SAFETY AND EFFICACY OF A CONTAGIOUS BOVINE PLEUROPNEUMONIA

INACTIVATED VACCINE FORMULATED WITH DIFFERENT ADJUVANTS

HARRISON OSUNDWA LUTTA

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DECLARATION

This thesis is my original work and has not been submitted for an award in any other institution.

Harrison Osundwa Lutta

Signature…………………………………………..Date…………………………………………….

This thesis has been submitted to the graduate school for examination with our approval as supervisors.

Dr. David Odongo

School of Biological Sciences, University of Nairobi

Signature………………………………………….Date…………………………………………

Dr. Jan Naessens

International Livestock Research Institute, Kenya

Signature………………………………………….Date…………………………………………..

Dr. Hezron O. Wesonga

Kenya Agricultural Research Institute-VRC, Muguga North

Signature………………………………………….Date…………………………………………..

DEDICATION

To my wife: Stella Lutta, children: Stallon, Alvin and Ashley for their love, support and endurance during my course work and preparation of this thesis.

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LIST OF ABBREVIATIONS

Ag	Antigen
ANOVA	Analysis of Variance
AUIBAR	African Union-InterAfrican Bureau for Animal Resources
CBPP	Contagious Bovine Pleuropneumonia
Cc	Conjugate Control
c-ELISA	Competitive Enzyme Linked Immuno-sorbent Assay
CFT	Complement Fixation Test
Cm	Monoclonal Control
FAO	Food and Agricultural Organization
IgG	Immunoglobin G
ISCOMS	Immuno-stimulating Complexes
KARI	Kenya Agricultural Research Institute
ml	Millilitre
*Mmm*SC	*Mycoplasma mycoides* subspecies *mycoides* Small Colony
NCST	National Council for Science and Technology
OD	Optical Density
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
p-ELISA	Protein Enzyme Linked Immuno-sorbent Assay
PI	Percentage Inhibition
rpm	Revolution per Minute
SAT	Slide Agglutination Test
VCM	Veronal Buffer with Calcium and Magnesium

ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is a respiratory disease of cattle caused by *Mycoplasma mycoides* subspecies *mycoides* Small Colony (*Mmm*SC) and is mainly controlled by quarantine and vaccination using live *Mmm*SC T1/44 and T1-SR strains. These vaccines while requiring cold chain for delivery, only provide protection for short durations, are unstable after reconstitution and are associated with post-vaccinal site reactions. This study assessed the safety, and protection threshold of an inactivated T1/44 *Mmm*SC vaccine formulated in different adjuvants. Cattle were randomly assigned into 7 groups of 10 animals and the experimental group vaccinated with different inactivated formulations. Animals in the negative control group were vaccinated with either adjuvant alone or Phosphate Buffered Saline, while the positive control group received the current live attenuated T1/44 vaccine. Twenty additional cattle were intubated and served as pathogen donors during the challenge experiment. After vaccination, no animal recorded fever, while the number of animals with swellings at the vaccination site were significantly higher (P<0.001) in the groups vaccinated with the inactivated vaccine formulations compared to the positive control group (T1/44 vaccine). Sero-conversion rates following vaccination were higher in groups that received the inactivated vaccine (KE2 and KE6). After challenge, a significant proportion of animals which received the inactivated vaccine formulations, developed fever and gross pathological lesions characteristic of CBPP, this compared to the live attenuated vaccine. Overall, the protection rate for animals which received the live attenuated T1/44 vaccine was higher. The results demonstrated that inactivated vaccine formulations although safe, did not provide protection levels similar to the current live attenuated T1/44 vaccine. There is need to explore alternative vaccine formulations and booster immunization regimes.

CHAPTER ONE:

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

Contagious Bovine Pleuropneumonia (CBPP) is a respiratory disease affecting cattle and is caused by *Mycoplasma mycoides* subspecies *mycoides* Small Colony (*Mmm*SC). CBPP is the only bacterial disease included in the Office International des Epizooties (OIE) list of prioritized communicable animal diseases and has a potential of spreading across national borders, with serious socioeconomic consequences (Rweyemamu and Benkirane, 1996).

The disease causes a significant constraint throughout most of sub-Saharan Africa, due to mortalities and productivity losses, and is a threat to food security and access to international markets in a continent where approximately 30 to 50% of livestock keepers in sub-Saharan Africa live below the poverty line (Thiaucourt *et al*., 2000; Thornton *et al.,* 2002; Tambi *et al.,* 2006).

CBPP was known to occur in Europe since the 16th century and its worldwide distribution occurred during the 19th century due to increased international trade in live cattle. The disease was eradicated in most of the European countries before the beginning of the 20th centuary (Provost *et al*., 1987).

It is believed that CBPP was introduced into southern Africa in the 19th century through importation of infected cattle from Europe. Likewise, the disease was introduced into East, Central and West Africa in 19th century by cattle from India (Provost *et al*., 1987). The history of CBPP in Kenya between 1900 to 1970 has been previously reported by Kariuki, (1971). African countries that have managed to eradicate CBPP include Zimbabwe and South Africa, where the disease was last reported in 1904 and 1924 respectively, while Botswana was officially declared free of CBPP in 2008 (http://www.oie.int/eng/info/en ppcb.htm).

*Mmm*SC is a bacterial pathogen belonging to the taxonomic class mollicutes, a class whose organisms are characterised by lack of a cell wall (Razin *et al*., 1998). This property makes it difficult to recommend treatment in mollicutes as compared to other bacteria that have a cell wall since most antimicrobials and antibiotics effect their action on bacteria by inhibiting cell wall synthesis. Table 1 shows some common mycoplasmas and their associated diseases.

Table 1: Common Mycoplasmas and their associated diseases

Host	Agent	Disease symptoms
Cattle	*M. mycoides* subsp. *mycoides* Small Colony	Causes CBPP
Cattle	*M. leachi sp. Nov.*	Related to cattle suffering from arthritis and mastitis disorders
Cattle	*M. dispar*	Causes calf pneumonia
Goats, sheep	*M. mycoides* subsp. *capri*	Causes mastitis, arthritis, keratitis and pneumonia
Goats, sheep	*M. capricolum* subsp. *capricolum*	Causes mastitis, arthritis, keratitis and pneumonia
Goats	*M. capricolum* subsp. *capripneumoniae*	Causes Contagious Caprine Pleuropneumonia (CCPP)
Human	*M. pneumoniae*	Primary atypical pneumonia
Human	*M. penetrans*	Weaken immune system (related to HIV disorders)
Human	*M. genitalium*	Involved in pelvic inflammatory disease

Source: Manso-Silvan *et al.,* (2009); https://en.wikipedia.org/wiki/mycoplasma

Mollicutes include species that cause disease in human, fish, goats, plants and insects (Minion, 2002). These parasites are tiny prokaryotes which hardly ever invade tissues, with notable exceptions being *Mycoplasma penetrans*, *M. pneumoniae* and *M. genitalium* which are intracellullar (Razin and Jacobs, 1992; Baseman, 1995; Pilo *et al.,* 2005). Mycoplasmas have a characteristic prokaryotic genome consisting of a plasma membrane, ribosomes and an extremely coiled circular double stranded DNA molecule (Razin, 1999). The genome size ranges from 580 kb for *M. genitalium* to 1380 kb in *Mycoplasma mycoides* subspecies *mycoides* Large Colony (Caporale *et al.,* 1996).

Mycoplasmas survive by adhering on the surface of the epithelial lining of respiratory tracts as a requirement for colonization and maintenance of infection, and adherence of *MmmSC* to bovine bronchial epithelial cells has been demonstrated using Flow Cytometry (Razin and Jacob, 1992; Razin *et al.,* 1998; Gunaserekera *et al.,* 2000). Attached mycoplasmas adsorb nutrients on the host while utilizing the hosts' fatty acids and cholesterol (Razin, 1999). Mycoplasmas are specific to the host and tissues they inhabit (Razin, 1992). It has been shown that *MmmSC* is more cytotoxic to bovine than caprine endothelial cells, signifying that the cytotoxicity reflects specificity for bovine species (Valdiviero-Garcia *et al.,* 1989).

The pathogenicity and virulence of *MmmSC* is poorly understood (Pilo *et al.,* 2007). A better understanding of the mechanism of pathogenesis of *MmmSC,* especially the early interactions between the organism and the host, may allow designing of strategies to prevent colonization and infection of respiratory airways of epithelial lining of the host by the parasite (Kelly *et al.,* 1999; Ofek *et al.,* 2003). It has been suggested that this should also be a consideration in designing of a safe and efficient vaccine against *MmmSC* (Pilo *et al.,* 2007).

Mycoplasma membranes contain galactan, a carbohydrate which is found in form of polysaccharides, lipopolysaccharides, glycolipids and glycoproteins (Buttery *et al.,* 1976). These carbohydrate components play a significant role in the interaction of the organism with the cell membrane of its host, and also play a role in its virulence (Razin, 1978). It has been proposed that in *Mycoplasma dispar*, the carbohydrate capsule plays a role in the pathogenesis of bovine pneumonia in calves by contributing to attachment process on the host (Gourley and Howard, 1978; Almeida and Rosenbusch, 1991). Several molecular mechanisms may contribute to the pathogenicity and virulence of *MmmSC* allowing mycoplasma to specifically adhere to the host tissue, exert cytotoxic effect, evade the host's immune defence, cause inflammation and disease symptoms in the host (Vilei *et al.,* 2007; Pilo *et al,* 2007). The attachment of mycoplasma to the endothelial host cells causes damage either by interfering with receptors of the host membrane or altering host cell's mechanisms (Debey and Ross, 1994).

1.2 Symptoms of CBPP

Contagious Bovine Pleuropneumonia manifests as clinical forms that include peracute, acute and chronic forms (Mariner *et al.*, 2006). The peracute form is characterised by sudden death while the acute form manifests as cough, persistent fever accompanied by laboured breathing. The chronic form manifests as loss of weight, and cough on exertion.

1.3 Diagnosis of CBPP infection

Diagnosis generally employs a combination of all or any of the following: clinical signs such as outbreaks of pneumonia, serological tests and post-mortem findings of affected lungs showing a grossly fibrinous broncho-pneumonia accompanied with pleuritis. The degree of severity varies proportionally according to different conditions (Wesonga and Thiaucourt, 2000). Other diagnostic tests available include immunoblot and molecular based diagnostic assays which involve use of Polymerase Chain Reaction (Pettersson *et al.*, 1996).

1.4 Transmission and economic impact of CBPP

Direct contact between infected and susceptible animals is the principal mode of transmission. The disease is characterized by its variable course and insidious nature (Mariner *et al.*, 2006). Prevalence of CBPP is highest among cattle in the pastoral systems where losses due to the social impact of the disease may by far outweigh economic losses. Economic losses in sub-Saharan Africa, although very difficult to calculate, have been estimated at US $2 billion per annum (Masiga *et al.*, 1996). This is a huge financial loss for a disease affecting livelihood of 24 million people of low income (Thomson, 2005). The losses are mainly due to mortalities, loss of production, and disruption of local as well as international trade and cost of control or eradication campaigns (Amanfu *et al.*, 1998; Windsor and Wood, 1998). Direct losses in Kenya are estimated at €4.78 million per annum (GOK, 2004; Tambi *et al.,* 2006).

1.5 Geographical distribution of CBPP

CBPP is widespread in Africa, occurring in 24 countries of sub-Saharan Africa (Figure 1). In western Africa, it is mainly enzootic or sporadic. In eastern and south-eastern Africa, CBPP is of major concern, placing South Africa which is free from the disease and exports meat to international markets under direct threat (Masiga *et al.,* 1996). CBPP is thought to have been present in East and West Africa prior to the colonial period (Windsor, 2000). In Africa, high incidences of the disease have been reported in Zambia, Tanzania, and Botswana (Iowa State University, 2004). The disease has however been eradicated from the western hemisphere, the UK and Australia and more recently in Spain, Portugal and Italy (Provost *et al.*, 1987; Regalla *et al.*, 1996).
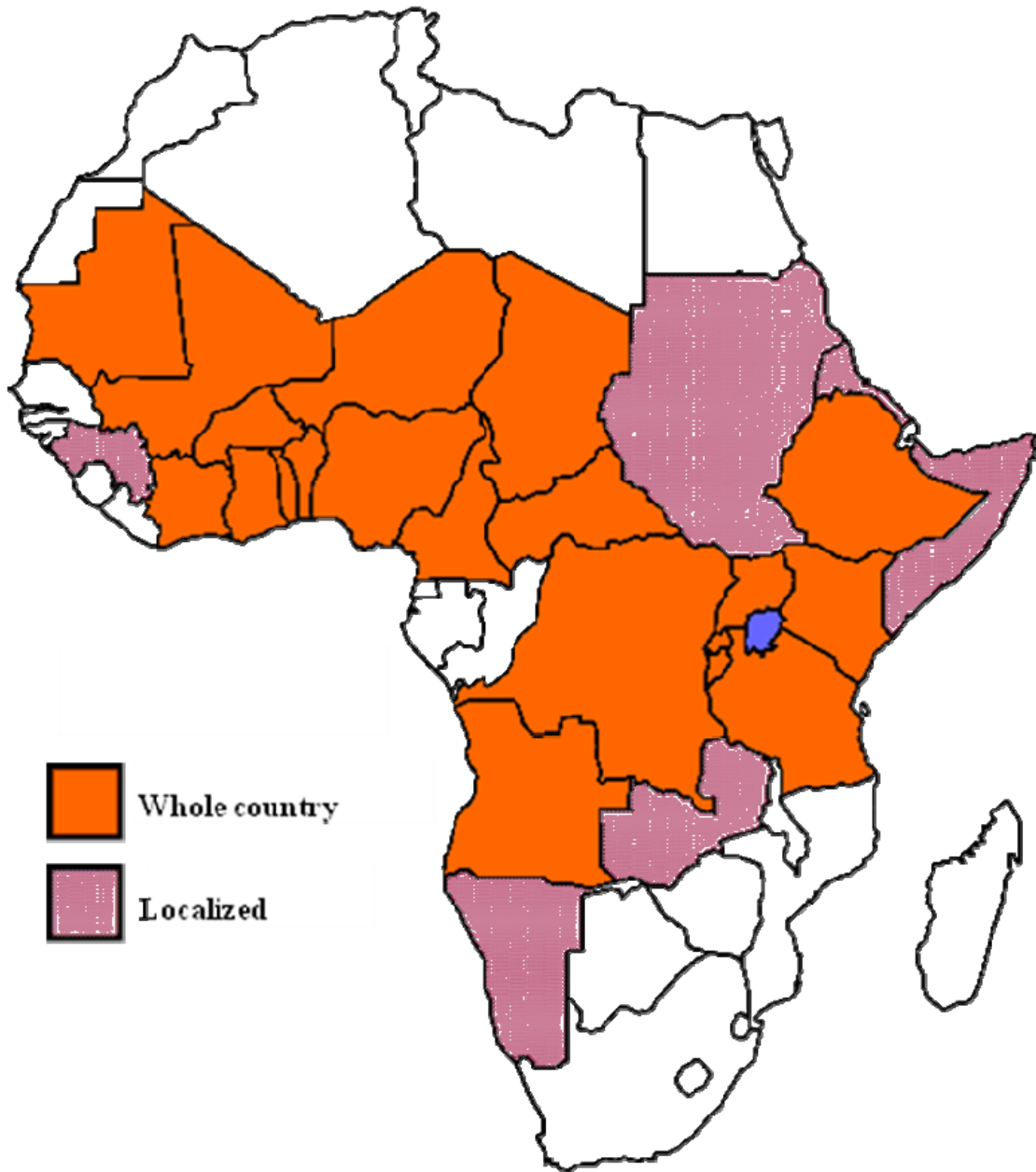
Figure 1: Distribution of CBPP in sub-Saharan Africa.
   (Source: Jan Naessens, from country reports, 2005 to 2010)

