A COMPARISON OF THE STABILITY, SAFETY AND EFFICACY OF TWO CONTAGIOUS BOVINE PLEUROPNEUMONIA VACCINE FORMULATIONS

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

The Nkando's family

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LIST OF ABBREVIATIONS

Ag	Antigen
ANOVA	Analysis of Variance
AUIBAR	African Union-InterAfrican Bureau for Animal Resources
$CaCl_2$	Calcium Chloride
CBPP	Contagious Bovine Pleuropneumonia
Cc	Conjugate Control
ccu	Colour changing units
c-ELISA	Competitive Enzyme Linked Immunosorbent Assay
CFT	Complement Fixation Test
cfu	Colony forming units
Cm	Monoclonal Control
cm	Centimetre
CO_2	Carbon dioxide
EMPRES	Emergency Prevention System
FAO	Food and Agricultural Organization
g	Gram
HEPES	Hydroxyethyl Piperazine Ethanesulfonic Acid
H_2O	Water
HS	Haemolytic System
H_2SO_4	Sulfuric Acid
IgG	Immunoglobin G
KARI	Kenya Agricultural Research Institute

$MgSO_4$	Magnesium Sulfate
$MgCl_2$	Magnesium Chloride
ml	Millilitre
MmmSC	Mycoplasma mycoides subspecies mycoides Small Colony
NaCl	Sodium Chloride
OD	Optical Density
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
PI	Percentage Inhibition
rpm	Revolution Per Minute
SAT	Slide Agglutination Test
TMB	Tetramethyl Benzidine
UK	United Kingdom
USA	United States of America
VCM	Veronal Buffer with Calcium and Magnesium
VRC	Veterinary Research Centre
VVPC	Veterinary Vaccine Production Centre

Abstract

A study was carried out to evaluate a modified contagious bovine pleuropneumonia (CBPP) vaccine against the standard (currently in use) vaccine. The objective was to determine whether the modified vaccine was superior to the standard one with a view to recommend for field use. Both vaccines were lyophilized preparations from the T_1 vaccine strain of *Mycoplasma mycoides* subsp. *mycoides* biotype small colony (*Mmm*SC), grown in different types of media and reconstituted in different types of diluents. The two vaccines were compared in terms of stability, safety and efficacy. To determine stability, the two vaccines were reconstituted, and then aliquoted into three parts each. One aliquot from each vaccine was then stored at +4°C, 25°C and 37°C respectively. The number of viable *Mycoplasma* microorganisms from each aliquot was then estimated at intervals of 0, 1, 2, 4, 8, 12, 24, 48, 72 and 168 hours using the microtitration method. The safety was determined by vaccinating two groups of randomized cattle at the tail-tip, and then determining changes in tail-tip circumferences and rectal temperatures. A control group that comprised of unvaccinated animals was included. The efficacy of the two vaccines was determined by challenging the vaccinated and the non-vaccinated animals each with 60ml of a *Mycoplasma* broth culture containing 3×10^8 colony forming units (CFU) of *Mmm*SC, three months post vaccination. The challenge was done using a predetermined nasotracheal intubation method aided by a bronchoscope. The challenge method had been predetermined by infecting sixteen animals each with 60ml of a *Mycoplasma* broth culture containing 3×10^8 CFU deposited at the tracheal bifurcation using a nasotracheal bronchoscope. The efficacy of the two vaccines was compared by their protection rates which were calculated by first determining the pathology score in each animal. The pathology score was calculated using a lesion score (determined by the size and severity of the lung lesion) and a factor determined by the lesion score and whether or

not *Mycoplasma* organisms were isolated from the lung lesion. The pathology scores were transformed into protection rates expressed as a percentage of one less the mean score of each group divided by the mean score of the control group.

From the results obtained in stability studies, it was observed that there was a significant ($P < 0.001$) titre drop in the standard vaccine at all temperatures and at all the intervals, compared to the modified vaccine. In the safety studies, changes in tail-tip circumferences in the animals vaccinated with the standard vaccine were more severe than those vaccinated with the modified vaccine ($P < 0.001$), while rectal temperatures in both groups remained within the normal range. From the results obtained in the efficacy studies, there was no difference in protection rates between the two vaccines ($P > 0.05$).

From the results of this study, it was concluded that the modified vaccine has a higher stability than the standard vaccine. Further, the modified vaccine was safer than the standard one. However, the modified vaccine was not superior to the standard vaccine in terms of efficacy. On the basis of higher stability and safety, it was therefore concluded that the modified vaccine should be recommended for field use to replace the standard one. However, further modifications of the vaccine are needed with a view to increase the efficacy.

CHAPTER ONE

1.0. GENERAL INTRODUCTION

Contagious Bovine Pleuropneumonia (CBPP) is a respiratory illness in cattle caused by *Mycoplasma mycoides* subsp. *mycoides* biotype small colony (*Mmm*SC), and characterized by the presence of serofibrinous interstitial pneumonia and chronic lesions (sequestra) in the lungs. It is the only bacterial disease in the list 'A' diseases of the Office International des Epizooties (OIE). The disease is a major cause of exclusion of infected countries and zones from international trade in cattle and cattle products (Dedieu *et al.*, 2005). In Africa, CBPP is considered to be the most important animal disease, affecting at least 27 countries (Anonymous, 2000; Roeder *et al.*, 1999). Due to its potential for transboundary transmissibility and serious socioeconomic consequences (Rweyemamu and Benkirane, 1996), it is one of the prioritised diseases under Food and Agricultural Organization Emergency Prevention System (FAO EMPRES). In addition, countries that are free of this epidemic are continuously threatened by reemerging infections.

In Africa, CBPP causes losses estimated at US$ 2 billion per annum (Masiga *et al.*, 1998). Efforts at controlling the disease in many of the affected African countries take most of the resources of the veterinary services. In Kenya, some regions have been under permanent quarantine for over 40 years due to CBPP (Wesonga, personal communication). Further, cattle from these regions have to undergo rigorous testing before they are allowed to move into clean or buffer zones, thus limiting their access to local markets and excluding them entirely from the international markets.

Contagious Bovine Pleuropneumonia is mainly controlled by vaccination, quarantine, movement control, and stamping out policies. However, non-vaccine based control measures cannot realistically be applied in Africa (March, 2004). This is due to poor economies, fragmentation or poor veterinary services, and nomadic and transboundary movements of cattle. Effective vaccination is therefore the only realistic policy for CBPP containment on the continent. Contagious Bovine Pleuropneumonia vaccines, including the current $T_1$44 and T_1sr (streptomycin-resistance) vaccines however, have limitations that make their delivery in the field difficult. The vaccines are prepared from broth cultures of live attenuated *Mycoplasma mycoides* subsp. *mycoides (Mmm*SC*)* and are generally considered to be of poor stability (Rweyemamu *et al.*, 1995; Thiaucourt *et al.*, 2000). Although the vaccines are freeze-dried to reduce the requirement for cold-chain transport in the field, they must however be used within 2 hours following reconstitution, to avoid titre loss in the high ambient temperatures found in African field conditions (Provost *et al.*, 1987). The short shelf-life is attributed to a rapid fall in the pH, which is known to affect the titre of the *Mycoplasma* organisms in the vaccines. The best growth of *Mmm*SC is achieved between pH 7 and 8, the optimum being pH 7.4; a decrease in pH to less than 6.5 causes cessation of growth and rapid death of cells (Rodwell and Mitchell, 1979). Further, the vaccines lack an indicator for pH changes. A modified CBPP vaccine has been developed to address these limitations. The modification is based on addition of a pH buffer system, N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), with a starting pH of 8.0 to the growth media. Further, the magnesium sulfate ($MgSO_4$) reconstitution fluid is substituted by phosphate buffered saline (PBS) which also contains a pH indicator. The modifications are based on observations that pH in both growth media and reconstitution fluid can reach levels suboptimal for survival of *Mmm*SC (Waite and March, 2001) and vaccine

efficacy (Pollack *et al.,* 1969). These changes are expected to buffer a drop in pH, and so increase the shelf-life of the vaccine especially after reconstitution. The inclusion of a pH indicator, phenol red, in the new vaccine will enable users to monitor pH changes and discard the vaccine if the pH falls below the critical level. These new features are expected to widen vaccine coverage in the field, including remote areas that are difficult to cover by the current vaccine. However, the expected improved shelf-life need to be validated before recommending the vaccine for field use.

In addition to the poor stability, the current $T_1$44 and T_1sr vaccines are also considered to be of poor efficacy. Despite vaccination campaigns using freeze-dried broth cultures of live attenuated *Mycoplasma mycoides* subsp. *mycoides* small colony biotype (*Mmm*SC) (strain $T_1$44 or T_1sr), there has been a substantial re-emergence of the disease (March, 2004). Observations from the field (Masiga *et al.,* 1996; 1998) and experimental studies (Thiaucourt *et al.,* 2000; Yaya *et al.,* 1999) have indicated that the current vaccines do not effectively protect cattle from outbreaks of disease, especially with primary vaccination. The development of an efficacious and safe vaccine is therefore important.

The efficacy of $T_1$44 has been evaluated on several occasions (Thiaucourt *et al.,* 2000); however, there is not enough data available on its biological safety and potential residual virulence. In addition, $T_1$44 is known to cause severe post-vaccinal reactions in some cattle.

It is therefore also necessary to establish if modifications in the new vaccine have any improvements on the safety and efficacy of the vaccine before recommending it for field use.

The determination of the efficacy of vaccine requires an efficient infection and challenge method. A validated *in vitro* or laboratory animal model lacks in CBPP experimental studies. A report by Smith (1971) cites the use of a mouse model. However, results obtained in a mouse model may not necessarily represent the situation in the target species (March *et al.*, 2006). This means that all CBPP experimental trials have to be performed on the natural bovine host. The natural method of infection is through close contact with infected animals (Provost *et al.*, 1987). Hudson and Turner (1963) infected animals by intrabronchial intubation with a virulent strain of *Mycoplasma mycoides*. However, other studies have reported relative difficulty in reproducing the disease by intubation (Gourlay and Howard, 1982; Lloyd and Etheridge, 1983; Wesonga and Thiaucourt, 2000). These previous procedures involved sedation of animals which necessitated them to lie on lateral recumbency; followed by insertion of a tube through the mouth into the trachea or bronchi, where the infection culture was deposited. This operation is lengthy as animals take a lot of time before recovery from anesthesia. A reliable and rapid challenge method is therefore needed for the evaluation of the efficacy of CBPP vaccines.

1.1. Justification

In Africa, CBPP is currently the most economically important disease of cattle. Its control takes most of the time and resources of the veterinary services. In Kenya, the disease is responsible for a permanent quarantine in some regions for over 40 years. Animals from these regions therefore have a restricted access to the local markets and are entirely excluded from international markets. Although several control measures have been employed (vaccination, quarantine, movement control, stamping out), the only realistic control measure in Africa is vaccination. This is due to economic, social and cultural conditions.

Current vaccines, which are live attenuated, are unstable under high environmental temperatures generally found in Africa. They also have a low efficacy and cause severe post-vaccinal reactions. In addition, it is unclear to what extent they can protect against newer isolates of the causal organism, for example *Mmm*SC.

These limitations have necessitated modifications in formulations of the vaccines. It is however necessary to establish whether the modified vaccine has an improved stability, safety and efficacy before recommending it for field use.

In order to determine the efficacy of the vaccine, vaccinated animals have to be challenged with live infective organisms. An effective and reproducible infection/challenge method is needed. The current method in use is endobronchial intubation. However, it has been found to be unreliable. There is therefore a need to investigate into a more reliable method.

The study will highly contribute to science and livelihoods of pastoral communities. The modified vaccine will be evaluated and if found superior, will be recommended for control of the disease on the African continent. Effective control of the disease would save time and resources. It would also open markets for local and international livestock trade, thereby improving the economy of the countries as well as livelihoods of pastoralist communities.

1.2. Goal

To enable safe and efficacious vaccination against CBPP, using a stable vaccine.

1.3. The Broad Objective

To establish whether the modified vaccine has improved stability, safety and efficacy before recommending it for field use.

1.4. Specific Objectives

a) To validate the stability of the modified vaccine following reconstitution.

b) To determine the effectiveness of the challenge model by naso-tracheal inoculation with *M.mycoides* (SC) aided by the use of a bronchoscope.

c) To determine the safety of the modified vaccine following vaccination.

d) To determine the efficacy of the modified vaccine 3 months following vaccination.

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Contagious Bovine Pleuropneumonia (CBPP)

Contagious Bovine Pleuropneumonia is such a highly destructive disease in cattle that it is the only bacterial disease included in the World Organization for Animal Health's (OIE) 'A'list of prioritized communicable animal diseases. Under the FAO Emergency Prevention System (EMPRES), CBPP is a priority disease because of its potential for transboundary transmissibility and serious socioeconomic consequences (Rweyemamu and Benkirane, 1996). Disease symptoms range from hyperacute through acute to chronic and subclinical forms (Egwu *et al.*, 1996). Clinical signs of the acute form involve anorexia, nasal discharge, a dry cough, and dyspnoea (Ross, 1993; Scudamore, 1995). The mortality rate in cattle with acute clinical signs varies between 10 and 90% (Kiarie *et al.,* 1996). At necropsy, extensive lesions of pleurisy and pneumonia characterize the disease (Provost *et al.,* 1987). Those surviving may develop chronic pleuropneumonia or recover. In some cattle that apparently recover, pulmonary foci of infection become encapsulated but later release organisms that are then transmitted to other cattle (Hudson, 1971). Cattle that actually recover develop immunity to reinfection (Gourlay, 1975; Masiga and Windsor, 1975; Wittlestone, 1976); the immunity lasting at least three years (Newton and Norris, 2000).

