THE EFFICACY OF CONVENTIONAL AND MODIFIED CONTAGIOUS BOVINE PLEUROPNEUMONIA T144 VACCINES SIXTEEN MONTHS POST VACCINATION

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A Thesis submitted in partial fulfillment of the requirements for the award of the degree of Master of Science (Immunology) in the School of Pure and Applied Sciences of Kenyatta University

MAY, 2014
DECLARATION

I declare that this thesis is my original work and has not been presented for a degree or any other award in any other university.

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Signature----------------------------------Date------------------------------------------

CANDIDATE

SUPERVISORS’ APPROVAL

This thesis has been submitted for examination with our approval as supervisors.

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DEDICATION

To my loving mother, Asenath Kavindu, who saw the need for educating me against all odds and who modeled my first encounter with excellence. And to my late grandmother, Elizabeth Kalunde who would never let me forget how special I am. May she find peace in her rest.
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To the entire Kenyatta University fraternity, I would like to register my appreciation for the assistance given in the course of my study. For the spice of both humor and much needed criticism, I am especially indebted to my colleagues. The challenges did appear manageable with your encouragement. Finally, I would like to honor my dear mother, Asenath Kavindu and my sisters, Faith Nzilani and Magaret Mwongeli for their unwavering support and encouragement during the entire period of the project. May all the glory go to the Lord God almighty for granting me the wish to see this project accomplished.
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<td>ANOVA</td>
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<td>Office International des Epizooties</td>
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<td>VRC</td>
<td>Veterinary Research Centre</td>
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<td>LAT</td>
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<td>CITRAD-EMTV</td>
<td>French Agricultural Research Centre for International Development of animal Production and Veterinary Medicine department</td>
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<tr>
<td>VVPC</td>
<td>Veterinary Vaccine Production Centre</td>
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<tr>
<td>FMD</td>
<td>Foot and Mouth Disease</td>
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ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP), caused by Mycoplasma mycoides subsp. mycoides biotype Small Colony (MmmSC) is a cattle respiratory disease. It is controlled using live vaccines, movement control and slaughter of affected cattle. The current CBPP vaccine, T144 is associated with several limitations that make delivery in the field difficult. One of main shortcomings is low efficacy and sub-optimum bacterial titers. This has necessitated modifications in formulation of the vaccine. The current study sought to compare the protection of cattle at sixteen months post-vaccination by T144 vaccine prepared in the media in current use and the medium modified to improve the vaccine efficacy. The modifications include, use of a buffer system based upon Hydroxyethyl Piperazine Ethanesulfonic Acid (HEPES), incorporation of a simple pH indicator to the culture medium and reconstitution of the vaccine culture in buffered saline instead of 1M MgSO4 as is common practice. Ninety zebu cattle were obtained from a CBPP free area and divided into two groups. One group of 46 cattle was used in a preceding study to determine the efficacy of the two T144 vaccine formulations at 3 months post vaccination. In the current study the remaining 44 cattle were divided into 3 groups. One group (control group) was given phosphate buffered saline while the other two groups were vaccinated with conventional vaccine and modified vaccine respectively. Challenge was performed 16 months after vaccination by endobronchial intubation using 60 ml of pure culture of the pathogenic MmmSC strain B237. For the first three months following vaccination the animals were bled once per week and on monthly basis thereafter. Serum was analyzed by the complement fixation test (CFT) and the competitive enzyme linked immunosorbent assay (c-ELISA) test. Efficacy of each vaccine was determined by comparing the disease outcome of the vaccinatnd control groups. The analysis was at 0.05 confidence level. In vitro results show that at the same storage temperature, titers of the modified
vaccine were higher than those of the conventional vaccine. This difference was significant including at 37°C (t = 2.394, df = 7, p < 0.05). The c-ELISA post challenge results showed that there was a significant difference in the reactions of the 3 groups (F = 32.21, df = 14, p < 0.05). Further analysis of the data using the tukey test indicated no significant difference between the two vaccinated groups (q = 1.151, p > 0.05). The vaccinated groups combined were however significantly different from the control unvaccinated group (q = 5.78, p < 0.05). The mean pathology scores of the animals vaccinated with the conventional and the modified T144 vaccine were 3.4 and 3.9 respectively. The difference was not significant (t = 0.0841, df = 13, P > 0.05). The protection conferred by the two vaccines was 61.8% and 56.1% for the conventional and the modified vaccine respectively. This study recommends the continued application of the conventional vaccine in the field. Further research is also recommended to find out how the high stability of the modified vaccine demonstrated in the in vitro study can be translated into improved vaccine efficacy.
CHAPTER ONE: INTRODUCTION

1.1 Background information

Contagious bovine pleuropneumonia (CBPP) is a highly infectious respiratory disease caused by *Mycoplasma mycoides* subspecies *mycoides* biotype Small Colony (MmmSC). According to FAO (2003), the disease is the cause of a myriad of problems in Africa which result in serious social-economic challenges. A study by Thompson (2005) reported that an estimated 24.4 million people in 19 African countries are at risk from the effects of CBPP. The disease is important due to its mortalities, loss of productivity, threat to food security and access to markets (FAO, 2004; Thiaucourt *et al*., 2004; Tambi *et al*., 2006). The fact that CBPP is included in the ‘A’ list of the World Organization for Animal Health of prioritized communicable animal diseases means that if it is present in a country, that country is excluded from international trade (FAO, 2003).