1.5.1 Distribution and prevalence of CBPP in Kenya

In Kenya, CBPP was first reported in 1901, following the pattern of disease outbreaks and the situation of the disease in the neighbouring countries. This led to zonation of the country with respect to CBPP (Kariuki, 1971). In 2002, three zones were recognized as follows: Zone I comprising of clean areas which included most of the country mainly the central and western highlands and part of the coast (Wanyoike, 1999). Zone II comprising areas under threat but with no disease and consisted of West Pokot, Baringo, Samburu, Kitui, Kilifi and Maasailand. Illegal movement of cattle from West Pokot to Busia, Bungoma and Siaya districts in the West led to outbreaks in these districts which thus led to the neighbouring districts of Trans-Nzoia and Kakamega being included into Zone II districts (Ministry of Livestock Development and Fisheries, 1978). Zone III included infected or severely threatened districts such as the entire north and eastern districts of Turkana, Marsabit, Mandera, Wajir, Isiolo, Garissa and Tana River.

A new zonation of the country into four (4) zones was done in 2003 (Figure 2) and included clean, surveillance, buffer and infected zones. Clean zones[1] also referred to as CBPP free zones, comprise the western highlands, central and coastal region while the surveillance zone comprise of mainly Machakos, Mwingi, Kitui and Makueni districts and Samburu district and the areas directly neighbouring the clean zone. The buffer zone comprises the northern part of Eastern province (Marsabit and Isiolo districts), northern part of Narok and the districts neighbouring the surveillance districts. The infected zone include Southern part of Narok, Turkana, West Pokot, Moyale, Mandera, Wajir, Garissa, Ijara and Lamu, Tana-river, Kajiado and Transmara districts (Wanyoike *et al.,* 2004).

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[1] CBPP free zone is a zone in which there is no evidence (clinical, pathological, laboratory) of disease occurring in cattle due to the disease causing agent (*Mmm*SC*)*; or where the particular agent has been eliminated (OIE, 2008). Besides revised zonation, a revised control policy was suggested in 2003 (Wanyoike *et al.*, 2004; GOK, 2003). However, declining state funds and adjustments in trade policy as well as increased need for transhumance has led to the break down of the strategy putting the whole country at risk of CBPP infection.
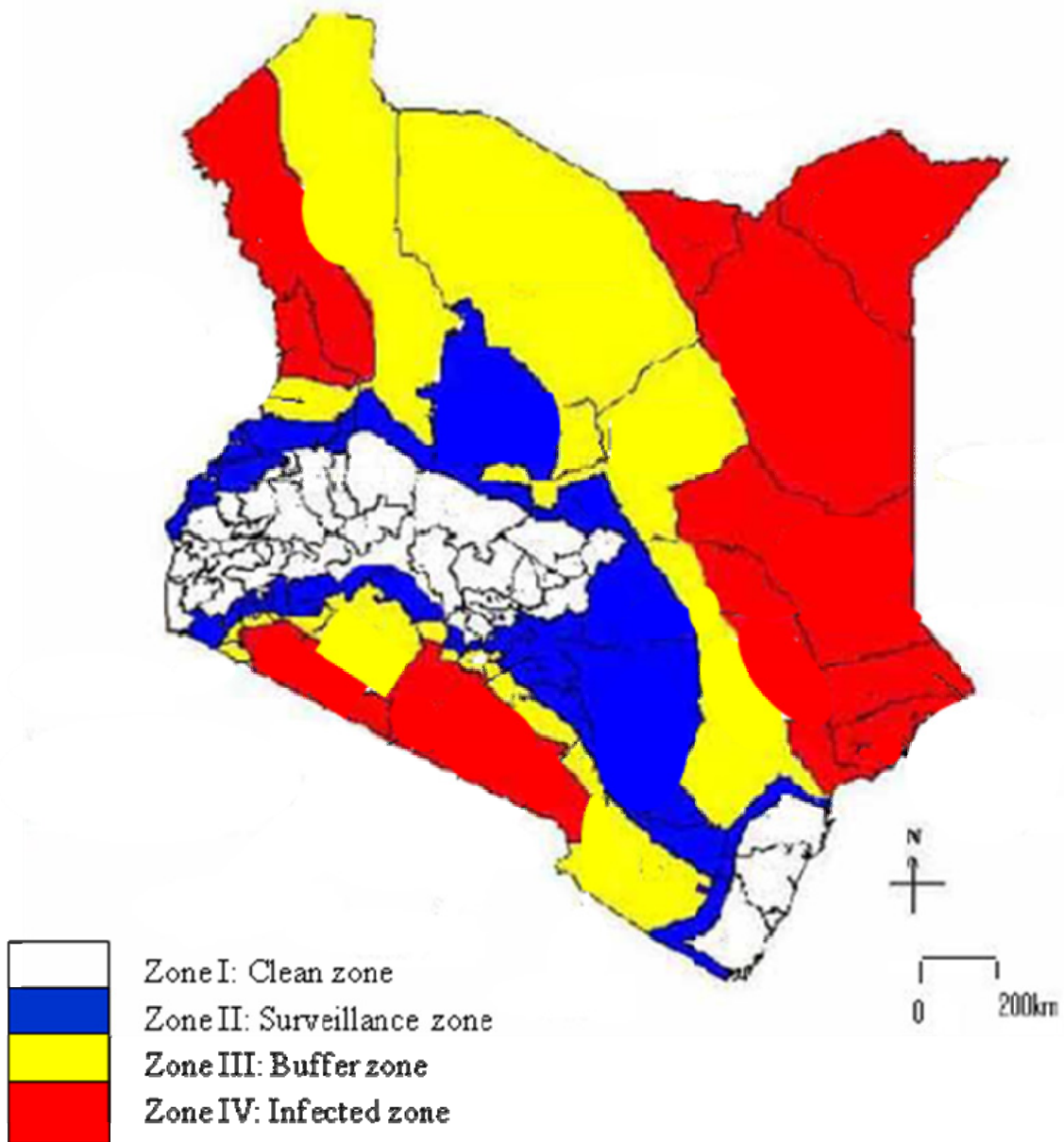
Figure 2: Distribution of CBPP in Kenya.

(Source: Adapted from Wanyoike, S.W. Consultative group meeting on CBPP report, 2003 in Rome, Italy)

The prevalence of CBPP in endemic and epidemic areas has been reported to be approximately 3% and 13% respectively and incidences of up to 47% can happen in erratic outbreaks; mostly in arid and semi-arid areas (Wanyoike, 1999; Gitau, 2001; Mugo, 2004).

1.6 Epidemiology of CBPP infection

The epidemiology of CBPP in Africa is dominated by four factors, namely: i) cattle which are the only domestic species affected, ii) Buffalos which are the wild animal reservoirs, iii) clinical cases or chronic carriers being the usual sources of infection, through direct contact, and iv) cattle movements which play an important role in the maintenance and spread of the disease (Turner, 1954; Masiga, 1996). Features of CBPP epidemiology include transient and inapparent infection and existence of persistently infected animals with encapsulated, infected sequestra (Mariner *et al.*, 2006). Following field-testing with the slaughter of acutely infected animals and mass vaccination in Maasailand, this area remained free of CBPP from 1968 to 1986. However with the purchase of ex-Garissa and ex-Isiolo cattle probably from Somalia in 1986/87, CBPP was re-introduced into Maasailand (Wanyoike, S.W. Consultative Group Meeting on CBPP Report, 2003). Epidemiological studies in Kenya in 1999 established that CBPP had partly been re-established in Maasailand and was now threatening the areas of Ukambani (Makueni, Machakos, Mwingi and Kitui), Nakuru and Thika districts. This is due to trans-human cattle movement by the Maasai as a result of the decrease in grazing area during dry season (Wanyoike, 1999).

1.7 Control of CBPP

CBPP is controlled through vaccination and quarantine. The control policy in Kenya has constantly been along the lines of mass vaccination in enzootic areas, increasing emphasis on field testing with slaughter of reactors within the enzootic areas, rigid control over movement out of the enzootic and suspect areas and rapid stamping out, by testing and possibly vaccination against the disease (Kane, 1975; GOK, 2003; Wanyoike *et al.,* 2004).

Constraints to the application of control procedures include: low efficacy of the current live vaccines, especially in Africa where the disease is controlled mainly through vaccination, supplemented only by quarantine and restriction of animal movement.

Policies formulated to control the disease through slaughter of cattle and restrictions of movement are difficult to implement in Africa for a variety of reasons including the cost of compensation and logistics, social, cultural and trade practices in pastoral communities (Egwu *et al.,* 1996; Litamoi and Seck, 1998; Radostits *et al.,* 2000). It would be difficult to compensate for stamped out cattle as most African countries cannot afford the cost, this besides the distress caused to farmers (Mullins *et al.,* 2000; McLeod and Rushton, 2007). Control intervention of CBPP through treatment of affected cattle has been discouraged, because treatment alleviates the clinical signs but does not prevent the spread of infection and might favour development of chronic carrier animals (Provost *et al.,* 1987; Mariner and Catley, 2004).

1.7.1 Live CBPP vaccines old formulations

Louis Willems, a Belgian scientist developed and tested the earliest vaccine against CBPP in early 1840s. This vaccine, consisting of pleural fluids from lungs of naturally infected cattle, was inoculated through the tail of naïve cattle and conferred a measure of immunity. However, this approach resulted in severe post-vaccination reactions referred to as Willem's reactions that caused vaccinated cattle to lose their tails (Fisher, 2003). Later vaccines that were developed in 1950s were egg-propagated, avianised and caprinised, but these had a short life span while still eliciting adverse post-vaccination reactions (Sheriff and Piercy, 1952; Hyslop, 1956). These vaccines were eventually replaced with live broth cultures of *Mmm*SC strains called KH$_3$J, V5 and T1 (Radostits *et al.,* 2000), which were later abandoned in favour of freeze-dried vaccines because they conferred poor immunity, had short shelf life and were heat sensitive (FAO EMPRES, 1995; Litamoi *et al.,* 2007).

Currently, two *Mmm*SC strains are used for preparing CBPP vaccines; strain T1/44 and a streptomycin resistant derivative, T1-SR (Provost, 1982). T1 vaccine was attenuated with 44 passages in embryonated eggs to produce T1/44 vaccine (Davies *et al.,* 1968). The use of *Mmm*SC strain T1/44 is often followed with reports of severe post-vaccinal reactions similar to those shown in Figure 3A while reports of these severe post-vaccinal reactions following the use of strain T1-SR are mild and uncommon (Figure 3B). These reactions manifest as oedema and often require antibiotic treatment with a proportion of animals dying if not treated (Thiaucourt *et al.*, 2000; Thiaucourt *et al.*, 2003).

