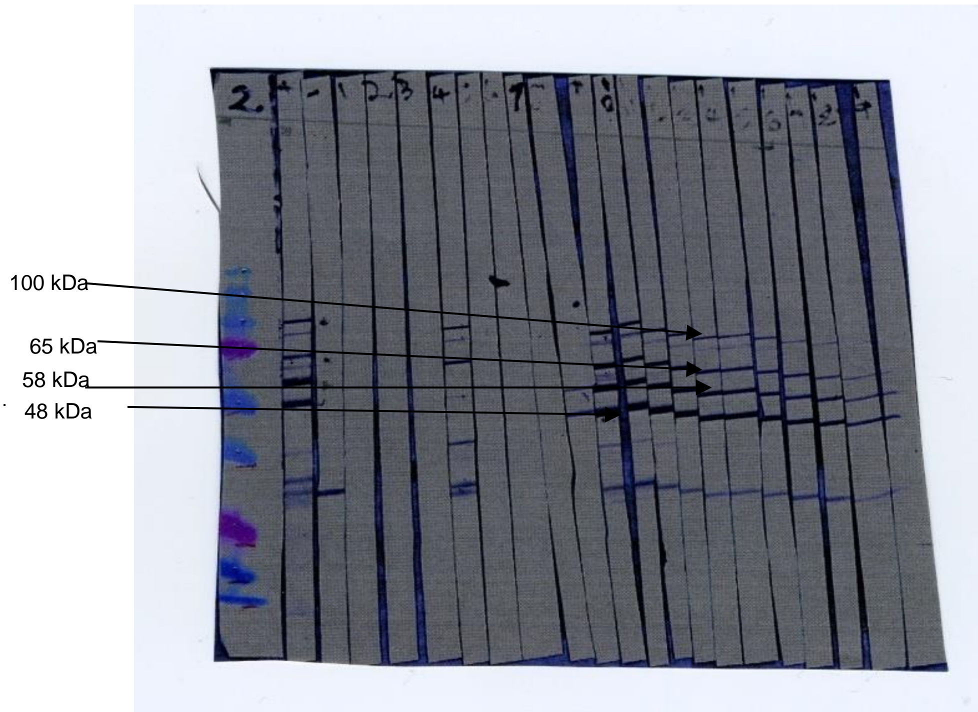


Fig.5.10. Humoral immune response to a local strain “Matapi”. Western-blot based on gradient SDS-PAGE illustrates the response of IgG2. The reactions of the sera taken sequentially every week from the control untreated animal (#165) and infected by contact are shown. The prominent bands shown on the right appeared between weeks 13 and 19 post infections; however, band 100 kDa did not appear at weeks 18 and 19.

Time in weeks

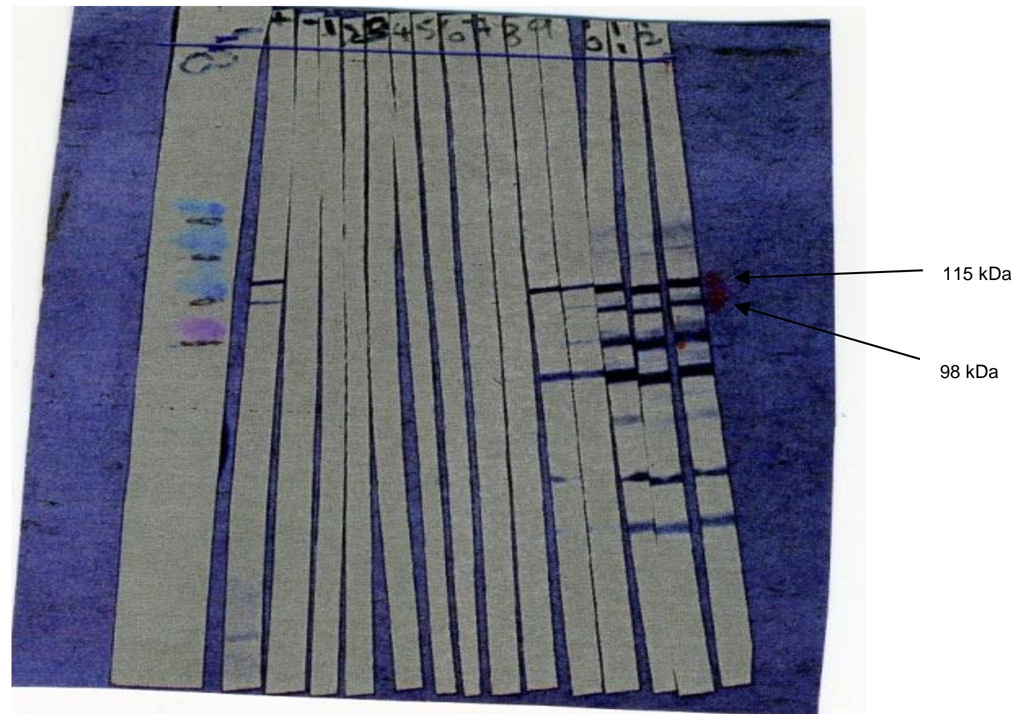


Fig.5.11. Humoral immune response to a local strain "Matapi". Western-blot based on gradient SDS-PAGE illustrates the response of IgG2. The reactions of the sera taken sequential every week from the animal vaccinated with Tween 20 cells (#194) and infected by contact are shown. Days post contact infection is given on top of each stripe. The major bands shown on the right reveal the appearance of 98 kDa which is not common to all *M. mycoides* subsp. *mycoides* SC strains and 115 kDa three weeks before death.

5.3.2.2 Determination of the immune response for animals in Biofilm group

Immunoblot analysis showed a strong IgM antibody response to 110 kDa protein. This antibody response appeared from week 5 post intubation for cow #78. There were no significant bands for the other animals. The control animals produced IgG1 antibody response to 141 kDa, 132 kDa and 87 kDa proteins for cow #183 while cow #159 produced antibodies to 138 kDa, 135 kDa, 87 kDa, 71 kDa and 56 kDa proteins. The antibody responses in both cattle appeared from week 10 post infections. Cow #138 was the only cow in this group which produced antibody responses for both IgG1 and IgG2 to 112 kDa, 74 kDa, 52 kDa, 44 kDa, 41 kDa proteins (IgG1) and 107 kDa, 87 kDa, 87 kDa, 68 kDa, 56 kDa, 46 kDa proteins for IgG2 (not shown).

5.4 Discussion

There is currently a lack of information on the nature of protective immunity in CBPP (Mulongo *et al.,* 2013). However, the little available information is associated with Mmm-specific IFN-γ secreting $CD4^{+}$ T cells and specific serum IgA responses (Dedieu *et al.,* 2005a; Tote *et al.,* 2008). Therefore, the aim of this research was to evaluate the role of the protective immune responses by characterizing the humoral immune responses in sera collected during the course of the experiment. The understanding of the protective immune responses would be ideal for the development of improved CBPP vaccines. The disparity in the humoral immune response exhibited by the animals of this study illustrated the complex interaction between the host and the pathogen. This interaction led to acute, sub-acute to chronic or symptomless stage of the disease. This chapter reports on the humoral immune responses observed on experimental animals. The sera used to define the immune responses were collected from vaccinated, non-treated in-contact control, intubated and biofilm animals. The humoral immune responses were evaluated by indirect ELISA, Complement Fixation Test and immunoblot analysis.

The statistical analysis of the immunoglobulins G1, G2 and M showed that the immune responses were affected ($p < .05$) by treatment (vaccine), week and treatment x week interactions. The IgA Mmm-specific response was affected ($p < .05$) only by treatment and week interactions. In general, the Tween 20 vaccine displayed a higher OD response for IgG1, IgG2 and IgM than the other groups. However, in IgA the PP vaccine maintained a higher response. On average, the immune response reached the first peak at around week 8 post infection possibly reflecting the onset of the pathological lesions.

The role of antibodies in protection against Mmm is controversial because of the lack of correlation between antibody responses, clinical signs and lung lesions (Dedieu *et al.,* 2005a; Nicholas *et al.,* 1996). The results of the indirect ELISA showed that the T1/44 sub-cutaneously vaccinated animals maintained steady immune responses in all four immunoglobulins. It seemed to be that the antibody elicited by the vaccine in the animals under this group mounted a protective immune response. Furthermore, animals in this group were less affected. Two animals had chronic pathological lesions with small lesions whilst three did not have any lesions. The vaccine elicited a protective immune response although it did not prevent the formation of the lung lesions in some animals. The T1/44 intranasally vaccinated group exhibited low antibody responses and had chronic pathological lesions.

Animals vaccinated with Purified Protein (recombinant vaccine candidate) did not have CF antibody titers at day 1 post infection. Most of the immunoglobulins appeared between weeks 6 and 12 post infection possibly reflecting severe disease. However, a persistent high IgA response was maintained. The vaccine-induced response was short lived and failed to produce a protective immune response. Conversely, the Tween 20 immunized animals had persisting high immune response in IgG1, IgG2 and IgM. Animals in the Purified Protein and Tween 20 groups had high IgA response with chronic pathological lesions. The immune response for control animals reached a first increased peak between week 8 and 9. Chronic pathological lesions were observed at post mortem. These lesions were not different from the ones observed in Purified Protein and Tween 20 groups.

Most of the animals under this study had high IgA response. The IgA appears to be an indicator of disease with a high response in animals with high clinical score. However, the study of Mmm specific antibody isotypes responses in Zebu cattle experimentally infected with Mmm revealed a high IgA response at both local and systemic in animals with sub-acute to chronic infections. The author postulated that IgA response is associated with protection (Niang *et al.,* 2006b). Studies dealt with other respiratory mycoplasmas highlighted the importance of IgA in host defence mechanism (Howard *et al.,* 1987; Gourlay and Howard, 1982).

The antibody response of IgM, IgG1 and IgG2 in indirect ELISA did not show any significant difference in terms of intensity and clinical signs among animals. The results suggested that these immunoglobulins are not correlated to protection. This finding is in accordance with a study done by Niang *et al.* (2006b). However, the role of IgG has been linked to protection in *M. bovis* (Gourlay and Howard, 1982). The presence of IgG1 and IgG2 in lung washings of mice infected with *M. pulmonis* correlated with protection (Gourlay and Howard, 1982).

Serological tools are important in the control of CBPP (Frey *et al.,* 1998). Currently, CFT is the official and most commonly used test. Although CFT is specific and sensitive in the acute phase of the infection, it detects about 70% of the chronically infected animals and nothing of the asymptomatic animals (Nicholas *et al.,* 1996). Additionally, CFT presented false positive results during sero monitoring of cattle in CBPP free area (Stärk *et al.,* 1995). CFT and cELISA are both recommended to be used as herd tests because of either false positive or false negative results associated with them in individual animals (Heller *et al.,* 2016).

The immunoblotting test (IB) is an OIE recommended test to ideally confirm CFT suspected false results (Anon, 2012). IB has higher sensitivity and specificity when compared to CFT (Anon, 2012). In this study, the Immunoblot was used to determine at which stage of the infection the key proteins appeared. Furthermore, the use of different immunoglobulins was to determine which immune response is worth stimulating in order to evoke protection. The information derived from IB may lead to the identification of the best protein(s) to be used in either for the development of a diagnostic test or for the formulation of a vaccine. Usually the sample is considered positive if it simultaneously shows a common immunological pattern that has immuno dominant antigens with molecular weight of 110, 98, 95, 62/60 and 48 kDa. However, as mentioned earlier, the aim of this study was to try to determine which protein appeared at what time during the course of the infection. An early and distinct reaction to 110 kDa was present for IgG1 and IgG2 in animals vaccinated sub-cutaneously and intranasally with T1/44. The reaction was persistent in two animals while in one animal it only lasted for the first 5 weeks post infection. A protein of molecular mass of 98 kDa was observed for IgG1, IgG2 and IgM Immunoblot in animals (T1/44 intranasally, control, Tween 20 and PP). The reaction occurred between week 4 and 18 post infection. Proteins of molecular mass of 95 kDa and 93 kDa were mainly present between weeks 8 and 18 post infection except in animals in T1/44 sub-cutaneous group where it was persistent from week 1 post infection. Immune bands of molecular mass of 85 and 87 as well as 80 kDa were observed in sera collected between weeks 5 and 18 post infection in animals in the non-treated in-contact control group (group A) and animals in the T1/44 intranasally group. A distinct reaction to the protein of molecular mass of 60/62 appeared in both IgG1 and

IgG2 in animals in the vaccine control, purified protein, Tween 20 and T1/44 intranasal groups. The reaction appeared for the last 12 weeks of the experiment.

A protein band at 45 kDa which is a glycerophosphate oxidase (GlpO) and associated with the induction of H_2O_2 toxicity and alleged to play a role in CBPP infected cattle (Pilo *et al.,* 2005) was present in IgM in two animals in the Purified Protein group from week 8 post infection. Possibly being the reason why animals in this group had severe pathological lesions.

The lipoprotein Q (LppQ) with a molecular mass of 48 kDa was identified to be associated with CBPP (Abdo *et al.,* 2000). The study done by Hamsten *et al.* (2010), indicated that protein MSC_1046 (LppQ) could be an important part of the immune response elicited by T1/44. Proteins with molecular mass between 47 and 49 kDa appeared at week 1 post infection in T1/44 subcutaneously vaccinated animals and from 5 to 18 post infection in non-treated in-contact control animals for IgG1 and IgG2. The LppQ is alleged to be specific to Mmm and was found in all strains (African, European, and Australian) (Abdo *et al.,* 2000). Furthermore, in the same groups of animals, a band of molecular mass between 69-71 kDa appeared between week 5 and 18 post infection whilst bands of molecular mass of 72 and 74 kDa appeared from week 13 to 18 post infections also in T1/44 vaccine and in in-contact vaccine control groups. The LppB protein that has a mass of 70 kDa is present in all strains including vaccine strains except in the European strains. Like any other membrane lipoproteins, it might play an indirect role in the virulence of the Mmm (Vilei *et al.,* 2000).