The disease which is transmitted by contact with infected cattle when droplets are inhaled or by contact with contaminated fodder and fomites, is characterized by severe, exudative pleuropneumonia that progresses to necrotic lobules and thickened pleura, cessation of rumination, a nasal discharge, a dry cough, and difficulty in breathing (Scudamore, 1995; EMPRES, 2002; Kusiluka and Sudi, 2003). In Africa, the mortality rate of CBPP ranges between 10% and 70% (Egwu *et al*., 1996). In most cases, it does not exceed 50% (EMPERES, 2002). Cattle that survive the disease may recover or have chronic pleuropneumonia. The chronically ill cattle constitute about 25% of affected cattle, they usually appear healthy despite having lung lesions and it is these so called ‘silent’ carriers that continue to spread the disease to the rest of the herd (EMPRES, 2002). In these
‘silent’ carriers, infected foci become encapsulated but later release organisms that are infectious. The disease is not transmitted through contact with excreta, animal housing and equipment or vehicles previously occupied by infected animals (Newton and Norris, 2000; Thiaucourt et al., 2004 a).

Contagious bovine pleuropneumonia is predominantly a disease of the genus Bos; both Bos taurus (European) and Bos indicus (Zebu) cattle are naturally infected (Provost and Joubert, 1970). There are many reported breed differences with respect to susceptibility. European breeds tend to be more susceptible than indigenous African breeds. Similarly, N’Dama cattle of Guinea and imported cattle from Europe are more susceptible than the Zebu (Provost et al., 1987). While almost zero mortality was reported in CBPP outbreaks in Europe, the pathological lesions were not different from those witnessed in outbreaks in Africa (Nicholas et al., 2000; Pilo et al., 2007). The phenomenon can be attributed to different mycoplasma strains, different cattle breeds as well as to the general health of the animal (Nicholas and Palmer, 1994; Nicholas et al., 2000; Vilei and Frey, 2004).

Contagious bovine pleuropneumonia occurs widely throughout the semi-arid regions of sub-Saharan Africa, in parts of Asia, and in Southern Europe (Sewell and Brocklesby, 1990). However, historically, it 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 by restriction of animal movement coupled with slaughter and compensation (Provost et al., 1987). The disease was eradicated from most parts of Western Europe in the beginning of
the 20th century but has been re-emerging every decade since then (Nicholas et al., 2000). During the 1980’s and 1990’s, countries such as France, Portugal, Italy and Spain reported CBPP outbreaks (OIE, 2009). The disease is believed to have been present in East and West Africa prior to the colonial era. It was introduced into South Africa from Europe in 1854 (Windsor, 2000). It is believed that CBPP could have been introduced into East Africa by cattle imported from India by British explorers in the 19th century (Masiga et al., 1996; Amanfu, 2009).

The colonial era and the subsequent two decades saw great progress in the control of CBPP in Africa. The greater part of western, southern and eastern Africa, were cleared of CBPP by slaughter and movement control (Egwu et al., 1996). When vaccines of moderate duration of immunity became available, CBPP control programmes became more inclined toward frequent vaccination and movement control. In the 1960’s and 1970’s, sustained research on CBPP in Kenya, Chad and other African countries as well as a massive international CBPP eradication campaign dumped ‘Joint Project 16’, resulted in the disappearance of clinical disease from most parts of Africa (FAO, 2002).