A **B**
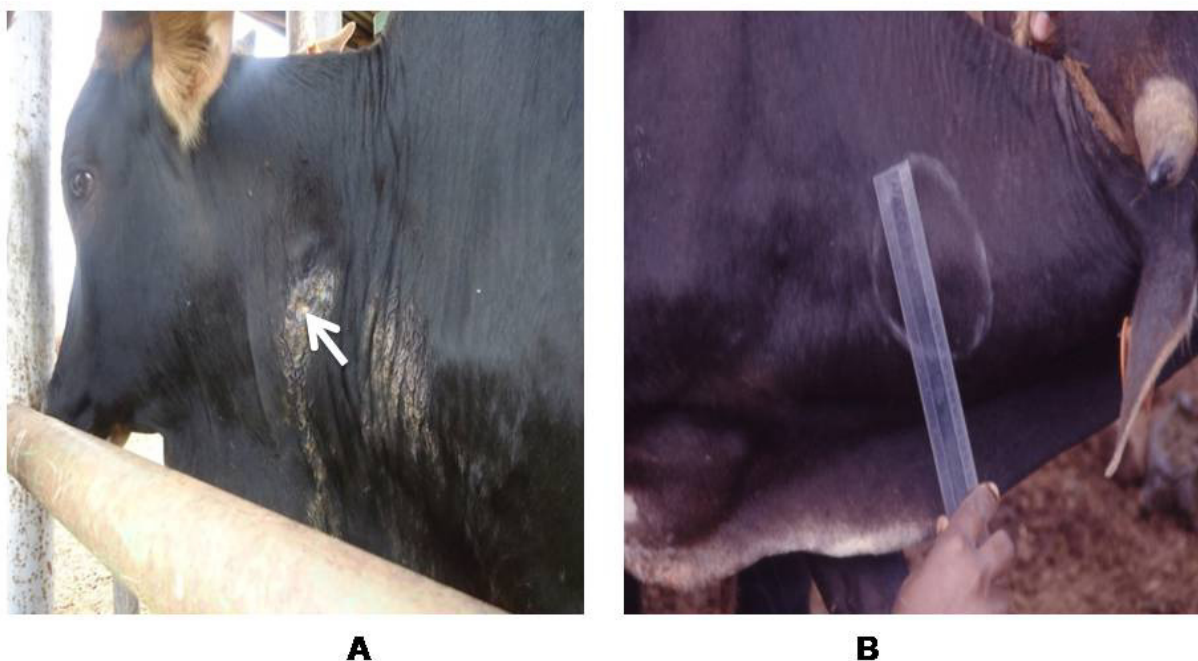
Figure 3: Post-vaccination reactions following vaccination with *Mmm*SC vaccine strains

> Panel A: Shows severe case of post-vaccination Willems reaction (indicated by white arrow) on the neck of an animal inoculated using *Mmm*SC vaccine strain T1/44 while

> Panel B: Mild Willems reaction following vaccination with *Mmm*SC strain T1-SR (white round mark)

In addition, these vaccines have been shown to retain some virulence (Thiaucourt *et al*., 2004). Previous experiments have demonstrated that a strain obtained from a post-vaccinal reaction site in an animal immunized with T1/44 vaccine elicited comparable pathological lesions to those of the pathogenic strain, suggesting that T1/44 strain may revert to virulence if it does not undergo sufficient attenuation (Wesonga *et al*., 2004). Experimental and field studies have demonstrated that the current CBPP vaccines do not effectively protect cattle from disease outbreaks, especially with first vaccination (Masiga *et al.,* 1998; Thiaucourt *et al.,* 2000).

However, strain T1/44 confers comparatively longer lasting immunity and requires less frequent repeat inoculations compared to T1-SR strain (Wesonga and Thiaucourt, 2000). Therefore, a better vaccine is needed for the control of contagious bovine pleuropneumonia in Africa. While vaccine coverage and protection rates of 80 to 90% are the target for vaccination campaigns to maintain a disease free status, the currently used CBPP vaccines only give protection rates between 30 to 60% after primary vaccination (Wesonga and Thiaucourt, 2000). This suggests that even with high levels of coverage, effective control of the disease will not be achieved with the current *Mmm*SC vaccine (Mariner *et al.,* 2006).

Limited knowledge on the nature of the disease itself has hampered the development of effective vaccines against CBPP. The study of CBPP immunity and immunopathology is complicated by the fact that experimental reproduction of the disease is difficult (Lloyd and Ethelridge, 1983) and studies rely mainly on endobronchial challenge (Thiaucourt *et al*., 2000). In addition, *Mmm*SC is pH sensitive (March *et al.*, 2002) and a loss of viability is thought to occur between reconstitution and administration of vaccine, if the vaccine is not used within 2 hours after reconstitution (Nkando, 2011; Provost *et al.,* 1987). The optimum pH is 7.4 with a decrease in pH to less than 6.5 stopping the growth of mycoplasma and causing rapid death of cells (Rodwell and Mitchell, 1979).

1.7.2 The use of inactivated CBPP vaccines

Inactivated CBPP vaccines were tested in Africa and shown to be relatively efficacious, but only when two large doses of MmmSC formulated in Freunds complete adjuvant were used (Gray *et al.*, 1986). The results have generally been uncertain, while detailed efficacy studies have not been conducted (Garba *et al.*, 1989). Recent developments in vaccinology and adjuvant formulations that are aimed at antigen presentation to host specific effector cells and modulation of host immune responses, have now opened new ways to explore new vaccine delivery platforms (Morein *et al.*, 1996).

1.7.3 CBPP vaccines in immuno-stimulating complexes (ISCOMS)

An MmmSC immuno-stimulating complex (MmmSC ISCOM) experimental vaccine was developed by solubilizing live MmmSC in Decanoyl-N-methylglucamide (MEGA-10) detergent. The solubilized proteins were added to cholesterol, phosphatidylcholine and Quil A in the ratio of 10:2:1:20 and dialyzed against PBS overnight at room temperature and for further 48 hours at 4°C to form ISCOM. The CBPP-ISCOM formulation was purified by sedimentation through a double layer of 10% and 20% sucrose and re-suspended in PBS then kept at -70°C until use (Abusugra *et al.*, 1997; Abusugra and Morein, 1999). When this vaccine was experimentally tested in mice and cattle, it induced strong humoral and CD8+ cytotoxic T cell responses in these animals (Abusugra *et al.*, 1997; Huebschle *et al.*, 2003).

The immunogenicity associated with ISCOMS is partly related to efficient presentation of antigens by antigen-presenting cells resulting in the production of pro-inflammatory cytokines such as IL-1, IL-6 and IL-12, and subsequently resulting in strong primary and secondary B and T cell responses (Morein *et al.*, 1995). The ISCOM exhibits its adjuvant activity by modulating T-helper cells towards a Th_1 response, resulting in IL-2 and IFN-γ production, and in some cases enhances the Th_2 cytokine, IL-4.

Therefore, both Th_1 and Th_2 responses can be induced by ISCOMS (Villacres-Eriksson, 1995). ISCOMS have been shown to be efficient antigen delivery systems for both parenteral (subcutaneous) and mucosal modes of immunization with ensuing immune responses after intranasal immunization being substantial both in serum and in the local lung secretions (Abusugra and Morein, 1999). Animals vaccinated against CBPP using ISCOM formulations show strong inflammatory reactions following challenge infection, and it is likely that a Th_1 response is involved in the pathogenesis of CBPP (Huebschle *et al*., 2003).

1.8 Justification

Contagious bovine pleuropneumonia causes high morbidity and mortality in cattle in sub-Saharan Africa and limits international trade. The control of CBPP is possible when massive vaccination with quality vaccines is attained. Current vaccines are based on 2 live strains of *Mmm*SC namely, T1/44 and T1-SR with both strains inducing post-vaccinal reactions at the inoculation site. In addition to the short duration of protection (6 months for TI-SR and one year for T1/44), these vaccines are unstable and expensive to deliver. Limited studies have been conducted on use of inactivated vaccines and where such studies have been undertaken, the details on delivery systems are lacking. Improved CBPP vaccine formulations are therefore required. The aim of this study was to provide information that would guide the selection of vaccine delivery platforms employing inactivated *Mmm*SC for vaccine development. The new vaccines if found safe and effective, would address the constraints of the current live CBPP vaccines that include post-vaccinal reactions at the inoculation site and low efficacy following primary vaccination.

1.9 General objective

The main objective of this study was to compare the safety and efficacy of a contagious bovine pleuropneumonia inactivated vaccine formulated with different adjuvants.

1.9.1 Specific objectives

The specific objectives to be addressed were:

1. To asses the safety of inactivated vaccines using different adjuvant formulations.

2. To determine the protective threshold of inactivated vaccines with different adjuvant formulations.

3. To compare safety and threshold of protection of currently used live vaccine against the inactivated vaccine formulated with different adjuvants.

4. To monitor and compare antibody responses in cattle following vaccination and challenge.

CHAPTER TWO:

MATERIALS AND METHODS

2.1 Cattle

Ninety male zebu cattle aged 2 to 3 years were purchased from Kakamega, a contagious bovine pleuropneumonia (CBPP) free zone. The animals were tested for CBPP using slide agglutination serum test (SAST) before purchase, and then transported to the Kenya Agricultural Research Institute-Kakamega station where they were ear-tagged, drenched and castrated.

Slide agglutination serum test (SAST) is a pen-side diagnostic test suitable for low resource laboratory settings. However, since this test is not specific and may give false positives and/or negatives, the serum samples that were negative by SAST were further confirmed by re-testing using Complement fixation test (CFT). Confirmed sero-negative animals were then transported to KARI-Veterinary Research Centre (VRC)-Muguga North, where they were confined for one month before commencement of the experiment. During this period they were vaccinated against Foot and Mouth Disease, black quarter and anthrax, and allowed to graze on secured paddocks.

2.2 Experimental design

The different proprietary vaccine formulations as indicated in Table 1 were prepared in CIRAD, Montpellier, France and send to KARI-VRC, Muguga North before the commencement of the experiment. Due to product ownership and protection rights, the compositions of these adjuvants could not be disclosed. Seventy cattle were randomly divided into groups of 10 and every animal within each group was inoculated subcutaneously on the left side of the neck with 0.5 ml of either of the vaccine formulations using a 5 ml syringe and a 19 gauge needle. Thereafter, the animals were examined daily for reaction to inoculation at the vaccination site and their rectal temperature recorded. The remaining twenty (out of the original 90 that were purchased) were experimentally infected and served as donors for infection during the challenge experiment.

Table 2: Vaccine formulations used to immunise experimental cattle in different groups

Group	N	Treatment
KE1	10	Phosphate Buffered Saline (PBS)
KE2	10	Inactivated antigen + Saponin
KE3	10	T1/44 commercial live attenuated vaccine
KE4	10	Inactivated antigen+Adjuvant1 (ISCOM1)
KE6	10	Inactivated antigen+Adjuvant2 (ISCOM2)
KE8	10	Adjuvant1
KE9	10	Adjuvant2

2.3 Collection of blood for serum preparation

Blood samples were collected from all experimental animals at weekly intervals up to 10 weeks following vaccination and thereafter for a further 14 weeks during the challenge period. The blood samples were obtained from the jugular vein and collected into labeled vacutainer tubes BD Vacutainer® tubes (Becton, Dickson & Company, U.S.A), then allowed to coagulate at room temperature for 2 to 4 hours. The coagulated blood was centrifuged to separate the serum which was then aliquoted into Nunc® CryoTubes® (Sigma-Aldrich®, Germany) and stored at -20°C until further analysis. Samples collected on the day of vaccination represented day zero of trial.

2.4 Preparation of inoculum for challenge

A culture of pathogenic strain of *Mmm*SC (Strain B237) was used as inoculum for infection and prepared using modified Newings tryptose broth (Appendix 1a). Five millilitres of broth was added to a 5 ml aliquot of a freeze dried culture, shaken and allowed to stand at room temperature for 30 min before dispensing 0.3 ml into Bijou bottles containing 2.7 ml of broth to make a 1:10 dilution.

The mixture was incubated at 37°C for 48 hrs to make sufficient quantities for animals at 60 ml/animal. Growth was monitored daily and confirmed by a change of pH, presence of filaments in the broth and through growth inhibition test on agar plates evidenced by fried egg-like colonies on the agar surface (Turner, 1960).

2.5 Experimental infection of cattle (Intubation)

Prior to inoculation, a sample of the culture was collected and used to estimate the number of mycoplasmas in the inoculums. Sixty millilitres of inoculum at a concentration of 10^8 cfu /ml was introduced through the nostril into the larynx and down to the trachea a procedure referred to as endobronchial inoculation or intubation. This was followed by 30 ml of 1.5% agar (Appendix 1d) suspended in distilled water and 30 ml of phosphate buffered saline (PBS) to flush down all the inoculum to the target site.

2.6 Contact transmission

Intubated animals were observed for clinical signs, including fever before being introduced to the vaccinated herd to allow for contact transmission. An animal was considered to have fever when it showed a rectal temperature of 39.5^{o}C and above, and was considered to show clinical signs of CBPP if fever persisted beyond 3 days. Both intubates and vaccinates were kept together for a period not less than 3 months.

2.7 Clinical examination

Rectal temperatures of each animal were recorded daily during the period of study for 2½ months after vaccination and for 3½ months following challenge. Post-challenge clinical examination included observation for nasal discharge, cough, and laboured breathing.

2.8 Necropsy, culture and isolation of mycoplasma

Animals were selected at random from each group and were killed by captive bolt and exsanguinations. A record of gross pathological changes in all organs was made with particular attention to the appearance of the lungs. Lung tissues from an area between the lesion and the grossly normal lung were cut and placed in sterile polythene bags then immediately transferred into a cool box and transported from the post-mortem room to the laboratory where they were processed and cultured for isolation of mycoplasma organisms.

2.9 Pathology scores and protection rates

Lesion scoring was carried out to determine severity of the disease in diseased animals using the pathology score described by Hudson and Turner, (1963). Briefly, using this method, the pathology score is calculated based on the size and duration of the lesion (whether adhesions are fibrous or fibrinous). Using the diameter, the presence of only encapsulated, resolving or fibrous lesions or the presence of pleural fibrous adhesions only were allocated a score of 1 regardless of the size. Other types of lesions including consolidation, acute, necrotic or sequestration were scored 2. In addition, if *Mmm*SC was isolated a value of 2 was added to the lesion score. The lesion score was then multiplied by an arbitrarily selected factor depending on average diameter of the lesion. A lesion size under 5cm was rated 1; that over 5cm and under 20cm was rated 2 while a lesion over 20cm was rated 3. Hence, the maximum pathology score was (2+2)3=12. The pathology score of each of the seven groups of cattle was obtained and used to calculate the protection rates by comparing pathological scores in the vaccinated and in the control groups. The score for indicators of disease and infection in vaccinates was divided by the score in controls and the result was subtracted from 1 then multiplied by 100 and percentage protection calculated as:

$$\text{Protection rate} = \left(1 - \left\{\frac{\textbf{Mean score of vaccinates}}{\textbf{Mean score of controls}}\right\}\right) \times 100$$

2.10 Characterization of cattle immune responses

Serum samples were analysed to determine antibody responses using three tests; competitive enzyme linked immuno-sorbent assay (c-ELISA), the complement fixation test (CFT) and an indirect recombinant protein ELISA (p-ELISA).