Besides the common immune bands of 110, 98, 95 and 74 kDa which appeared in some animals in this study, there were bands of lower molecular mass between 37 and 39

kDa which were present in either IgG1, IgG2 or IgM in some animals of the T1/44 intranasal group, vaccine non-treated in-contact control group and the Purified Protein group between week 6 and 18. Proteins of low molecular mass of between 36 and 20 appeared mostly for five weeks towards the end of the experiment in IgG1 for animals in the T1/44 intranasal group and the vaccine non-treated in-contact control group.

In the biofilm experiment, the in-contact control animals were severely affected compared to the intubated animals. Two in-contact control animals had high IgA response which started at the same period as CFT between weeks 11 and 14 post infection. All the animals had persistent IgM response. Bands of molecular weight of 110 and 74 kDa were prominent in sera collected toward the end of the experiment.

This study concurs with previous reviews (Lloyd, 1967; Gourlay, 1975; March, 2004) that it is difficult to correlate antibody response with clinical signs and lung lesions. However, it was found that most of the animals with high IgA response had high clinical scores and lung lesions at post mortem. Therefore, the presence of IgA response could be responsible for disease development and needs further investigation.

The immunoblot which was successfully used in the campaign to eliminate CBPP in Portugal whilst other control measures presented problems (Nicholas *et al.,* 2008) is an OIE recommended test. Although Immunoblot gives high level of sensitivity and specificity (Schubert *et al.,* 2011), it is not suitable for mass screening.

In summary, this study has shown that both IgG1 and IgG2 seemed to be the most immunoglobulins to be stimulated during the Mmm infection. It might be useful to formulate a vaccine that will stimulate these immunoglobulins. Additionally, proteins with

molecular mass of 110, 95 and 48 kDa might be important part of immune response since these proteins were present in T1/44 sub-cutaneously vaccinated animals and animals in this group were better protected compared to others.

CHAPTER SIX

GENERAL DISCUSSION AND FUTURE WORK

Contagious Bovine Pleuropneumonia (CBPP) caused by *Mycoplasma mycoides* subsp. *mycoides* (Mmm) is a pulmonary disease of cattle and Water buffalo (Provost *et al.,* 1987). The disease has great economic effects on the cattle industry in Africa (Windsor, 2000). The decrease in Government subsidised vaccination programmes in some African countries, resulted in the spread of the disease. Countries which successfully eradicated the disease experienced reappearance of CBPP in the last two decades (Marobela-Raborokgwe, 2011; Windsor, 2000). CBPP was eradicated in U.S and Australia through strict control of cattle movement, culling and compensation. These methods are not feasible in many African countries due to lack of governmental and civil resources to forcefully restrict movement of cattle and the financial resources needed to compensate cattle owners (Gull *et al.,* 2013). Since there is no official treatment for CBPP, vaccination with live attenuated strain T1/44 that induces short-lived immunity and causes severe post-vaccine lesions is the only feasible control method currently available (Pilo *et al.,* 2007; Mbulu *et al.,* 2004; Tulasne *et al.,* 1996) amongst other limitations mentioned in section 1.9.1. Although mycoplasma species are sensitive to some antibiotics (Yaya *et al.,* 2003; Ayling *et al.,* 2000), chemotherapy is not officially allowed in the control of CBPP for possible development of a carrier state and the spread of residues that might results into bacterial resistant in the environment (Yaya *et al.,* 2003). However, contrasting opinions exist on the role of antibiotic therapy in the control of CBPP. Researches on the use of antibiotics have proved that antibiotic

treatment minimizes the numbers of mycoplasma that are shed by infected animals and therefore minimizes the chances of infection transmission (Hübschle *et al.,* 2006; Nicholas *et al.,* 2012).

The diagnosis of CBPP is hampered by diagnostic tests which are not sensitive enough whilst requiring sophisticated equipment. Therefore, there is a critical need to develop a better vaccine and an easy to use robust diagnostic test. The development of a better vaccine requires an understanding of the immune response. Thus, the aim of this study was to experimentally evaluate the performances of two novel vaccines whilst comparing them to T1/44 vaccine and to evaluate the performance of T1/44 as mucosal vaccine. Furthermore, the role of Mmm biofilm *in vivo* was assessed. The sera collected during the duration of the experiment were used for the evaluation of the performance of the newly developed penside tests (LFD) in comparison to the already existing serological tests and to assess the Mmm-specific humoral immune response.

6.1 Experimental study of the vaccines

As no therapeutic treatment for CBPP is available, vaccination is currently the preferred control method (Gull *et al.,* 2013). Animal vaccination against CBPP has been in practice since the time of Willems (Provost *et al.,* 1987), when parental CBPP infective lymph was used as an inoculum and placed into an area of thick connective tissue (Provost *et al.,* 1987). This method of vaccination was discouraged due to side effects such as loss of tail (Bygrave *et al.,* 1968). Multiple vaccine formulations have been developed, however they have drawbacks such as the induction of undesirable side

effects and lack of consistent protective or long-lived immunity (Thiaucourt *et al.,* 2003). Although sub-cutaneous method of vaccination with T1/44 is practiced, side effects such as gross lesions at the site of the inoculation due to improper vaccine administration and death in some instances are still present (Nicholas *et al.,* 2008). Despite these side effects, attempts to control CBPP in Africa are with T1/44 vaccine strain that has been isolated from Tanzania and passaged 44 times in eggs in order to reduce the virulence factors (Nicholas *et al.,* 2008). The limitation of this vaccine is well documented (Nicholas *et al.,* 2008; Thiaucourt *et al.,* 2000). Previous attempts to develop new vaccines have been reported by Nicholas *et al*. (2005). These were the use of a whole cell mycoplasma which was inactivated with saponin and the use of recombinant subunit vaccine prepared from lipoprotein Q (LppQ). These two vaccines failed to elicit protective immunity and rather exacerbated the pathology in the vaccinated animals compared to the in-contact unvaccinated animals (Nicholas *et al*., 2005).

Other control measures such as strict animal movement and test and slaughter are difficult to implement due to transhumance pastoralism, fragmented veterinary services and lack of or insufficient resources for compensation as well as for activities related to the control of CBPP (Amanfu, 2009). However, Aligaz and Munganga (2019) suggested three possibilities of controlling the disease by (1) treating 85.7% of infectious cattle with antibiotics, without vaccinating healthy cattle, (2) vaccinating 80% of susceptible cattle within a period of 49 days, without treating the infectious cattle and (3) assuming that 50% of susceptible cattle are vaccinated within 73 days and 50% of infectious are treated with antibiotics. The option of using the combination of vaccination with antibiotics is the one likely to be successful (Aligaz and Munganga, 2019). However, for

this option to be a success, vaccines and antibiotics should be readily available to farmers of which cattle are at risk of exposure to infection.

It is therefore urgent to develop a vaccine that ideally elicits protection for more than two years with a single dose of injection and with minimal adverse reactions without requiring cold storage (Ayling, 2013).

It was against the issue of the ineffectiveness of the currently used vaccines that this study was conducted. The vaccinated and non-treated in-contact control animals were placed together with the intubated cattle. Inoculation of cattle by endobronchial intubation gives accurate determination of quantity of organism and incubation period; however, it is not natural and could overload the cattle with large and unnatural infection, thereby unfairly challenging the vaccines. Despite that, it is the only way of emulating the transmission of the infection in the absence of naturally existing sick animals. Nine out of ten intubated cattle became infected (Table 3.2). The effectiveness of this route of challenge was evident in the unvaccinated control animals whereby all five animals were serologically positive with typical CBPP lesions at post mortem (Table 3.2). The chronic pathological lesions ranged from lung hepatisation, sequestra of various sizes, pleural fluids and pericardiac fluids. It can therefore be assumed that the vaccinated animals received sufficient challenge to possibly assess the effectiveness of the vaccines used.

Of the three vaccine preparations used in the experiment described here, the T1/44 performed better, with a good protection as evidenced by a low total integrated score particularly in the sub-cutaneously vaccinated animals (Table 3.2). However, the presence of pleural and pericardiac fluid as well as small sequestra in some animals of this group would suggest that these vaccinated animals were theoretically capable of

infecting susceptible cattle, although the duration of their infectivity is unknown. Jores *et al.* (2013) reported that the live attenuated vaccines currently in use; do not prevent the progression of pathomorphological lesions after challenge. Although T1/44 is not recommended to be administered through the nostril it was nonetheless tried. The intranasal route of administration was less effective with three vaccinated cattle showing significant CBPP lesions (Table 3.2) suggesting that the mucosal immunity was not sufficiently stimulated. It might be that either mucosal immunity plays a less important role in protection to CBPP than was hoped or that a higher dose might have stimulated a better response as the efficacy of the vaccine is dose depended (Thiaucourt *et al.,* 2004).

The results of the two novel vaccines (Purified Protein and Tween 20) showed that the Purified Protein vaccine provided no protection as the total integrated score was slightly higher than the unvaccinated control animals (Table 3.2). It appeared that at least one of the proteins in the recombinant protein vaccine exacerbated the disease (Table 3.2 and figure 3.1). All of the five animals in this group had chronic pathological lesions with large sequestra which varied between 8 and 25cm in diameter (Table 3.2). Figure 3.1 depicts a typical chronic pathological lesion of encapsulated necrotic core in an animal vaccinated with purified protein. Enhanced pathology has been reported in animals vaccinated with a recombinant form of the LppQ protein formulated with ISCOM as adjuvant and challenged by contact (Nicholas *et al.,* 2004). Due to severe pathological lesions of the LppQ vaccine, it was thought that a cocktail of purified proteins might cover a wider range of potentially protective proteins and the LppQ would be at a lower concentration than that previously tried. However, it was not the case in this study.

The effects of the Tween 20 vaccine were more ambiguous. Three of the five vaccinated animals had low integrated scores (Table 3.2) which could be that they may have not been infected. However, judging by the success of the challenge in the unvaccinated and purified protein groups, it appears that this was unlikely and that Tween 20 vaccine elicited some protection although it failed to prevent the formation of chronic pathological lesions. The total integrated score was lower than the in-contact and the animals vaccinated with purified protein. It may be rewarding to further refine this vaccine preparation in an effort to improve its efficacy.

The work undertaken in this study confirmed earlier findings that subunit or inactivated vaccines may actually exacerbate the effects of CBPP (Nicholas *et al.,* 2004). The T1/44 vaccine is probably effective because cattle are exposed to a live semi-virulent preparation given in a site where the mycoplasma is generally incapable of initiating infection in the lungs (Mbulu *et al.,* 2004). However, the occasional outbreaks of CBPP in closed vaccinated herds may be the result of the live vaccine reaching the lung via the blood stream (Mbulu *et al.,* 2004).

6.2 The role of *Mycoplasma mycoides* subspecies *mycoides* biofilm *in vivo*

Although mycoplasmas have small genome size, they cause wide range of diseases in animals and humans but little is known about their virulence factors and their ability to persist in the hosts. When a wide range of mycoplasmas (*Mycoplasma putrefaciens*, *M. cottewii*, *M. yeatsii*, *M. agalactiae*, *M. bovis* and Mmm) were examined for their abilities to form biofilms, all other mycoplasmas except the highly pathogenic Mmm produced

prolific biofilms (McAuliffe *et al.,* 2006). However, Mmm was able to produce a biofilm *in vitro* using other substrates (McAuliffe *et al.,* 2007, 2008).

Even though the ability of Mmm to form biofilm *in vitro* has been evaluated (McAuliffe *et al.,* 2008), the evolution of the disease in the host caused by biofilm mycoplasma has not yet been assessed. Therefore, this necessitated the *in vivo* experiment of biofilm mycoplasma.

Although fewer cattle were used in the biofilm group compared to vaccine group, the ratio (1:1) of intubated to in-contact control cattle was higher which made the opportunity for Mmm to spread from intubated to in-contact cattle more effective. An unexpected lower integrated score was observed in the biofilm intubated cattle (Table 3.2). Apart from pericardiac fluid and lung hepatisation, no other typical CBPP pathological lesions were observed in the intubated animals (Table 3.2). Interestingly, one animal in the in-contact control group died due to an acute form of CBPP infection whilst a second animal within the same group had a large sequestrum of 15 to 17 cm in diameter with a high serological positive response (Table 3.2). This suggested that the intubation was effective in initiating the infection. The difference observed in the intubated group may be either due to slightly different infection dose, a different response to intubation or reasons that are not yet explained. Although the in-contact control animals were severely affected compared to intubated cattle in the biofilm group, they had less secondary exposure than their counterparts in the vaccine group. It is possible that, when the infection in the vaccine trial had taken hold, all animals were potentially spreading infection amongst the group, giving a higher rate of infection. The death of one animal and the presence of the sequestrum in another animal at post mortem may

indicate that passage of the isolate to in-contact cattle changes the pathogenic status of the isolate from its biofilm state.

The finding of the biofilm study is inconclusive and further study with more animals and a higher dose of inoculum is needed in order to arrive at meaningful results. However, the preliminary finding shows that animals intubated with biofilm grown Mmm present less clinical disease than the in-contact control animals.