Between the year 2006 and 2008, OIE reported contagious pleuropneumonia outbreaks in a record 21 African countries (OIE, 2009). Kenya in particular has seen an upsurge of the disease over the past few years (FAO, 2004). The disease has since 1990 spread to Narok District and the Nyanza region as well as to Tanzania (Masiga et al., 1996). In 1992, the disease spread to Uganda. The disease was introduced into southern Tanzania in 1994
At present, Kenya, and indeed Africa is faced with the option of either accepting CBPP, in which case increased antibiotic use will ultimately lead to a reduction in both production and income or to eradicate the disease by slaughter and movement restrictions (FAO, 2004). A classic example of the implications of the latter choice is Botswana.
(where the disease was eradicated in 1996) where 320,000 cattle were slaughtered at a cost of US$100 million and an estimated US$400 million indirect losses (Geering et al., 1999) including increased malnutrition in children (Windsor and Wood, 1998; Boonstra et al., 2001). This is not considered a reasonable method of CBPP control in Africa (Jores et al., 2008; Dedieu et al., 2009). Restrictions on movement control are challenging to enforce due to the nomadic lifestyle of pastoralists. The only workable option for Africa is vaccination (Hubschle et al., 2003). However, the development of new vaccines would be costly, the financiers are not easy to come by and the process could take several years (FAO, 2004). Therefore the solution lies with the improvement of existing vaccines by improving their thermal stability, viability and immugenicity. There is also need for the development and validation of new CBPP control strategies and diagnostic tests (FAO, 2007).

Almost without exception, all effective CBPP vaccines have been based upon live versions of the disease-causing mycoplasma either attenuated or none attenuated. Current vaccine strains (T1 44 and T1 SR) for CBPP are made from freeze dried broth cultures of live attenuated Mycoplasma mycoides subspecies mycoides, biotype Small Colony (Mmm SC) strain and are generally considered to exhibit poor efficacy and also poor stability (Rweyemamu et al., 1995; Thiaucourt et al., 2004b).

1.2 Statement of the problem

In the last decade, there has been a substantial resurgence of contagious bovine pleuropneumonia in Kenya and in Africa at large, despite vaccination campaigns. In
1996, the Food and Agriculture Organization recommended the exclusive use of attenuated live *M. mycoides mycoides* SC T\textsubscript{1} 44, a streptomycin variant of the vaccine strain T\textsubscript{1} (OIE, 2008a). The T\textsubscript{1} 44 vaccine efficacy is not in doubt considering it was successfully applied in Namibia (Banhare, 2001). The T\textsubscript{1} 44 vaccine has been shown to confer protection for approximately 1 year (Hudson, 1971; Egwu, 1996). March *et al.* (1999) attributes the poor efficacy to apparent regression in manufacturing standards. March *et al.* 2002 sites problems in existing protocols; the need for buffering the growth medium so that neutral pH can be maintained, thereby enabling attainment of a minimum level of $10^8$ viable organisms per dose of vaccine, and the need for a pH indicator in the vaccine so that deleterious acidification would be visibly obvious and ineffective vaccine could be discarded and the need to substitute the use of 1 molar MgSO\textsubscript{4} vaccine reconstitution. The modified T\textsubscript{1} 44 formulation in this study had phenal red pH indicator and a buffer (HEPES). There was therefore a need to find out if advantages of the media modification could be passed on to the vaccine production process.

### 1.3 Justification of the study

Contagious bovine pleuropneumonia is a disease that causes high morbidity and mortality in cattle. Paskin (2003) and Geering *et al.* (1999) observe that CBPP is an animal disease emergency with a wide range of effects; compromising food security through loss of protein, major production losses, increased costs of production due to costs of disease control, disruption of livestock/product trade, inhibition of sustained investment in livestock production and painful suffering of animals. The disease also retards genetic improvement besides limiting the ability of cattle to work (Masiga *et al.*, 1996).
Tambi et al. (2006) estimates the total economic cost of CBPP in twelve sub-Saharan African countries (direct and indirect production losses plus disease control costs) at 44.8 million euros (3.7 million euros per country). An investment of 14.7 million euros to control CBPP would prevent a loss of 30 million euros. The financial return on investment in CBPP control is positive, with cost-benefit ratios that range from 1.61 in Ghana to 2.56 in Kenya (Tambi et al., 2006). The T₄₄ vaccine was successfully used to control CBPP in Namibia (Banhare, 2001). One vaccine vial of the modified vaccine costs 2750 Kenya shillings. It can be used to protect 50 cattle per year since it contains 50 doses. The cost of CBPP control will be 55 Kenya shillings per animal per year. Control of CBPP is therefore important as a way to salvage the losses and increase the incomes of cattle owners.

1.4 Research questions

a) What is the effect of the conventional T₄₄ vaccine and the modified T₄₄ vaccine on protection of cattle against CBPP?

b) What is the effect of the conventional T₄₄ vaccine and the modified T₄₄ vaccine on the duration of protection of cattle against CBPP?

1.5 Null Hypotheses

a) T₄₄ vaccine prepared in modified media or in conventional media does not enhance the protection of cattle against CBPP.

b) There is no difference in the longevity of the protection conferred by the
conventional and modified T$_1$ 44 vaccines.

c) The CFT and the c-ELISA tests are not equally good for the detection of antibodies against CBPP in vaccine trials for determination of vaccine efficacy.