2.10.1 Competitive Enzyme Linked Immuno-sorbent Assay

One hundred micro litres of dilution buffer was dispensed into each well of the dilution plates and the controls added into appropriate wells as follows: 110 µl of dilution buffer into two conjugate control wells, 11 µl of undiluted strong positive in four wells (monoclonal antibody 117/5), 11 µl of undiluted positive control in two wells, 11 µl of undiluted negative control in two wells and 110 µl of diluted detection solution into four wells, 11 µl of undiluted samples were added into the remaining wells and 110 µl of diluted detection solution dispensed into each well of the dilution plate except the conjugate control. One hundred micro litres from each well of the dilution plate was transferred to the appropriate 96-well polysorp Nunc-Immunoplates (Nunc Laboratories, USA) pre-coated with lysed *Mmm*SC antigen solution, then covered with aluminium foil and incubated at 37°C for 1 hr with gentle agitation. The contents in each well were then poured off and washed twice with 300 µl of wash solution (PBS-Tween 20). One hundred micro litres of diluted conjugate (1:100 in dilution buffer) was added in each well and micro plates covered with aluminium foil and incubated at 37°C for 30 min with gentle agitation. The wells were washed three times with wash solution. After the final wash, 100 µl of Tetramethylbenzidine (TMB) substrate was added into each well and the plate incubated at 37°C for 20 min in the dark place. One hundred micro litres of stop solution added into each well and the plate was shaken by gentle tapping, and optical densities (OD) readings taken at a wavelength of 450 nm using a spectrophotometer.

The results were expressed as percentage inhibition (PI) calculated as follows:

$$PI = \left\{ \frac{OD\ Cm - OD\ Test}{OD\ Cm - OD\ Cc} \right\} \times 100$$

Where, OD Cm is the mean optical density in the monoclonal control wells, OD Test, the optical density in the test wells and OD Cc the mean optical density in the conjugate control wells. A percentage inhibition value equal or above 50% was considered positive.

2.10.2 Complement Fixation Test (CFT)

The CFT was carried out as described by Campbell and Turner, (1953) with minor modifications as recommended by OIE. Briefly, the process involved preparation of *Mmm*SC antigen, sensitized sheep red blood cells (SRBCs), Veronal buffer and PBS. Positive control sera were titrated prior to the commencement of the test. Complement (C′) and hemolytic system (HS) were commercially obtained from CIRAD-EMVT, France.

2.10.2.1 Preparation of CFT antigen

Two litres of tryptose broth was inoculated with T1/44 strain *Mycoplasma Mycoides* subsp. *mycoides* Small Colony and incubated at 37°C for 7 days. The culture was centrifuged at 3000 rpm for 30 min, the supernatant poured off and the pellet re-suspended in 20 ml of 0.85% NaCl and mixed vigorously using sterile glass beads. The mixture was autoclaved for 10 min and allowed to cool, followed by agitation in an 'atomix' for 5 min to achieve an even suspension. To preserve this suspension and make it isotonic, 0.05g of phenol and 0.085g NaCl was added to each 10 ml. The antigen suspension was stored at $+4^{o}$C for 6 weeks, with shaking at weekly intervals, to allow the antigen to stabilize.

2.10.2.2 Preparation of sensitized sheep Red Blood Cells

Sheep blood (50 ml) was collected in a graduated bottle containing 50 ml Alsever solution (Appendix 1b) and stored at 4°C for use within 7 days. Prior to use, the blood was centrifuged at 800g for 10 min and decanted and the cells washed three times with 9 volumes of VCM buffer (Appendix 1c) and the red blood cell sediment finally reconstituted to make a 6% suspension. Separately, hemolytic serum was diluted (10 ml of buffer: 14 µl hemolytic serum) and an equal amount then added to the 6% red blood cells. The 3% RBCs was then left on the bench with gentle agitation for 30 min to allow sensitization of the red blood cells.

2.10.2.3 The CFT micro titration procedure

Twenty five micro litres of test sample and 25 µl of control serum in 1:10 dilution were transferred to a test plate. Individual wells of a 96 well Nunc polysorp test plate were coated with 25µl of diluted antigen. To the 50µl of mixture, 25µl of diluted pre-titrated compliment was added and the plate incubated at 37°C for 30 min with gentle agitation. Twenty five micro litres of sensitized red blood cells was then added followed by incubation at 37°C for 30 min with vigorous shaking. The plates were stored at 4°C overnight and scored on the following day based on the size of the Sensitized Red Blood Cells (SRBCs) sediment. Scores were classified as follows: 4: complete fixation (no haemolysis), 3: almost complete fixation (very slight haemolysis), 2: Partial fixation (partial haemolysis), 1: very slight fixation (almost complete haemolysis) and 0: no fixation (complete haemolysis). Results were interpreted according to the OIE recommended procedure. At 1:10 dilutions, samples with a reading of 3 or 4 were considered positive and were re-titrated to get the end point titre. Samples with readings of 1 and 2 at the 1:10 dilution were considered negative.

2.10.3 Recombinant protein indirect ELISA

Wells of polysorp 96-micro-well plates (Nalge International, Roskilde Denmark) were pre-coated with 150 µl of either 1.2 µg/µl LPPB or 1.4 µg/µl PtsG recombinant antigens in PBS pH 7.2 for 2 hrs at 37°C. The wells were blocked with 300 µl of blocking buffer (0.2% casein) for 20 min at 37°C and washed 3 times in washing buffer (PBS+0.1% Tween 20). Serum test samples were diluted 1:400 in 1% skimmed milk in PBS, 150 µl added in duplicate on the plate and incubated at 37°C for 40 min washed 5 times in washing buffer. One hundred and fifty micro litres of HRP-conjugated anti-bovine IgG (Svanova, Sweden) was added at a dilution of 1:10, 000 and plate incubated at 37°C for 30 min to detect bovine anti-LPPB/anti-PtsG antibodies. The diammonium salt 2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) was used as chromogen and H_2O_2 as substrate for peroxidase, and the plate incubated in the dark with gentle shaking for 30 min before OD readings were obtained using an Immunoskan ELISA plate reader at 405 nm.

Before performing the CFT and p-ELISA immune-assays, 80 serum samples (from naïve, vaccinated and challenged cattle) were randomly selected from a group of many and antibody responses determined for comparison of sensitivities of the tests.

2.11 Data analysis

Data were entered into MS Excel software for calculation of means. The means were then exported into SPSS 16.0 data document for statistical analysis, and Analysis of Variance (ANOVA) used to compare the means of neck diameter, rectal temperatures, pathology scores and antibody responses between groups. Significance was determined at 95% confidence interval. McNemar's chi-square was used to compare means between serological tests at 95% confidence interval.

CHAPTER THREE:

RESULTS

3.1 Vaccine safety

3.1.1 Temperature recording post-vaccination

No fever was detected and rectal temperatures for all the animals were below $39.5^{\circ}C$ during the 88 days post-vaccination observation period. The mean rectal temperatures for KE1, KE2, KE3, KE4, KE6, KE8 and KE9 groups were $37.53^{\circ}C$, $37.64^{\circ}C$, $37.50^{\circ}C$, $37.58^{\circ}C$, $37.71^{\circ}C$, $37.55^{\circ}C$ and $37.58^{\circ}C$ respectively. Groups KE1 and KE3 body temperatures were not significantly different ($p>0.05$) at post-vaccination and post-challenge stages, this in comparison to KE2, KE4, KE6, KE8 and KE9.

3.1.2 Diameter of the swelling at the site of inoculation

Most of the animals vaccinated with different adjuvant formulations, showed swellings at the site of inoculation on the left side of the neck. The average swelling of each group recorded at the inoculation site is shown in Figure 4. Swellings were observed between the 2nd to 47th day post-inoculation in the seven groups (Appendix 2), and in most cases the reactions regressed by day 21. Only one animal developed Willems reaction in the group inoculated with T1/44 (KE3) vaccine and was treated. A large proportion (80%) of the animals in group KE2 showed signs of irritation after inoculation, as displayed by severe shaking of head while cattle vaccinated with inactivated vaccine formulated with different adjuvants had the highest reactions with all the ten animals in the groups showing swellings at the inoculation site. Group KE1 (negative control) was significantly different ($p<0.001$) from KE2, KE4, KE6, KE8 and KE9 but not significantly different from KE3 (positive control).

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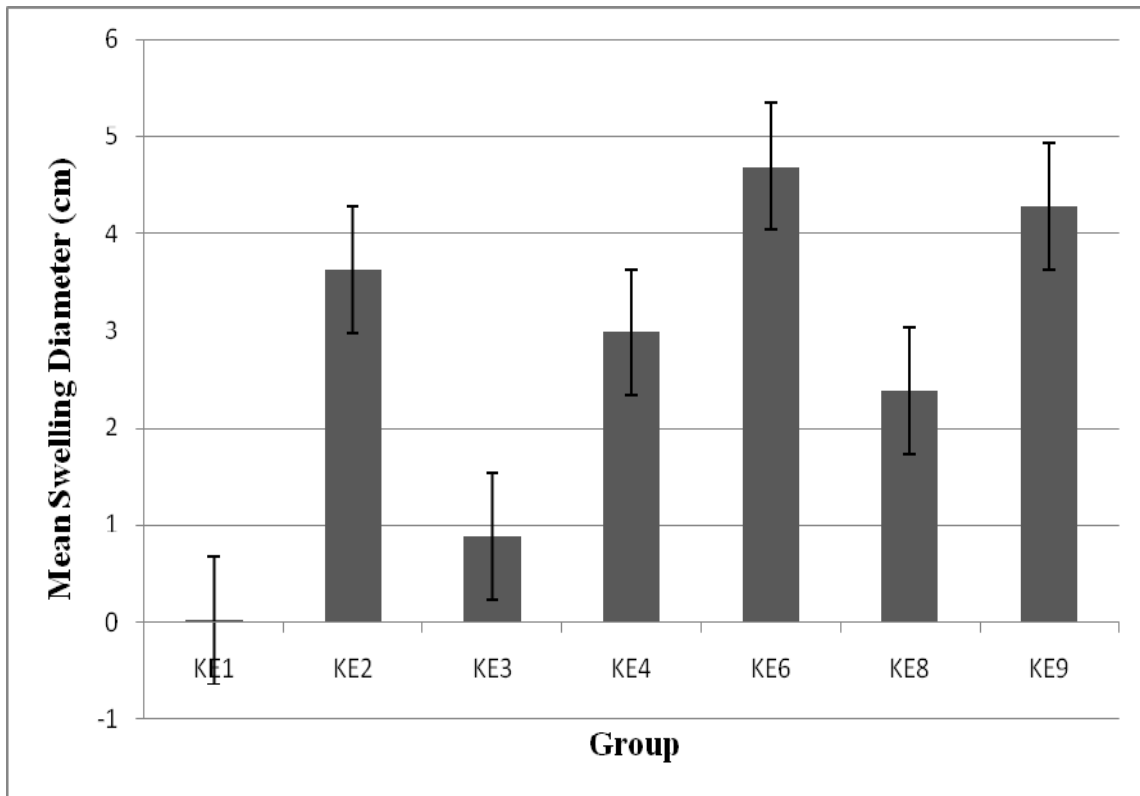
Figure 4: Mean measurements of the swellings of groups KE1 to KE9 over 48 days period

Cattle in each of the groups were vaccinated according to the following treatment;

KE1: Phosphate Buffered Saline; **KE2**: Inactivated antigen formulated in Saponin; **KE3**: T1/44 commercial live attenuated vaccine; **KE4**: Inactivated antigen formulated in ISCOM Adjuvant 1; **KE6**: Inactivated antigen formulated in ISCOM Adjuvant 2; **KE8**: ISCOM Adjuvant 1 and **KE9**: ISCOM Adjuvant 2.

3.2 Vaccine efficacy

3.2.1 Clinical response post-challenge

Out of the 20 intubated animals, 14 animals (66.67%) exhibited fever for at least two days during the experimental period. Fever commenced in the first animal one day post intubation, while in 13 others fever was observed for the duration of 2 to 14 days. One of the animals showed fever intermittently for fourteen days, starting from the second day post-inoculation. Three other animals showed fever for only two days. Generally, fever was intermittent in all animals and ranged between 39.5^{o}C to 41.1^{o}C.