6.3 Comparison of LFD to cELISA, LAT and LPPQ ELISA

The cELISA and CFT are the OIE recommended tests. Both tests have low sensitivity but are good at detecting antibodies at herd level (Marobela-Raborokwe *et al.,* 2003). The latex agglutination test is easy to perform but has the same sensitivity as CFT (Ayling *et al.*, 2005) which is really promising for a rapid field test compared with a laborious expensive laboratory test. The lack of more sensitive and easy to use field tests necessitated research into the development of a robust field test. The performance of the newly developed LFD as a possible field test for CBPP was evaluated and compared to LAT, cELISA and LPPQ ELISA. As mentioned earlier (section 4.2.1), it was not possible to analyse all of the samples using LPPQ ELISA.

In this study, the LFD displayed a low detection level of positive samples at a 50% cut-off when comparing positive results in all three tests. However, at a 30% cut-off point it compared well with the cELISA and the LAT. The majority of the negative sera picked up by LFD were of sera collected from T1/44 sub-cutaneously vaccinated animals. This might suggest that the LFD is able to distinguish the antibody response elicited by T1/44

vaccine from natural CBPP infection. However, it needs to be tested in a real outbreak situation where vaccination as a control measure is practiced. Comparing the LFD to the current field test, LAT, the LFD has the advantage of using a portable reading device which would rule out the possibility of ambiguous interpretation of results.

The cELISA had more positive results in animals during the chronic stage of infection (Muuka *et al*, 2011). Cattle in the conventionally intubated group were positive 9 days after infection. Provost *et al.* (1987), reported that antibodies can be detected from about 10 days after the onset of the clinical signs and remain detectable for few months. This finding is in agreement with the observations in this study. The cELISA is an established test with a defined cut-off point of 50% (as per manufacturer instructions). The LAT gives either a positive or negative results based on agglutination. The cut-off value for the LFD needed to be established and for this study it was set at 30%. Based on the results of the sera tested in the cELISA, LAT and LFD, the agreement between the three tests in detecting positive samples was low. Work done earlier (Ayling *et al.,* 2007) using positive and negative reference sera showed that LFD compared well with other serological tests.

It was noted that none of the three tests have the sensitivity to detect all infected cattle at all stages of the disease. Similar findings were reported (Enyaru *et al.,* 2012, Goncalves *et al.,* 2008). Therefore, continued research for a test that is capable of detecting CBPP during all stages of the disease is important. The recently developed LFD appeared to be a good alternative field test as it is not subjective. However, it needs to be refined using different conjugates and re-evaluated.

6.4 Assessment of Mmm-specific humoral immune

The work presented here shows that in experimental challenge and field studies, humoral immune response against Mmm has been variable. It is important to understand the characteristics of the protective immune responses for the development of vaccines (Niang *et al.,* 2006b). Although in CBPP, vaccines elicit short term immunity and recovered animals developed long term immunity, the protection mechanism is still not known (Tulasne *et al.,* 1996; Provost *et al.,* 1987). Knowing that cattle recovering from CBPP infection in the field mount long term immunity, Dedieu-Engelman (2008) studied the immune response of acute and convalescent animals and revealed that the latter had stronger and persisting IgA response as well as higher Mmm-specific CD4+ T-cell response with the production of gamma interferon detected in the blood and respiratory lymph nodes. This might suggest the involvement of both cell-mediated and antibody-mediated immune responses in the protection against CBPP.

Despite the fact that the antibody-mediated immune response plays a role in protection against Mmm, it is still not clear at what stage during the infection the antibody is effective because of the lack of correlation between antibody response, clinical signs and lung lesions (Dedieu *et al.,* 2005a; Nicholas *et al.,* 1996). Animals with high antibody titers may have no visible lesions and lesions might be present in animals with negative serological responses (Nicholas *et al.,* 1996) making it difficult to establish the role of humoral in immune response in CBPP infected animals.

The statistical analysis of the immunoglobulins G1, G2 and M showed that the immune responses were affected ($p < .05$) by treatment (vaccine), by week and by treatment

times week interactions. The IgA Mmm-specific response was affected ($p < .05$) only by treatment and by week interactions. In general, the Tween 20 vaccine displayed a higher OD response for IgG1, IgG2 and IgM than the other groups. However, in IgA the PP vaccine maintained a higher response. On average, the immune response reached the first peak at around week 8 post infection possibly reflecting the onset of the pathological lesions however; it would be good to do a time course study.

Animals vaccinated with Purified Protein (recombinant vaccine candidate) did not have CF antibody titers at day 1 post infection. Most of the immunoglobulins appeared between week 6 and 12 post infection possibly reflecting severe disease. However, a persistent high IgA response was maintained. The vaccine-induced response was short lived and failed to produce a protective immunity. Conversely, the Tween 20 immunized animals had persistent high immune response in IgG1, IgG2 and IgM. Animals in the Purified Protein and Tween 20 groups had high IgA response with chronic pathological lesions. The immune response for control animals reached a first increased peak between week 8 and 9.

The majority of the animals in this study exhibited chronic pathological lesions such as lung adhesions, sequestra and either pleural fluid. Furthermore, a high IgA response was observed in the chronically affected animals. This finding is in agreement with the study done in Zebu cattle where a high IgA response at both local and at systemic level was observed in animals with sub-acute to chronic infections (Niang *et al.,* 2006b). This finding led the author to postulate that IgA response is associated with protection (Niang *et al.,* 2006b).

There was no significant difference observed in humoral immune responses of IgM, IgG1 and IgG2. Similar results were observed by Niang *et al.* (2006b). These findings here indicate that these immunoglobulins are not correlated to protection. However, in *M. bovis* IgG is stated to have a role in protection (Gourlay and Howard, 1982). In addition, the presence of IgG1 and IgG2 in lung washings of mice infected with *M. pulmonis* correlated with protection (Gourlay and Howard, 1982).

In the current study, it was observed that animals with smaller pathological lesions had early but low serological response, possibly indicating a protective response. However, in some cases the production of pleural fluid and pericardiac fluid appeared in the absence of sequestra. Conversely, the high CFT antibody responses observed in severely affected animals might be an indication that the host response is damaging. Whether the presence of pleural fluid or pericardiac fluid prevents the formation of sequestra or result in less stress to the host is unknown. In addition, when antibody responses were monitored for almost a year following Mmm infection a correlation between the response of different immunoglobulins and clinical signs as well as lung lesions could not be established (Niang *et al.,* 2006b).

6.5 Assessment of antibody response with Immunoblotting

Humoral immune responses of different immunoglobulins (Ig) were evaluated with IBT and dominant immunogenic proteins with molecular masses of 110, 98, 95, 85, 80, 72, 62, 48 and 39 kDa were identified (Abdo *et al.,* 1998).

The analysis of Mmm strains by immunoblotting was successfully carried out from European isolates (Gonçalves *et al.,* 1998). An EU FAIR funded research that involved

several European countries has led to the use of immunoblotting for CBPP as a diagnostic tool and IBT was successfully used in the eradication of CBPP in Portugal in 1990 (Nicholas *et al.,* 2008).

A protein band at 45 kDa which is a glycerophosphate oxidase (GlpO) and associated with the induction of H_2O_2 toxicity and alleged to play a role in CBPP infected cattle (Pilo, *et al.,* 2005) was present in some animals. Furthermore, lipoprotein Q (LppQ) with a molecular mass of 48 kDa was identified to be associated with CBPP (Abdo *et al.,* 2000). The study of Hamsten *et al,* (2010), indicated that protein MSC_1046 (LppQ) might be important part of the immune response elicited by T1/44 vaccine. Animals vaccinated sub-cutaneously with T1/44 and the in-contact control animals in the vaccine group revealed bands with molecular mass between 47 and 49 kDa from week 4 to week 7 post infections for IgG1 and IgG2. The LppQ is alleged to be specific to Mmm and was found in all strains (African, European, and Australian) (Abdo *et al.,* 2000). Bands with molecular mass between 69-71 kDa were as well present. The LppB protein that has a mass of 70 kDa is present in all CBPP strains including vaccine strains but not in the European strains. Like any other membrane lipoproteins, this might play an indirect role in the virulence of the Mmm (Vilei *et al.,* 2000).

Besides the common proteins of 110, 98, 95 and 74 kDa which appeared in several animals under this study, there were bands of lower molecular mass between 39 and 20 kDa which appeared mostly for five weeks towards the end of the experiment.

The biofilm in-contact control animals were severely affected compared to the intubated animals. Two in-contact control animals had high IgA response which started at the same period as CFT between weeks 10 and 13 post infection. All the animals had a

persistent IgM response. Bands of molecular weight of 110 and 74 kDa among others were seen in sera collected toward the end of the experiment in the biofilm in-contact control animals.

The immunoblot which was successfully used to eliminate CBPP in Portugal while other control measures presented problems (Nicholas *et al.,* 2008) is an OIE recommended test and is highly sensitive and specific (Schubert *et al.,* 2011), however it lacks reproducibility and robustness. Furthermore, the inter-laboratory proficiency test carried out in 2009 showed that the test is not reproducible as the results were varied among the participating laboratories (Gaurivaud and Poumarat, 2012) because there is no standardized protocol; the French protocol is different from the Portuguese Gonçalves method. To ensure reproducibility of the immunoblotting results, standardisation of the protocol for the production of antigen will be essential.

6.6 FURTHER WORK

As a result of the pathological lesions observed during post mortem examination, no vaccine conferred appreciable protection. The Purified Protein that consisted of a cocktail of five proteins and was assumed to work against the Vmm exacerbated the disease. The AIOH emulsified adjuvant was used for both novel vaccines. It is possible that further research in the adjuvant and vaccine area is still needed. Improved understanding of the ISCOM might help improvements in the formulation of a subunit vaccine. Since Mmm enters the host through mucosal route, it might be necessary to look into the possibility of constructing a mucosal vaccine for CBPP or better yet to

evaluate the current T1/44 with a higher dose. Vaccination with BEN-1 eradicated CBPP in China (Xin *et al.,* 2012). It could be useful to try the efficacy of this vaccine in Africa as suggested by Jores *et al.* (2013).

The development of an effective vaccine for CBPP has been hampered by a lack of understanding of host-pathogen interactions during the infection (Schieck *et al.,* 2015). The results of transposon mutagenesis (transfer of genes to a host organisms' chromosome) techniques were inconclusive (Schieck *et al.,* 2016). However, with the studies on genome synthesis of M. *mycoides* subsp. *capri* (Mmc) that is closer to Mmm (Gibson *et al.,* 2010) and other molecular techniques (Noskov *et al.,* 2010; Lartigue *et al.,* 2007; Lartigue *et al.,* 2009) have made it easier to understand the biology and the pathogenesis of these bacteria (Schieck *et al.,* 2016).

In addition to evaluating the two novel vaccines, the effect of the biofilm-associated Mmm to induce the disease when it is endo-bronchially administered was evaluated. The abilities of mycoplasma species to form biofilm as well as their survival and environmental persistence were established (McAuliffe *et al.,* 2006; 2008). The severity of the disease manifestation was higher in non-treated control animals and less in the intubated animals. Clearly, there is a need to determine why biofilm-associated Mmm was more virulent in the control animals rather than in the intubated animals. Furthermore, there is a need to analyse protein profiles in samples from endo-bronchially intubated and in-contact control animals in order to determine the changes that might be responsible for the virulence factors in none intubated animals.

In this thesis the performance of the newly developed penside test, LFD in detecting positives and negatives animals in experimental animals was evaluated against cELISA

and LAT. The LFD was found to be an easy field test that does not require sophisticated equipment except a handheld battery operated device. The use of the device would make it easier for even untrained community health workers to handle it as it displays the value. Furthermore, it will ease the problem of ambiguous results associated with LAT. The LFD needs further refining using a different conjugate.

Specific protective antibody responses to Mmm proteins have not been identified (Schieck *et al.,* 2016). Furthermore, the non-production of antibodies that neutralize l-α-glyserol-3-phosphate oxidase during the chronic stage of CBPP infection that is associated with presence of viable mycoplasma suggests that mycoplasma evade the host immune system (Schieck *et al.,* 2014; Mulongo *et al.,* 2013). To gain a better understanding on the role of antibody immune response, the author investigated the humoral immune response in experimental cattle using Indirect ELISA, IBT and CFT. Although IBT is reported to be the most sensitive and specific serological tests available for CBPP (Schubert *et al.,* 2011), the test is cumbersome and it lacks reproducibility. The dominant immunogenic antigens revealed in the study of Abdo *et al.* (1998) and in the study reported here differ. Therefore, a comparison of the studies is difficult. One possible noteworthy finding is that bands of lower molecular masses between 39 and 20 kDa appeared mostly for five weeks towards the end of the experiment. Hence, research into an easy to perform IBT with short steps and standardised reagents is needed.

REFERENCES

Abdo el-M., Nicolet, J., Miserez, R., Gonçalves, R., Regalla, J., Griot, C., Bensaide, A., Krampe, M., Frey, J. (1998). Humoral and bronchial immune responses in cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* type. Veterinary Microbiology. **59:** 109-122.

Abdo el-M., Nicolet, J., Frey, J. (2000). Antigenic and Genetic Characterization of Lipoprotein LppQ from *Mycoplasma mycoides* subsp. *mycoides* SC. Clinical and Diagnostic Laboratory Immunology. **7 (4):** 588-595.

Abusugra, I., Wolf, G., Bölske, G., Thiaucourt, F., Morein, B. (1997). ISCOM vaccine against contagious bovine pleuropneumonia (CBPP). Biochemical and immunological characterization. Veterinary Immunology and Immunopathology. **59 (1-2):** 31-48.

Abusugra, I., F., Morein, B. (1999). ISCOM is an efficient delivery system for *Mycoplasma mycoides* subsp. *mycoides* (MmmSC) antigens inducing high mucosal and systematic antibody response. Immunology and Medical Microbiology. **23:** 5-12.