2.1.1. Aetiology

Mycoplasma mycoides subsp. *mycoides* biotype small colony (*Mmm*SC) is the aetiological agent of CBPP (Cottew and Yeats, 1978; Nicholas and Bashiruddin, 1995). The organism is an extracellular pathogen that lives in close association with the host cells. It has a genome size of

1,211 kb (Westburg *et al.*, 2004) and is phylogenetically a member of the *Mycoplasma mycoides* cluster which are pathogens of ruminants. Members of the *M.mycoides* cluster include *M. mycoides* subsp. *mycoides* large colony (*Mmm*LC), *M. mycoides* subsp. *capri*, *M. capricolum* subsp. *capripneumoniae*, *M. capricolum* subsp. *capricolum* and *Mycoplasma* bovine group 7 which is an unnamed group of bovine *Mycoplasma* isolates (Pettersson *et al.*, 1996; Nicholas and Bashiruddin, 1995; Weisburg *et al.*, 1989). *Mycoplasmas* belong to the class *Mollicutes*, whose members lack a cell wall and are pleomorphic. In young cultures they tend to appear as branching filaments, and in old cultures as small coccoid bodies. They are the smallest known self-replicating organisms (Maniloff *et al.*, 1992; Razin *et al.*, 1998).

2.1.2. Host Susceptibility

Naturally, *Mmm*SC affects only the ruminants of the *Bos* genus, i.e. both *Bos taurus* and *Bos indicus* (zebu). There are many reports on breed differences in susceptibility. In general, European breeds are more susceptible than indigenous African breeds (Provost *et al.*, 1987). Differences in age susceptibility have also been reported. Animals under three years are less resistant to experimental challenge (Masiga and Windsor, 1978).

Mycoplasma mycoides (SC) has been isolated from buffaloes (*Bubalus bubalis*) in Italy (Santini *et al.*, 1992), and from sheep and goats in Africa and more recently in Portugal (Brandão, 1995; De Santis *et al.*, 1999; Kusiluka *et al.*, 2000; Srivastava *et al.*, 2000). Although *Mmm*SC has been isolated from pneumonic goats and sheep, their exact role in epidemiology of the disease is unknown at present (Brandão, 1995). Among wild animals, one single case has been reported in American buffaloes (*Bison bison*) but none in African buffaloes (*Syncerus caffer*) (Shifrine,

1967) or other wild ruminants (Leach, 1957). Although it has been reported that the domestic buffalo (*Bubalus bubalis*) is susceptible, the disease is difficult to reproduce experimentally in this species (Provost, 1988). The disease has also been reported in yaks (*Bos grunniens*) and bison (*Bison bonasus*) in zoological gardens (Leach, 1957).

2.1.3. Transmission

Direct and repeated contact between sick and healthy animals is the principal mode of transmission. Alternative routes, however, like wind-borne and indirect transmission, cannot be excluded (Masiga *et al.*, 1996; Regalla and Lefèvre, 1996a). Infection by contamination of inanimate objects is unlikely under natural conditions, but it has been effected experimentally, by feeding cattle with hay contaminated with *Mmm*SC (Windsor and Masiga, 1977). Out of six animals that were infected in this manner, five developed complement-fixing antibody in their sera and three had unequivocal lesions of CBPP where *Mmm*SC was recovered after slaughter. Though several authors (Mahoney, 1954; Martel *et al.*, 1985; Provost *et al.*, 1987 and Egwu *et al.*, 1996) have suggested involvement of chronic carriers in the perpetuation of the infection, the issue is still not resolved. Risk factors for its spread include high-density confinement in night housings and use of common grasslands and watering places (Provost *et al.*, 1987). In Africa, contagion between zones or between countries is essentially related to cattle movements caused by trade, transhumance and social conflicts (Roeder and Rweyemamu, 1995).

2.1.4. Distribution

Historically, CBPP was a disease of Europe, North America and Asia but was eradicated from the United States, Canada and most of Europe in the 19th century prior to the identification of the

causative agent (Provost *et al.*, 1987). The disease is however still endemic in the Iberian Peninsula, Eastern Europe, Asia and Africa. CBPP is present in most sub-Saharan countries such as Uganda and Kenya, where it had been eradicated in the 1970s (Egwu *et al.*, 1996). In addition, Angola, Benin, Cameroon, Chad, Eritrea, Ivory Coast, Ghana, Nigeria, Sudan, Togo and Zaire are infected (Nicholas and Bashiruddin, 1995). The introduction of CBPP into southern Africa from Europe in 1854 and its subsequent spread as far north as Angola are well documented (Windsor, 2000). The East African focus has advanced south into Tanzania (Bolske *et al.*, 1995) and subsequently spread throughout most regions of that country. The long-standing focus in Angola and northern Namibia has again invaded Zambia (Mariner *et al.*, 2006b). Contributory factors to this current resurgence are thought to include the breakdown of veterinary services (Provost, 1996), increased and unrestricted cattle movements due to drought, war, and civil strife (Windsor and Wood, 1998), and a lack of vaccine efficacy.

In Kenya, the disease is endemic in the North and Northeast of the country from where it periodically spreads through uncontrolled cattle movement to cause epidemic outbreaks in the Central and Southern parts of the country. Narok District, which is contiguous with neighbouring Ngorongoro district of Tanzania is endemically infected (Masiga *et al.*, 1998).

2.1.5. Economic Importance

Annual economic losses arising from the disease in the African continent have been estimated at US$ 2 billion (Masiga *et al.*, 1998), arguably making it the most important cattle disease in Sub-Saharan Africa. It causes greater losses in cattle than any other disease, including rinderpest (OIE, 1995). The direct losses are from mortalities, reduced milk yield, vaccination costs,

treatment costs, disease surveillance and research programmes. The indirect costs are due to the chronic nature of the disease, which causes loss in production and reproduction, and loss of cattle trade due to quarantines. To some communities like the Maasai in Kenya, sociological losses probably outweigh financial losses.

2.1.6. Diagnosis

A diagnosis based on history of contact with infected animals, clinical findings, serology, necropsy findings and microbiological examination is necessary. Detection requires measurement of serum antibodies (Dannacher *et al.*, 1986) or isolation and identification of *M.mycoides* subsp. *mycoides* from infected organs (Bashiruddin *et al.*, 1994). Serological diagnosis is difficult because of complex cross-reactions with other *Mycoplasma* strains within the mycoides cluster (Nicholas and Bashiruddin, 1995; Poumarat *et al.*, 1992; Taylor *et al.*, 1992).

Whereas many tools are available for CBPP diagnosis, including Polymerase Chain Reaction (PCR) techniques, there is no single test that can detect all infected animals.

The current OIE-prescribed test for the diagnosis of CBPP is the modified complement fixation test (CFT) (Campbell and Turner, 1953; OIE, 2002). Although the test is highly specific, it is relatively expensive to perform, slow and requires highly trained personnel to perform it accurately and consistently. In addition, it is less effective at detecting the disease in early or chronic stages of infection (OIE, 2002). A number of tests have recently been described, including biochemical (Rice *et al.*, 2000) indirect and competitive enzyme-linked immunosorbent assay (ELISA) (Le Goff and Thiaucourt, 1998; Nicholas *et al.*, 1996), immunoblotting (Nicholas *et al.*, 1996; Regalla *et al.*, 2000), and PCR (Bashiruddin *et al.*, 1994; Miserez *et al.*, 1997). Rapid slide agglutination tests for CBPP diagnosis by using *Mmm*SC CFT antigen mixed with a

drop of whole blood or serum have previously been described (Priestley, 1951; Turner and Etheridge, 1963). Diagnosis of CBPP however still requires culture and identification of *M. mycoides* SC though PCRs can facilitate diagnosis. Clinically and even pathologically, CBPP may be confused with other pneumonic conditions, most especially bovine pasteurellosis. However, bovine pasteurellosis would likely spread much more rapidly and consequently the epidemiologic picture may be different.

2.1.7. Treatment

Treatment of affected cattle with antimicrobial drugs is officially discouraged. However, in many African countries, treatment of clinical CBPP cases is now a standard field practice; and livestock owners have attested to its beneficial effects. Many antibiotics have been shown to be active in *in vitro* assays. This is the case of tetracyclines, macrolides, lincosamines, streptogramines and quinolones (Ayling, 2000). In the field, such antibiotics were used successfully to treat post-vaccinal reactions when using strain T1 44 (Lindley, 1971). Recent studies by Mariner *et al.*, (2006a) reveal that using treatment to reduce the infectious period by 50% resulted in a 64% reduction in mortality and a reduction in the prevalence of infected herds from 75.4% to 33.2%. In light of this, effective control of CBPP using feasible treatment regime can reduce transmission by decreasing the duration of infection and the effective reproductive number of *Mycoplasma* organisms (Tambi *et al.*, 2006). Although antibiotics, including tetracycline have been used in many African countries to treat CBPP (Mariner and Catley, 2004), their use is not recommended because treatment does not completely eliminate *M.mycoides* subsp. *mycoides* colonization, resulting in carriers that can infect susceptible cattle (Hudson, 1971). There is also a fear that they can promote the re-emergence of resistant *Mycoplasma* strains and antibiotic

residues in human food (Wesonga and Thiaucourt, 2000). However, tylosin is highly effective in control of excessive vaccination reactions and should be of value in the treatment of clinical cases (Egwu *et al.*, 1996).

2.1.8. Prevention and Control

Successful control of the spread of CBPP rests on removing susceptible animals from any possible contact with CBPP-infected animals, whether clinically affected or subclinical carriers. There are four essential tools in CBPP control and eradication. These are vaccination, quarantine, animal movement control and stamping out. Each control measure acts by reducing the effective reproductive number of the agent in the population (Tambi *et al.*, 2006). However, not all these measures are used by countries to control CBPP. The current policy advocated by the African Union Interafrican Bureau for Animal Resources (AUIBAR) (AU/IBAR, 2002) for the control of CBPP requires collection of epidemiological data and information to determine and detect foci of infection; effective control of animal movements from and towards these foci; mass vaccination of cattle regularly for at least 5 consecutive years; and repeat vaccination of the same animal each year.

Ideally, CBPP control is achieved by eliminating the whole cattle herd population wherever the disease is detected i.e. stamping-out. However, this may not prove realistic as it is considered too costly and logistically difficult to apply. Many governments cannot afford the cost of compensation to the cattle owners whose cattle are slaughtered. Eradication has recently been achieved by stamping out policies in Botswana (Masupu *et al.*, 1997) but its cost has been

tremendous and it is unlikely that other African countries can afford it (Wesonga and Thiaucourt, 2000).

Effective control of animal movements is impossible in most sub-Saharan Africa. This is mainly due to pastoralism and transhumance (Dedieu *et al.*, 2005) practiced by many ethnic groups in the arid and semi-arid areas of Africa. An inter-tribal or inter-clan cattle raiding is also a great hindrance to an effective animal movement control in the region. Other factors behind poor animal movement control strategies are logistical, financial and political difficulties that render animal health authorities increasingly incapable of enforcing laws and regulations in relation to cattle movements (Thomson, 2005).

Based on a combination of stamping-out policy, control of cattle movement and quarantine, CBPP has been eradicated from numerous countries such as Australia, USA and many European countries. These measures are impracticable in Africa where nomadism and transhumance are a necessity (Dedieu *et al.*, 2005). In Africa, the only realistic prophylaxis has to rely on vaccination.

The history of CBPP vaccination dates back to 1852, when Willems established that subcutaneous inoculation of CBPP-infective lymph into thick connective tissue protected cattle against contact challenge (Willems, 1852). This immunization procedure was widely used in Europe and South Africa until the policy of eradication by stamping-out was adopted (Turner, 1959). Since then, various types of vaccines have been tested (Turner, 1959; Provost, 1974; Provost *et al.*, 1987). Vaccination with either inactivated (Garba and Terry, 1986; Gray *et al.*, 1986) or attenuated (Brown *et al.*, 1965; Hudson, 1965; Masiga and Windsor, 1974; Garba *et al.*,

1991) vaccines has been used to prevent CBPP. The attenuated CBPP vaccine has been used in combination with the rinderpest vaccine (Provost, 1970) demonstrating its use in multivalent vaccines (Kiarie *et al.*, 1996). According to official documents at least 5 different vaccine formulations have been used for CBPP control with varying degrees of success (Huebschle *et al.*, 2004). Amongst these vaccines were the Kabete vaccine strain (1930's), vaccine strain KH3J (1970's), vaccine strain V_5 (1970's and 1980's), vaccine strain T_1 sr (1980's and early 1990's) and presently vaccine strain T_1 44 (mid 1990's to date). The live vaccines strains which have been used in Africa are the KH3J and the T1 (Karst, 1971; Howard and Taylor, 1985). They offer short-term immunity, but can cause adverse reactions and even CBPP itself (Mbulu *et al.*, 2004). When these vaccine strains were adapted to grow in streptomycin-containing media, less pathogenic variants were obtained, designated KH3J-SR and T_1-SR. The $T_1$44 and T_1-SR were constrained by lower potency and efficacy (Tulasne *et al.*, 1996; Masiga and Domenech, 1995). The KH3J and its variants is no longer used. Changes of vaccine formulations were brought about either due to serious side reactions or due to poor immune response as judged by failing protection (Huebschle *et al.*, 2004). Nowadays CBPP prophylaxis in Africa relies on the use of empirically attenuated T_1 strain (Sheriff and Piercy, 1952). Two strains are used for preparing CBPP vaccines; strain $T_1$44 (a naturally mild strain isolated in 1951 by Sheriff and Piercy in Tanzania, and passaged 10 times in embryonated eggs and then 44 times in broth culture), and streptomycin-resistant derivative (T1sr) (Brown *et al.*, 1965; Provost, 1982). However, the efficacy of the T_1 vaccines is low and they induce only short-term protection, making annual vaccination necessary to achieve a sufficient level of protection (Thiaucourt *et al.*, 2000). In addition, these vaccines are known to retain some virulence (Revell, 1973; Thiaucourt *et al.*,

2004b). Consequently, the development of an improved vaccine is a prerequisite for the eradication of CBPP in Africa.