In the challenged groups, fever was first recorded in one animal in group KE4 on the 40th day post-contact and in two additional on day 41. By day 42 three animals had fever. On 46th day five animals had fever respectively. Thereafter, the animals showing fever increased with subsequent days. The proportion of animals with fever ranged between 6-10 between the groups and the duration of fever ranged between 1-16 days. All animals in group KE2 developed fever. Figure 5 below shows a representative animal showing fever for 13 days.
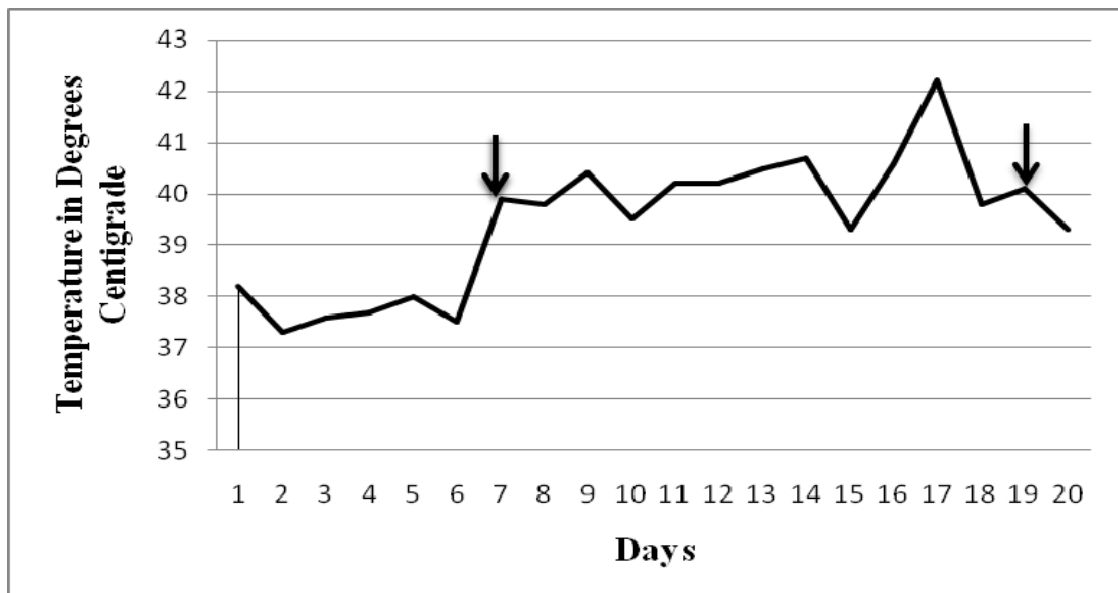
Figure 5: A representative animal showing fever after challenge.
(The 13 day duration of fever is indicated between the arrows)

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3.2.2 Necropsy, culture and isolation

Some of the pathological lesions observed following post-mortem examination are represented in Figure 6. Post-mortem examination revealed gross pathological lesions characteristic of CBPP in all groups with the pleural cavity containing copious amounts of yellowish coloured clear fluid (Figure 6A). Lesions included both fibrous and fibrinous adhesions of the pleura within the chest wall, diaphragm or the pericardial sac (Figure 6B), and consolidation of lung tissues (Figure 6C) showing characteristic marbling and hepatization (Figure 6D). Heavy deposits of fibrin flocculates were also encountered as were unilateral pleuropneumonic lesions, pleuritis as well as developed sequestra (Figure 6E). The normal lung from an animal that was protected is shown in Figure 6F.
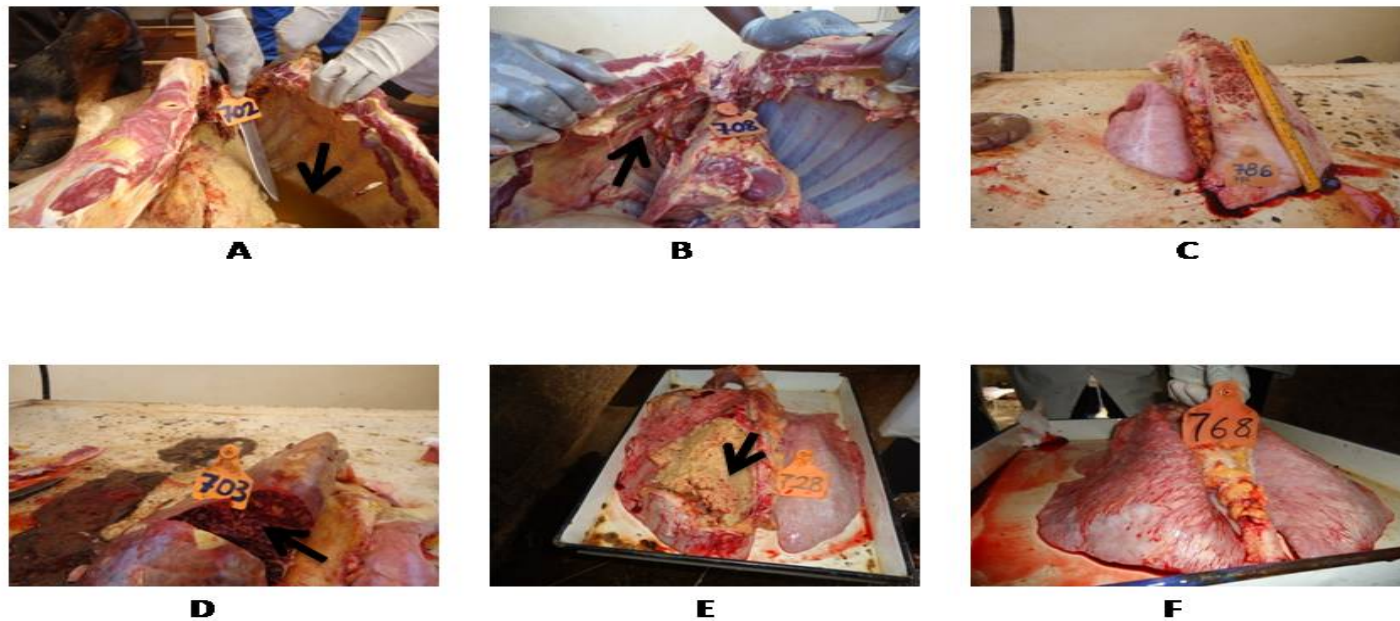
Figure 6: Representative post-mortem examination results with pathological lesions characteristic of CBPP

A) Pleural fluid in the thoracic cavity of an animal vaccinated with KE4, B) Fibrous adhesions on the thoracic cavity of a zebu bull vaccinated with KE8, C) Enlarged right lung with normal left lung in a zebu bull vaccinated with KE9, D) Cut surface of the lung with grey and red hepatisation in a zebu bull vaccinated with KE4, E) Entire lung showing sequestra in a zebu bull vaccinated with KE4 and F) Normal lung in a zebu bull vaccinated with KE3.

Pathological scores between vaccinated groups KE2, KE3, KE4, KE6, KE8, KE9 and the non-vaccinated controls were not significantly different. Table 2 is a summary of post-mortem results of animals in different groups. Briefly, groups KE2, KE4, KE6 and KE9 had the highest number of animals (4) showing presence of pleural fluid in the thoracic cavity while group KE3, KE8 and KE1 had 3, 2 and 1 animals respectively with pleural fluid.

Fibrous adhesions were observed in a total of 29 animals with groups KE8 and KE9 having the most (6 animals in each group) and groups KE3 and KE6 having the least (2 animals in each group). Fibrinous adhesions were observed in a total of 31 animals with group KE6 having the most (7 animals) and groups KE3 and KE8 having the least (3 animals in each group).

Hepatisation was observed in a total of 29 animals with group KE2 recording the most animals (7/10) while no hepatisation was observed in group KE3. Sequestration was observed in a total of 34 animals with group KE3 and KE8 having the most (7 animals in each group) and groups KE1, KE2 and KE6 having the least (3 animals in each group).

Normal lungs with no lesions were observed in total of 7 animals with 3 animals in group KE3, 2 animals in KE2 while groups KE6 and KE8 had one animal in each group with a normal lung. The *Mmm*SC isolation was carried out from pleural fluid (if present) and the lung of each animal in each group of animals with lesions and those without lesions on the lung.

Table 3: Post-mortem results of animals in different treatment groups

Groups	KE1	KE2	KE3	KE4	KE6	KE8	KE9
Presence of pleural fluid	3	4	1	4	4	2	4
Fibrous adhesions	3	5	2	5	2	6	6
Fibrinous adhesions	5	5	3	4	7	3	4
Hepatization	5	7	0	4	6	3	4
Sequestrae	3	3	7	5	3	7	6
Normal lung	2	0	3	1	1	0	0

3.2.3 Pathology scores and protection rates

The number of animals with lesions and range of pathology scores in different groups is shown in Table 3 below. Group KE2 had the highest pathology score while group KE3 had the least pathology score. Group KE3 was significantly different ($p < 0.05$) from all other groups.

Table 4: Lesions and pathological scores in different experimental groups

Groups	KE1	KE2	KE3	KE4	KE6	KE8	KE9
Number of animals with lesions	8/10	10/10	7/10	9/10	9/10	10/10	10/10
Pathology score equal to 12	5/10	9/10	1/10	6/10	7/10	4/10	6/10
Range of pathology score	2-12	8-12	2-12	2-12	2-12	8-12	8-12
Average pathology score	8.8	11.6	5.4	9.8	10.2	9.6	10.4

Protection rates in all groups following challenge at 58 days post-vaccination ranged between -31.20 to 38.64. Group KE3 had the highest protection rate (38.64) as compared to the rest while group KE2 had the least protection rate (-31.2). The protection rates for groups KE4, KE6, KE8 and KE9 were: -11.36, -15.91, -9.10 and -18.18 respectively.

3.2.4 Antibody responses as determined by c-ELISA assay

Blood was obtained from cattle, sera separated and tested before vaccination. Those cattle which showed sero-conversion pre-vaccination were excluded from results on sero-converting animals. Sero-conversion was not observed in any animal in the control groups KE1 and KE8 post-vaccination but was however observed in KE2, KE3, KE4, KE6 and KE9. Group KE6 had the highest sero-conversion after vaccination followed by KE2. The earliest sero-conversion was observed one week post-vaccination in groups KE2 and KE6, while animals in groups KE3 and KE4 showed sero-conversion in the second week post-vaccination and those in group KE9 sero-converted by the 3rd week. Post-challenge results showed that a large proportion of animals sero-converted in almost all groups; KE4 sero-converting most followed by KE2. Table 4 shows percentages of cattle that showed sero-conversion. There was no significant difference (p>0.05) in antibody responses by c-ELISA between groups at post-vaccination and post-challenge stages.

Table 5: Proportion (%) of cattle showing sero-conversion based on c-ELISA

Groups	KE1	KE2	KE3	KE4	KE6	KE8	KE9
Pre-vaccination	10%	10%	20%	30%	20%	50%	20%
Post-vaccination	0%	44%	38%	29%	80%	0%	11%
Post-challenge	80%	89%	88%	100%	80%	60%	88%

Sero-conversion (detection of specific antibodies) was determined as percentage inhibition (>50%) by the c-ELISA assay. Figure 7 below is a representative graph of a sero-converting animal. Vaccinated cattle showed evidence of sero-conversion beginning from the second week up to the 10th week post-vaccination. Animals exposed to challenge started sero-converting at the 6th week post-challenge with the highest percentage inhibition recording on the 8th week.
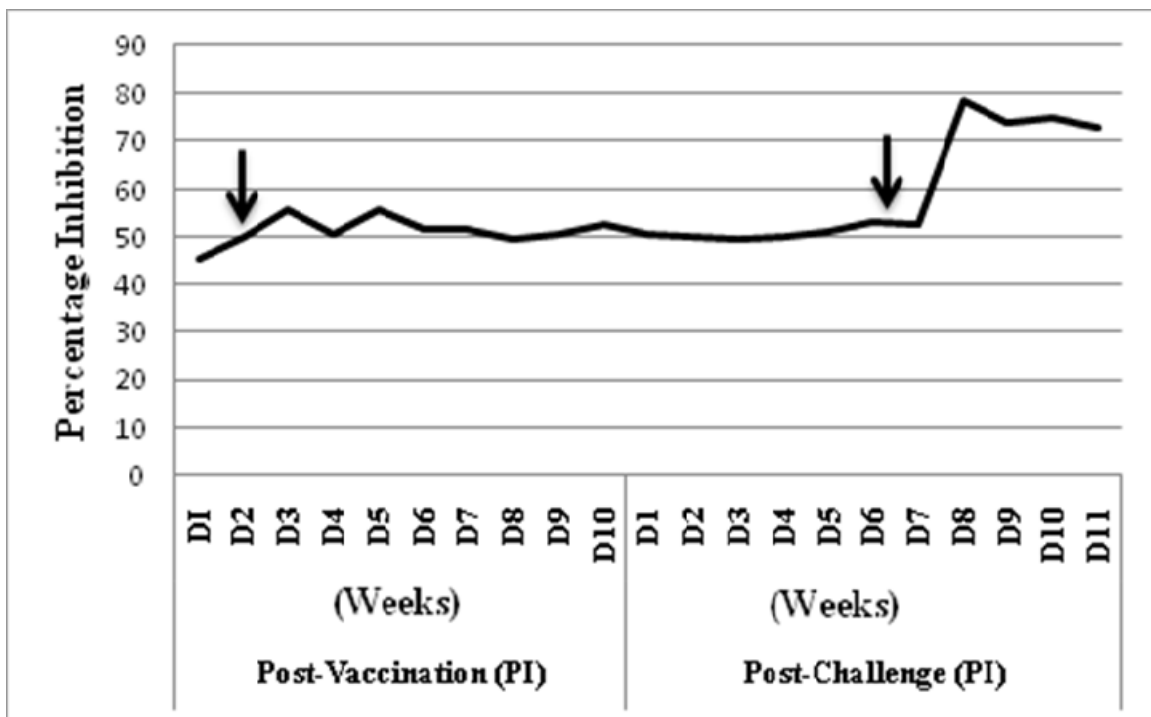
Figure 7: A representative sero-converting animal (753 of KE4) as determined by c-ELISA (arrows indicate the week when sero-conversion occurred)

3.2.5 Antibody responses as determined by CFT assay

The CFT assay was done on randomly selected animals based on three disease clinical outcomes namely: lesion free cases, presence of sequestrae and acute cases. CFT antibody titres were detected as early as the second week after vaccination and persisted for 6-9 weeks. The antibody titres ranged between 1:10 and 1:80 and were found in some but not all animals. Low antibody titres ranging between 1:10 to 1:20 were observed in some animals in KE4 and KE6 and persisted for 6-9 weeks. The highest sero-conversion was observed in lesion free cases after vaccination while the highest sero-conversion was observed in cattle with sequestrae after challenge.

Table 5 below shows percentages of cattle that showed sero-conversion. None of the animal had sero-converted prior to vaccination. There was significant difference ($p<0.05$) between acute cases and lesion free cases but no significant difference ($p>0.05$) between acute cases and sequestrae cases and no significant difference between post-vaccination and post-challenge stages.