1.6 Objectives

1.6.1 General objective

To determine the efficacy of the conventional and modified contagious bovine pleuropneumonia T$_1$ 44 vaccines at sixteen months post vaccination.

1.6.2 Specific objectives

a) To determine if cattle vaccinated with the modified vaccine are still protected 16 months after vaccination.

b) To compare the efficacy of the T$_1$ 44 vaccine prepared in conventional media and that prepared in modified media at 16 months following vaccination and challenge.

c) To compare the CFT and the c-ELISA tests in the detection of antibodies against *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony in sera.

1.7 Significance of the study

Livestock plays a central role in the social, cultural and economic lives of the African people. The cattle respiratory disease, CBPP, has been in the continent for many decades.
The current CBPP vaccines do not just have sub-optimal bacteria titres, they have low efficacy as well. While new vaccines have not been forthcoming, the HEPES based buffer system has been demonstrated to boost culture viability. Should the incorporation of this buffer in the T\textsubscript{1}44 vaccine boost its efficacy and stability then the continent will enjoy not just increased food security but a more vibrant economy as well.
CHAPTER TWO: LITERATURE REVIEW

2.1 Contagious bovine pleuropneumonia (CBPP)

2.1.1 The causative organism

*Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (MmmSC), the causative agent of CBPP, is a member of the bacteria division *f Firmicutes*, class *Mollicutes* (*mollis*-soft; *cutis*-skin) order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Mycoplasma* (Johansson and Pettersson, 2002; Westberg et al., 2004). *Mollicutes* lack a cell wall and are known as the smallest self-replicating organisms (Westberg et al., 2004; Pilo et al., 2007). A lipoprotein plasma membrane encloses the cell (Egwu et al., 1996).

Only a relatively small number of the genera *Mycoplasma*, *Ureaplasma*, and *Acholeplasma* have been isolated from animals. The most important pathogens are found in the *Mycoides* ‘cluster’ (Nicolet, 1996). They include the agents of contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), and agents of pneumonia, arthritis and mastitis in goats and sheep (Nicholas and Ayling, 2003). Animal mycoplasmas are extracellular parasites with a special affinity for mucous membranes where they adhere firmly to epithelial cells (Wise et al., 2006). Pathogenic mycoplasmas also demonstrate preference for certain tissues and organs, particularly the respiratory system, the urogenital tract, the mammary gland and the serous membranes (Razin and Tully, 1995). This is because *Mollicutes* have evolved from other bacteria by regressive evolution leading to reduced genomes and as a result a limited metabolic capacity (Pollack et al., 1997; Razin et al., 1998). Most of the pathogenic mycoplasmas are
adapted to one main host in which they are pathogenic since they are also highly host specific (Nicolet, 1996). Mycoplasma pneumoniae causes pharygitis and pneumonia in human beings (Murray et al., 2009). Mycoplasma genitalium, Mycoplasma hominis and Mycoplasma Ureaplasma species are also human pathogens (Waite et al., 2005). Mycoplasma mycoides subsp. mycoides SC was the first member of the mycoplasma cluster (Table 2.1) to be isolated in 1898 (Norcard and Roux, 1898).

Table 2.1 The Mycoplasma mycoides cluster (Murray et al., 2009)

<table>
<thead>
<tr>
<th>Mycoplasma species</th>
<th>Disease</th>
<th>Main host</th>
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<tr>
<td>M. mycoides SC</td>
<td>Pneumonia</td>
<td>Cattle (also sheep and goats)</td>
</tr>
<tr>
<td>M. mycoides</td>
<td>Arthritis and pneumonia</td>
<td>Goats and sheep</td>
</tr>
<tr>
<td>Large colony</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. capricolum subsp. Capricolum</td>
<td>Arthritis, mastitis and Pneumonia</td>
<td>Goats and sheep (also cattle)</td>
</tr>
<tr>
<td>M. capricolum subsp. Capricopneumonia</td>
<td>CCPP (pleuropneumonia)</td>
<td>Goats (also sheep)</td>
</tr>
<tr>
<td>M sp. Bovine group 7</td>
<td>Arthritis, mastitis and calf pneumonia</td>
<td>Cattle (also goats and sheep)</td>
</tr>
</tbody>
</table>
The cluster consists of six closely related mycoplasmas that are pathogenic for ruminants. Besides MmmSC, other species are *M. mycoides* subsp. *mycoides* biotype large colony (MmmLC), *M. mycoides* subspecies *Capri*, *M. capricolum* subsp. *Capricolum*, *M. capricolum* subsp. *capripneumoniae*, and *mycoplasma* sp. Bovine serogroup 7 (Mboser 7). However, in the year 2009 *Mycoplasma mycoides* biotype large colony type was combined with *Mycoplasma mycoides* subsp *capri* into a single subspecies while Bovine serogroup 7 (Mboser 7) strains were assigned to a separate species *Mycoplasma leachii* (Manso-Silvan et al., 2009). *Mycoplasma mycoides* subsp. *mycoides* biotype small colony forms an intermediate branch between the *Mycoplasma capricolum* species group and the *Mycoplasma capri* species group (Petterson et al., 1996).