Aligaz, A. M., Munganga, J. M. W. (2019). Mathematical Modelling of Transmission Dynamics of Contagious Bovine Pleuropneumonia with Vaccination and Antibiotic Treatment. Journal of Applied Mathematics. 1-10.

Amanfu, W., Masupu, K. V., Adomu, E. K., Raborokgwe, M.V., Bashuriddin, J. B. (1998). An outbreak of contagious bovine pleuropneumonia in Ngamiland district of north-western Botswana. Veterinary Record. **143:** 46-48.

Amanfu, W., Sediadie, S., Masupu, K.V., Raborokgwe, M.V., Benkirane, A., Geiger, R., Thiaucourt, F. (2002). Comparison between cELISA and CFT in detecting antibodies to *Mycoplasma mycoides* biotype SC in cattle affected by CBPP in Botswana. Annals of the New York Academy of Sciences. **16:** 364-369.

Amanfu, W. (2009). Contagious bovine pleuropneumonia (Lungsickness) in Africa: Onderstepoort Journal of Veterinay Research. **76:** 01.

Anon. (1956). Animal Disease and Parasite Act, 1956 (Act 13 of 1956).

Anon. (2001). European Commission. Diagnostic tests for Contagious Bovine Pleuropneumonia (CBPP). Report of the Scientific Committee on Animal Health and Animal Welfare. 1-33.

Anon. (2002). FAO Animal Health Organisation, Recognizing contagious bovine pleuropneumonia. No. 13 Revision. 1.

Anon (2003). Directorate on Veterinary Services of Namibia, annual report.

Anon. (2004). OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.

Anon. (2005 & 2007a). OIE World Animal Health Information Database.

Anon. (2007b). Workshop on CBPP Control in the SADC Region. Dar Es Salaam. September 24-26.

Anon. (2008). OIE manual of diagnostic tests and vaccines for terrestrial animals.

Anon. (2016). Directorate on Veterinary Services of Namibia, Epidemiology.

Ayling, R. D., Baker, S. E., Nicholas, R. A. J., Peek, M. L., Simon, A. J. (2000). Comparison of *in vitro* activity of danofloxacin, florofenicol, oxytetracycline, spectinomycin and tilmicosin against *Mycoplasma mycoides* subspecies *mycoides* small colony type. Veterinary Record. **26**: 243-245.

Ayling, R.D. (2002). Diagnosis and Control of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides* small colony in cattle. PhD thesis. University of London.

Ayling, R. D., Bisgaard-Frantzen, S., March, J. B., Godinho, K., Nicholas, R. A. (2005). Assessing the in vitro effectiveness of antimicrobials against *Mycoplasma mycoides s*ubsp. *mycoides* small-colony type to reduce contagious bovine pleuropneumonia infection. Antimicrobial Agents Chemotherapy. **49:** 5162-5165.

Ayling, R. D., Godinho, K., Nicholas, R. A. J. (2007). Comparative studies on the *in vitro* antimicrobial sensitivities of *Mycoplasma mycoides* subsp. *mycoides* SC type and *Mycoplasma bovis*. CBPP control: Antibiotics to the Rescue. FAO-OIE-AU/IBAR-IAEA. Consultative Group Meeting on CBPP in Africa 2006. FAO, Rome, pp. 51-61.

Ayling, R. (2013). Meeting Report, Contagious Bovine Pleuropneumonia. Workshop, ILRI, Addis Ababa. IOM Newsletter. **37:** 10.

Barber, T. L., Stone, S. S., DeLay, P. D. (1970). Antibody in Cattle Experimentally Infected with Contagious Bovine Pleuropneumonia. Journal of American Society for Microbiology. **2:** 617-622.

Bashiruddin, J.B., Taylor, K.T., Gould, R.A. (1994). A PCR-based test for the specific identification of *Mycoplasma mycoides* subsp*. mycoides* SC. Journal of Veterinary Diagnostic Investigation. **6:** 428-434.

Bashiruddin, J. B., Santini, F. G., De Santis, P., Visaggio, M. C., Di Fransesco, G., D'Angelo, A., Nicholas, R. A. J. (1999). Detection of *Mycoplasma mycoides* subspecies *mycoides* SC in tissues from an outbreak of contagious bovine pleuropneumonia by culture, immunohistochemistry and polymerase chain reaction. Veterinary Record. **145:** 271-274.

Bashiruddin, J.B., de Santis, P., Persson, A., Ball, H., Regalla, J. (2005). Detection of *Mycoplasma mycoides* subsp. *mycoides* SC in bovine lung and lymph node tissues by culture, sandwich ELISA and polymerase chain reaction systems. Research in Veterinary Science. **78:** 199-205.

Blanchard, A., Browning, G. (2005). Mycoplasmas: molecular biology, Pathogenicity and strategies for control. Pp 485-514.

Blancou, J. (1996). Early methods of surveillance and control for contagious bovine pleuropneumonia. *Revue scientifique et Technique*. **15:** 4, 1263-1282.

Boonstra, E., Lindbaek, M., Fidzani, B., Bruusgaard, D. (2001). Cattle eradication and malnutrition in under five's: a natural experiment in Botswana. Public Health Nutrition. **4**: 877–882.

Brandao E. (1995). Isolation and identification of *Mycoplasma mycoides* subsp. *mycoides* strains in sheep and goats. Veterinary Record, **136:** 98-99.

Bruderer, U., Regalla, J., Abdo, E.M., Huebschle, O.J.B., Frey, J. (2002). Serodiagnosis and monitoring of contagious bovine pleuropneumonia (CBPP) with an indirect ELISA based on the specific lipoprotein LppQ of *Mycoplasma mycoides* subsp. *mycoides* SC, Veterinary Microbiology. **84:** 195-205.

Bygrave, A. C., Moulton, J. E., Shrine, M. (1968). Bulletin of Epizootic Diseases of Africa. **16:** 21.

Churchward, C. P., Flint, J., Danks, C., Humbert, I., Hashem, E., Nicholas, R. A. J., Ayling, R. D. (2007). Development and initial evaluation of a lateral flow device for the rapid detection of contagious bovine pleuropneumonia. In: Proceedings of the World Association of Veterinary Laboratory Diagnosticians – 13th International Symposium, Melbourne, Australia, 12-14 November 2007.

Costerton, J. W.; Geesey, G. G.; Cheng, K. J. (1978). How bacteria stick. Scientific American **238:** 86-95.

Davis, R. E., **Dally, E. L., Gundersen, E., Lee, I. M., Habili, N. (1997).** *Candidatus* Phytoplasma australiense, a new phytoplasma taxon associated with Australian grapevine yellows. International Journal of Systematic Bacteriology. **47:** 262-269.

Dedieu, L., Balcer-Rodrigues, V., Yaya, A., Hamadou, B., Cisse, O., Diallo, M., Niang, M. (2005a). Gamma interferon-producing CD4 T-cells correlate with resistance to *Mycoplasma mycoides* subsp. *mycoides* infection in cattle. Veterinary Immunology and Immunopathology. **107:** 217-233.

Dedieu, L., Chapey, E. T., Balcer-Rodrigues, V. (2005b). *Mycoplasma mycoides* subsp. *mycoides* Biotype Small Colony-Secreted Components Induce Apoptotic Cell Death in Bovine Lecucocytes. Scandaninavian. Journal of Immunology. **62:** 528-538.

Dedieu, L., Balcer-Rodrigues, V., Cisse, O., Diallo, M., Niang, M. (2006). Characterisation of lymph node immune response following *Mycoplasma mycoides* subsp. *mycoides* infection in cattle. Veterinary Research. **37:** 579-591.

Dedieu-Engelmann, L. (2008). Contagious bovine pleuropneumonia: A rationale for the development of a mucosal sub-unit vaccine. Comparative Immunology, Microbiology and Infectious Deseases. **31:** 227-238.

Dupuy, V., Manso-Silvàn, L., Barbe, V., Thebault, P., Dordet-Frisoni, E., Citti, C., Pumarat, F., Blanchard, A., Breton, M., Sirand-Pugnet, Thiaucourt, F. (2012). Evolutionary History of Contagious Bovine Pleuropneumonia Using Next Generation

Sequencing of *Mycoplasma mycoides s*ubsp. *mycoides* ''Small Colony''. PLoS ONE. **7 (10) :** e46821

Dyson, D. A., Smith, G. R. (1975). Contagious bovine Pleuropneumonia: mouse-protective antibody in the sera of infected and vaccinated cattle. Research in Veterinary Science. **19:** 8-16.

Edward, D., Freundt, E. A. (1956). The classification and Nomenclature of Organisms of the Pleuropneumonia Group. Journal of General Microbiology. **14:** 197-207.

Egwu, G. O., Nicholas, R. A. J., Ameh, J. A., Bashiruddin, J. B. (1996). Contagious Bovine Pleuropneumonia: An Update. Veterinary Bulletin. **66:** 9, 875-888.

Enyaru, J. C., Olaho-Mukani, K., Matovu, E., Monrad, J., Etoori, A., Ongor, C., Galiwango, T., Jamugisanga, P., Otim-Onapa, M., Twinamatsiko, E. (2003). Evaluation of polymerase chain reaction technique for the diagnosis of Contagious Bovine Pleuropneumonia (CBPP) in Uganda. Uganda Journal of Agricultural Sciences. **10:** 175-180.

Enyaru, J. C. K., Biryomumaisho, S., Balyeidhusa, A. S. P., Ebong, C., Musoni, A., Manzi, M., Rutagwenda, T., Zimurinda, J., Asiimwe, T., Gahakwa, D. (2012). Comparison of Competitive ELISA, PCR and Loop Mediated Isothermal Amplification of Mycoplasmal DNA in Confirmatory Diagnosis of an Outbreak of Contagious Bovine Pleuropneumonia in Eastern Rwanda. International Journal of Animal and Veterinary Advances. **4**(1): 22-28.

Fisher, J. (2003). To kill or not to kill: the eradication of CBPP in Western Europe. Medical History. **47:** 314-331.

Freundt, E. A. (1993). Culture media for classic mycoplasma, In: Methods in Mycoplasmology. (S. Razin and J. G. Tuly eds.) pp. 127-135. Academic Press, New York.

Frey, J., **Abdo, el-M., Miserez, R., Regalla, J., Gonçalves, R., Griot, C., Bensaide, A., Krampe, M., Nicolet, J. (1998).** Humoral and bronchial immune responses in cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* SC. In: Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular

genetics. (Leori, G., Santini, F., Scanziani, E., Frey, J.). Published by the European Commission Directorate-General Science, Research and Development Environment research programme B-1049 Brussels. **2:** pp 40-43.

Gaurivaud, P., **Poumarat, F. (2012).** Serodiagnosis of contagious bovine pleuropneumonia by immunoblotting. Euro Reference/Journal of Reference. 8: 17-20.

Geering, W. A., Roeder, P. I., Obi, T. U. (1999). Manual on the Preparation of National Disease Emergency Plans. FAO.

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., Benders, G. A., Montangue, M. G., Ma, L., Moodie, M. M., Merryman, C., Vashee, S., Krishnakumar, R., Assad-Garcia, N., Andrews-Pfannkoch, C., Denisova, E. A., Young, L., Qi, Z. Q., Seqall-Shapiro, T. H., Calvey, C. H., Parmar, P. P., Hutchison, C. A 3rd ., Smith, H. O., Venter, J. C. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. Science. **329:** 52-56.

Gonçalves, R., Regalla**, J., Penha Gonçalves, A. (1994).** Immunoblot and Electrophoretic analysis of *Mycoplasma mycoides* subsp. *mycoides* SC strains isolated from bovine and small ruminants. IOM Letters 3, 64-65.

Goncalves, M. R., Regalla, J., Nicolet, J., Frey, J., Nicholas, R.A.J. Bashiruddin, J. B., De Santis, P., Goncalves, P. A. (1998). Antigenic heterogeneity among *Mycoplasma mycoides subspecies* subsp. *mycoides* SC isolates: discrimination of major surface proteins. Veterinary Microbiology. **63:** 13-28.

Gonçalves, R., Regalla, J., Ayling, R. D., Nicholas, R. A. J. (2008). Impact of *Mycoplasma bovis* infection on sero-surveillance for contagious bovine pleuropneumonia. Veterinary Record. **163:** 632-633.

Gourlay, R. N., Palmer R. F. (1965). Further studies on the allergic reaction in CBPP. Journal of Comparative Pathology: **76.**

Gourlay, R. N. (1975). CBPP-protection following natural infection and vaccination. Developments in biological standardization. **28:** 586-589.

Gourlay, R. N., Howard, C. J., (1982). Respiratory mycoplasmosis. Advances in Veterinary Sciences and Comparative Medicine. **26:** 289-332.

Gull, T., French, R. A., Gorton, T. S., Burrage, T. G., Prozesky, L., Geary, S. J., Adams, L. G. (2013). Models of Contagious Bovine Pleuropneumonia: Evaluation of Two Novel Strains. The Open Veterinary Science Journal. **7:** 23-33.

Hamsten, C., Westberg, J., Bölske, G., Ayling, R., Uhlén, M., Persson, A. (2008). Expression and immunogenicity of six putative variable surface proteins in *Mycoplasma mycoides* subsp. *mycoides* SC. Microbiology **154**: 539–549.

Hamsten, C. (2009). Protein based approaches to understand and prevent contagious bovine pleuropneumonia. PhD thesis, School of Biotechnology, Royal Institute of Technology (KTH), Stockholm, Sweden.

Hamsten, C., Tjipura-Zaire, G., McAuliffe, L., Hübschle, O. J. B., Scacchia, M., Ayling, R., Westberg, J., Persson, A. (2010). Protein-Specific Analysis of Humoral Immune Responses in a Clinical Trial for vaccines against Contagious Bovine Pleuropneumonia. Clinical and Vaccine Immunology, **May:** 853-861.