CHAPTER THREE

3.0. COMPARISON OF THE STABILITY OF THE STANDARD AND THE MODIFIED CBPP VACCINES FOLLOWING RECONSTITUTION

Abstract

A study was carried out to compare the stability of two contagious bovine pleuropneumonia (CBPP) vaccines, namely the standard (currently in use) and the modified vaccine. Both vaccines were lyophilized preparations from the T_1 vaccine strain of *Mycoplasma mycoides* subsp. *mycoides* biotype small colony (*Mmm*SC), grown in different types of media. The *Mmm*SC that were used to make the standard vaccine were grown in the standard media while those that were used to make the modified vaccine were grown in media modified by addition of a pH buffer. The standard vaccine was reconstituted in the recommended normal saline while the modified vaccine was reconstituted with the recommended phosphate buffered saline (PBS). The two vaccines were then aliquoted and stored at +4°C, 25°C and 37°C. The number of viable microorganisms was then estimated at intervals of 0, 1, 2, 4, 8, 12, 24, 48, 72 and 168 hours using the microtitration method. From the results obtained it was observed that there was a significant ($P<0.001$) titre drop in standard vaccine at all temperatures (+4°C, 25°C and 37°C) and for all the intervals, compared to the modified vaccine. High titres (≥ 10^7 viable CFU) were observed in the modified vaccine at 37°C for a period of 72hours as compared to the standard vaccine. From these results it was concluded that the modified vaccine exhibits a higher stability than the standard vaccine. Titres of the modified vaccine generally remained above the OIE-recommended immunizing dose of 10^7 CFU.

The considerable stability of the modified vaccine culture particularly at higher temperatures would reduce the incidence of vaccine failure. In addition, immediate use of the reconstituted freeze-dried vaccine would not be necessary in the field.

3.1. Introduction

Contagious Bovine Pleuropneumonia vaccine ($T_1$44 and T_1sr) is a live attenuated vaccine, and therefore its stability under field conditions and its correct administration are very critical to vaccine efficacy. For a long time, liquid culture vaccines were successfully used to control CBPP especially in East Africa and Australia (Brown *et al.,* 1965; Hudson, 1965). However, because *Mycoplasmas* are heat and acid labile such fluid vaccines had to be kept refrigerated and used within one month from the time of harvest (Hudson, 1965).

With a view to increase the shelf-life and also to facilitate ease of handling, freeze-drying has progressively been adopted and is now the most widely employed method for preserving such liquid vaccine cultures (Provost, 1970). As a result, all CBPP vaccines in current use in Africa are lyophilized products. Although relatively heat resistant, freeze-dried vaccines would still need to be preserved frozen (-20°C or below) in order to conserve the viability of the *Mycoplasmas* (Litamoi *et al.,* 2005). CBPP vaccines stored in such a manner remain potent for up to 2 years without significant loss of titre (Litamoi, unpublished data). Recently, Litamoi *et al.* (2005), demonstrated use of a system (code-named *xerovac*) of CBPP vaccine dehydration that renders the product more heat resistant with a view to decreasing dependence on vaccine cold storage. Although the technique seems to yield a relatively thermostable vaccine for CBPP, the long lyophilization period involved would require high-energy consumption and thus increased vaccine production cost.

One of the major factors behind poor vaccine efficacy is likely to be sub-optimum bacterial titres (March *et al.,* 2002). It has been reported that many vaccine production laboratories do not reach the OIE recommendation of delivering a vaccine at 10^8 viable *Mycoplasmas* per animal dose (which allows for losses during lyophilization, storage and transport) (Rweyemamu *et al.*, 1995;

Litamoi and Seck, 1999). To reduce the requirement for cold-chain transport in the field and to allow for longer-term storage at -20°C, current vaccines are freeze-dried. Reconstitution is performed using 1M solution of magnesium sulfate (O.I.E, 2000), which has been reported to exhibit a thermoprotective effect compared to the use of saline or distilled water alone (Provost, 1970; Provost *et al.,* 1987). It has been shown that aqueous solutions of certain divalent cations e.g. 1M $MgSO_4$ confer a certain degree of heat stability to CBPP vaccine when used as the vaccine reconstitution fluid (Provost *et al.,* 1987). However, March *et al.,* (2002) observed a significant drop in pH when vaccines were reconstituted in 1M Mg SO_4, thus reducing rapidly the viability of *Mycoplasma* cultures (up to 6 $\log_{10}$ in titre). Subsequently, following reconstitution, the vaccines must be used within 2 hours to avoid unacceptable titre loss in the high ambient temperatures found in African field conditions (Provost *et al.,* 1987). This has necessitated improvement in vaccine formulation including the use of different growth media and diluents to improve titres and thermal stability (March, 2004). In this study however, it is worth noting that normal saline was used as a diluent for the standard vaccine instead of a molar solution of $MgSO_4$; since no country advocates the use of $MgSO_4$ as a diluent for the CBPP vaccines in the field.

The aim of the present study was to verify whether the modification of the vaccine in terms of the growth media which contains the buffer, and the different diluent, increases the stability of the vaccine, as compared to the standard vaccine.

3.2. Materials and Methods

3.2.1. Vaccine Reconstitution and Titration

Five (5ml) of each diluent (normal saline and PBS for the standard and the modified vaccine respectively) were used to reconstitute each of the freeze-dried vaccine vial. Each of the reconstituted vaccine was transferred to a universal bottle. From each vaccine, a 10-fold dilution series from 10^{-1} to 10^{-10} was prepared in 4 ml bottles containing 2.7ml of the standard Gourlay media. The remainder of the reconstituted vaccine was aliquoted into three bottles and placed at +4°C, 25°C and 37°C respectively for preparation of more dilutions at 1, 2, 4, 8, 12, 24, 48, 72 and 168 hours post reconstitution. The viable *Mycoplasma* contents in the serial dilutions were then determined using microtitration method.

3.2.2. Determination of Viable *Mycoplasma* Content of the Vaccine

Determination of *Mmm*SC viable counts was carried out immediately after preparation of serial dilutions. Microtitration was performed using the method of Thiaucourt and Di Maria, (1992), as follows:

From stock Gourlay medium, 100µl was dispensed into each well of columns 1 to 10 of the 96-well microtitre plate. Two hundred (200µl) of the same media was also dispensed into each well of column 11 and 12. To each well of row H from columns 1 to 10, 100µl of vaccine dilution 10^{-10} was added. This process was repeated for each dilution from row G (10^{-9}) to A (10^{-3}). Dilution 10^{-1} and 10^{-2} were not used. Thereafter, each microplate was sealed and incubated at 37°C and at an atmosphere of 5% CO_2 for 10days. The microplate cultures were examined daily using a plate-reading mirror for evidence of growth as indicated by colour change of the medium in the wells.

from pink to yellow. This procedure was repeated for all the dilutions made at respective temperatures and intervals.

3.2.3. Calculation of Titre

Following titrations on microplates, the viable *Mycoplasma* content was estimated by using Spearman-Karber Formula. The 50% end points calculated using this formula were expressed as colour changing units (ccu_{50} log_{10}) (Litamoi *et al.*, 1996). The titres given were geometric means of tests.

According to the Spearman-Karber formula:

Log_{10} Median Dose = $(X_0 - (d/2) + d(\Sigma r_i/n_i)$

Where: X_0 = Log_{10} of the reciprocal of the lowest dilution at which all test inocula are positive.

d = log_{10} of the dilution factor (i.e. the difference between the log dilution intervals)

n_i = number of test inocula used at each individual dilution (after discounting accidental losses)

r_i = number of positive test inocula (out of n_i)

$\Sigma(r_i/n_i)$ = $\Sigma(P)$ = Sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive result.

Summation is started at dilution X_0

3.2.4. Statistical Analysis

Analysis of variance (ANOVA) was used to determine if there were differences in titres between the two vaccines at different temperatures and periods. GenStat statistical software (9^{th} edition) was used in the analysis.

The difference between means in the variables (vaccine, temperature and time) was analyzed.

P-values less than 0.001 were considered significant.

3.3. Results

3.3.1. Microtitration

Figure 3.1a, b and c refers to the titres of the two vaccines. Overall titres of cultures of the modified vaccine were higher than those of the standard vaccine at all temperatures and for all periods.

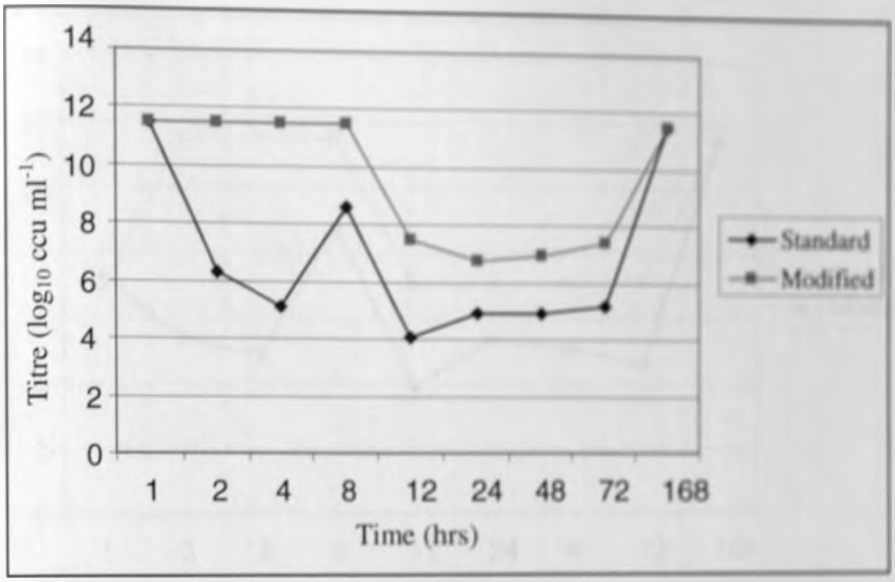

Figure 3.1a: Titres of *Mycoplasma* organisms for the standard and the modified vaccine incubated at +4°C after reconstitution, at various intervals between hr 1 and 168.

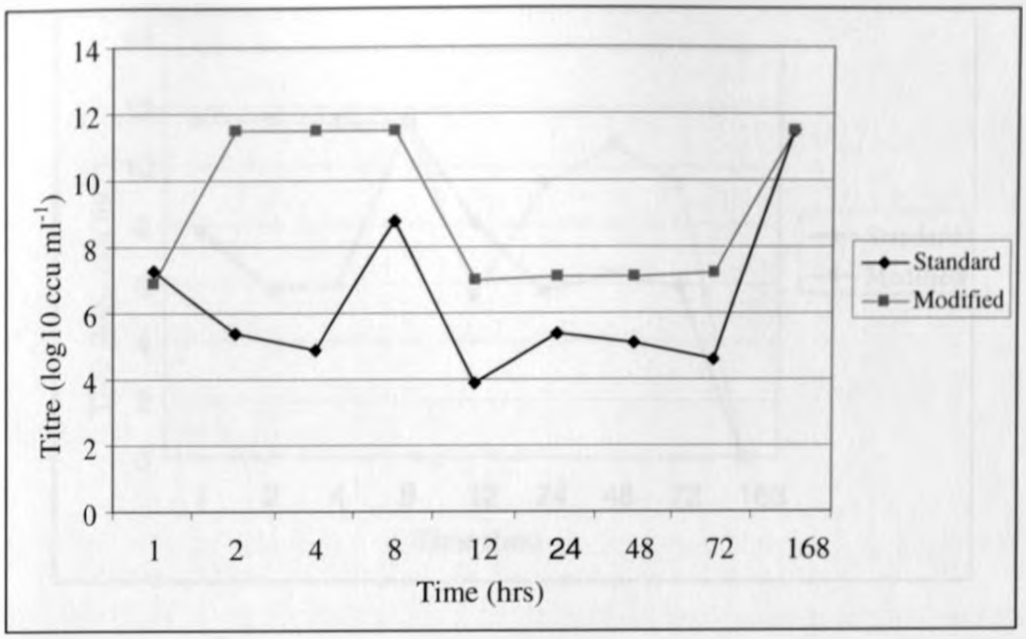

Figure 3.1b: Titres of *Mycoplasma* organisms for the standard and the modified vaccine incubated at 25°C after reconstitution, at various intervals between hr 1 and 168.