Table 6: Proportion (%) of cattle showing sero-conversion based on CFT assay

Time-point	Animals		
	Without lesion	With sequestrae	Acute (lesion>20cm)
Pre-vaccination	0%	0%	0%
Post-vaccination	71%	30%	13%
Post-challenge	57%	90%	63%

3.2.6 Antibody responses as determined by recombinant protein ELISA

Animals were randomly selected as described in section 3.2.5 and serum samples tested against two recombinant protein antigens. Prior to the test, 11 positive and 11 negative serum samples (confirmed by c-ELISA) were used to determine the cut off point for the antigen. A doubling dilution of the sera from 1:50 to 1: 1600 was used to determine the optimum dilution of 1:400 for both antigens that allowed discrimination of positive and negative test serum samples.

The recombinant, LPPB was obtained at a concentration of 1.2μg/μl and titrated at a doubling dilution of between 0.3-0.002μg/μl to find the optimal titre of 0.01μg/μl. Similarly, PtsG antigen obtained at 1.4μg/μl was titrated and obtained an optimal titre of 0.02μg/μl. Using the optimum values above, 53 CBPP positive and 27 negative test sera (confirmed by c-ELISA) were used to carry out recombinant protein ELISA test with both antigens. An optical density (OD) reading of less than or equal to the cut off point of 0.35 was found to represent a negative sample while that above 0.35 represented a positive sample. The results show that both antigens showed sero-conversion in lesion free cases and sequestrae cases. However, there was no sero-conversion in acute cases (Table 6). There was no significant difference ($p > 0.05$) between the absorbance of two proteins at post-vaccination and post-challenge stages.

Table 7: Proportion (%) of cattle showing sero-conversion based on p-ELISA

Randomly selected animals			Without lesion	With sequestrae	Acute (lesion>20cm)
Pre-vaccination	a)	LPPB	50%	100%	0%
	b)	PtsG	0%	100%	0%
Post-vaccination	a)	LPPB	50%	100%	0%
	b)	PtsG	45%	100%	0%
Post-challenge	a)	LPPB	55%	100%	0%
	b)	PtsG	65%	100%	0%

Post-vaccination results showed that the animal started sero-converting from the first week up to the tenth week (Figure 8). Post-challenge results showing that the animal started sero-converting from the first week, with the highest percentage inhibition recorded during the 7th and 8th week.
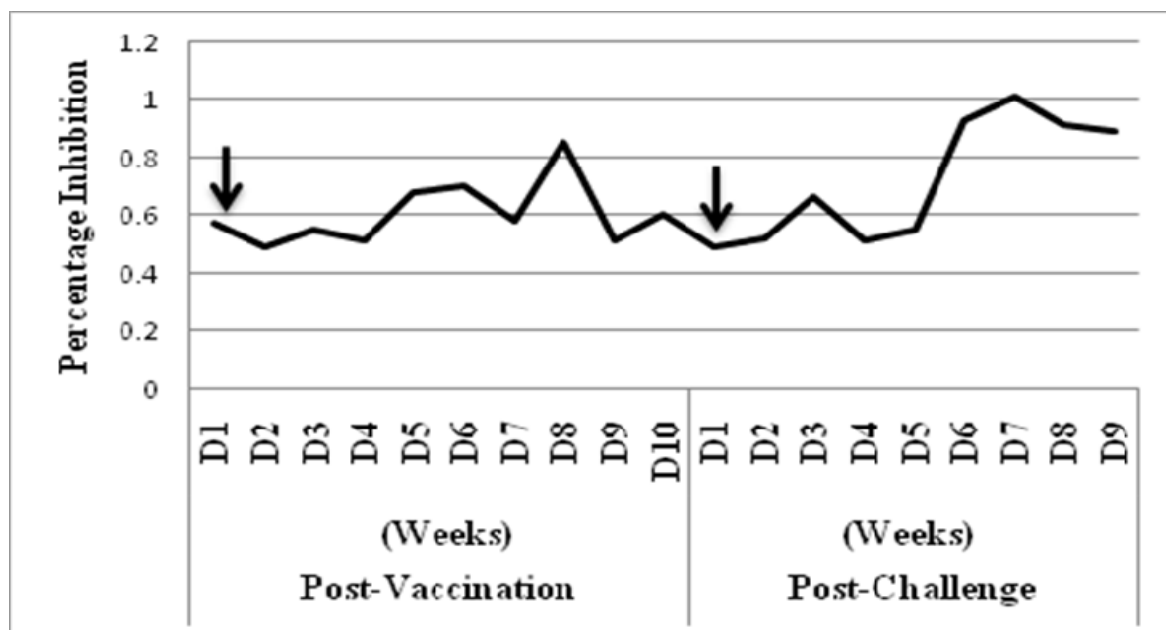
Figure 8: A representative sero-converting animal (753 of KE4) as determined by p-ELISA (arrows indicate the week when sero-conversion occurred)

The sensitivities of c-ELISA, CFT and two recombinant antigens of p-ELISA were compared on 80 serum samples (5 from naïve, 40 from vaccinated and 35 from challenged cattle). The 35 serum samples from challenged cattle were confirmed to be true positives because earlier, *Mmm*SC had been isolated from cultures of lung samples of cattle from which these serum samples were obtained. From these samples, c-ELISA, CFT, LPPB antigen of p-ELISA and PtsG antigen of p-ELISA tests detected antibody responses as follows: 53(66.25%), 13(16.5%), 42(52.5%) and 40(50%) respectively. There were significant associations between CFT, c-ELISA and two antigens of recombinant p-ELISA as shown in Tables 7, 8 and 9.

Table 8: Comparison between CFT and c-ELISA results using 80 randomly selected serum samples

CFT	c-ELISA N=80		
	Positive	Negative	Total
Positive	13	0	13
Negative	40	27	67
Total	53	27	80

There was a significant (p<0.001) McNemar's association between CFT and c-ELISA. The two methods are different as shown in the table above. Competitive ELISA detected more positive serum samples than CFT.

Table 9: Comparison between CFT and LPPB antigen of p-ELISA results using 80 randomly selected serum samples

CFT	LPPB N=80		
	Positive	Negative	Total
Positive	13	0	13
Negative	29	38	67
Total	42	38	80

There was a significant (p<0.001) McNemar's association between CFT and LPPB antigen for p-ELISA. The two methods are different as shown in the table above. LPPB antigen for protein ELISA detected more positive serum samples than CFT.

Table 10: Comparison between CFT and PtsG antigen of p-ELISA results using 80 randomly selected serum samples

CFT	PtsG N=80		
	Positive	Negative	Total
Positive	13	0	13
Negative	27	40	67
Total	40	40	80

There was a significant (p<0.001) McNemar's association between CFT and PtsG antigen for p-ELISA. The two methods are different as shown in the table above. PtsG antigen for protein ELISA detected more positive serum samples than CFT.

CHAPTER FOUR:

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 Discussion

This study compared the safety and threshold of protection of live vaccines in current use with that of inactivated *Mmm*SC vaccine strain formulated with different adjuvants. This was expected to address the limitations of live vaccines in current use that include residual virulence, short term and incomplete immunity (Rweyemamu *et al.,* 1995). The purpose of this study was therefore to test and select the best inactivated vaccine formulated with adjuvants for control of CBPP.

Vaccine safety of inoculants was assessed by comparing rectal temperatures and swellings at the site of inoculation between the different groups. Rectal temperatures for all groups remained within the normal physiological range after vaccination, implying that all products did not cause adverse reactions; as measured by temperature changes.

The number of animals with post-vaccinal reactions in groups inoculated with inactivated *Mmm*SC formulated with different adjuvants were significantly higher (P<0.001) than those inoculated with the live T1/44 vaccine strain in current use. Only one animal in the group inoculated with live T1/44 vaccine strain showed post-vaccinal reaction, with a severe lesion of Willems reaction and was treated (Thiaucourt *et al.,* 2000; Thiaucourt *et al.,* 2003). However, many animals in the groups inoculated with inactivated vaccine products did not require treatment because the swellings regressed without rapture. The swellings following inoculation with inactivated products lasted longer than 48 days, making the animals less acceptable on the market.

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Post-vaccination reactions on the neck of animals vaccinated with the inactivated vaccine formulated with different adjuvants were similar to those elicited by pro-inflammatory reactions as reported by Huebschle *et al.,* (2003). The safety values observed with the live T1/44 vaccine were expected under experimental conditions. However, under field conditions more or less severe post-vaccinal reactions may be expected (Sori, 2005). Reasons for this include stress or vaccination of animals at varying stages of the disease since some may be free from the disease while others may be incubating or carriers.

The efficacy of several vaccine candidate products was evaluated on the basis of protection as measured by development of clinical signs (fever), lesion or pathology score. The negative control group (KE1) showed a rise in rectal temperature between the 8th and 14th week post-contact similar to the vaccinated groups (between 8th and 14th week). These results are supported by similar observations in cattle inoculated with inactivated products (Huebschle *et al.,* 2003).

The prominent manifestation of CBPP in animals inoculated with inactivated vaccine formulated with adjuvants compared well with negative controls. This is in contrast with those vaccinated with live vaccine which although showing similar response of lesions, showed a lower average pathology score, suggesting some protection using live vaccine. These findings differ from those by Gray *et al.,* (1986); Nicholas and Ayling, (2003).

However, the protection demonstrated by these authors was based on techniques with multiple inoculations and high doses where cattle were vaccinated twice with very large doses of inactivated *Mmm*SC mixed with Freund's complete adjuvant. In previous results reported by Nicholas and Ayling, (2003), and working with *Mycoplasma bovis*, a higher dose of saponized inactivated products (1 ml compared to 0.5 ml used in this study) was used on two separate vaccination regimes, 6 weeks apart while Gray *et al.,* (1986), inoculated cattle subcutaneously with 20 ml of inactivated *Mmm*SC mixed with Freund's complete adjuvant.

In a separate more recent study, Thiaucourt *et al.,* (2012) observed that cattle re-vaccinated with inactivated vaccine formulated with ISCOMS adjuvants, prepared in the same batch and used in Mali, improved efficacy compared to the live T1/44 vaccine in current use; suggesting that re-vaccination would most likely have conferred protection and improved efficacy in this study.

A single inoculation of a much lesser dose of inactivated *Mmm*SC mixed with Freund's incomplete adjuvant gave no significant protection which was the case in this study. A minimum protective dose and re-vaccination should therefore be defined when vaccinating cattle with inactivated vaccine formulated with adjuvants (Gray *et al.,* 1986).

The findings here also differ from those reported by Huebschle *et al.,* (2003) who showed that animals vaccinated with inactivated vaccine formulated with an adjuvant had reduced mortality and clinical symptoms. On post-mortem, animals in this study that had no lesions at killing included those that did not show fever after challenge. One animal in the negative control group did not develop lesions; suggesting evidence of innate resistance. There was no distinction between control and vaccinated animals in this study as to the development of sequestrae, similar to observations by Huebschle *et al.,* (2003).

In this study, animals inoculated with live T1/44 vaccine strain showed the least pathology score, suggesting that they were better protected compared to groups that received inactivated vaccine formulated with different adjuvants and the negative control. The protection rate observed with live T1/44 vaccine here is comparable to that observed by Thiaucourt *et al.,* (2004) using live T1/44 vaccine strain in animals receiving a single vaccination and challenged 3 months later. He reported a protection rate of 40-60% following primary vaccination, increasing to between 80 and 95% upon re-vaccination. The protection rate recorded in this study by live T1/44 vaccine strain was 38.64% and was found to be higher than that reported by Yaya *et al.,* (1999) of 30%.

All groups receiving killed products showed no protection at all, with killed products appearing to sensitize the animals leading to a more severe disease. Disregarding whether responses to these inoculants confer immunity or not, a successful immunization should elicit a stronger immune response upon contact with a pathogen.

Serology using c-ELISA and CFT showed that animals in two groups (KE2 and KE6), inoculated with inactivated vaccine mixed with adjuvant, had higher antibody responses after vaccination which persisted up to 10th week. These same animals in groups KE2 and KE6 were not protected on challenge, suggesting that the antibodies were not protective, observations that are similar to those reported previously by Abusugra *et al.,* (1997) where ISCOMs were found to induce non protective antibody responses.

A few animals in all groups in the present study were euthanized following signs of severe disease after challenge but no antibodies were detected. The cause of this was not established, but could be due to antibody eclipsing as a result of excess circulating antigens (March *et al.,* 2003).

The two serological tests used (Complement Fixation Test and competitive Enzyme Linked Immuno-sorbent Assay) in this study are those currently recommended by the OIE for serological diagnosis of CBPP. These tests however, have drawbacks necessitating the need to develop more robust tests. CFT has high specificity but takes long to perform and requires more elaborate training of staff. Additionally, it is less effective at diagnosing animals in the early stages of the disease or of animals with chronic lesions (Rurangirwa, 1995). The c-ELISA test on the other hand, is simpler to carry out. However, validation studies are still insufficient to make good conclusions about its use in surveillance studies where vaccinations are undertaken in enzootic areas of infected zones. In addition, the test is sensitive but its specificity is uncertain (Sidibé *et al.,* 2012). There is therefore a need for development of another test as sensitive as c-ELISA and as specific as CFT.

Preliminary studies have been initiated to use sub-cellular components of the mycoplasma genome sequence of *Mmm*SC strain PG1 (Westberg *et al.,* 2004) to develop an alternative test. They offer an emerging possibility to improve both diagnostic and therapeutic approaches with selected antigens. These sub-cellular components have been employed in a suspension bead array platform and mixed for ELISA setting (Hamsten *et al.,* 2010).