Heller, M., Sachse, K., Schubert, E. (2007). Contagious bovine pleuropneumonia-a nearly forgotten disease? Deutsche Tierärztliche Wochenschrift. **114:** 2, 43-49.

Heller, M., Gicheru, N., Zaire-Tjipura, G., Muriuki, C., Yu, M., Botelho, A., Naessens, J., Jores, J., Liljander, A. (2016). Development of a Novel Cocktail Enzyme-Linked Immunosorbent Assay and a Field-Applicable Lateral-Flow Rapid Test for Diagnosis of Contagious Bovine Pleuropneumonia. Journal of Veterinary Microbiology. **54:** 1557-1565.

Houshaymi, B. M., Miles, R. J., Nicholas, R. A. J. (1997). Oxydation of glycerol differentiates African from European isolates of *Mycoplasma mycoides* subspecies *mycoides* Small Colony (small colony). Veterinary Record. **140**: 182-183.

Howard, C. J., Taylor, G. (1985). Immune response to mycoplasma infections of respiratory tract. Veterinary Immunology of Immunopathology. **10:** 1, 3-32.

Howard, C. J., Thomas, L. H., Parsons, K. R. (1987). Immune response of cattle to respiratory mycoplasmas. Veterinary Immunology of Immunopathology. **17:** 401-412.

Hübschle, O. J. B., Tjipura-Zaire, G., Abusugra, I., Di Francesca, G., Mettler, F., Pini, A., Morein, B. (2003). Experimental field trial with an immunostimulating complex (ISCOM) vaccine against CBPP. Journal of Veterinary Medicine. **50:** 298–303.

Hübschle, O. J. B., Bamhare, C., Tjipura-Zaire G. (2004). The situation of CBPP in Namibia in view of a recent outbreak in the Caprivi region. FAO Report of the Third Meeting of the FAO/OIE/OAU-IBAR Consultative Group. FAO, Rome 2003, pp 172-175.

Hübschle, O. J. B., Ayling, R. D., Godinho, K., Lukhele, O., Tjipura-Zaire, G., Rowan, T. G., Nicholas, R. A. J. (2006). Danofloxacin (Advocin ^{TM}) reduces the spread of contagious bovine pleuropneumonia to healthy in-contact cattle. Research in Veterinary Science. **81:** 304-309.

Hudson, J. R., Etheridge J. R. (1965). Contagious bovine pleuropneumonia: Experiments with the antibiotic Tylosin. Australian Veterinary Journal. **41(5):** 130-135.

Jores, J., Nkando, I., Sterner-Kock, A., Haider, W., Poole, J., Unger, H., Muriuki, C., Wesonga, H., Taracha, E. L. (2008). Assessment of *in vitro* interferon-gamma responses from peripheral blood mononuclear cells of cattle infected with *Mycoplasma* subsp*. mycoides* type. Veterinary Immunology and Immunopathology **124:** 1-2, 192-197.

Jores, J., Mariner, J. C., Naessens, J. (2013). Development of an improved vaccine for contagious bovine pleuropneumonia: an African perspective on challenges and proposed actions. Veterinary Research. **44:** 122, 1-5.

Kabilika, S. H., Songolo, A. C., Chisembele, C. (2007). Contagious bovine pleuropneumonia in Zambia, Historical trends and current situation: Workshop on CBPP Control in the SADC Region. Dar Es Salaam. September 24-26.

Krause, D. C., Taylor-Robinson, D. (1992). Mycoplasmas which infect humans. In: Mycoplasmas: Molecular Biology and Pathogenesis (McElhaney, R. N., Finch, L. R. Baseman, J. B. eds) pp. 417-444. American Society for Microbiology. Washington, D.C.

Kusiluka, L. J. M., Semuguruka, W. D., Kazwala, R. R., Ojenyiyi, B., Friis, N. F. (2000). Demonstration of Mycoplasma capricolum subsp. capripneumonia and *Mycoplasma mycoides* subsp*. mycoides* type in outbreaks of caprine pleuropneumonia in eastern Tanzania. Acta Veterinaria Scadinavica. **41:** 3, 311-319.

Lanzavecchia, A., **Sallustro, F. (2005).** Understanding the generation and function of memory T-cell subsets. Current Opinion in Immunology. **17:** 326-332.

Lartigue, C., **Glass, J. I., Alperovich, N., Pieper, N., Parmar, P. P., Hutchison, C. A 3rd ., Smith, H. O., Venter, J. C. (2007).** Genome transplantation in bacteria: changing one species to another. Science. **317:** 632-638.

Lartigue, C., **Vashee, S., Algire, M. A., Chuang, R.Y., Benders, G. A., Ma, L., Noskov, V. N., Denisova, E. A., Gibson, D. G., Assad-Garcia, N., Alperovich, N., Thomas, D. W., Merryman, C., Hutchison, C. A 3rd ., Smith, H. O., Venter, J. C., Glass, J. I. (2009).** Creating bacterial strains from genomes that have been cloned and engineered in yeast. Science. **325:** 1693-1696

Le Goff, C., Thiaucourt, F. (1998). A competitive ELISA for the specific diagnosis of contagious bovine pleuropneumonia. Veterinary Microbiology. **60:** 2-4, 179-191.

Lesnoff, M., Laval, G., Bonnet, P., Abdicho, S., Workalemahu, A., Kifle, D., Peyraud, A., Lancelot, R., Thiaucourt, F. (2004). Within-herd spread of CBPP in Ethiopian highlands. Preventive Veterinary Medicine **64:** 1, 27-40.

Littell, R. C., Henry, R. C., Ammerman, C. B., (1998). Statistical analysis of repeated measures data using SAS procedures. Journal of Animal Science. **76:** 1216-1231

Lloyd, L. C. (1967). An attempt to transfer immunity to M. *mycoides* infection with serum. Bulletin of Epizootic Diseases of Africa. **15:** 1, 11-17.

London, C. A**., Perez, V. L., Abbas, A. K. (1999).** Functional characteristics and survival requirements of memory $CD4^+$ T lymphocytes in vivo. Journal of Immunology. **162:** 776-773.

Lorenzon, S., Arzul, I., Peyraud, A., Hendrikx, P., Thiaucourt, F. (2003). Molecular epidemiology of contagious bovine pleuropneumonia by multilocus sequence analysis of *Mycoplasma mycoides* subspecies *mycoides* biotype SC strains. Veterinary Microbiology 93**:** 4, 319-33.

Mabey, B., Peeling, R. W., Ustianowski, A., Perkins, M. D. (2004). Diagnostics for the developing world. Nature Reviews Microbiology. **2 (3):** 231

Mair, G., Vilei, E. M., Wade, A., Frey, J., Unger, H. (2013). Isothermal loop-mediated amplification (lamp) for the diagnosis of contagious bovine pleura-pneumonia. BMC Veterinary Research. **9:** 108.

Manso-Silván, L., Vilei, E. M., Sachse, K., Djordjevic, S. P., Thiaucourt, F., Frey, J. (2009). *Mycoplasma leachii* sp *nov.* as a new species designation for *Mycoplasma* sp. Bovine group 7 of Leach, and reclassification of *Mycoplasma mycoides* subsp. *mycoides* LC as serovar of *Mycoplasma mycoides* subsp. c*apri*. International Journal of Systematic and Evolutionary Microbiology. **59:** 1353-1358.

March, J. B., Waite, E. R., Litamoi, J. K. (2000). Re-suspension of T1/44 vaccine cultures of *Mycoplasma mycoides* subsp. *mycoides* SC in 1 molar MgSO4 causes a drop in pH and a rapid reduction in titre. FEMS Immunology and Medical Microbiology. **34:** 2, 97-103.

March, J. B., Harrison, J. C., Borisch, S. M. (2002). Humoral immune responses following experimental infection of goats with *Mycoplasma capricolum* subsp. *capripneumoniae*. Veterinary Microbiology 84, 29-45.

March, J. B., Kerr, K., Lema, B. (2003). Rapid detection of contagious bovine pleuropneumonia by a *Mycoplasma mycoides* subsp. *mycoides* SC capsular polysaccharide-specific antigen detection latex agglutination test. Clinical and Diagnostic Laboratory Immunology. **10:** 2, 233-40.

March, J. B. (2004). Improved formulations for existing CBPP vaccines-recommendations for change. Vaccines. **22:** 4358-4364.

Marobela-Raborokwge, C., Nicholas, R. A. J., Ayling, R. D., Bashiruddin, J. B. (2003). Comparison of complement fixation test, immunoblotting, indirect ELISA and competitive ELISA for detecting antibodies to *Mycoplasma mycoides* subspecies *mycoides* Small Colony (SC) in naturally infected cattle from the 1995 outbreak in Botswana. Onderstepoort Journal of Veterinary Research. **70:** 21-27.

Marobela-Raborokwge, C. (2011). Contagious bovine pleuropneumonia in Botswana: experience with control, eradication, prevention and surveillance. Veterinaria Italiana. **47 (4):** 397-405.

Masinga, W. N., Roberts, D. H., Kakoma, I., Rurangirwa, F. R. (1975). Passive immunity to CBPP. Research in Veterinary Science, **19**: 330-332.

Masinga, W. N., Domenech, J. (1995). Overview and epidemiology of contagious bovine pleuropneumonia in Africa. Revue Scientifique et Technique. **14:** 3, 611-30.

Masinga, W. N., Domenech, J., Windsor, R. S. (1996). Manifestation and epidemiology of contagious bovine pleuropneumonia in Africa. Revue Scientifique et Technique **15:** 4, 1283-1308.

Mbulu, R. S., Tjipura-Zaire, G., Lelli, R., Frey, J., Pilo, P., Vilei, E. M., Mettler, F., Nicholas, R. A. J., Hübschle, O. J. B. (2004). Contagious bovine pleuropneumonia (CBPP) caused by vaccine strain T1/44 of *Mycoplasma mycoides* subsp. *mycoides* SC. Veterinary Microbiology. **98:** 229-234.

McAuliffe, L., Ellis, R. J., Miles, K., Ayling, R. D., Nicholas, R. A. J. (2006). Biofilm formation by mycoplasma species and its role in environmental persistence and survival. Veterinary Microbiology. **152:** 913-922.

McAuliffe, L., Ayling, R. D., Ellis, R. J., Nicholas, R. A. J. (2007). Biofilm-grown *Mycoplasma mycoides* subsp. *mycoides* SC exhibit both phenotypic and genotypic variation compared with planktonic cells. Veterinary Microbiology. **129:** 315-324.

McAuliffe, L., Ayling, R. D., Ellis, R. J., Nicholas, R. A. J. (2008). The role of biofilm formation in the virulence and persistence of *Mycoplasma mycoides* subsp. *mycoides* SC. Abstract: 17th International Organisation for Mycoplasmology.

Messick, J. B., Smith, G., Berent, L., Cooper, S. (2000). Genonme size of *Eperythrozoon suis* and hybridization with 16S rRNA gene. Canadian Journal of Microbiology **46:** 11, 1082-1086.

Messick, J. B. (2004). Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Veterinary and Clinical Pathology, **33:** 2-13.

Musisi, F. L., Dungu, B., Thwala, R., Mogajane, M. E, Mtei, B. J. (2003). The threat of contagious bovine pleuropneumonia and challenges for its control in the SADC region. Towards sustainable CBPP control programmes for Africa. FAO-OIE-AU/BAR-IAEA

Consultative Group on Contagious Bovine Pleuropneumonia Third meeting, Rome, 12-14.

Muuka, G., Hang'ombe, B.M., Nalubamba, K.S., Kabilika, S., Mwambazi, L., Muma, J. B. (2011). Comparison of complement fixation test, competitive ELISA and LppQ ELISA with post-mortem findings in the diagnosis of contagious bovine pleuropneumonia (CBPP). Tropical Animal Health Production.**43:** (5), 1057-62.

Mulongo, M. M., Frey, J., Smith, K., Schnier, C., Wesonga, H., Naesens, J., Mckeever, D. (2013). Cattle immunized against the pathogenic L-alpha-glycerol-3-phosphate oxidase of *Mycoplasma mycoides* subsp. *mycoides* fail to generate neutralizing antibodies and succumb to disease on challenge. Vaccine.**31:** 5020-5025.

Naseem, S., Meens, J., Jores, J., Helle, M., Du bel, S., Hust, M., Gerlach, G. (2010). Phage display-based identification and potential diagnostic application of novel antigens from *Mycoplasma mycoides* subsp. *mycoides* type. Veterinary Microbiology. **142**: 285–292.

Niang, M., Cisse, O., Diallo, M., Doucoure, M., Kone, M., Simbe, C. f., Amanfu, W., Thiaucourt, F. (2006a). Effect of antibiotic therapy on the pathogenesis of CBPP. Experimental transmission of the disease by contact from infected animals treated with oxytetracycline. In: FAO Animal Production and Health. CBPP Control: Antibiotics to the rescue? Pp 25-32.

Niang, M., Diallo, M., Cisse, O., Kone, M., Doucoure, M., Roth, J. A., Balcer-Rodrigues, V., Dedieu, L. (2006b). Pulmonary and serum antibody responses elicited in zebu cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* SC by contact exposure. Veterinary Research 37: 733-744.

Niang, M., Serry, A., Doucoure, M., Kone, M., Diaye, N., Amanfu, W., Thiaucourt, F. (2010). Experimental studies on the effect of long-acting oxytetracycline treatment in the development of sequestra in contagious bovine pleuropneumonia-infected cattle. Journal of Veterinary Medicine and Animal Health. **2 (4):** 35-45.