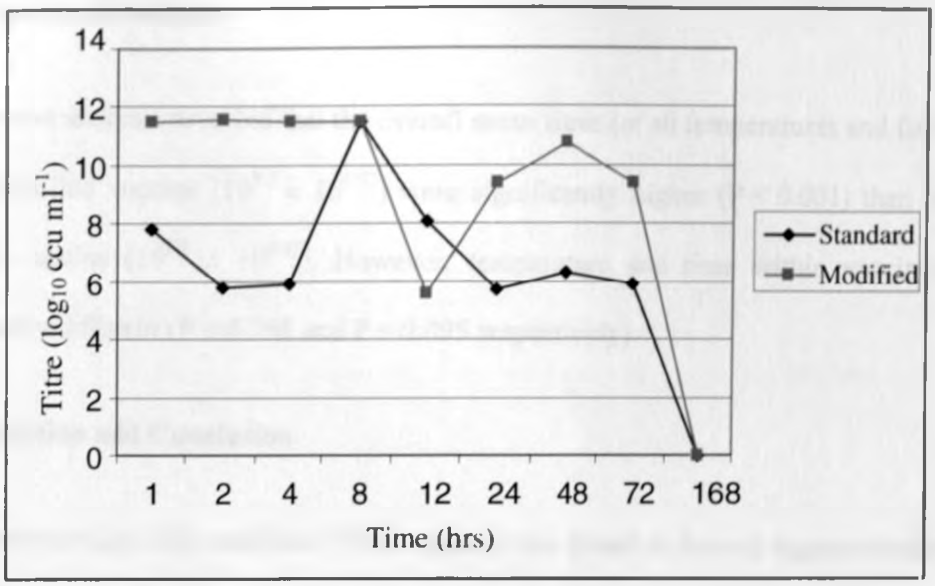

Figure 3.1c: Titres of *Mycoplasma* organisms for the standard and the modified vaccine incubated at 37°C after reconstitution, at various intervals between hr 1 and 168.

3.3.2. Statistical Analysis

Comparative analysis revealed that the overall mean titres (at all temperatures and for all periods) of the modified vaccine ($10^{9.3} \pm 10^{0.97}$) were significantly higher ($P < 0.001$) than those of the standard vaccine ($10^{6.5} \pm 10^{0.97}$). However, temperature and time within vaccines were not significantly different ($P = 0.765$ and $P = 0.095$ respectively).

3.4. Discussion and Conclusion

In the current study, the modified CBPP vaccine was found to have a higher stability than the standard vaccine. The modification of the vaccine involved incorporation of a buffer (HEPES) in the growth media, and substituting the normal saline diluent with PBS which also contained a pH indicator. When the two vaccines were titrated at various temperatures and intervals, a significant ($p<0.001$) drop in titre at all temperatures (4°C, 25°C and 37°C) and for all periods was observed. Although the titres for the modified vaccine dropped rapidly after around 8hrs, they generally remained above the OIE-recommended immunizing dose of 10^7 (OIE, 2000). The higher stability of the modified vaccine was particularly evident at 37°C incubation. At this temperature, high titres ($> 10^7$ viable *Mycoplasmas*) were observed for a period of 72hours. This higher stability was an indication that the type of media in which the vaccine is grown and thereafter the resuspension fluid have a marked effect on the viability of *Mycoplasma.*

Although in this study normal saline was used instead of $MgSO_4$ as a diluent for the standard vaccine, the results obtained are consistent with those of March *et al.,* (2002) who found a notable drop of vaccine titre when $MgSO_4$ was used as a diluent for the standard vaccine.

The modified vaccine when reconstituted with PBS exhibited a considerable thermostability at temperatures up to 37°C. This would enable the vaccine to remain viable under high environmental temperature conditions such as those found in Africa. Further, the need for immediate use of reconstituted vaccine would not be necessary. This would increase the ease of use in the field and also reduce wastage. Overall, this would reduce both production costs and the incidence of vaccine failure. It was therefore concluded that the modified vaccine should be recommended for field use to replace the standard one

CHAPTER FOUR

4.0. CHALLENGE MODEL BY NASOTRACHEAL INOCULATION USING A BRONCHOSCOPE

Abstract

A study was carried out to assess the effectiveness of a bronchoscope in infecting cattle with a pathogenic field strain of *Mycoplasma mycoides* subsp. *mycoides* (SC). The objective was to establish a reliable and rapid challenge method for use in evaluation of the efficacy of CBPP vaccines. Sixteen animals were each inoculated with 60ml of a *Mycoplasma* broth culture containing 3×10^8 cfu of *Mmm*SC, grown and harvested on day 4. The culture was deposited at the tracheal bifurcation using a bronchoscope. The effectiveness of the infection method was assessed by clinical, pathological, bacteriological and serological findings following inoculation. Out of sixteen animals inoculated, 10 (62.5%) showed clinical disease as evidenced by fever as early as 2 days post inoculation. At postmortem examination, 15 (93.8%) animals displayed typical lesions of CBPP from which *Mmm*SC was isolated. Antibodies to *Mmm*SC were detected in 10 (62.5%) animals by CFT and 11 (68.8%) animals by c-ELISA test. The results obtained demonstrate that nasotracheal inoculation of cattle with a pathogenic strain of *Mmm*SC with the aid of a bronchoscope leads to early onset of clinical disease, with higher numbers of animals showing clinical disease than observed in similar previous studies. This nasotracheal inoculation using a bronchoscope should therefore be adopted for use in CBPP experimental challenge infections of cattle.

4.1. Introduction

Contagious Bovine Pleuropneumonia is an important disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* (SC). In Africa, the disease is controlled mainly by vaccination. In the evaluation of the efficacy of CBPP vaccines an experimental infection/challenge method is needed. The infection/challenge has to be performed on the natural bovine host due to lack of a validated *in vitro* or laboratory animal model for the disease. The use of mice as laboratory models (Smith, 1971) is questionable as virulence factors of *Mmm*SC and protection in cattle certainly depend on specificities of bovine immune response (Wesonga and Thiaucourt, 2000).

In earlier times (the pre-1900), most CBPP experiments designed to measure the degree and duration of immunity following vaccination were performed by inoculating virulent culture or tumour-lymph subcutaneously behind the shoulder (Willems, 1900). Campbell, (1938a) exposed the animals at various intervals after vaccination to aerolized virulent culture. However, present studies have employed the bovine host, necessitating the use of large numbers of cattle. Challenge of vaccinated cattle has traditionally employed a technique that more closely resembles natural infection. In this method, vaccinated and unvaccinated (control) cattle are placed in close and continued contact with experimentally infected cattle which serve as donors of infective material (Hudson and Turner, 1963; Masiga and Read, 1972; Davies *et al.*, 1968; Masiga and Windsor, 1974; Wesonga and Thiaucourt, 2000). This method of transmission has its drawback, producing unpredictable rate of transmission from the infected animals to the challenge groups, with some trials failing due to low transmission rates while others have high rates. This is in addition to the costly length of time required to maintain the animals while waiting for the transmission to take place. Further, the infection methods that have been used to

produce the disease in animals that serve as source of infective material have been inefficient. The method involves sedation of animals followed by inoculation of infective material endobronchially. This has been reported to cause death in some animals due to inspiration of ruminal contents before recovery from sedation (Hudson and Turner, 1963). In addition, the operation takes more time as the sedative has to be allowed to take effect before intubation and thereafter the sedation to wear out. In some cases, it has been found difficult to reproduce the disease by intubation (Gourlay and Howard, 1982; Lloyd and Etheridge, 1983). In previous studies, 2-3 weeks were required for endobronchially-infected animals to develop the disease (Yaya *et al.*, 1999; Wesonga and Thiaucourt, 2000).

These problems coupled with the need to carry out challenge trials on newly developed vaccines requires that a more efficient infection model is established. The method should deliver the infective material more effectively and therefore save on costs of cattle as well as limit the time for running an experimental trial.

The purpose of the study was therefore to determine the effectiveness of a challenge model by naso-tracheal inoculation of cattle with *Mmm*SC aided by the use of a bronchoscope.

4.2. Materials and Methods

4.2.1. Animals

Sixteen head of male cattle (Bos or East African Zebu), aged 2-3 years, were purchased from Kakamega district of Western province, Kenya; historically known to be free from CBPP. Before purchase, they were ear-tagged, bled and tested for CBPP using slide agglutination test (SAT), complement fixation test (CFT) and competitive enzyme linked immunosorbent assay (c-ELISA)

test. The SAT was performed at the site. Briefly, an antigen suspension of stained *Mycoplasma mycoides* subsp. *mycoides* was mixed with a drop of blood on a glass slide. The slide was gently swirled for a minute and then grossly observed for agglutination. Any animal showing agglutination was considered positive. For the CFT and c-ELISA test, serum was transported to the research station for analysis. The tests were performed as described later (4.2.5.1 and 4.2.5.2 respectively).

All animals tested and found negative for antibodies to *Mycoplasma mycoides* subsp. *mycoides*, were then transported to the experimental station (KARI-Veterinary Research Centre, Muguga). The animals were dewormed with Nilzan plus cobalt® (Cooper, Nairobi, Kenya) and vaccinated against Foot-and-Mouth disease, lumpy skin disease, black quarter and anthrax. The animals were grazed during the day and confined in a paddock at night. Hay, water and mineral supplements were also provided. For a period of one month before infection, the animals were bled weekly for pre-infection serum samples.

4.2.2. Preparation of *Mmm*SC Infection Culture

Infection culture of a pathogenic local isolate of *Mmm*SC (from a CBPP outbreak in Kenya), confirmed to be *Mmm*SC by polymerase chain reaction (PCR) assay was prepared for naso-tracheal inoculation as follows: A 5ml aliquot of frozen culture was thawed for 30min at room temperature and 10-fold dilutions made into bijou bottles containing prewarmed Gourlay broth. A part of these dilutions were plated on Gourlay agar plates while the remainder was incubated at 37°C for 48hrs. The dilutions were then pooled and used to make 10 fold dilutions in larger quantities which were then scaled up every 24 hrs in 500 ml culture bottles holding a total of 1.8litres of infection culture. At every stage of scaling up, the cultures were incubated for 48hrs

at 37°C. Growth was monitored daily based on turbidity and colour change, and appearance of filaments.

4.2.3. Intubation

All the animals were restrained in a crush and their rectal temperatures and weight recorded. They were also bled for pre-infection serum samples. In a standing position, a bronchoscope (VFS-2A, Swiss Precision, USA) was passed through the nostrils to the larynx and down to the tracheal bifurcation. The bronchoscope was kept in this position until completion of intubation. Sixty (60ml) of *Mmm*SC culture containing 3×10^8 CFU of *Mmm*SC was then introduced through the bronchoscope into the trachea using a syringe, followed by 15ml of 1.5% agar suspended in distilled water. This was followed by 35ml of phosphate buffered saline (PBS) to flush down all the material to the target site. The animals were housed and fed as described previously (4.2.1).

4.2.4. Clinical Examination

Rectal temperature was taken daily at 8.00am. The animals were also observed for other clinical signs such as cough, nasal discharge and laboured breathing. Observations on feeding behaviour were made between 9.00am and 3.30pm, the time when the animals were grazed.

4.2.5. Serological Examination

All the animals were bled as a routine once a week for the whole period of the experiment. Samples of blood were taken from the jugular vein into vacutainer tubes, allowed to clot at room temperature overnight and then centrifuged to collect serum. Serum samples were stored at -20°C

until the end of the experiment. Samples from each animal were then tested serially for presence of antibodies using CFT (4.2.5.1.) and c-ELISA test (4.2.5.2).

4.2.5.1. Complement Fixation Test (CFT)

The CFT carried out was according to Campbell and Turner (1953), with some modifications. The microtitration plate method as recommended by the OIE was followed. Complement fixing *Mmm*SC antigen, sheep red blood cells (SRBC), positive control sera and buffer were prepared locally. Complement (C') and hemolytic system (HS) were commercially obtained from CIRAD-EMVT, France.

Preparation of Antigen for CFT

Two litres of tryptose broth were inoculated with T_1 strain *M. Mycoides* and incubated at 37°C for 7 days. The culture was centrifuged at 40,000g. The packed organisms were resuspended in 20ml of 0.85% NaCl and mixed vigorously after adding some sterile glass beads. The preparation was then autoclaved for 10min at 15lbs pressure and allowed to cool. Thereafter it was agitated in an 'atomix' (Measuring and scientific equipment, London, England) for 5min in order 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 10ml. The antigen suspension was stored at +4°C for 6weeks, with shaking at weekly intervals, to allow the antigen to stabilize.