The mycoplasma genome is a double stranded DNA molecule (Westberg et al., 2004). The genome for *Mycoplasma mycoides* SC was found to be 1212kb (Westberg et al., 2004). Most mycoplasmas express surface proteins that undergo reversible changes to alter the antigenic repertoire at the cell and population level. However, only two variable surface proteins are known in *M. mycoides* SC (Razin et al., 1998; Gaurivaud et al., 2004). The organism is easily inactivated under high temperatures. Heat sensitivity however depends on the medium in which the organism is suspended. For instance, in
lung fluid, MmmSC will be inactivated in 4 hours at 40°C and in 2 minutes at 60°C. Although inactivation is accelerated in normal saline, hypertonic solutions have a thermostabilising effect (Provost et al., 1987).

Mycoplasma is readily inactivated by high temperatures hence adherence to the absolute requirements of a cold chain during transportation, reconstitution and use in the field is very important (Nicholas et al., 2000). This is the only way the minimum number of mycoplasmas in each dose of the vaccine is preserved. The temperature conditions in the tropics are intolerable to the organism which can only survive for two or three days of exposure (Masiga et al., 1996). Resistance of *Mycoplasma mycoides* subspecies *mycoides* biotype small colony (MmmSC) to antibiotics is low.

The organism, like most other mollicutes grows facultatively (in aerobic and anaerobic environments) at a pH of 7.6 to 7.8. Anaerobically, it ferments glucose with the production of an acid, it also utilizes other energy sources like fructose (Nicholas and Bashirudin, 1995). Supplementing the media with uracil, thymine and guanine is important for MmmSC growth (Nicholas and Bashirudin, 1995; Egwu, 1996). Generally, mycoplasmas depend on the host for all amino acids except for cysteine, aspartic acid and glutamic acid (Razin et al., 1998). The addition of inhibitors such as penicillin and thallium acetate is necessary to avoid growth of other bacteria. The medium can be used as broth or solid medium with 1% to 1.2% agar (OIE, 1992).
Mycoplasma Mycoides SC can be identified by growth inhibition tests, immunofluorescent based methods, monoclonal antibodies (MAbs) and biochemical tests (OIE, 2010; Brooks et al., 2009). A Taqman-based real time PCR and a multiplex PCR for the detection of Mycoides mycoides subsp. mycoides biotype Small Colony have also been tested (Vilei and Frey, 2010; Schnee et al., 2011). The bacteria is relatively easy to grow, taking two to four days. It is evidenced by the appearance of small one millimeter colonies with characteristic ‘fried eggs’ appearance and a dense apparently elevated center in agar media or homogenous cloudiness often coupled with a silky fragile filament in fluid media (OIE, 2004).
Plate 2.1: The characteristic fried egg appearance of *Mycoplasma mycoides* subspecies *mycoides* biotype Small Colony (OIE, 2004).

The filaments whirl when shaken. The CBPP causing mycoplasma variant, MmmSC is culturally and antigenically similar to *M. mycoides mycoides* biotype large colony (MmmLC) which causes polyarthritis, pleuritis and pneumonia in goats. However, MmmLC does not affect cattle. The two variants also differ in their proteolytic activity and fermentation of sorbitol. The small colony variant also possesses the enzyme alpha gycosidase-transcarbamylase (Egwu, 1996). In a study seeking for a tool to distinguish African strains from the less virulent strain of *M. mycoides* SC, a non-synonymous single nucleotide polymorphism (SNP) coding for 6-phospho-ß-glucosidase was found in the Bgl gene (Vilei and Frey, 2004). In another study, Vilei *et al.* (2007) found that the strains expressing the Bgl isoform Val\textsubscript{204} included nearly all highly virulent African strains and showed high cytotoxicity towards embryonic bovine lung cells in the presence of ß-D-glucosides which are sugars that could result from innate immune responses in the host lung infection.