Nicholas, R. A. J., Bashiruddin J. B. (1995). *Mycoplasma mycoides* subspecies *mycoides* (Small Colony Variant): the agent of CBPP and Member of the "*Mycoplasma*

mycoides subspecies *mycoides* Cluster". Journal of Comparative Pathology. **113:** 1, 1-27.

Nicholas, R. A. J., Santini, F. G., Clark, K. M., Palmer, N. M. A., De Santis P. (1996). A comparison of serological tests and gross lung pathology for detecting CBPP in two groups of Italian cattle. Veterinary Record. **139:** 4, 89-92.

Nicholas, R., Baker, S. (1998). Recovery of mycoplasmas from animals. In: Methods in Molecular Biology. Mycoplasma Protocols. Pp 37-43. (Miles, R. and Nicholas, R. eds.) Humana Press, Totowa, New Jersy.

Nicholas, R., Bashiruddin, J., Ayling, R., Miles, R. (2000). Contagious bovine pleuropneumonia: a review of recent developments. Veterinary Bulletin. **70:** 827-837.

Nicholas, R.A.J., Ayling, R. D., Stipkovits, L. (2002). An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis.* Vaccine 20, 3569-3575.

Nicholas, R. A. J., Tjipura-Zaire, G., Mbulu, R. S., Scacchia, M., Mettler, F., Frey, J., Abusugra, I., Huebschle, O. J. B. (2004). An inactivated whole cell vaccine and LppQ subunit vaccine appear to exacerbate the effects of CBPP in adult cattle; p. 91-97. Proceedings of the 3rd Meeting of the FAO-OIE-OAU/IBAR-IAEA Consultative Group on CBPP. Towards Sustainable CBPP Control Programmes for Africa (Rome, 12–14November 2003). FAO; Rome, Italy.

Nicholas, R. A. J., Tjipura-Zaire, G., Scacchia, M., Frey, J., Huebschle, O. J. B. (2005). An inactivated vaccine against CBPP exacerbates disease in cattle; Proceedings of FAO/ OIE/AU Consultative Meeting on CBPP. November, FAO, Rome: 91-97

Nicholas, R., Ayling, R., McAuliffe, L, (2008a). Contagious Bovine Pleuropneumonia. In: Mycoplasma diseases of Ruminants. CABI. pp 69-97.

Nicholas, R., Ayling, R., McAuliffe, L, (2008b). Antigenic Analysis of Mycoplasmas. In: Mycoplasma diseases of Ruminants. CABI. pp 53-57.

Nicholas, R., Ayling, R., McAuliffe, L, (2009). Contagious bovine pleuropneumonia: a reminder. Veterinary Record. **165**: 756-75.

Nicholas, R., Ayling, R., Tjipura-Zaire, G., Rowan, G. (2012): Treatment of contagious bovine pleuropneumonia. **171:** London. BMJ Publishing Group.

Nicolas, M. M., Stalis, I.S, Clippinger, T. L., Busch, M., Nordhausen, R., Malouf, G., Schrenzel, M. D. (2005): Systemic Disease in Vaal Rhebok (Pelea capreolus) caused by Mycoplasmas in the Mycoides Cluster. Journal of Clinical Microbiology, **43:** 1330-1340.

Nkando, I., Perez-Casal, J., Mwirigi, M., Prysliak, T., Townsend, H., Berberov, E., Joseph, K., Mugambi, J., Soi, R., Liljander, A., Jores, J., Gerdts, V., Potter, A., Naessens, J., Wesonga, H. (2016): Recombinant *Mycoplasma mycoides* proteins elicit protective immune responses against contagious bovine pleuropneumonia. Veterinary Immunology and Immunopathology, **171:** 103-114.

Nocard, R., Roux, E. R. (1898). Le microbe de la peripneumonia. Annala Institute Pasteur. **12:** 240-262.

Noskov, V. N., Segall-Shapiro, T. H., Chuang, R. Y. (2010). Tandem repeat coupled with endonuclease cleavage (TREC): a seamless modification tool for genome engineering in yeast. Nucleic Acids Research. **38:** 2570-2576.

Olabode, H. O. K., Mailafia, S., Adah, B. M. J., Nafarnda, W. D., Ikpa, L. T., Jambalang, A. R., Bello, R. H. (2013). Serological Evidence of Contagious Bovine Pleuro-Pneuminia antibodies in trade cattle (Bos Indicus) sold in Kwara state –Nigeria. Online International Journal of Microbiology Research. **1:** 14-19.

Onono, J. O., Wieland, B., Suleiman, A., Rushton, J. (2017). Policy analysis for delivery of contagious bovine pleuropneumonia control strategies in sub-Saharan Africa. Scientific and Technical Review of the Office International des Epizooties (Paris). **36 (1):** 195-205.

Persson, A., Jacobsson, K., Frykberg, L., Johansson, K. E., Poumarat, F. (2002). Variable Surface Protein Vmm of *Mycoplasma mycoides* subsp. *mycoides* Small Colony Type. Journal of Bacteriology. **184:** 13, 3712-3722.

Pilo, P., Vilei, E. M., Peterhans, E., Bonvin-Klotz, L., Stoffel, M. H., Dobbelaere, D., Frey, J. (2005). A metabolic enzyme as a primary virulence factor of *Mycoplasma mycoides* subsp. *mycoides* small colony. Journal of Bacteriology. **18:** 19, 6824-31.

Pilo, P., Frey, J., Vilei, E. M. (2007). Molecular mechanism of pathogenicity of *Mycoplasma mycoides* subsp. *mycoides SC*. Veterinary Journal. **174 (3):** 513-521

Poumarat, F., Solsona, M., Boldini, M. (1994). Genomic, protein and antigenic variability of *Mycoplasma* bovis. Veterinary Microbiology 40, 305-321.

Provost, A., Perreau, P., Breard, A., Le Goff, C., Martel, J. L., Cottew, G. S. (1987). Contagious Bovine Pleuropneumonia. Veterinary Record. **63:** 427-429.

Rana, R., Srivastava, N. C. (2001). Cell-mediated immune response in goats vaccinated with killed *Mycoplasma mycoides* subsp. *capri*. Saponin vaccines. Indian Journal of Animal Sciences. **(7):** 198-199.

Razin, S., Yogev, D., Naot, Y. (1998). Molecular Biology and Pathogenicity of Mycoplasmas. Microbiology and Molecular Biology Reviews. **62:** 4, 1094-1156.

Regalla, J., Caporale, V., Giovannini, A., Santini, F., Martel, J. L., Concalves, A. P. (1996). Manifestation and epidemiology of contagious bovine pleuropneumonia in Europe. Revue Scientifique et Technique. **15:** 4, 1309-1329.

Regassa, L. B., French, F. E., Stewart, K. M., Murphy, A. C., Jandhyam, H. L., Beati, L. (2011). A Costa Rican bacterial spiroplasma biodiversity survey in tabanid flies reveals new serogroups and extends United States ranges. International Journal of Biodiversity and Conservation. **3:** 8, 338-344.

SAS Institute, Inc., (2003). SAS/STAT user's guide.

Salmon, D. E. (1896). Infectious disease of cattle. In. Special report on Diseases of cattle and cattle feeding. U.S. Department of Agriculture, Washington, DC, pp. 376-388.

Santini, F. G., D'Angelo, A. R., Scacchia, M., Di Giannatale, E., Visaggio, M. C., Farinelli, G., Di Francesco, G., Guarducci, M. (1992). Sequestro Polmonare in un bufalo domestico da *Mycoplasma mycoides* subsp. *mycoides*: Isolamento, quadro anatomo-istopatologico ed immunoistochimico. Veterinaria Italiana. 4-10.

Scacchia, M., Sacchini, F., Filipponi, G., Luciani, M., Lelli, R., Tjipura-Zaire, G., Di Provvido, A., Shiningwane, A., Ndiipanda, F., Pini, A., Caporale, V., Hübschle, O. J. (2007). Clinical, humoral and IFN gamma responses of cattle to infection with *Mycoplasma mycoides* var. *mycoides* small colony and attempts to condition the pathogenesis of the infection. The Onderstepoort journal of veterinary research. **74:** 251-263.

Scacchia, M., Sacchini, Tjipura-Zaire, G., Lelli, R., Sacchini, F., Pini, A. (2011). Contagious bovine pleuropneumonia: humoral and pathological events in cattle infected by endotracheal intubation or by exposure to infected animals. Veterinaria Italiana, **47** (4): 407-413.

Scanziani, E., **Paltrinieri, S., Boldini, M., Grieco, V., Monaci, C., Giusti, A. M., Mandelli, G. (1997).** Histopathological and immunohistochemical findings in thoracic lymph nodes of cattle with Contagious Bovine Pleuropneumonia. Journal of Comparative Pathology. **117:** 127-136.

Schieck, E., Lartigue, C., Frey, J., Vozza, N., Hegermann, J., Miller, R. A., Valguarnera, E., Muriuki, C., Meens, J., Nene, V., Naessens, J., Weber, J., Lowary, T. L., Vashee, S., Feldman, M. F., Jores, J. (2016). Galactofuranose in *Mycoplasma mycoides* is important for membrane integrity and conceals adhesins but does not contribute to serum resistance. Molecular Microbiology. **99 (1):** 55-70.

Schieck, E., Liljander, A., Hamsten, C., Gicheru, N., Scacchia, M., Sacchini, F., Schnee, C., Sterner-Kock, A., Hlinack, A., Naessens, J., Poole, J., Persson, A., Jores, J. (2014). High antibody titres against predicted Mycoplasma surface proteins do not prevent sequestration in infected lung tissue in the course of the experimental contagious bovine pleuropneumonia. Veterinary Microbiology. **172:** 284-293.

Schnee, C., Heller, M., Jores, J., Tomas, H., Neubauer, H. (2011). Assessment of novel multiplex real-time PCR assay for the detection of the CBPP agent *Mycoplasma mycoides* subsp. *mycoides* SC through experimental infection in cattle. BMC Veterinary Research. **7:** 47.

Schneider, H. P. (1994a). State Veterinary Disease Control. In: Animal Health and Veterinary Medicine in Namibia. AGRIVET. pp 253-260.

Schneider, H. P., Van der Lugt, J. J., Hübschle, O. J. B. (1994b). In: Infectious Diseases of Livestock with special reference to Southern Africa Vol II, (Coetzer, J. A. W., Thomson, G. R., Tustin, R. C. eds.) pp 177**:** 1483-1494. Oxford University Press.

Schubert, E., Sache, K., Jores, J., Heller, M. (2011). Serological testing of cattle experimentally infected with *Mycoplasma mycoides* subsp. *mycoides* using four different tests reveals a variety of seroconversion pattern. BMC Veterinary Research. **7:** 72.

Simmons, W. L., Dybvig, K. (2009). Mycoplasma biofilms *ex vivo* and *in vivo*. Federation of European Micribiological Societies. Research Letter.

Smith, G. R. (1967). *Mycoplasma mycoides* var*. mycoides* SC: production of bacteriaemia and demonstration of passive immunity in mice. Journal of Comparative Pathology. **77:** 203-209.

Smith, G. R. (1971). *Mycoplasma mycoides* var. *mycoides* SC: Immunity and mouse-protective antibody. Journal of Comparative Pathology. **81:** 267-278.

Ssematimba, A., Jores, J., Mariner J. C. (2015). Mathematical modelling of the transmission dynamics of contagious bovine pleuropneumonia reveals minimal target profiles for improved vaccines and diagnostic assays. PLoS One **10:**e0116730. http://dx.doi.ord/10.1371/jpurnal.pone.0116730.

Stärk, K. D. C., Vicari, A., Tontis, A., Nicolet, J. (1995). Epidemiological investigations of contagious bovine pleuropneumonia in Switzerland. Schweizer Archiv für Tierheilkunde. **137:** 92-100.

Sylla, D., Litamoi, J., Rweyemamu, M. M. (1995). Vaccination strategies for the contagious bovine pleuropneumonia in Africa. Revue Scientifique et Technique. **14:** 3, 577-92.

Tambi, N. E., Maina, W. O., Ndi C. (2006). An estimation of economic impact of contagious bovine pleuropneumonia in Africa. Revue Scientifique et Technique. **25:** 3, 999-1011.

Tardy, F., Maigre, L., Pumarat, F., Citti, C. (2009). Identification and distribution of genetic markers in three closely related taxa of the *Mycoplasma mycoides* cluster :

refining the relative position and boundaries of the *Mycoplasma* sp. Bovine group 7 taxon (*Mycoplasma leachii*). Microbiology. **155:** 3775-3787.

Teshale, S., Aschalew, Z., Esayas, G., Fikru, R. (2005). I solation and identification of *Mycoplasma mycoides* subspecies *mycoides* Small Colony biotype in Eastern Ethiopia. International Journal of Applied Research in Veterinary Medicine. **3:** (1). 30-34.

Thiaucourt, F., Lorenzon, S., David, A., Tulasne, J. J., Domenech, J. (1998). Vaccination against contagious bovine pleuropneumonia and the use of molecular tools in epidemiology. Annals of the New York Academy of Sciences. **849:** 146-51.

Thiaucourt, F., Yaya, A., Wesonga, A., Hübschle, O. J. B., Tulasne, J. J., Provost, A. (2000). CBPP: A Reassessment of the efficacy of vaccines used in Africa. Annals of the New York Academy of Sciences. **916:** 70-80.

Thiaucourt, F., Dedieu, L., Maillard, J. C., Bonnet, P., Lesnoff, M., Laval, G., Provost, A. (2003). Contagious bovine pleuropneumonia vaccine, historic highlights, present situation and hopes. Developments in Biologicals. **114:** 147-160.

Thiaucourt, F., Aboubakar, Y., Wesonga, H., Maanso-Silvan, L., Blanchard, A. (2004). Contagious bovine pleuropneumonia vaccines and control strategies: recent data. Developmental Biology. **119:** 99-111.