Preparation of Veronal Buffer (with calcium and magnesium) -VCM

This was used as a diluent for all CFT reagents. It was prepared by dissolving 5.75g of barbitone (di-ethyl barbituric acid) into 500ml of hot distilled water. Eighty five (85g) of NaCl was then

added and the solution made up to 1400ml with sterile distilled water. Two (2.0g) grams of sodium barbitone was dissolved in 500ml distilled water and then added to the NaCl-barbitone solution. This was then made up to 2000ml with distilled water followed by addition of 1.68g $MgCl_26H_2O$ and 0.28g $CaCl_2$. This solution constituted the VCM buffer and was stored at +4°C. The buffer was diluted 1 in 5 with sterile double distilled water before use.

Preparation of Sensitized Sheep Red Blood Cells (SRBC)

One sheep (reared at KARI-Muguga) was aseptically bled from jugular vein directly into Alsevers solution (2.05%w/v glucose, 0.42%w/v sodium chloride, 0.8w/v trisodium citrate, 0.555%w/v citric acid, distilled water to 100% in equal proportions).The blood was kept at 4°C for at least two days (but not more than 4 days) before use. On the day of use, it was centrifuged at 700g for 5minutes and the supernatant and the buffy coat were sucked out. The SRBC were washed 3 times with VCM buffer. A 6% cell suspension was then made in VCM. The SRBC suspension was added to an equal volume of the HS to make a final cell concentration of 3% SRBC. The HS had been diluted in VCM to the recommended titre (12 haemolytic doses read at 50% end-point). To allow for enough sensitization, the suspension was continuously mixed on a rotating mixer for 30 minutes at room temperature before use.

The CFT Procedure

Prior to the test, all the reagents (hemolysin, complement, antigen and sheep red blood cells) were standardized according to OIE, (2000) to determine optimal concentrations.

Briefly, a 1:10 dilution of each serum sample and controls (positive and negative) to be tested was prepared by transferring 20µl of each into 180µl of VCM buffer in a pre-plate microtitre

plate. Twenty five (25µl) of each was then transferred into the working plate. The controls were diluted further in doubling dilutions up to 1:640. The antigen (25µl) was added to each well. This was followed by addition of complement (25µl) to each well. The plate was incubated for 30 minutes at 37°C with gentle shaking. Twenty five (25µl) of haemolytic system was then added to each well followed by incubation at 37°C for 30 minutes, with gentle shaking. The plates were incubated overnight at 4°C after which they were examined with a plate reader. Depending on the size of the SRBC button, the results were classified as follows:

a) ++++ → complete fixation (no haemolysis)

b) +++ → almost complete fixation (very slight haemolysis)

c) ++ → Partial fixation (partial haemolysis)

d) + → very slight fixation (almost complete haemolysis)

e) 0 → no fixation (complete haemolysis)

Thereafter, any sample showing any degree of fixation of complement (C′) was titrated further in order to determine the actual CFT titre. Briefly, a 1/10 to 1/640 doubling dilutions of the test sera and controls was prepared. The CFT was then completed as described above. The controls included were: a positive control (bovine sera from naturally infected animals) (diluted up to 1:640), negative control (healthy bovine serum) (diluted up to 1:640), antigen control (to detect any anti-complementally activity of the Ag) (25µl VCM + 25µl Ag + 25µl C′ + 25µl HS), complement control (to show that enough complement is present to lyse the cells) (50 µl VCM + 25µl C′ + 25µl HS) and haemolytic system control (to show that the cells do not lyse spontaneously) (75 µl VCM + 25µl HS). The end point of titration was taken as the highest serum dilution giving 50% fixation of complement.

4.2.5.2. Competitive ELISA Test

A commercial c-ELISA test kit (CIRAD/Institut POURQUIER) was used. The kit contained 96-well polysorp Nunc-Immunoplates (Nunc Laboratories, USA) pre-coated with lysed *Mmm*SC antigen solution, mouse monoclonal antibody against *Mmm*SC, polyclonal rabbit antimouse immunoglobulin conjugated to horse radish peroxidase, Tetramethyl Benzidine (TMB) substrate, and various reagents and controls (Positive and negative control sera, conjugate control and monoclonal control). The test was carried out following the recommended bench protocol. The optical densities (OD) were read spectrophotometrically at a wavelength of 450 nm. 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 PI value equal or above 50% was considered positive.

4.2.6. Necropsy and Sample Collection

Cattle were selected using a pre-determined schedule, sacrificed and a post mortem examination carried out. The selected animals were stunned by use of a captive bolt pistol and then exsanginated. Blood for serum was collected in vacutainer tubes; and on opening the carcass, pleural fluid, where present, was aspirated into a 10ml syringe and immediately stored in a cool box. The lungs were then examined for CBPP lesions. The size of lesions (diameter in cm) was then recorded. Pieces of lung from an area between the lesion and the grossly normal were cut

and placed in sterile polythene bags, transferred to a cool box and transported to the laboratory where they were processed and cultured for isolation of *Mycoplasma* organisms.

4.2.7. Lesion Scoring

Lesion scoring was carried out to determine severity of the disease in individual animals using the method of Hudson and Turner, (1963). Briefly, the presence of only encapsulated, resolving or fibrous lesions or pleural adhesions only, are rated 1. The presence of other types of lesions like consolidation, necrosis or sequestration was rated 2. If in addition, *Mmm*SC was isolated, a 2 was added to the above rating. The resulting score was then multiplied by a factor depending on the lesion size e.g. multiply by factor 1 if the lesion size is under 5cm diameter, by factor 2 if it is over 5cm and under 20cm, and by factor 3 if it is over 20cm in diameter. Hence, the maximum pathology score would be (2+2)3=12.

4.2.8. Bacteriological Examination

Briefly, the lung tissue samples were sliced into small pieces using sterile scalpels. The pieces were then put into Gourlay media (broth with added penicillin and thallium acetate) contained in bijou bottles. The following day, 1ml of the supernatant was diluted using a 10-fold dilutions series from 10^{-1} to 10^{-3} in bijou bottles. From these dilutions, 0.2ml was plated onto agar plates with Gourlay media. The remaining (0.8ml) broth cultures were incubated aerobically at 37°C and examined daily for ten days for evidence of growth; indicated by colour change from pink to yellow, and in some, filamentous growth. The plates were incubated at 37°C in a humidified chamber and examined under inverted microscope at X25 magnifications for *Mycoplasma* microcolonies. The examination was done at day 1, 5 and 10 respectively.

4.3. Results

4.3.1. Clinical Findings

Of the 16 animals, 10 (62.5%) exhibited fever for at least one day in the experimental period (Table 4.1). Fever commenced in the first animal 2 days post inoculation. In eight out of the 10 animals, fever was observed for duration of 2-9 days. Two animals, each showed fever intermittently for thirteen days, starting from the second day post inoculation. One animal showed fever for only one day. Fever was generally intermittent in all animals and ranged from 39.5°C to 42.0°C. Cough was recorded in 6 (37.5%) animals.

4.3.2. Serological Response

In the CFT, 10 (62.5%) animals were positive with titres ranging from 1/10 to 1/640 (Table 4.1) while in c-ELISA test, 11 (68.8%) were positive, with PI values ranging from 50.9 to 94.5%.

Table 4.1: The duration of fever and serological reactions in CFT and c-ELISA in animals inoculated with *Mmm*SC using a bronchoscope.

Animal ID	Duration of fever(days)	CFT	c-ELISA
505	0	-	-
506	1	-	+
509	9	+(1:20)	+
513	9	+(1:80)	+
515	0	-	-
519	0	+(1:160)	+
520	0	+(1:10)	-
522	0	+(1:40)	+
524	13	+(1:640)	+
525	8	+(1:40)	+
527	2	+(1:320)	+
532	2	-	+
539	0	-	-
542	13	+(1:640)	+
543	8	+(1:160)	+
544	2	-	-

Key

\+ indicates animal seroconverted

− indicates animal did not seroconvert

4.3.3. Necropsy and Pathology Index

Out of the 16 animals, 15 showed lung lesions characteristic of CBPP. Of these, eleven had a high pathology score of between 8 and 12 (Table 4.2). One animal (6.3%) died on day 28 post inoculation, 5 (31.3%) were killed *in extremis* and the rest (62.5%) survived up to 45days, the period after which they were sacrificed. In those showing lesions, gross changes included unilateral pleuropneumonia, with pleuritis and well-developed sequestra in some. In two of them, the pleural cavity contained copious amounts of yellowish-coloured fluid. In addition, the lung tissue was consolidated and in some places, with characteristic marbling. Enlargement of bronchial and mediastinal lymph nodes was observed in all the animals killed *in extremis*. Eleven of them had high pathology scores (8-12) with the remaining having a score of 2 each.

4.3.4. Bacteriological Findings

Mycoplasma mycoides (SC) was isolated from lung samples of 15/16 cattle (Table 4.2), including those that did not show lesions at post-mortem or serological response in neither CFT nor c-ELISA test.

Table 4.2: Pathology scores and *Mycoplasma* culture results in animals inoculated with *Mmm*SC using a bronchoscope.

Animal ID	Day Sacrificed (post infection)	Pathology Score	Culture results
505	44	8	+
506	47	2	+
509	29	12	+
513	29	12	+
515	44	2	+
519	47	8	+
520	47	0	–
522	47	12	+
524	28	12	+
525	29	12	+
527	44	8	+
532	47	9	+
539	47	2	+
542	29	8	+
543	44	8	+
544	44	2	+

Key

\+ Indicates *Mmm*SC isolated

– Indicates *Mmm*SC not isolated

4.4. Discussion and Conclusion

This study was conducted to determine the effectiveness of reproducing CBPP in cattle by intubation using a bronchoscope. From the results obtained, the method is rapid and reliable. Out of 16 cattle that were infected, 10 (62.2%) developed clinical disease as evidenced by fever. Seroconversion was observed in 10 and 11 animals in the CFT and c-ELISA test respectively. At post-mortem, 15 (93.8%) had lung lesions while *Mmm*SC was isolated from 15 out of the 16 animals. Previous methods used in reproducing the disease through intubation have been inefficient. The methods, which involved sedation of animals followed by inoculation of infective material endobronchially without the aid of a bronchoscope, reportedly caused death in some animals due to inspiration of ruminal contents before recovery from sedation (Hudson and Turner, 1963). In addition, the methods were slow since it took time for sedation to take effect and also to wear out. In some cases, it has been found difficult or completely impossible to reproduce the disease (Gourlay and Howard, 1982; Lloyd and Etheridge, 1983; Thiaucourt, *et al.*, 2000). Wesonga and Thiaucourt, (2000) reproduced only a few cases of clinical disease (5/40) and late development of disease in the rest. In other studies, 2-3 weeks were required for the infected animals to develop the disease (Yaya *et al.*, 1999; Wesonga and Thiaucourt, 2000). Thiaucourt *et al.*, (2000) completely failed to reproduce the disease therefore necessitating a repeat of the intubation. In the current study, the method used was safe since animals were intubated in a standing position. Further, majority of the animals succumbed to clinical disease which was observed as early as 2 days post inoculation. Majority of animals also had lesions at post-mortem.

Infection of cattle with *Mmm*SC using a bronchoscope and without sedation is therefore a rapid, efficient and reliable method of reproducing the disease. This method would be useful as a challenge method in assessment of efficacy of CBPP vaccines.

CHAPTER FIVE

5.0. DETERMINATION OF SAFETY AND EFFICACY OF THE STANDARD AND THE MODIFIED VACCINES

Abstract

A study was carried out to evaluate the safety and efficacy of a modified contagious bovine pleuropneumonia (CBPP) vaccine against the standard (currently in use) vaccine. The objective was to determine whether the modified vaccine was superior to the standard one in terms of safety and efficacy, with a view to recommend it for field use. The safety was determined by vaccinating two groups of randomized cattle at the tail-tip, and then determining changes in tail-tip circumferences and rectal temperatures. A control group comprising of unvaccinated animals was included. The efficacy was determined by challenging vaccinated and non vaccinated animals at three months post vaccination, each with 60ml of a *Mycoplasma* broth culture containing 3×10^8 CFU of *Mmm*SC. The challenge was done using a predetermined nasotracheal intubation method aided by a bronchoscope. The efficacy of the two vaccines was compared by calculating the protection rate in each group. The protection rates were calculated from pathology indices obtained from each animal and calculated using lesion scores. The latter were determined by the size and severity of the lung lesions. In the safety studies, changes in tail-tip circumferences in the animals vaccinated with the standard vaccine were more severe than those vaccinated with the modified vaccine ($P<0.001$), while rectal temperatures in both groups remained within the normal range. From the results obtained in the efficacy studies, there was no difference in protection rates between the two vaccines ($P>0.05$).

From the results of this study, it was concluded that the modified vaccine is safer than the standard one. However, the modified vaccine is not superior to the standard vaccine in terms of efficacy. On the basis of the improved safety, it was therefore concluded that the modified vaccine should be recommended for field use to replace the standard one. However, further modifications of the vaccine are needed with a view to increase the efficacy.

5.1. Introduction

Vaccine strain $T_1$44, an attenuated live *Mmm*SC vaccine, has been used for control of CBPP since 1956, although its safety and efficacy has been questionable (Rweyemamu *et al.*, 1995; Thiaucourt *et al.*, 2000; March, 2004). It had been observed that the pH in both the vaccine's growth media and reconstitution fluid can reach levels suboptimal for survival of *Mmm*SC (Waite and March, 2001) and vaccine efficacy (Pollack *et al.*, 1969). March, (2004) carried out a modification which involved an addition of a buffer system (HEPES) to the growth media of the vaccine strain, addition of a pH indicator to the reconstitution fluid and the use of phosphate buffered saline (PBS) for reconstitution of the freeze-dried vaccine in the field.