The sub-cellular components of the mycoplasma are variable surface proteins, generally believed to enhance colonization of the host tissue and help evade host immune responses by antigenic variation. Five such lipoproteins studied in detail have been reviewed in Pilo *et al.,* (2007): LppA (Monnerat *et al.,* 1999), LppQ (Abdo *et al.,* 2000), LppB (Vilei *et al.,* 2000), Vmm, a variable surface protein (Persson *et al.,* 2002) and LppC (Pilo *et al.,* 2003). These selected recombinant antigens may qualify as highly specific sero-diagnostic markers for CBPP.

In this study, a few test serum samples that had been tested and confirmed by c-ELISA were re-tested against 2 recombinant lipoproteins LPPB and PtsG. The preliminary findings from this showed that the recombinant protein ELISA test was more sensitive than CFT and almost like c-ELISA. However, the specificity of these recombinant antigens was not determined.

4.2 Conclusions

Adjuvant formulations at the dose regimen used in this study, did not change the safety of inactivated *Mmm*SC vaccines; and they did not improve the efficacy of inactivated *Mmm*SC vaccines. Reactions observed in inactivated vaccine formulated with different adjuvants were considered mild since they did not warrant treatment, suggesting that the vaccines were safer than the live vaccine. However, the swellings may limit the market value of live animals, especially due to the long duration it takes for them to regress.

The efficacy of inactivated *Mmm*SC vaccine formulated with different adjuvants showed that the inoculants did not confer protection in their present formulation and protocol of administration. Further work is needed before they can be recommended to qualify as vaccine candidates against CBPP. Whereas these observations were made on cattle inoculated only once, it is possible that booster vaccination may confer higher protection and improve efficacy, similar to observations made in other studies.

4.3 Recommendations

1. There is need to explore the use of booster immunization regimes as carried out in parallel studies elsewhere which gave positive responses to immunization.

2. Alternative formulations of ISCOMS adjuvants should be tested when assessing and optimizing inactivated vaccines.

3. Recombinant protein ELISA should be explored as a possible additional CBPP sero-diagnostic test for early detection of CBPP.

REFERENCES

Abdo, E. M., Nicolet, J. and Frey, J. (2000). Antigenic and genetic characterization of lipoprotein, LppQ from *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*SC). *Clinical and Diagnostic Laboratory Immunology,* **7:** 588–595.

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

Abusugra, I., Wolf, G., Bolske, G., Thiaucourt, F. and Morein, B. (1997). Immuno-stimulating complex (ISCOM) vaccine against CBPP. Biochemical and immunological characterization. *Veterinary Immunology and Immunopathol*ogy, **59:** 31-48.

Almeida, R. A. and Rosenbusch, R. F. (1991). Capsule like surface material of *mycoplasma dispar* induced by in-vitro growth in culture with bovine cells is antigenically related to similar structures expressed in vivo. *Infection and Immunity,* **59:** 3119-3125.

Amanfu, W., Sediade, S., Masupu, K. V., Benkirane, A., Geiger, R. and Thiaucourt, F. (1998). Field validation of competitive Enzyme Linked Immuno-sorbent Assay (c-ELISA) for the detection of contagious bovine pleuropneumonia in Botswana. *Revue. dElevage et de medecine veterinaire des Pays Tropicaux (Paris),* **51:** 189-193.

Baseman, J. B., Lange, M., Criscimagna, N. L., Giron, J. A. and Thomas, C. A. (1995). Interplay between mycoplasmas and host target cells. *Microbiol Pathogenesis,* **19:** 105-116.

Buttery, C., Lagergard, T. and Titchen, D. A. (1976). Acute respiratory, circulatory and pathological changes in the calf after intravenous injection of the galactan from *Mycoplasma mycoides* subsp. *Mycoides* Small Colony. *Journal of Medical Microbiology,* **9:** 379-391.

Campbell, A. D. and Turner, A. W. (1953). Studies of Contagious Bovine Pleuropneumonia of cattle. An improved complement fixation test. *Australian Veterinary Journal,* **29:** 154-163.

Caporale, R. J., Giovannini, A., Santini, F., Martel, J. L. and Goncalves, A. P. (1996). Manifestation and epidemiology of CBPP. *European Review Science Technology,* **15:** 1309-29.

Davies, G. (1991). Contagious Bovine Pleuropneumonia: What every veterinarian ought to know. *State Veterinary Journal,* **1:** 9-11.

Davies, G., Masiga, W. N., Shifrine, M. and Read, W. C. S. (1968). The efficacy of T1 strain broth vaccine against contagious bovine pleuropneumonia: Preliminary in-contact trials. *Veterinary Record,* **83:** 239-244.

Debey, M. C. and Ross, R. F. (1994). Ciliostasis and loss of cilia induced by Mycoplasma hyopneumoniae in porcine tracheal organ cultures. *Infection and immunity,* **62:** 5312-5318.

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

Food and Agricultural Organization EMPRES. (1995). Concept paper: The emergency control of Contagious Bovine Pleuropneumonia in Eastern and Southern Africa. Joint FAO EMPRES and OAU-IBAR regional workshop, 4th-6th July, 1995, Arusha, Tanzania. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

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

Garba, S. A., Terry, R. J., Adegboye, D. S., Lamorde, A. G. and Abalaka, J. A. (1989). The choice of adjuvants in mycoplasma vaccines. *Microbios,* **57:** 15-19.

Gitau, G.K. (2001). Background information on contagious bovine pleuropnemonia in Kenya. Consultancy report produced by AU/IBAR/PACE. Nairobi, Kenya. African Union Inter African Bureau for Animal Resources (AU/IBAR).

Government of Kenya. (2003). Contagious Bovine Pleuropneumonia Policy, strategy and zonation. Ministry of Livestock and Fisheries Development. Nairobi, Kenya.

Government of Kenya. (2004). National Policy for the sustainable development of the Arid and Semi-Arid lands of Kenya. Nairobi, Kenya. Government printer.

Gourley, R. N. and Howard, C. J. (1978). Isolation and pathogenicity of mycoplasmas from the respiratory tract of calves. *Current Topics Veterinary Medicine,* pg. 295-304.

Gray, M. A., Simam, P. and Smith, G. R. (1986). Observations on experimental inactivated vaccines for CBPP. *Journal of Hygiene (Camb),* **97:** 305-315.

Gunasekera, T. S., Attfield, P. V. and Veal, D. (2000). A Flow Cytometry Method for Rapid Detection and Enumeration of Total Bacteria in Milk. *Applied and Environmental Microbiology,* **66:** 1228-1232.

Hamsten, C., Tjipura-Zaire, G., McAuliffe, L., Otto J. B. Huebschle, J. B., Massimo Scacchia, M., Ayling, R. D. and Persson, A. (2010). Protein Specific Analysis of Humoral Immune Responses in a Clinical Trial for Vaccines against Contagious Bovine Pleuropneumonia. *Clinical and vaccine immunology*, **Vol. 17:** 853–861.

Hudson, J. R. and Turner, A. W. (1963). Contagious Bovine Pleuropneumonia: A comparison of the efficacy of two types of vaccines. *Australian Veterinary Journal,* **39:** 373- 385.

Huebschle, O. J. B., Tjipura-Zaire, G., Abusugra, I., Difrancesca, G., Mettler, F., Pini, A. and Morein, B. (2003). Experimental Field Trial with an Immuno-stimulating Complex (ISCOM) Vaccine against Contagious Bovine Pleuropneumonia. *Journal of Veterinary Medicine, B 50:* 298-303.

Hyslop, N. S. G. (1956). Duration of immunity of cattle vaccinated with egg-adapted Contagious Bovine Pleuropneumonia vaccine. *British Veterinary Journal,* **112:** 519-522.

Iowa State University. (2004). *Status of CBPP worldwide.*

Kane, K.W.S. (1975). The control of contagious bovine pleuropneumonia in Kenya. *Bulletin of the Office of International Epizootics,* **84:** 421-428.

Kariuki, D. P. (1971). History of CBPP in Kenya. *Bulletin of Epizootic Diseases of Africa,* **19:** 111-116.

Kelly, C. G., Younson, J. S., Hikmat, B. Y., Todryk, S. M., Czisch, M., Haris, P. I., Flindall, I. R., Newby, C., Mallet, A. I., Ma, J. K. and Lehner, T. (1999). A synthetic peptide adhesion epitope as a novel antimicrobial agent. *Nature Biotechnology,* **17:** 42-47.

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

Litamoi, J. K. and Seck, B. M. (1998). Vaccine quality issues in Africa, including safety and efficacy: In report of the first meeting of the FAO/OIE/OAU-IBAR Consultative group on Contagious Bovine Pleuropneumonia. 5th-7th October, 1998. Rome, Italy; pg. 65-75. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Litamoi, J. K., Ayelet, G. and Rweyemamu, M. M. (2007). Preparation of heat tolerant CBPP vaccine. An initial assessment of the sustainability of xerovac process: In CBPP control.

Lloyd, L. C. and Etheridge, J. R. (1983). Contagious bovine pleuropneumonia produced by aerosol artificially generated from cultures of *Mycoplasma mycoides* subsp. *Mycoides* Small Colony. *British Veterinary Journal,* **139:** 330-337. Antibiotics to the Rescue. Proceedings of FAO-OIE-AU/IBAR-IAEA Consultative group meeting on CBPP in Africa. 6th-8th November, 2006. Rome, Italy, p67-71. Food and Agriculture Organization of the United Nations (FAO), Rome Italy.

Manso-Silván, L., Vilei, E. M., Sachse, K., Djordjevic, S. P., Thiaucourt, F. and 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* Large Colony as a serovar of *Mycoplasma mycoides* subsp. *capri. International Journal of Systematic and Evolutionary Microbiology,* **59:**1353-1358.

March, J. B., Kerr, K. and 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):** 233–240.

March, J. B., Waite, E. R. and Litamoi, J. K. (2002). Re-suspension of T1/44 vaccine Cultures *Mycoplasma mycoides* subsp. *mycoides* SC in 1 molar MgSO4 causes a drop in PH and a rapid reduction in titres. *FEMS Immunology and Medical Microbiology,* **34:** 97-103.

Mariner, J. C. and Catley, A. (2004). The dynamics of CBPP endemism and development of effective control strategies: In towards sustainable CBPP control programmes for Africa. Proceedings of the third meeting of FAO-OIE-AU/IBAR-IAEA Consultative group on Contagious Bovine Pleuropneumonia 12th-14th November, 2003. Rome, Italy, p76-80. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Mariner, J. C., McDermott, J., Heesterbeek, J. A. P., Thomson, G. and Martin, S. W. (2006). A model of contagious bovine pleuropneumonia transmission dynamics in East Africa. *Preventative Veterinary Medicine,* **73:** 55-74.

Masiga, W. N., Domenich, J. and Windsor, R. S. (1996). Manifestation and epidemiology of contagious bovine pleuropneumonia in Africa. *Reviews of Science and Technology Office of International Epizootics,* **15(4):** 1283-1308.

Masiga, W. N., Rossitor, P. and Bessin, R. (1998). Vaccines and vaccination: the impact of withdrawing rinderpest vaccination on CBPP control. Report of the FAO/OIE/OAU-IBAR CBPP consultative group meeting, Rome, Italy 5th-7th October, 1998. Rome: FAO publication X3960-E, **1999:** 81-85.

Mcleod, A. and Rushton, J. (2007). Economics of animal vaccination. *Revue Scientifique et Technique; Office International des Epizooties,* **26:** 313-326.

Minion, F. C. (2002). Molecular Pathogenesis of Mycoplasma Animal Respiratory Pathogens. *Frontiers in Bioscience,* **7:** 1410-1422.

Ministry of Livestock Development and Fisheries (MoLD & F). (1978). *Veterinary Departmental Reports*, Nairobi.

Monnerat, M. P., Thiaucourt, F., Poveda, J. B., Nicolet, J. and Frey, J. (1999). Genetic and serological analysis of lipoprotein LppA in *Mycoplasma mycoides* subsp. *mycoides* Large Colony and *Mycoplasma mycoides* subsp. *capri. Clinical and Diagnostic Laboratory Immunology,* **6:** 224–230.

Morein, B., Lovgren-Bengtsson, K. and Cox, J. (1996). Modern adjuvants: Functional aspects. In: Concepts in Vaccine Development *(Kaufmann, S.H.E., Ed.),* pp. 243-263. Walter de Gruyter, Berlin.

Morein, B., Lovgren, K., Ronberg, B., Sjolander, A. and Villacres-Ericksson, M. (1995). Immuno-stimulating complexes: Clinical potential in vaccine development. *Clinical and experimental Immunology,* **3:** 461-475.

Mugo, P. M. (2004). Determination of the prevalence of contagious bovine pleuropneumonia in Kenya: comparison of complement fixation test and bacterial culture. Master of science Thesis, Kenyata University.

Mullins, G. R., Fidzani, B. and Kolanyane, M. (2000). At the end of the day: The socio-economic impacts of eradicating Contagious Bovine Pleuropneumonia from Botswana. *Annals of the New York Academy of Science,* **916:** 333-344.

Naessens, J. Distribution of CBPP in sub-Saharan Africa. Country reports (2005-2010).

Nicholas, R. A. J. and Ayling, A. D. (2003). Mycoplsma bovis, disease, diagnosis and control. *Research in Veterinary Science,* 7**4:** 105-112.

Nkando, I., Ndinda, J., Kuria, J., Naessens, J., Mbithi, F., Schnier, C., Gicheru, M., McKeever, D. and Wesonga, H. (2011). Efficacy of two vaccine formulations against contagious bovine pleuropneumonia (CBPP) in Kenyan indigenous cattle. *Research in Veterinary Science,* **93(2):** 568-573.

Ofek, I., Hasty, D. L. and Sharon, N. (2003). Anti-adhesion therapy of bacterial diseases: prospects and problems. *Immunology and Medical Microbiology,* **38:** 181-191.

Office International des Epizooties. (2008). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 6th Edition. Chapter: 2.4.9. Contagious bovine pleuropneumonia. Page, 712.