The strains expressing the Bgl isoform Ala\textsubscript{204} showed virtually no cytotoxicity and included low virulent strains such as strains T\textsubscript{1} SR, PG1 and T\textsubscript{1} 44. The study reported that Bgl isoform Val\textsubscript{204} is an indirect virulence factor as strains expressing this isoform are more prone to survive in elevated levels of these sugars which may be produced in the host lung during infection (Vilei *et al.*, 2007). The observed differences in cytotoxicity were attributed to the 6-phospho-ß-glucosidase activity affecting *M. mycoides* SC viability in environments with high ß-D-glucosides levels.
A phenomenon in which CBPP outbreaks in Europe resulted in almost zero mortalities was explained by among other reasons, presence of mycoplasma strains different from those in Africa (Nicholas et al., 2000; Vilei and Frey, 2004; Pilo et al., 2007). Molecular epidemiological studies conducted by Lorenzon et al. (2003) on 44 strains of MmmSC indicated that there are three distinct lineages of MmmSC circulating in Africa. This is consistent with earlier reports that CBPP occurred in the northern part of Africa prior to the well-documented introduction of the disease to South Africa in 1853 by one or two bulls imported from the Netherlands (Thiaucourt et al., 2004a).

2.1.2 Pathogenesis of contagious bovine pleuropneumonia

Studies on pathogenesis of the disease have been carried out (Nicolet, 1996; Westberg et al., 2004; Pilo et al., 2005). Genes encoding classical virulence factors such as adhesins, invasions or toxins have not been reported in the genome sequence of M. mycoides SC (Westberg et al., 2004). There has been no report on the existence of surface receptors mediating adhesion for M. Mycoides SC. However, other pathogenicity factors have been the subject of recent review (Pilo et al., 2007). Studies have shown that the mycoplasmas induce the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukins and interferon gamma (IFN-γ) upon their interaction with macrophages and monocytes (Nicolet, 1996).

The development of CBPP lesions seems to be dependent on the induction of auto-
immune and hypersensitivity reactions since agglutination antibodies are directed toward galactan (capsular polysaccharide) which is antigenically similar to the components of the normal lung (Kakoma et al., 1973; Logos, 1986). It has been suggested that the amount of galactan (a surface antigen) produced by a mycoplasma strain is directly proportional to its virulence (Lloyd et al., 1971). March et al. (1999) found strain T144 to contain the second largest capsular polyssacharide (CPS) content after Tan 8. Other strains included Gladysdale, T1 SR, V5, N6, Afade, Tan1 and M375.

A study by March et al. (2000) showed that the M375 strain of M. Mycoides SC isolated in Botswana produced less bacteriaemia in comparison to strains that produce larger amounts of CPS. The M. Mycoides SC genome has been reported to have redundancies in the capsule biosynthesis genes, which suggests the significance of the capsule as a virulence factor (Westberg et al., 2004). Dedieu et al. (2009) suggested the neccessity of a better understanding of the pathogenesis of CBPP and the protein specific protective immune responses.

Other antigens of MmmSC have also been characterized (Monnerat et al., 1999; Vilei et al., 2000). They include membrane lipoproteins, lipoprotein A (LppA) and lipoprotein B (LppB). These lipoproteins contain only a few epitopes which are specific to MmmSC (Monnerat et al., 1999). Another lipoprotein, lipoprotein Q (LppQ) is a predominant antigen of M. mycoides SC (Bonvin-Klotz et al., 2008). It induces an early immune response in cattle with CBPP which persists long after other immune responses have faded (Abdo et al., 2000). The LppQ N-terminal area is located on the outer surface of
the membrane and is strongly antigenic (Abdo et al., 2000), a character which together with LppQs high specificity, have made the lipoprotein to be exploited in the development of an indirect ELISA test for serological diagnosis and epidemiological investigations of CBPP (Bruderer et al., 2002).

Mycoplasmas are known to cause oxidative damage of host tissue by secretion of hydrogen peroxide (Tryon and Baseman, 1992). Hydrogen peroxide has been shown to accumulate from the oxidation of glycerol by *M. mycoides* SC (Miles et al., 1991). The glycerol catabolism system and its product, hydrogen peroxide, have been identified as potential virulent factors (Pilo et al., 2005). The system consists of ATP-binding cassette (ABC), transporter (GtsABC), and a membrane bound glycerol-phosphate oxidase (GlpO). In *M. mycoides* SC, the ATP-binding cassette (ABC) transporter proteins are involved in both active glycerol uptake and oxidative phosphorylation to dihydroxyacetone phosphate (DHAP) releasing hydrogen peroxide which is highly toxic (Vilei and Frey, 2001).

The pathogens ability to colonize the host tissue and to evade the host immune response by antigenic variation is enhanced by variable surface proteins (Persson et al., 2002). Only two variable surface proteins have been characterized in *M. Mycoides* SC, Vmm and PtsG (Gaurivaud et al., 2004). The variable surface protein, Vmm, is a small lipoprotein whose expression is at transcription level (Westberg et al., 2004).
2.1.3 Disease symptoms and pathology of Contagious bovine pleuropneumonia

Healthy animals placed in a CBPP-infected herd may begin showing signs of disease 20 to 123 days later (Masiga et al., 1996; Egwu et al., 1996) while experimentally, subsequent introduction of large quantities of infective material at the tracheal bifurcation, the incubation period is 2 to 3 weeks. This long incubation period during which the animal could be actively secreting the organism as well as cattle movement due to drought make CBPP particularly difficult to control (Masiga et al. 1996).