Contagious Bovine Pleuropneumonia post-vaccinal reactions (termed Willems reactions) has been well documented since the mid 19th century (Litamoi and Seck, 1999). The site of inoculation has been shown to be the main predisposing factor (Sori, 2005). It is characterized by an oedema at the site of injection; 10 to 20 days post inoculation. This oedema may vary in size and in some cases it may be extensive enough to cause death of the animal (Wesonga and Thiaucourt, 2000). For this reason, the vaccine is often given at the tail-tip. However, the tail-tip may become necrotic and slough off. The proportion of the adverse reactions and their pathogenesis has not been in agreement among researchers. The reactions have often been a cause of rejection of the vaccine by animal owners and subsequently a burden for veterinary services. These reactions occur with an unpredictable frequency up to 11% in some instances (Lindley, 1971). The effects can be minimized by administering the vaccine at the tail-tip, so if a severe reaction occurs, the tail-tip can be amputated, thereby saving the animal's life. Alternatively, antibiotics can be used to treat the reaction (Egwu *et al.*, 1996). The post vaccinal

reactions have not been observed with the T1sr vaccine (Thiaucourt *et al.*, 2003). This strain is also completely devoid of residual pathogenicity, unlike strain $T_1$44 which has been found to induce lung lesions in exceptional cases (Huebschle *et al.*, 2002). Strain T1sr is therefore an alternative choice to $T_1$44, although the duration of immunity is shorter. Strain T1sr was found to be ineffective in controlling outbreaks in southern Africa leading to its suspension (Tulasne *et al.*, 1996).

Contagious Bovine Pleuropneumonia has been eradicated from most developed countries through rigorous policy of restriction of cattle movement, slaughter and financial compensation. However, for socio-cultural and economic reasons, such measures cannot be implemented in most African countries. For these reasons, it is believed that the only realistic way of controlling CBPP in the third world, including African countries, is by widespread and repeated vaccination (Rweyemamu *et al.*, 1995; Tulasne *et al.*, 1996). However, the currently used vaccines throughout Africa induce immunity of short duration. Observations from the field (Masiga *et al.*, 1996; 1998) and experimental studies (Yaya *et al.*, 1999; Thiaucourt *et al.*, 2000) have indicated that the current vaccines do not effectively protect cattle from outbreaks of disease, especially with primary vaccination.

Despite vaccination campaigns using freeze-dried broth cultures of live attenuated *Mmm*SC (strain $T_1$44 or T_1sr), there has been a substantial re-emergence of the disease (March, 2004). The purpose of this study was to establish if modifications in the new vaccine have any improvements on the safety and efficacy of the vaccine before recommending it for field use.

5.2. Materials and Methods

5.2.1. Animals

All the cattle used in the experiment were obtained from a CBPP-free area. They were pretested, transported, dewormed, vaccinated, housed, observed, sampled and fed as described previously (chapter 4).

5.2.2. Randomization, Vaccine Preparation, Vaccination and Follow-up

Ninety cattle were randomly selected and divided into three groups of 30 each. The groups were prefixed A, B and C for the animals vaccinated with the standard and the modified vaccine, and the controls respectively. Their weight was determined using a weighing band. All the animals were bled for pre-vaccination serum samples. Tail-tip circumference of each animal was taken using a thread, transferred to the mathematical ruler, measured and recorded.

The standard and the modified freeze-dried $T_1$44 vaccine strain of *Mmm*SC (batch number 02/06 and 03/06 respectively) obtained from KARI-Veterinary Vaccine Production Centre (KARI-VVPC) were prepared according to the manufacturer's recommendations. This was as follows:

For the standard vaccine, the vial was reconstituted with 7.5ml of normal saline to make 15 doses of 0.5ml each. The modified vaccine was reconstituted with 50ml of PBS to make 100 doses of 0.5ml each. The vaccines were reconstituted while placed on ice and at the crush where the animals were to be vaccinated.

The animals were restrained in the crush each group at a time. Each animal in group A and B received 0.5ml of the standard and the modified vaccine respectively. This was done at the tip of the tail using an automatic syringe (HSW, ROUX-REVOLVER®, Germany) and a gauge '14' hypodermic needle. The third group (control) received 0.5ml of phosphate buffer saline, at the

tail-tip. The three groups of animals were grazed in separate paddocks at least 200m apart. Rectal temperature was taken daily and the animals bled weekly for serum samples. Tail measurements were taken daily for 30 days post vaccination.

5.2.3. Challenge

Three months post vaccination, 15 animals were randomly selected from each group. However, one selected animal from group B was sacrificed (following fracture of hind limb) thereafter leaving 14. The remaining 15 animals in each group were preserved for a different study later. The selected animals were then challenged by nasotracheal intubation with the aid of a bronchoscope as described previously (chapter 4). They were also housed and fed as described previously (chapter 4).

5.2.4. Clinical Examination

Inoculated animals were restrained in a crush daily at the same hour (between 8.00 and 10.00am) and the rectal temperature of each was recorded. Temperatures of 39.5°C and above were considered to constitute a fever. Other appropriate clinical observations recorded included cough, nasal discharge and dyspnoea.

5.2.5. Serological Examination

This was carried out using CFT and c-ELISA test as described previously (chapter 4).

5.2.6. Necropsy, Sample Collection and Lesion Scoring

This was carried out as described previously (chapter 4). However, animals were slaughtered after showing fever (≥ 39.5°C) for ten days consecutively. Slaughtering was carried out at random, without prior knowledge as to which group the animal under post-mortem examination belonged.

5.2.7. Bacteriological Examination

This was carried out as described previously (chapter 4).

5.2.8. Determination of Protection

The protection was calculated according to Hudson and Turner, (1963) 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.

The formula is given as;

$$\textbf{Protection Rate} = \left(1 - \left\{ \frac{\textbf{Mean Score of Vaccinates}}{\textbf{Mean Score of Controls}} \right\} \right) \times 100$$

5.2.9. Statistical Analysis

Comparative analysis of the changes in tail-tip circumferences and rectal temperatures between animals vaccinated with the standard vaccine, the modified vaccine, and the controls was performed using ANOVA.

In order to determine if there was a difference in protection afforded by the two vaccines, pathology scores were evaluated. The difference between the mean pathology scores in the three groups (vaccinates and controls) was analyzed using ANOVA.

In order to determine if there was a difference in antibody response (in both CFT and c-ELISA test) between the two vaccines, comparison of the proportions of the animals (vaccinates and controls) that responded was performed using Chi-square (χ^2) test.

5.3. Results

5.3.1. Vaccine Safety

5.3.1.1. Tail-tip Circumferences

The daily mean tail-tip circumferences of the vaccinates and the control animals are shown in figure 5.1. The clinical findings were either circumscribed swellings or a bulb of variable diameter at the site of inoculation. In eleven cases of the animals vaccinated with the standard vaccine, oedema was markedly extended anteriorly to the tail. Development of the swellings ranged between 3-21 days post-inoculation in both vaccinated groups. The reactions in most cases regressed by day 21. In some animals, no obvious swelling appeared at the site of the vaccination. In the control group, swellings which were attributable to trauma appeared for the first two days post vaccination in all animals. The analysis of variance in tail-tip circumferences between the animals vaccinated with the standard vaccine, the modified vaccine, and the controls indicated that there were changes in the vaccinates. Comparison of the means for the vaccinates (4.66 ± 0.057 and 4.26 ± 0.059 for the standard and the modified vaccine respectively) with that

of the controls (3.35 ± 0.027) indicated that the means for the vaccinates were significantly higher than that of the controls (P<0.001). A comparison between the means for the vaccinated groups indicated that the mean for the standard group was higher than the mean for the modified group (P<0.001).

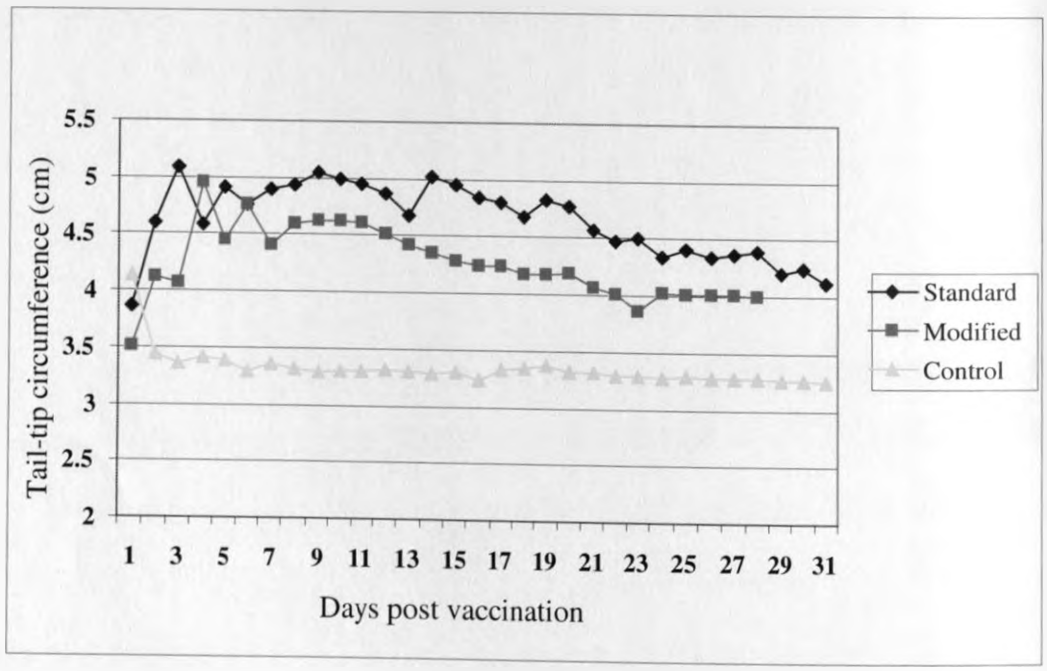

Figure 5.1: Daily mean tail-tip circumferences over a thirty day period of the animals vaccinated with the standard and the modified vaccine, and the controls.

5.3.1.2 Rectal Temperatures

The rectal temperatures for all the animals remained within the normal range during the 30 days post vaccination observation period. However, comparison between the means for the vaccinates (38.0 ± 0.04 and 38.2 ± 0.02 for the animals vaccinated with the standard and the modified vaccine respectively), with that of the controls (38.1 ± 0.03), indicated that there were changes in the vaccinates. A comparison between the means for the vaccinated groups indicated that the mean for the modified group was higher than the mean for the standard group ($p < 0.001$).

5.3.2. Vaccine Efficacy

5.3.2.1. Serological Response Post Vaccination

In the CFT, seroconversion was observed in 19 (63.3%) and 5 (16.7%) animals that received the standard and the modified vaccines respectively. In the c-ELISA test, seroconversion occurred in 5 (16.7%) of the animals vaccinated with either the standard or the modified vaccine. Considering both tests, the earliest seroconversion was detected 2 weeks post vaccination. Seroconversion was not observed in any animal in the control group with both tests.

The analysis of the CFT antibody titres between the animals vaccinated with the standard vaccine, the modified vaccine and the controls indicated that the proportion reacting after vaccination was significantly different ($\chi^2 = 33.07$, $P < 0.001$ with 2 df). A comparison between the proportions that reacted in the vaccinated groups indicated that the proportion for the group given the standard vaccine was significantly higher ($\chi^2 = 13.61$, $P < 0.001$ with 1 df).

The analysis of the c-ELISA antibody titres between the animals vaccinated with the standard vaccine, the modified vaccine, and the controls indicated that there were responses in the

vaccinates (χ^2 = 5.62, P = 0.06 with 2 df)). However, there was no difference between the animals vaccinated with the standard and the modified vaccine (χ^2 = 0.00, P = 1.000 with 1 df).

5.3.2.2. Clinical Response Post Challenge

Post challenge clinical responses included nasal discharge, cough, laboured breathing, disinclination to move, and postures that indicated oxygen deficiency (Fig. 5.2). Several animals also exhibited anorexia. The earliest fever was recorded on day 7 post inoculation in fourteen animals. Of these, 4 (26.7%) were from the control group and 5(33.3%) and 5(35.7%) were from the group vaccinated with the standard and the modified vaccine respectively. The fever recorded at any given time ranged between 39.5-40.6°C.