Thiaucourt, F., Van Der Lugt, J. J., Provost, A. (2005). Contagious bovine pleuropneumonia. Infectious Disease of Livestock, 2nd edition, **3:** pp 2045-2059.

Thomson, G. R. (2005). Contagious bovine pleuropneumonia and poverty. A strategy for addressing the effect of the disease in sub-Saharan Africa. Animal Health Programme. Center for Tropical Veterinary Medicine, University of Edinburgh, UK.

Tjipura-Zaire, G., Hübschle, O., Scacchia, M., McAuliffe, L., Nicholas, R., Fielder, M., Ayling, R. D. (2008). Comparison between Complement Fixation Test and Lateral Flow Device in Detecting Antibodies to *Mycoplasma mycoides* subsp. *mycoides* Small colony. 17th IOM, China.

Totté, P., Rodrigues, V., Yaya, A., Hamadou, B., Cisse, O., Diallo, M., Niang, M., Thiaucourt, F., Dedieu, L. (2008). Analysis of cellular responses to *Mycoplasma*

mycoides subsp. *mycoides* biotype associated with control of contagious bovinepleuropneumonia. Veterinary research. **39:** 1, 8.

Trichard, C. J. V., Basson, P. A., Van der Lugt, J. J., Jacobsz, E. P. (1989). An outbreak of CBPP in the Owambo Mangetti area of South West Africa/Namibia. Microbiological Immunofluorescent, Pathological and Serological findings. Onderstepoort Journal of Veterinary Research. **56:** 4, 277-284.

Tulasne, J. J., Litamoi, J. K., Morein, B., Dedieu, L., Palya, V. J., Yami, M., Abussugra, I., Sylla, D., Bensaid, A. (1996). CBPP vaccines: the current situation and the need for improvement. Revue Scientifique et Technique. **15:** 1373-1396.

Vilei, E. M., Abdo, E.M., Nicolet, J., Botelho, A., Gonçalves, R., Frey, J. (2000). Genomic and antigenic differences between the European and African/Australian clusters of *Mycoplasma mycoides* subsp. *mycoides.* Microbiology. **(146):** 477-486.

Vilei, E. M., Frey, J. (2010). Detection of *Mycoplasma mycoides* subsp. *mycoides* SC in bronchoalveolar lavage fluids of cows based on a TaqMan real-time PCR discriminating wild type strains from an lppQ- mutant vaccine strain used for DIVA-strategies. Journal of Microbiology Methods. **81:** 211-218

Waite, E. R., March, J. B. (2001). The effect of HEPES Buffer systems upon the pH, growth and survival of *Mycoplasma mycoides* subsp. *mycoides* (MmmSC), Vaccine cultures. FEMS Microbiology letters. **201:** 2, 291-294.

Westberg, J., Persson, A., Holmberg A., Goesmann, A., Lundeberg, J., Johansson, K. E., Pettersson, B., Uhlén, M. (2004). The Genome Sequence of *Mycoplasma mycoides* subsp. *mycoides* Type Strain PG1 T, the causative Agent of Contagious Bovine Pleuropneumonia (CBPP). Genome Research. **14:** 2, 221-221.

Williamson, D. L., Gasparich, G. E., Regassa, L. B., Saillard, C., Renaudin, J., Bovè, J. M., Whitcomb, R. F. (2011). Family II. Spiroplasmatacea. In: Bergey's Manual of Systematic Bacteriology vol 4; Krieg, N. R., Ludwig, W., Whitman, W. B., Hedlund, B., Paster, B. J., Staley, J. T., Ward., N., Brown, D. R., Parte, A. eds. (springer, New York), pp. 654-686.

Windsor, R. S., Masinga, W. N (1977). Investigation into the role of carrier animals in the spread of CBPP. Research in Veterinary Science. **23:** 2, 224-229.

Windsor, R. S., Wood, A. (1998). Contagious bovine pleuropneumonia: The cost of control in Central/Southern Africa. Annals of the New York Academy of Science. **849:** 299-306.

Windsor, R. S. (2000). The eradication of Contagious Bovine Pleuropneumonia from South Western Africa: A plan for action. Annals of the New York Academy of Science. **916:** 326-32.

www.afrivip.org/sites/default/files/cbpp_complete

www.bacterio.clct.fr/e/eperythrozoon.html

www.bacterio.clct.fr/m/mycoplasma.html

Xin, J., Li, Y., Nicholas, R. A., Chen, C., Liu, Y., Zhang, M. J., Dong, H. (2011). A history of the prevalence and control of contagious bovine pleuropneumonia in China. Veterinary Journal. **191:** 166-170.

Zreik, L. P. Carle. J. M., Bove, J. M. Garnier, M. (1995). Characterization of the mycoplasmalike organism associated with wirches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* Phytoplasma aurontifoloia". International Journal of Systematic Bacteriology. **45:** 449-453.

APPENDIX 1: CBPP Lung form

Trial n. _____________ Animal Id.N°________ Date ________ Central Veterinary Laboratory, Namibia-IZSAM, Italy

Left Lung **Right Lung**

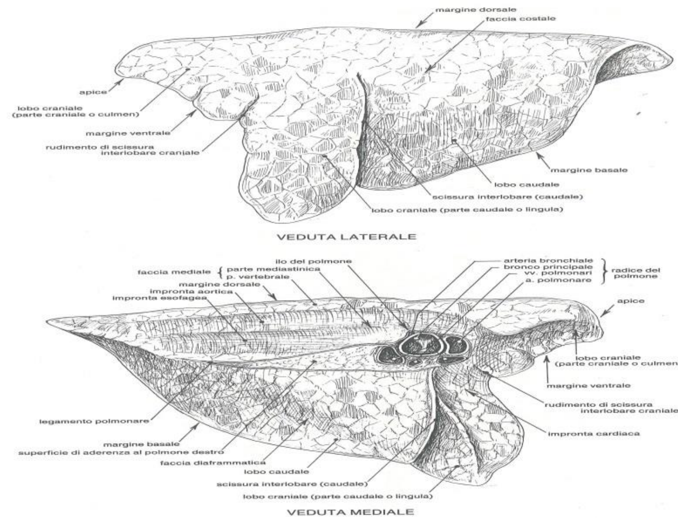


Fig. 294
POLMONE SINISTRO DI BOVINO
ISOLATO DOPO FISSAZIONE IN SITU

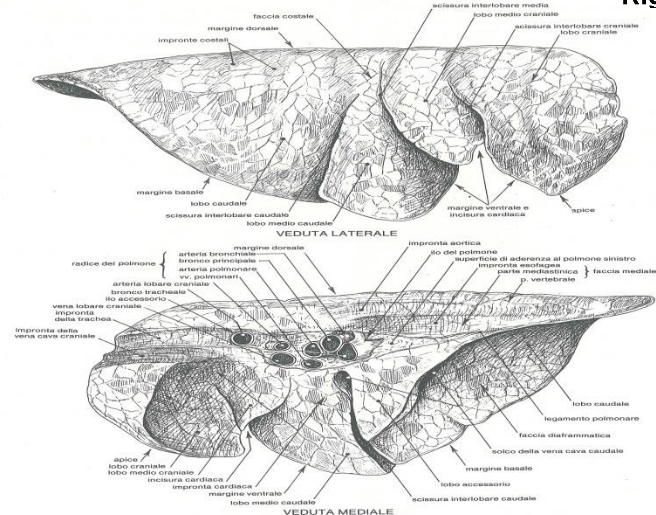


Fig. 293
POLMONE DESTRO DI BOVINO
ISOLATO DOPO FISSAZIONE IN SITU

	Engorg.	Red Hepat	Marble Policr	Necrosis	Sequestra	Gray Hepat	Resolut	Pseudomembr	Fibr. adhesion	Sinechie	Pleura fluid
L. apical											
R. apical											
L. cardiac											
R. Cardiac											
Acc Lobe											
L. diaphr											
R. diaphr											
Pleura											

APPENDIX 2: Comparisons of the results of the three serological tests

Table 4.4. Comparisons of the results of the three serological tests in sera collected from in-contact control cattle in the vaccine group

Cattle No	188				175				177				145				165			
Date in Weeks	cELISA %	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT
2	44	5	nd	nd	51	0	nd	nd	51	0	nd	nd	38	0	nd	nd	40	10	nd	nd
3	45	6	21.0	-	59	0	14.0	-	46	0	9.5	-	44	1	18.4	-	38	5	13.5	-
4	48	2	21.7	-	51	1	16.5	-	47	2	6.6	-	38	0	18.9	-	41	6	4.9	-
5	42	4	11.6	-	56	1	15.6	-	44	1	10.5	-	37	1	14.9	-	37	6	9.1	-
6	52	4	9.7	-	60	2	10.3	-	41	0	9.3	-	43	0	18.8	-	64	64	121.6	++
7	33	nd	21.7	-	54	nd	38.9	-	47	nd	17.7	-	34	nd	29.4	-	67	nd	194.2	+++
8	36	nd	38.5	-	60	nd	25.2	-	38	nd	21.6	-	0	nd	12.0	-	74	nd	78.3	+++
9	45	nd	30.6	-	42	nd	14.3	-	29	nd	19.2	-	5	nd	23.1	-	73	nd	113.8	+++
10	43	nd	15.5	-	54	nd	13.9	-	46	nd	22.0	-	8	nd	23.7	-	82	nd	56.5	+++
11	38	nd	53.0	+++	72	nd	50.4	-	19	nd	13.0	-	25	nd	16.4	-	79	nd	42.1	+
14	73	nd	115.9	++	79	nd	84.5	+++	72	nd	115.0	+++	35	nd	29.9	+	77	nd	27.4	+
17	72	nd	72.0	+++	72	nd	30.0	+++	73	nd	64.0	+++	85	nd	36.6	++	76	nd	23.5	+

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.5. Comparisons of the results of the three serological tests in sera collected from cattle vaccinated with Purified Protein

Cattle No	**155**				**150**				**189**				**161**				**162**			
Date in weeks	**cELISA %**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**
0	27	1	nd	nd	45	0	nd	nd	26	0	nd	nd	25	2	nd	nd	46	-1	nd	nd
1	24	1	nd	nd	38	2	nd	nd	43	6	nd	nd	29	5	nd	nd	41	1	nd	nd
2	35	4	nd	nd	53	0	nd	nd	47	4	nd	nd	44	2	nd	nd	41	0	nd	nd
3	45	0	30.6	-	47	0	43.9	-	47	1	10.8	-	41	2	14.7	-	38	1	14.0	-
4	40	1	17.6	-	53	0	11.0	-	55	0	31.8	-	43	2	25.8	-	44	-1	30.1	-
5	40	1	18.9	-	49	0	17.0	-	48	1	16.3	-	47	2	15.8	-	43	0	23.8	-
6	42	1	21.5	-	46	1	27.2	-	65	3	33.7	++	48	2	6.6	-	42	0	15.8	-
7	21	nd	16.3	-	36	nd	14.8	-	61	nd	112.7	+++	22	nd	10.8	-	37	nd	7.8	+
8	45	nd	22.6	-	46	nd	25.2	-	84	nd	147.6	+++	39	nd	16.2	-	50	nd	157.8	+++
9	68	nd	139.9	+++	34	nd	32.2	-	75	nd	144.7	+++	39	nd	14.0	+	52	nd	196.7	+++
10	85	nd	281.7	+++	58	nd	43.7	+	84	nd	154.4	+++	33	nd	11.3	-	77	nd	206.9	+++
11	86	nd	176.6	+++	72	nd	36.2	++	81	nd	132.1	++	55	nd	16.4	-	77	nd	89.9	+++
14	86	nd	69.0	+++	77	nd	41.8	+++	83	nd	73.7	++	67	nd	118.9	++	76	nd	55.4	+++
15	93	nd	69.2	+++	87	nd	51.5	+++	88	nd	73.3	++	75	nd	104.0	++	84	nd	46.6	+++
17	85	nd	82.1	+++	89	nd	34.4	+++	84	nd	93.5	++	77	nd	93.2	++	83	nd	57.4	+++

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.6. Comparisons of the results of the three serological tests in sera collected from cattle vaccinated with Tween 20 cells

Cattle No	**139**				**158**				**194**				**170**				**182**			
Date in weeks	**cELISA %**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**	**cELISA%**	**LppQ %**	**LFD**	**LAT**
0	42	-1	nd	nd	65	0	nd	nd	52	93	nd	nd	37	-2	nd	nd	46	15	nd	nd
1	53	0	nd	nd	61	0	nd	nd	44	130	nd	nd	29	0	nd	nd	48	24	nd	nd
2	52	2	nd	nd	63	1	nd	nd	55	67	nd	nd	37	0	nd	nd	41	11	nd	nd
3	53	2	30.2	-	63	1	25.8	-	46	74	26.8	-	37	2	49.9	-	45	13	37.5	-
4	45	1	22.0	-	62	1	29.1	-	47	80	25.0	-	31	1	29.6	-	43	10	35.8	-
5	50	2	35.8	-	64	0	27.8	-	76	134	58.4	-	39	-1	29.9	-	45	8	36.4	-
6	72	72	22.4	-	64	0	17.5	-	93	173	108.4	+++	43	3	19.9	-	51	3	34.7	-
7	91	nd	125.4	+++	42	nd	23.0	-	87	nd	199.0	+++	40	nd	47.2	+++	0	nd	35.9	-
8	90	nd	165.9	+++	43	nd	18.9	-	91	nd	128.2	+	30	nd	168.0	+++	0	nd	48.3	-
9	92	nd	142.9	+++	40	nd	13.1	-	92	nd	145.5	++	51	nd	147.8	+++	0	nd	34.1	-
10	74	nd	108.3	+++	46	nd	18.2	+++	89	nd	120.7	+	59	nd	104.8	+++	0	nd	50.0	-
11	92	nd	64.8	++	53	nd	15.8	-	nd	nd	nd	nd	72	nd	77.4	+++	82	nd	16.8	-
12	93	nd	92.6	++	39	nd	10.0	-	nd	nd	nd	nd	71	nd	54.8	++	82	nd	94.8	+
13	90	nd	57.4	++	33	nd	17.0	-	nd	nd	nd	nd	0	nd	56.5	++	83	nd	81.2	+
14	90	nd	70.5	++	35	nd	19.6	-	nd	nd	nd	nd	39	nd	64.6	+++	91	nd	73.1	+
15	96	nd	68.5	++	47	nd	23.4	-	nd	nd	nd	nd	76	nd	33.6	++	93	nd	104.5	+
16	92	nd	52.9	++	32	nd	33.4	-	nd	nd	nd	nd	73	nd	44.5	+	nd	nd	nd	nd
17	92	nd	74.7	++	41	nd	14.9	-	nd	nd	nd	nd	68	nd	64.1	+	nd	nd	nd	nd