Persson, A., Jacobsson, K., Frykberg, L., Johansson, K. E. and Poumarat, F. (2002). Variable surface protein, Vmm of *Mycoplasma mycoides* subsp. *mycoides* Small Colony type. *Journal of Bacteriology,* **184:** 3712–3722.

Pettersson, B., Leitner, T., Ronaghi, M., Bölske, G., Uhlén, M. and Johansson, K. E. (1996). Phylogeny of the *Mycoplasma mycoides* cluster as determined by sequence analysis of the 16S rRNA genes from the two rRNA operons. *Journal of Bacteriology,* **178:** 4131–4142.

Pilo, P., Frey, J. and Vilei, E. M. (2007). Molecular mechanisms of pathogenicity of *Mycoplasma mycoides* subsp. *mycoides* Small Colony. *Veterinary Journal,* **174:** 513–521.

Pilo, P., Martig, S., Frey, J. and Vilei, E. M. (2003). Antigenic and genetic characterisation of lipoprotein LppC from *Mycoplasma mycoides* subsp. *mycoides* Small Colony. *Veterinary Research,* **34:** 761–775.

Pilo, P., Vilei, E. M., Peterhans, E., Bonvin-Klotz, L., Stoffel, M. H., Dobbelaere, D. and Frey, J. (2005). A Metabolic Enzyme as a Primary Virulence Factor of *Mmm*SC. *Journal of Bacteriology,* **187:** 6824-6831.

Provost, A. (1982). Advantages of a joint campaign against rinderpest and bovine pleuropneumonia. *Reviews of Science and Technology Office of International Epizootics*, **3:** 687-704.

Provost, A. (1987). Cotagious Bovine Pleuropneumonia. *Reviews of Science and Technolgy Office of International Epizootics,* **6:** 625-679.

Radostits, O. M., Gray, C. C., Blood, D. C. and Hinchcliff, K. W. (2000). Veterinary medicine. A text book of the diseases of Cattle, Sheep, Pigs, Goats and Horses, London, UK, Saunders.

Razin, S. (1978). The Mycoplasmas. *Microbiological Reviews,* **42:** 414-470.

Razin, S. (1992). Mycoplasma taxonomy and ecology, pg. 3-32. In J. Maniloff, R. N. McElhaney, L. R. Finch and J. B. Baseman (ed.), Mycoplasmas: molecular biology and pathogenesis. American Society for Microbiolgy, Washington, D. C.

Razin, S. (1999). Adherence of Pathogenic Mycoplasmas to Host Cells. *Bioscience Reports,* 1**9:**5.

Razin, S. and Jacobs, E. (1992). Mycoplasma Adhesion. *Journal of General Microbiology,* **138:**407.

Razin, S., Yogev, D. and Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews,* **62:** 1094-1156.

Regalla, J., Caporale, V., Giovannini, A., Santini, F.G., Mantel, J.L., and Goncalves, A.P. (1996). Manifestation and epidemiology of contagious bovine pleuropneumonia in Europe. *Scientific and Technical Review*, **15:** 1309-1329.

Rodwell, A. W. and Mitchell, A. (1979). Nutrition, growth and reproduction. In: The Mycoplasmas. I. Cell Biology (Barile, M.F and Razin, S, Eds.), pg. 103-139. *Academic Press, New York.*

Rurangirwa, E. R. (1995). Uses of serology for the diagnosis of contagious bovine pleuropneumonia. *Reviews of Science and Technology Office of International Epizootics,* **1 4 (3):** 603-609.

Rweyemamu, M. M. and Benkirane, S. (1996). Global impact of infections with organisms of the "*Mycoplasma mycoides* cluster" in ruminants. In: Frey, J., Sarris, K (Eds), COST 826 Agriculture and Biotechnology Mycoplasmas of Ruminants: Pathogenicity, Diagnostics, Epidemiology and Molecular Genetics, EUR 16934, Luxembourg, pg. 1-11.

Rweyemamu, M. M., Litamoi, J., Palya, V. And Sylla, D. (1995). Contagious bovine pleuropneumonia vaccines: the need for improvements. *Reviews of Science and Technology Office of International Epizootics,* **14(3):** 593-601.

Sheriff, D. and Piercy, S. E. (1952). Experiments with an Avianised Strain of the organism of Contagious Bovine Pleuropneumonia. *Veterinary Records,* **64:** 615-612.

Sidibé, C. A., Grosbois, V., Thiaucourt, F., Niang. M., Lesnoff. M. and Roger, F. (2012). Performance evaluation of two serological tests for CBPP) detection in an enzootic area using a Bayesian framework. *Tropical Animal Health and Production,* **44(6):** 1233-8.

Sori, T (2005). Contagious Bovine Pleuropneumonia (CBPP) Post-Vaccinal Complication in Ethiopia. *International Journal of Applied Research in Veterinary Medicine,* **3:** 344-350.

Tambi, N. E., Maina, W. O. and Ndi, C. (2006). An estimation of the economic impact of contagious bovine pleuropneumonia in Africa. *Revue Scientifique et Technique; Office International des Epizooties,* **25:** 999-1012.

Thiaucourt, F., Aboubakar, Y., Wesonga H., Manso-Silvan, L. and Blanchard, A. (2004). CBPP vaccines and control strategies: recent data. In: Schudel, A and Lombard, M (eds.) Control of Infectious Animal Diseases by Vaccination. *Developments in Biologicals*, **119:** 99-111.

Thiaucourt, F., Yaya, A., Wesonga, H., Huebschle, O. J., Tulasne, J. J. and Provost, A. (2000). Contagious Bovine pleuropneumonia. A Reassessment of the Efficacy of Vaccines Used in Africa. *Annals of the New york Academy of Sciences,* **916:** 71-80.

Thiaucourt, F., Dedieu, L., Maillard, J. C., Bonnet, P.M., Lesnoff, M., Laval, G. and Provost, A. (2003). Contagious bovine pleuropneumonia vaccine, historic highlights, present situation and hopes. In: *Vaccines for OIE List A and Emerging Animal Diseases.* Brown, F and Roth, J (Eds). *Developments in Biologicals*, **114:** 147-160.

Thiaucourt, F., Niang, M., Sery, A., Wesonga, H., Wade, A., Muuka, G., Fekadu, K., Manso-Silván, L., Totte, P. and Rodrigues, V. (2012). Poster on Contagious Bovine Pleuropneumonia Need for different types of vaccines? 19th meeting of the International Organization for Mycoplasmology, 15th-19th July, 2012, Toulouse, France.

Thomson, G. R. (2005). Contagious Bovine Pleuropneumonia and poverty: A strategy for addressing the effects of the disease in sub-Saharan Africa. Research report, DFID animal health programme centre for tropical veterinary medicine. University of Edinburgh, UK.

Thornton, P. K., Kruska, R. L., Henniger, N., Kristjanson, P. M., Reid, R. S., Atieno, F., Odero, A. N. and Ndegwa, T. (2002). Mapping poverty and livestock in the developing world. Nairobi, Kenya. International Livestock Research Institute (ILRI).

Turner, A. W. (1954). Epidemiological characteristics of CBPP. *Australian Veterinary Journal,* **30:** 312-317.

Turner, A. W. (1960). Growth-inhibition tests with Mycoplasma mycoides as a basis for chemotherapy and selective culture media. *Australian Veterinary Journal,* **36:** 221-224.

Valdiviero-Garcia, A., Rosendal, S. and Serebrin, S. (1989). Adherence of *Mycoplasma mycoides* subspecies *mycoides* to Cultured Endothelial Cells. *Zentralblatt fur Bakteriologie,* **272:** 210-215.

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

Vilei, E. M., Correa, I., Ferronha, H., Bischof, D. and Frey, J. (2007). B-D-Glucoside utilization by *Mycoplasma mycoides* subsp. *mycoides* Small Colony: possible involvement in the control of cytotoxicity towards bovine lung cells. *BMC Microbiology,* **7:**31.

Villacres-Eriksson, M. (1995). Antigen presentation by naïve macrophages, dendritic cells and B cells to primed T lymphocytes and their cytokine production following exposure to immunostimulating complexes. *Clinical and Experimental Immunology,* **102:** 46-52.

Wanyoike, S. W. (1999). Assessment and Mapping of CBPP in Kenya: Past and present. Master of Science Thesis. *Freie Universitat Berlin and Addis Ababa University*.

Wanyoike, S. W. (2003). Report of consultative group meeting on CBPP in Rome, Italy.

Wanyoike, S. W., Bendat, A. K. and Lung'aho, W. (2004). The use of the field CFT in the diagnosis and control of CBPP-The Kenyan experience-successes and failures: In towards sustainable CBPP control Programmes for Africa. Proceedings of the third meeting of Food and Agricultural Organization-Office International des Epizooties-African Union/InterAfrican Bureau for Animal Resources-International Atomic Energy Agency (FAO-OIE-AU/IBAR-IAEA). Consultative group meeting on CBPP. 12th-14th November, 2003. Rome, Italy. Page 184-195.

Wesonga, H. O. and Thiaucourt, F. (2000). Experimental studies on efficacy of T1-SR and T1/44 vaccine strains of Mycoplasma mycoides subsp. mycoides Small Colony. *Revue. d'elevage et de medecine veterinaire des pays tropicaux,* **53:** 313-318.

Wesonga, H., Manso Silvan, L. and Thiaucourt, F. (2004). CBPP vaccine strain T1/44: possible reversion to virulence. Report of the Third Meeting of the Food and Agricultural Organization-Office International des Epizooties-African Union/ InterAfrican Bureau for Animal Resources-International Atomic Energy Agency. Consultative Group meeting on Contagious Bovine Pleuropneumonia (CBPP). Rome, Italy. 12-14 November 2003. FAO, Rome, pg. 98-99.

Westberg, J., Persson, A., Holmberg, A., Goesmann, A., Lundeberg, J., Johansson, K. E., Pettersson, B. and Uhle´n, M. (2004). The genome sequence of *Mycoplasma mycoides* subsp. *mycoides* Small Colony type, strain PG1T, the causative agent of contagious bovine pleuropneumonia (CBPP). *Genome Research,* **14:** 221–227.

Windsor, R. S. and Wood, W. A. (1998). Contagious bovine pleuropneumonia. The costs of control in Central/Southern Africa. *Annals of the New York Academy of Science,* **29;849:** 299-306.

Windsor, R. S. (2000). The eradication of Contagious bovine pleuropneumonia from South Western Africa: a plan of action. *Annals of the New York Academy of Science,* **916:** 326-332.

Yaya, A., Golsia, R., Hamadou, B., Amaro, A. and Thiaucourt, F. (1999). Comparative trial of two vaccine strains for CBPP, T1/44 and T1-SR, in Cameroon, R*evue d elevage et de medecine veterinaire des* pays *tropicaux,* **52:** 171-179.

APPENDICES

Appendix 1:

a) Preparation of Newings tryptose broth (quantities per 1 Litre)

Tryptose (20gms), Glucose (5gms), Sodium chloride (5gms), Thallous acetate (0.5gms), Disodium hydrogen phosphate (2.5gms), Glycerol (5mls), Penicillin ½ vial (50ml), 0.5% Phenol red (4mls), Fresh yeast extract (10mls), 25% Sodium pyruvate (2gms), Pig serum (150cc). These are added to distilled water and made up to 1 litre. Adjust PH to between 7.6-7.8 with NaOH and HCL. Then sterilise by filtration.

b) Alsever solution for Complement Fixation Test (quantities per 1 Litre)

Glucose (18.66gms), Sodium chloride (4.18gms), Tri-sodium citrate (8.00gms), Citric acid (0.55gms). Add water up to 1000mls and sterilise by filtration.

c) Veronal buffer with calcium and magnesium (VCM)

Sodium chloride (8.5gms), Barbitone (0.575gms), Sodium barbitone (0.185gms), anhydrous calcium chloride (0.028gms), $6H_2O$ Magnesium chloride (0.168gms). Top up with 1000mls distilled water. Adjust pH to 7.2.

d) Tryptose agar (quantities per 1 Litre)

Tryptose sugar (20gms), Sodium chloride (5.0gms), Glycerol (5.0mls), Anhydrous disodium phosphate (2.5gms). Add 1000mls distilled water and adjust pH between 7.6 to 7.8 with 1M NaOH. Add Bacteriological Agar 15gms. Mix well and dissolve by heating. Autoclave at 120°C for 15 minutes. Pig serum is added to the above autoclaved portion. It is prepared as follows: Sterilized pig serum (900mls), Penicillin 1,000,000 each (1vial), Glucose (15gms), Fresh yeast extract-30ml, and 1% thallous acetate-3 ml (0.3gms). Mix and filter to sterilize the serum before addition to the autoclaved portion.

Appendix 2: Number of animals with swellings and mean of measurements of the swellings in groups KE1 to KE9 over a period of 48 days post-vaccination

Groups	KE1	KE2	KE3	KE4	KE6	KE8	KE9
Number of animals with swellings	1	10	1	10	10	8	10
Date							
Pre-vaccination	0	0	0	0	0	0	0
4.8.11	0	1	0	3.3	5.9	4.7	5.7
6.8.11	0	4.9	0	3.8	6	3.4	5.8
9.8.11	0	6.4	0	3.3	5	2.8	6.2
12.8.11	0.1	6.9	0	5.6	7.1	2.3	4.8
14.8.11	0	5.9	0	5.7	6.8	2.1	5.6
20.8.11	0.1	3.8	0.6	3.7	4.2	2.9	5.6
28.8.11	0	3	2	2.1	3.3	1.7	3.3
9.9.11	0	2.3	2.4	1.5	3.8	2.2	2.7
17.9.11	0	1.8	2.1	1.3	3.4	1.8	2.1
18.9.11	0	1.8	2	1.2	3.3	1	2.7
20.9.11	0	2.1	0.6	1.3	2.8	1.3	2.6