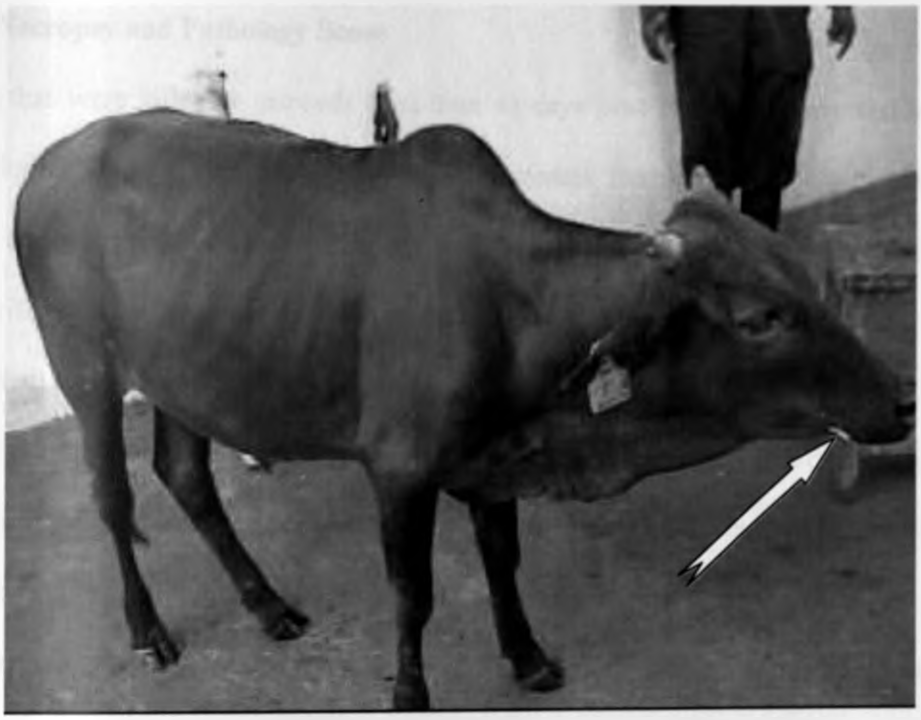

Figure 5.2: A zebu bull vaccinated with standard $T_1$44 vaccine and challenged with 3×10^8 CFU of *Mmm*SC 3 months later. The animal is showing clinical signs of CBPP, namely, extended neck, abducted elbows, mouth frothing (arrow) and dullness.

5.3.2.3. Necropsy and Pathology Score

Animals that were killed *in extremis* (less than 45 days post challenge) were sixteen (36.4%). This included 3(18.8%), 4(25%) and 9(56.3%) animals from the group vaccinated with the standard and the modified vaccine, and the controls respectively. The rest survived up to and were sacrificed at day 45 post challenge.

Post-mortem examination showed gross pathological lesions characteristic of CBPP. The lesions observed included; unilateral pleuropneumonic lesions, pleuritis and well-developed sequestra (Fig. 5.3). In some cases, the pleural cavity contained copious amounts of yellowish-coloured clear fluid. Other lesions included adhesions of the pleura with the chest wall, diaphragm or the pericardial sac (Fig 5.4), and consolidation of lung tissues. The consolidated tissues had the characteristic marbling and hepatization (Fig. 5.5). Heavy deposits of fibrin flocculates were also encountered. Enlargement of bronchial and mediastinal lymph nodes were regularly observed in affected animals. A high percentage (93.3%) of animals in the control group had lesions in contrast to 66.7% and 42.9% in the group vaccinated with the standard and the modified vaccine respectively.

In the group vaccinated with the standard vaccine, the overall mean pathology score was 1.9 (Table 5.1). Ten (66.7%) of the animals had pulmonary lesions and the pathology scores ranged between 1 and 8. In the group vaccinated with the modified vaccine, the mean pathology score was 4. The number of animals with pulmonary lesions was 6 (42.9%) with a pathology score range of 1-12. Out of this, four animals had a very high pathology score of 12 each. The overall mean pathology score in the control group was 8.3. The number of animals with pulmonary lesions was 14 (93.3%) with a pathology score ranging between 1 and 12 (Table 5.1). Although animals in the vaccinated group generally had lesions of lower magnitude than the control group,

four animals in the group vaccinated with the modified vaccine displayed lesions of similarly high magnitude to those observed in most of the control group.

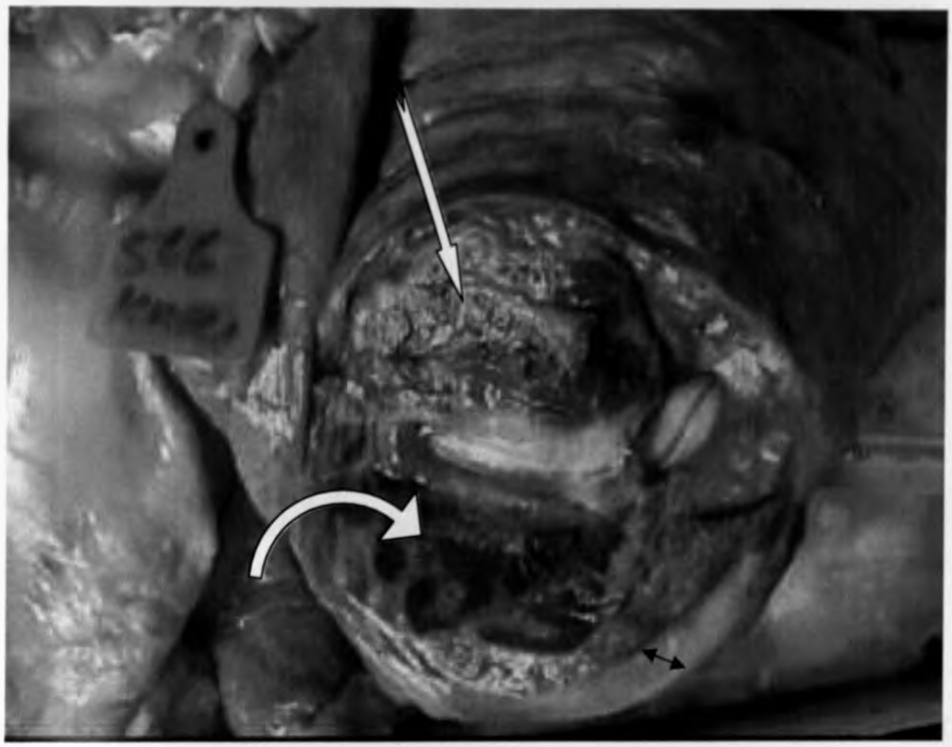

Figure 5.3: A cut surface of a sequestrum in a lung of non-vaccinated cattle challenged with 3×10^8 CFU of *Mmm*SC. It shows necrotic material (straight arrow), viable lung tissue (curved arrow) and fibrous capsule (double arrows).

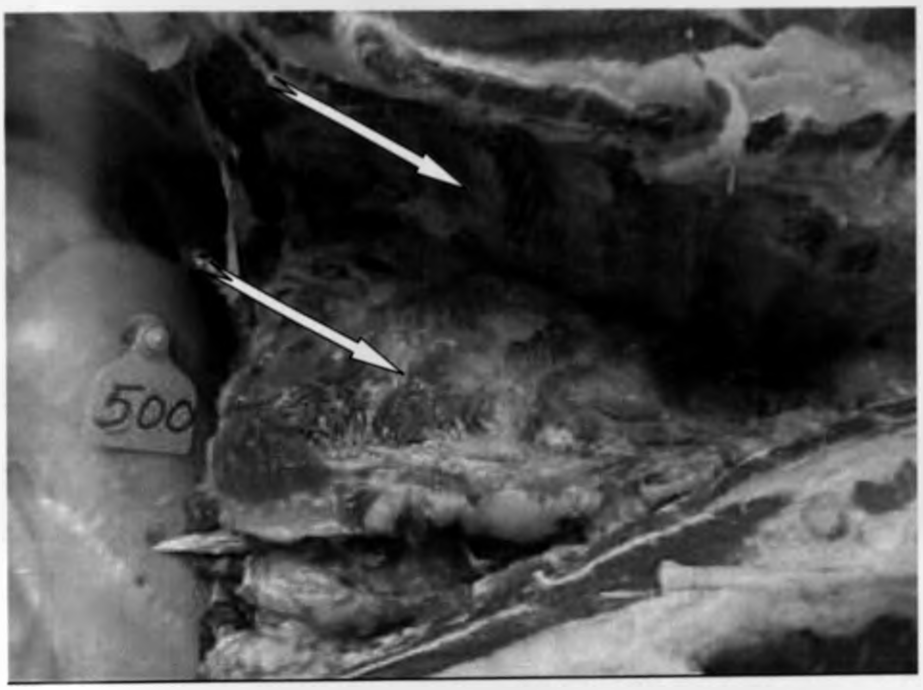

Figure 5.4: A thoracic cavity of non-vaccinated cattle challenged with 3×10^8 CFU of *Mmm*SC. The picture shows adhesions between parietal (upper arrow) and visceral pleura (lower arrow) in an advanced case of CBPP.

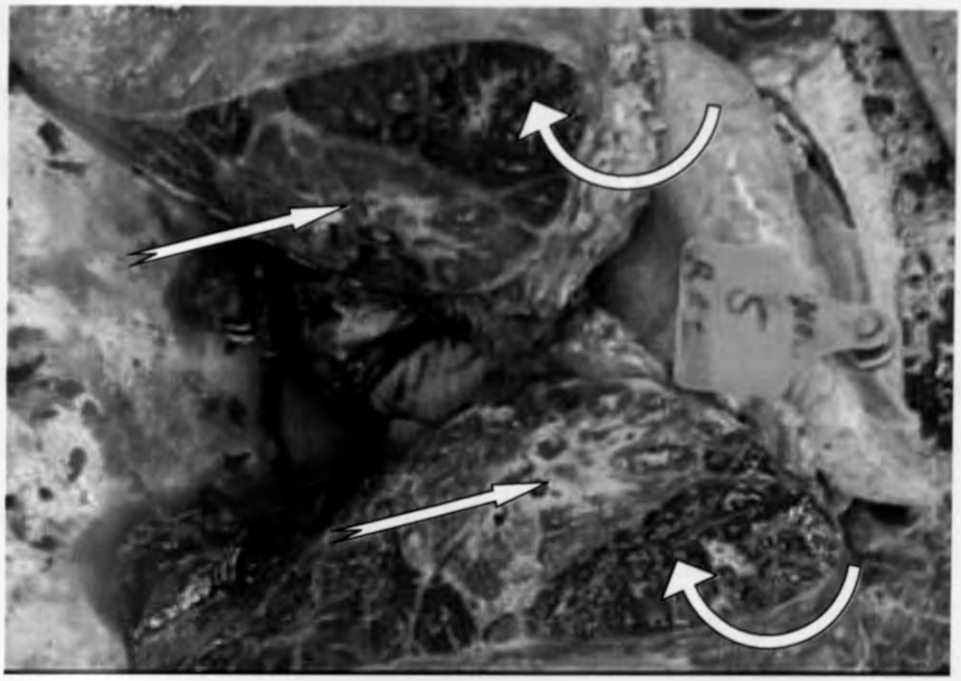

Figure 5.5: A cut surface of a lung of cattle vaccinated with the modified $T_1$44 vaccine and challenged with 3×10^8 CFU of *Mmm*SC 3 months later. The picture shows gray hepatization (straight arrows) and red hepatization (curved arrows).

5.3.2.4. Bacteriological Findings

Isolation of *Mmm*SC was made from 18 cases of the animals with lesions and from one animal without a lesion.

5.3.2.5. Vaccines Protection Rates

A summary of the protection rates by the two vaccines following challenge three months post vaccination is given in table 5.1. The rates were 77.1% and 51.8% for the standard and the modified vaccine respectively.

Table 5.1: Summary of the protection rates in cattle vaccinated with the standard and the modified vaccine, following challenge three months later with 3×10^8 CFU of *Mmm*SC.

Group	No. of cattle	Mean Pathology Score	Protection Rate
Control	15	8.3	
Standard	15	1.9	77.1
Modified	14	4	51.8

5.3.2.6. Statistical Analysis

Comparative analysis revealed that the proportion of the protected animals was not significantly different ($P > 0.05$) between the group vaccinated with the standard vaccine and the one given the modified vaccine. However, comparison of the vaccinates and the controls showed that protection was significantly higher ($P < 0.001$) in vaccinated groups (1.93 ± 0.56 and 4.0 ± 1.47 for the standard and the modified vaccinated groups respectively) than in controls (8.27 ± 1.23).

5.4. Discussion and Conclusion

The present study was carried out to compare the safety and efficacy between the modified and the standard CBPP vaccine formulations. From the results obtained it was concluded that the modified vaccine was safer than the standard vaccine but there was no difference in the efficacy between the two. In the evaluation of safety, although the rectal temperatures remained within the normal physiological values following vaccination, there was however, a difference in tail-tip circumferences. The tail-tip circumferences were significantly higher ($P < 0.001$) in the animals vaccinated with the standard vaccine than in those inoculated with the modified vaccine. This was an indication that the standard vaccine elicited much more severe post-vaccinal reactions than the modified one. The reactions observed in both vaccinates were however considered mild, and since no complications were encountered, they therefore did not warrant treatment. Under normal conditions, CBPP vaccines need to be given to healthy animals. This situation may be difficult to attain in the field, whereby some animals may be incubating the disease, and others are carriers. In both cases the post-vaccinal reactions may therefore be expected to be more

severe under field conditions. Other field conditions that may contribute to a much more severe reaction include low hygiene standards. Strict adherence to the use of sharp needles and of the right gauge coupled with changing them after vaccination of few animals is important. Failure to respect these procedures can cause contamination of the vaccine and inoculation of contaminants into animals (Sori, 2005). As a result, abscesses may occur at the site due to secondary bacterial infection.