The disease is characterized by a primary broncho-pneumonic invasion due to inhalation of infective droplets, early secondary lymphatic involvement and tertiary vascular lesions, which lead to the characteristic necrosis and encapsulation. Adhesions connecting thickened visceral and parietal pleura are common (Egwu et al., 1996; Amanfu et al., 2000; FAO, 2002). Animals that succumb to the disease normally show extensive and marked inflammation of the lung and associated pleurae. In most cases this inflammation is unilateral (Cottew, 1979; Wesonga, personal communication). In an affected herd, CBPP may be seen in hyperacute, acute, subacute or chronic forms. About 21% of animals are resistant to the disease (CITRAD-EMTV, 1992).

2.1.3.1 Hyper Acute form of Contagious bovine pleuropneumonia

This form occurs during the onset of an outbreak (Masiga et al., 1996). It is rapidly fatal with no clinical manifestation. Some animals may die in one to three days with no sign of pneumonia (Scudamore, 1995). Death may be as a result of asphyxia, toxaemia or heart
failure. Those that survive develop acute lesions which may in turn become sequestra.

2.1.3.2 Acute form of Contagious bovine pleuropneumonia

The acute form of the disease manifests during the early stages of an outbreak. The first clinical sign noted may be an increase in body temperature (Masiga et al., 1996). This is followed by severe exudative pleuropneumonia that progresses to necrotic lobules and thickened pleura (Hudson, 1971), cessation of rumination, nasal discharge, a dry cough, and difficulty in breathing. A few days later the temperatures rise to 40°C or higher, accompanied by a fall in milk yield (in cows), anorexia and cessation of rumination. Chest pain becomes evident and affected animals stand with the elbows abducted and the back arched, the head extended and the nostrils dilated. Breathing becomes short and rapid (FAO, 2002). In the severe form of the disease, the mouth remains wide open and may contain foam. Mucoid discharge from the nostrils may occur. Exercise will aggravate the respiratory distress. Complications like arthritis, pericarditis, peritonitis and arbotion may occur.

According to Masiga et al. (1996) mortality rate can reach 50% but varies according to the severity of the disease. Many cases develop into the chronic form. Chronic lung lesions of up to 30 cm in diameter may develop and become encapsulated (these are what are referred to as sequestra). Some acutely ill animals recover completely and as a result there may be scars. In the acute form, the lesion is characterized by a severe fibrinous pneumonia with pleural exudates. The volume of the pleural exudates varies but can sometimes be several litres. The presence of yellow fibrin (up to 2-3cm thick, with an
‘omelette’ look) causes the lung to adhere to the costal wall (Egwu et al., 1996). The lung parenchyma appears pink to dark red in color, turning to grey with time. The marbled appearance of the lung is typical of CBPP (EMPERES, 2002).

2.1.3.3 Subacute and symptomless form of Contagious bovine pleuropneumonia

The subacute and symptomless forms are very frequent and are characterized by mild signs or no clinical signs at all. These initial signs are also not easy to distinguish from those of any severe pneumonia with pleuritis and as a result clinical diagnosis of CBPP is considered difficult (OIE, 2008a). Animals with these forms of CBPP are able to transmit the infection and therefore it is possible that they are the most dangerous of all (Masiga et al., 1999).

2.1.3.4 Chronic form of Contagious bovine pleuropneumonia

According to Masiga et al. (1999) the evolution into chronic form is very common towards the end of an epidemic and it evolves from the acute form. The affected animals may show unspectacular signs, with mild respiratory distress on exercise, but can also exhibit a violent and prolonged cough (FAO, 2002). The animal may remain in poor condition for a long time, depending on the size of the chronic lung lesion. Contagious bovine pleuropneumonia lesions are distinctive and therefore surveillance of CBPP involving Lung lesions is a useful method of monitoring the disease (OIE, 2008b). Fever is intermittent and the temperature is never high.
An animal with sequestrum may appear normal. Sometimes a palpation of the lung is necessary to detect sequestra that are located deeper in the lung (Wesonga and Thiacourt, 2000). Some chronically ill animals may recover completely leaving either scars or no scars. Some small sequestra may be replaced by fibrotic scars evidenced by a small fibrous attachment to the thoracic wall and an area of fibrosis in the lung (Masiga et al., 1999). The presence of *M. mycoides* varies with the stage of the disease and it may be difficult to isolate from chronic lesions especially where antibiotics have been administered (OIE, 2008b).