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.7. Comparisons of the results of the three serological tests in sera collected from cattle vaccinated with T1/44 sub-cutaneously

Cattle No	146				192				179				181				178			
Date in weeks	cELISA %	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT
0	61	88	nd	nd	28	1	nd	nd	39	41	nd	nd	57	78	nd	nd	50	30	nd	nd
1	68	88	nd	nd	42	0	nd	nd	45	37	nd	nd	71	70	nd	nd	68	13	nd	nd
2	57	39	nd	nd	32	0	nd	nd	42	19	nd	nd	60	54	nd	nd	55	14	nd	nd
3	67	30	25.4	+++	34	0	22.0	-	63	11	11.2	-	78	33	13.3	+	20	20	24.1	+++
4	58	47	24.2	+++	43	0	21.8	-	53	14	19.5	-	67	30	11.5	++	60	7	13.0	++
5	57	25	17.0	+++	47	0	35.5	-	59	9	16.1	-	66	57	18.7	++	60	13	18.7	++
6	54	53	20.1	+++	63	39	145.6	+	53	14	10.0	-	62	51	12.7	+++	57	10	6.5	++
7	45	nd	34.7	+++	0	nd	273.1	++	27	nd	23.7	++	53	nd	18.3	++	46	nd	31.6	++
8	46	nd	14.5	++	33	nd	203.2	++	42	nd	17.9	+	68	nd	20.3	+	65	nd	8.5	++
9	32	nd	33.7	+++	30	nd	91.9	+++	32	nd	15.6	+	59	nd	15.2	+	40	nd	12.1	-
10	38	nd	16.8	++	21	nd	159.9	++	34	nd	23.8	-	60	nd	17.4	-	48	nd	18.5	+
11	59	nd	19.4	+	18	nd	122.1	+	32	nd	23.4	-	50	nd	14.5	+	46	nd	14.6	+
14	65	nd	35.4	++	23	nd	57.1	-	27	nd	19.6	+	59	nd	19.5	++	45	nd	25.7	-
17	51	nd	26.9	++	43	nd	45.5	-	60	nd	25.6	-	61	nd	8.6	++	48	nd	15.3	++

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.8. Comparisons of the results of the four serological tests in sera collected from cattle vaccinated with T1/44 intranasal

Cattle No	186				176				147				195				152			
Date in weeks	cELISA %	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT
0	26	2	nd	nd	18	2	nd	nd	39	4	nd	nd	12	3	nd	nd	12	2	nd	nd
1	24	19	nd	nd	28	0	nd	nd	47	3	nd	nd	26	6	nd	nd	18	3	nd	nd
2	29	0	nd	nd	37	2	nd	nd	46	0	nd	nd	30	10	nd	nd	32	1	nd	nd
3	31	0	6.5	-	44	0	18.9	-	54	0	24.4	++	33	-1	11.5	-	36	0	21.8	-
4	31	1	12.0	-	42	0	22.4	-	49	1	18.4	++	40	0	19.3	-	34	1	16.6	-
5	31	1	14.9	-	36	-4	3.4	-	43	0	16.7	+++	38	4	19.8	-	31	0	8.7	-
6	37	0	10.4	-	42	2	19.5	+	55	1	20.1	+	47	15	27.9	-	32	-1	41.2	-
7	33	nd	27.4	-	20	nd	17.2	+	49	nd	17.6	++	59	nd	26.6	+++	23	nd	17.4	-
8	45	nd	16.2	-	23	nd	20.4	+	48	nd	16.0	+	63	nd	225.3	+++	45	nd	16.4	-
9	23	nd	13.3	-	11	nd	16.6	+	40	nd	29.3	+	66	nd	172.2	+++	28	nd	11.5	-
10	47	nd	32.3	-	29	nd	47.7	-	57	nd	19.8	+	74	nd	137.0	+++	19	nd	16.6	-
11	45	nd	14.6	-	22	nd	16.8	-	53	nd	14.7	+	81	nd	99.2	++	26	nd	12.1	-
17	78	nd	60.5	++	50	nd	13.8	++	86	nd	56.9	+++	59	nd	40.2	++	23	nd	6.1	++

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is measured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.9. Comparisons of the results of the four serological tests in sera collected from cattle intubated with biofilm grown cells

Cattle No	11				20				62				67				78			
Date in weeks	cELISA %	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT
4	59	0	14.4	_	50	1	7.7	_	51	0	12.2	_	90	137	237.8	++	56	1	20.5	_
5	58	0	25.5	_	45	1	19.8	_	46	0	22.1	_	92	126	219.8	++	61	0	13.2	_
6	58	-11	18.9	_	52	1	35.3	_	53	0	20.0	_	91	120	174.4	+++	51	0	36.9	_
7	39	nd	24.4	_	33	nd	19.0	+	42	nd	21.1	_	93	nd	129.6	+++	42	nd	28.3	+
8	33	nd	8.3	_	22	nd	11.3	+	18	nd	4.3	_	84	nd	70.1	+++	8	nd	16.0	+
9	34	nd	10.4	_	35	nd	32.3	_	40	nd	22.2	_	88	nd	82.4	+++	37	nd	14.3	+
10	44	nd	19.0	_	33	nd	28.2	_	52	nd	18.2	_	92	nd	79.8	+++	27	nd	19.1	_
11	50	nd	18.0	_	43	nd	12.8		41	nd	14.4	_	90	nd	79.9	+++	43	nd	16.3	_
17	50	nd	9.5	+	37	nd	3.8	+	18	nd	3.1	-	80	nd	25.1	++	54	nd	18.5	++

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is mswasured by the degree of clumping as expressed either + to +++ depending on the degree of clumping.

Table 4.10. Comparisons of the results of the four serological tests in sera collected from in-contact control cattle in the biofilm group

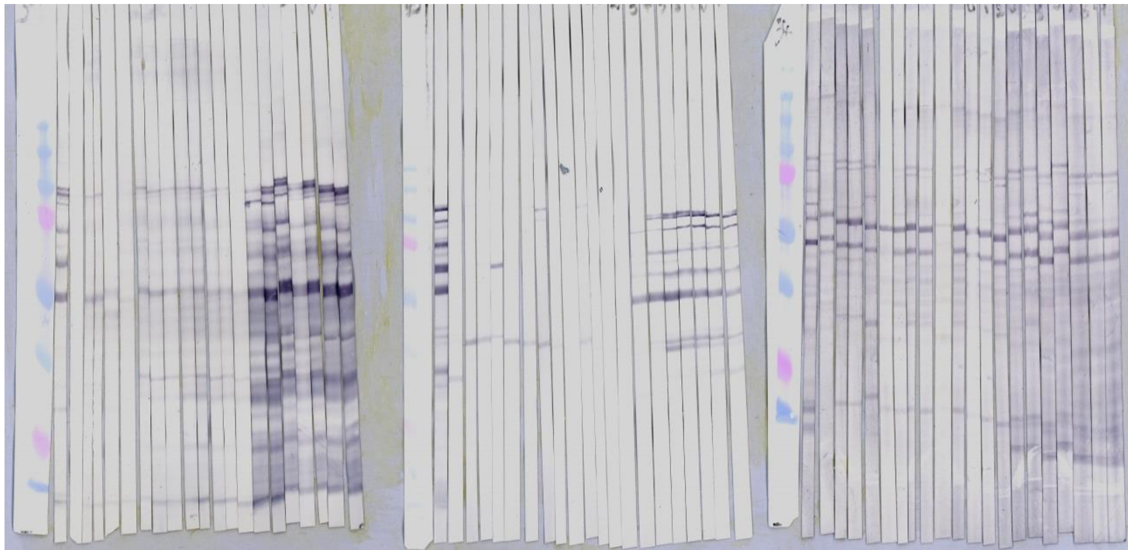
Cattle No	180				183				138				154				159			
Date in weeks	cELISA %	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT	cELISA%	LppQ %	LFD	LAT
4	43	1	nd	nd	46	5	nd	nd	51	0	nd	nd	38	23	nd	nd	33	5	nd	nd
5	41	1	nd	nd	33	2	nd	nd	41	12	nd	nd	35	30	nd	nd	45	6	nd	nd
6		2	nd	nd	29	2	nd	nd	51	1	nd	nd	37	27	nd	nd	37	6	nd	nd
8	38	nd	11.6	-	28	nd	7.2	-	16	nd	4.2	-	12	nd	10.7	+	43	nd	5.8	+
9	24	nd	13.3	nd	28	nd	14.2	nd	33	nd	8.8	nd	5	nd	19.6	nd	17	nd	13.0	nd
10	25	nd	28.7	-	28	nd	15.8	-	50	nd	146.5	-	0	nd	15.0	+	19	nd	22.2	+
11	30	nd	nd	-	17	nd	nd	nd	73	nd	nd	+++	5	nd	nd	-	14	nd	nd	-
17	41	nd	nd	-	39	nd	nd	-	nd	nd	nd	nd	25	nd	nd	++	80	nd	nd	++

cELISA is measured in percentage inhibition (%) at a cut-off point of 50%. The LFD is measured in percentage and the aim here is to determine the cut-off point while the LAT is mswasured by the degree of clumping as expressed either + to +++ depending

APPENDIX 3: Immunoblot and Immunoblot Profile

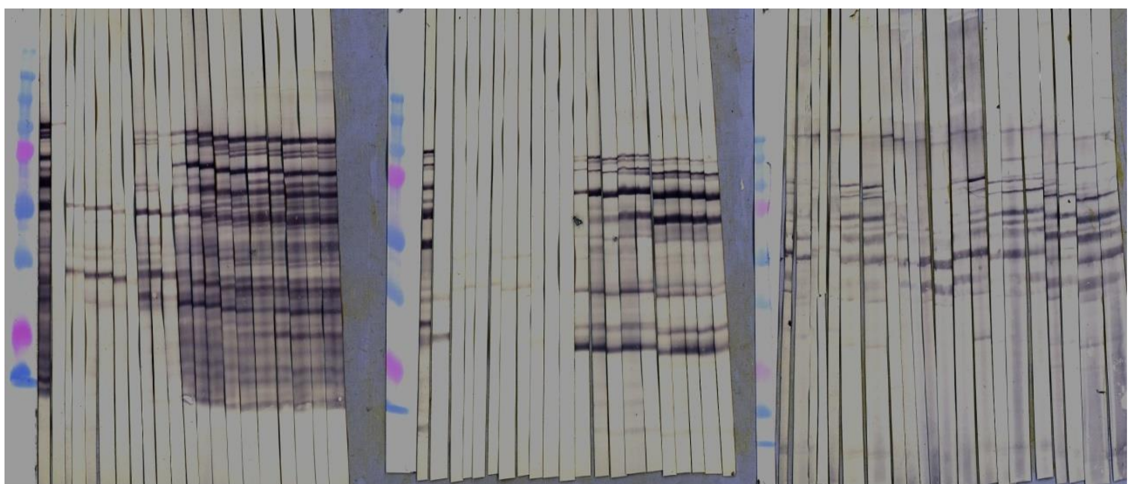
Animal # 175 (Vaccine in-contact)

IgG1 IgG2 IgM



Animal # 195 (T1/44 intranasal)

IgG1 IgG2 IgM



Immunoblot profile

IgG1 Immunoblot (kDa) profile from animals in vaccine group

Control					T1/44 i/s			T1/44 s/c			Tween 20			Purified Protein		
188	**175**	**177**	**145**	**165**	**186**	**176**	**147**	**146**	**192**	**178**	**194**	**170**	**182**	**155**	**150**	**161**
126	81	110	87	95	189	120	87	102	49	110	126	91	120	117	126	120
100	58	91	60	85		98	74	93		93	115	89	78	115		
74	48	74	51	69		56	60	71		55	79	69	65			
60		60		68		46	54					60	54			
50		35		56		37	32					55				
36		20		49			30					50				
				30			21									

IgG2 and IgM Immunoblot (kDa) profile from animals in vaccine group

Control					T1/44 i/s		T1/44 s/c	Tween 20			Purified Protein		Tween 20 IgM	Purified Protein IgM		
188	**175**	**177**	**145**	**165**	**186**	**147**	**178**	**194**	**170**	**182**	**161**	**162**	**182**	**189**	**161**	**162**
57	93	110	129	100	174	112	155	115	55	115	120	123	123	100	91	120
	91	91	72	65	132	95	126	98		98	97	85	110	79	58	87
	72	74	66	58	100	74	91			76	95		93	59	50	
	59	60	60	48	76	60	48			62			89	50	45	
	54	55			39	55				50			79	47	39	
	39	35				36								37		