The efficacy of the two vaccines was evaluated on the basis of seroconversion in CFT and c-ELISA, and on the basis of pathology scores. In serology, there was a higher seroconversion with the standard vaccine in CFT, while with the c-ELISA, there was no difference between the two vaccines. In both tests, both groups showed a decline in antibody titres two months post vaccination. This observation is in line with previous experiments concerning vaccinations using T_1 derived vaccines (Yaya *et al.*, 1999; Wesonga and Thiaucourt, 2000).

There was no difference observed in the pathological scores between the groups vaccinated with the standard and the modified vaccine. In the present study, the classical Hudson and Turner (1963) scoring system was used to measure the protection rate, as it has frequently been the case in the past (Gilbert *et al.*, 1970). The severity of lesions was expressed as pathology scores which were then transformed into percent protection rates. Although the standard vaccine indicated a better protection (77.1%) compared to the modified vaccine (51.8%), the difference was not statistically significant ($P > 0.05$).

The protection rates observed in the present study are similar to those observed in previous studies on protection afforded by T_1 vaccine strain by a single vaccination against a challenge 3

months post vaccination in Kenya, where Thiaucourt *et al.*,(2000) reported a protection rate of 60%. Similarly, Mariner *et al.*, (2006a and b) estimated efficiency of $T_1$44 vaccine to lie between 50 and 80%, while Thiaucourt *et al.*, (2004a) offered a value of 40-60% following primary vaccination, increasing to 80-95% upon revaccination. However, the present protection rates are much higher than those reported by Yaya *et al.*, (1999) who reported a protection rate of 30% in a similar experiment in Cameroon. Although the two vaccines do not show a difference in efficacy under the controlled conditions of this study, there may be differences in efficacy under field conditions where the vaccines will be subjected to environmental stress. Further, in the present study, the modified vaccine showed higher safety and can therefore be recommended for field use.

CHAPTER SIX

6.0. GENERAL DISCUSSION AND CONCLUSION

Contagious Bovine Pleuropneumonia is an economically important transboundary disease, widely present in sub-Saharan Africa. Social, cultural and economic factors determine that effective vaccination is the only viable control method at present (March, 2004). However, the CBPP vaccines currently in use are reportedly of low efficacy, stability and safety (Rweyemamu *et al.*, 1995; Thiaucourt *et al.*, 2000).

Thermostability of CBPP vaccines has been an area of great concern considering that the vaccines have to be transported over long distances under high environmental temperatures, to remote areas where cold chain maintenance is difficult.

Post-vaccinal reactions of the vaccines currently in use are characterized by an oedema (Willems reactions) at the site of injection. Subsequent secondary bacterial infections may result in sloughing-off of tail and sometimes death of the animal. Besides the economic losses, the reactions have often been a cause of rejection of the vaccine by animal owners and subsequently a burden for veterinary services.

The current used vaccines throughout Africa induce immunity of short duration and do not protect cattle from outbreaks of disease, especially with primary vaccination (Masiga *et al.*, 1996; 1998; Yaya *et al.*, 1999; Thiaucourt *et al.*, 2000). The low immunity causes outbreaks which leads to death of animals as well as loss of markets. In addition to the need for annual vaccinations, this causes serious economic losses as well as making the disease expensive to control.

The need for more effective and safer vaccines cannot therefore be overemphasized.

A modified CBPP vaccine has been developed by March, (2004) with a view to address mainly the issue of stability. The modification includes the use of HEPES–buffer systems in the growth media and the inclusion of pH indicators in the reconstitution fluid, together with the use of PBS as a vaccine diluent. These modifications are expected to enhance the viability of *Mycoplasma* organisms thereby enhancing the efficacy of the vaccines. These modifications may indirectly influence safety.

In the present study, the modified vaccine was found to have a higher environmental stability than the standard vaccine at all the three temperature conditions. On this basis, the modified vaccine should be recommended for field use. The considerable enhanced survival of the modified vaccine culture, particularly at higher temperatures, would reduce both production costs and the incidence of vaccine failure. In addition, the immediate use of reconstituted freeze-dried vaccine would not be necessary in the field, since modified vaccine when reconstituted with PBS exhibit a considerable thermostability at temperatures up to 37°C. This would increase both the ease of use in the field, and lead to a reduction in wastage.

From the results of safety studies, it was concluded that the modified vaccine is safer than the standard one. Although both vaccines elicited untoward local reactions, a more pronounced reaction was seen in the animals vaccinated with the standard vaccine; despite the use of vaccine derived from the same seed strain. The higher safety would lead to less severe post-vaccinal reactions, therefore making the vaccine more acceptable by cattle owners and subsequently

reducing economic losses as well as the cost of controlling the disease. However, there is a need to further investigate the mechanisms of the enhanced safety.

From the efficacy studies, the modifications made in the vaccine did not influence the efficacy. A possible explanation is that variations in protection could be linked to the presence of some highly susceptible animals in one or the other group. The relatively limited size of each group and the degree of variations and individual susceptibility may not have allowed for observation of any significant trend. Another possible explanation is that the experiment was conducted under conditions that did not allow for expression of the instability of the current vaccine, since the two vaccines were administered immediately after reconstitution. Under field conditions therefore, the efficacy of the current vaccine may be significantly lower than the modified one and further investigation is needed.

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APPENDICES

Appendix 3.1a: Titres of *Mycoplasma* organisms for the standard vaccine incubated at 4°C, 25°C and 37°C after reconstitution, at various intervals between hr 1 and 168.

Time(hrs)	Vaccine	4°C	25°C	37°C
1	Standard	$10^{11.5}$	$10^{7.3}$	$10^{7.8}$
2	Standard	$10^{6.3}$	$10^{5.4}$	$10^{5.8}$
4	Standard	$10^{5.1}$	$10^{4.9}$	$10^{5.9}$
8	Standard	$10^{8.6}$	$10^{8.8}$	$10^{11.5}$
12	Standard	$10^{4.1}$	$10^{3.9}$	$10^{8.1}$
24	Standard	$10^{4.9}$	$10^{5.4}$	$10^{5.7}$
48	Standard	$10^{4.9}$	$10^{5.1}$	$10^{6.3}$
72	Standard	$10^{5.2}$	$10^{4.6}$	$10^{5.9}$
168	Standard	$10^{11.5}$	$10^{11.5}$	nil

Appendix 3.1b: Titres of *Mycoplasma* organisms for the modified vaccine incubated at 4°C, 25°C and 37°C after reconstitution, at various intervals between hr 1 and 168.

Time(hrs)	Vaccine	4°C	25°C	37°C
1	Modified	$10^{11.5}$	$10^{6.9}$	$10^{11.5}$
2	Modified	$10^{11.5}$	$10^{11.5}$	$10^{11.6}$
4	Modified	$10^{11.5}$	$10^{11.5}$	$10^{11.5}$
8	Modified	$10^{11.5}$	$10^{11.5}$	$10^{11.5}$
12	Modified	$10^{7.5}$	10^{7}	$10^{5.6}$
24	Modified	$10^{6.8}$	$10^{7.1}$	$10^{9.4}$
48	Modified	10^{7}	$10^{7.1}$	$10^{10.8}$
72	Modified	$10^{7.4}$	$10^{7.2}$	$10^{9.4}$
168	Modified	$10^{11.5}$	$10^{11.5}$	nil

Appendix 5.1: Average tail-tip circumferences (cm) of the vaccinates and the non-vaccinates (controls) thirty days post vaccination.

Day	Standard	Modified	Control
1	3.9	3.5	4.1
2	4.6	4.1	3.5
3	5.1	4.1	3.4
4	4.6	5	3.4
5	4.9	4.5	3.4
6	4.8	4.8	3.3
7	4.9	4.4	3.4
8	4.9	4.6	3.3
9	5.1	4.6	3.3
10	5.1	4.6	3.3
11	5	4.6	3.3
12	4.9	4.5	3.3
13	4.7	4.4	3.3
14	5	4.3	3.3
15	5	4.3	3.3
16	4.9	4.3	3.2
17	4.8	4.2	3.3
18	4.7	4.2	3.3
19	4.8	4.2	3.4
20	4.8	4.2	3.3
21	4.6	4.1	3.3
22	4.5	4	3.3
23	4.5	3.9	3.3
24	4.3	4	3.3
25	4.4	4	3.3
26	4.3	4	3.3
27	4.4	4	3.3
28	4.4	4	3.3
29	4.2	–	3.3
30	4.2	–	3.3
31	4.2	–	3.3

KEY

– indicates temperature record not available

Appendix 5.2a: CFT titres of the animals vaccinated with the standard vaccine.

	Week Post Vaccination								
Animal ID	WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9
1	0	0	1:20	1:20	1:20	1:10	1:10	0	0
10	0	0	1:10	1:10	1:10	1:10	0	0	0
11	0	0	1:40	1:40	1:20	1:20	0	0	0
20	0	0	1:20	0	0	0	0	0	0
502	0	0	1:20	0	0	0	0	0	0
504	0	0	1:40	1:20	1:20	1:20	1:10	0	0
512	0	1:20	1:40	1:20	0	0	0	0	0
533	0	0	1:20	0	0	0	0	0	0
538	0	0	1:10	0	0	0	0	0	0
541	0	0	0	1:10	0	0	0	0	0
545	0	0	1:40	1:40	1:20	1:20	1:10	1:10	0
548	0	0	1:80	1:40	1:40	0	0	0	0
556	0	0	0	1:10	0	0	0	0	0
557	0	0	1:10	0	0	0	0	0	0
563	0	0	1:80	1:80	1:40	1:40	1:40	1:20	1:20
564	0	1:40	1:160	1:80	1:80	1:80	1:40	1:40	1:40
571	0	0	1:40	0	0	0	0	0	0
586	0	0	1:20	1:20	0	0	0	0	0
587	0	0	0	1:20	0	0	0	0	0

Appendix 5.2b: CFT titres of the animals vaccinated with the modified vaccine.

	Week Post Vaccination								
Animal ID	WK 1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9
2	0	0	1:10	0	0	0	0	0	0
23	0	0	1:20	1:10	1:10	1:10	0	0	0
24	0	0	1:20	0	0	0	0	0	0
573	0	0	1:40	1:20	1:10	0	0	0	0
578	0	0	1:20	1:20	1:10	0	0	0	0

Appendix 5.3: Hudson and Turner pathology scores of the vaccinates and the non-vaccinates (controls) challenged with 3×10^8 CFU of *Mmm*SC three months post vaccination.

Animal ID	Lesion Size(cm)	Lesion score	Isolation	Factor	Pathology Score
5	43×21	2	2	3	12
7	44×25	1	2	3	6
10	13×10	2	0	2	4
11	-	0	0	1	0
13	27×21	2	2	3	12
15	24×11	2	2	3	12
16	2×0.2	1	0	1	1
17	58×34	2	2	3	12
19	6×2	1	0	2	2
20	4×3	2	0	1	2
500	52×30	2	2	3	12
501	19×14	2	2	2	8
502	-	0	0	1	0
504	-	0	0	1	0
511	-	0	0	1	0
516	7×1.7	1	0	2	2
521	-	0	0	1	0
526	5×1.5	1	0	2	2
529	34×39	2	2	3	12
530	-	0	0	1	0
531	-	0	0	1	0
536	21×16	2	2	3	12
541	8×4.5	2	2	1	4
546	31×25	2	2	3	12
547	-	0	0	1	0
549	-	0	0	1	0
551	-	0	0	1	0
553	20×15	1	2	3	9
554	4×3	2	2	3	12
555	66×39	2	2	3	12
556	6×5	1	0	1	1
557	-	0	2	1	2
558	-	0	0	1	0
559	23×15	2	2	3	12
562	-	0	0	1	0
563	2×2	2	0	1	2
564	15×1	1	0	2	2
566	34×33	2	0	3	6
569	9×0.5	1	0	2	2
574	12×1	1	0	2	2
578	-	0	0	1	0
580	20×14	2	2	3	12
584	-	0	0	1	0
588	10×0.5	2	2	2	8

KEY

– no lesion

Appendix 5.4a: Pathology scores and the number of days the animal showed fever in the group vaccinated with the standard vaccine.

Animal ID	Pathology Score	Duration of Fever (Days)
10	4	3
11	0	1
19	2	3
20	2	3
501	8	5
502	0	0
504	0	0
541	4	4
551	0	2
556	1	0
557	2	0
563	2	1
564	2	0
574	2	1
584	0	0
MEAN	**1.9**	**1.5**

Appendix 5.4b: Pathology scores and the number of days the animal showed fever in the group vaccinated with the modified vaccine.

Animal ID	Pathology Score	Duration of Fever (Days)
5	12	9
7	6	9
13	12	7
17	12	10
511	0	0
521	0	0
530	0	2
531	0	0
547	0	0
549	0	0
559	12	4
562	0	0
569	2	2
578	0	1
MEAN	**4**	**3.1**

Appendix 5.4c: Pathology scores and the number of days the animal showed fever in the control group.

Animal ID	Pathology Score	Duration of Fever (Days)
15	12	1
16	1	0
500	12	11
516	2	0
526	2	0
529	12	10
536	12	10
546	12	8
553	9	5
554	12	6
555	12	10
558	0	0
566	6	1
580	12	8
588	8	4
MEAN	**8.3**	**4.9**