2.1.4 Mode of transmission of contagious bovine pleuropneumonia

Clinical cases are responsible for the spread of the infection because they can harbour the mycoplasma in the pharyngeal region and act as a source of infection (EMPERES, 2002). However it is also believed that chronic carriers, also called ‘lungers’ may break down and shed organisms into the bronchus and then into the environment (Provost et al., 1987). The main source of infection under natural conditions is the excretion of flugge-type droplets by the coughing animal. These droplets may be spread as far as 200 meters away (Masiga et al., 1996).

The type of husbandry adopted plays a major role in the epidemiology of the disease. High-density confinement in night shelters and use of common grasslands and watering places are major risk factors for the spread of the disease (Provost et al., 1987; Mariner et al., 2006a; Mariner et al., 2006b). Cattle movement plays a pivotal role in CBPP transmission across herds, regions, and countries (Domenech, 1988; Masiga and Domenech, 1995). These cattle movements are the norm in pastoralism, a major
production system in the arid and semi arid areas of Kenya (GOK, 2007b). The introduction of new cattle that have not been screened has also been cited as a doorway for the entry of CBPP in commercial ranches (GOK, 2007b).

2.1.5 Immune response to contagious bovine pleuropneumonia

Scientific research on immune response to CBPP has been ongoing (Dedieu et al. 2005; Totte et al., 2008; Dedieu, 2008). Protective immunity against CBPP seems to rely on both humoral and cell mediated immune responses (Dedieu, 2008). In a study dated 2008, in-vitro proliferation of CD4+ T cells and B cells was achieved by stimulating the cells using heat-killed *M. mycoides* SC. The cells came from cultures from animals with chronic CBPP (Totte et al., 2008). In an earlier study, Howard and Taylor (1985), found neither intradermal reactions nor lymphocyte proliferation correlated with protective immunity in CBPP, just like in other lung diseases.

The serum antibody response of MmmSC-infected cattle was however found to follow the usual course of IgM, IgG and IgA isotype-switch progression while the passive transfer of convalescent serum to naïve cattle conferred protection against subcutaneous challenge with MmmSC. In a study aimed at characterization of the humoral response to CBPP, Niang et al. (2006) reported that there were higher specific immunoglobulin A titers in animals with subacute to chronic infections. However, no correlation between antibody titers and clinical signs or lung lesions was found. Protective immunity has reportedly been conferred on calves following passive transfer of sera from cattle recovered from CBPP (Masiga et al., 1975).
The role of cell mediated immune responses in pathogenesis and control of *M. mycoides* SC has been investigated (Dedieu *et al.*, 2005; Totte *et al.*, 2008; Totte *et al.*, 2010). A study conducted by Dedieu *et al.* (2005) suggested that cell mediated responses to CBPP are specifically induced. Murray *et al.* (2009) indicates that the outcome of *M. mycoides* SC infection is dependent on the type of immune response raised, since IFN-\(\gamma\) promotes T\(_{H1}\) responses and inhibits T\(_{H2}\) responses. A persisted *M. mycoides* SC specific response mediated by IFN-\(\gamma\) secreting CD4\(^+\) T cells has also been reported in lymph nodes of all recovering cattle (Dedieu *et al.*, 2006). Here, the responses were higher in completely recovered animals than in recovering animals with lung sequestra. These IFN-\(\gamma\) producing CD4\(^+\) T cells that homed to the lymph nodes were postulated as memory T cells responsible for protective responses in these animals.

Although the high IFN-\(\gamma\) secretion in animals surviving CBPP have been the basis for the studies attaching significance to the cell mediated immune response, questions have been raised on the role of IFN-\(\gamma\) secreting CD4\(^+\) T cells in immunity (Hubschle *et al.*, 2003). There is consensus among researchers that cell mediated immunity appears to be important (Dedieu *et al.*, 2005; Dedieu *et al.*, 2006; Totte *et al.*, 2008; Totte *et al.*, 2010). A recent report by Sacchini *et al.* (2011) found that CD4\(^+\) T cells play a minor role in the control of a primary infection of cattle with Mycoplasma mycoides subspecies mycoides Small Colony biotype. Indeed, a subset of pathogen specific bovine CD4 cells with central memory cell characteristics were defined by Totte *et al.* (2010).
2.1.6 Diagnosis of contagious bovine pleuropneumonia

Provisional diagnosis of CBPP can be made on basis of clinical and pathological findings. However, due to the high percentage of CBPP infected animals that show no detectable clinical signs, the disease is often misdiagnosed and mishandled (Blancou, 1996). Pilo et al. (2007) site the difficulty in diagnosing the subacute and chronic phases of the disease as an outstanding challenge in the control of CBPP whether by serology or isolation. According to OIE (2008a), serological diagnosis of CBPP is only valid at herd level since individual animals may be in the early stage of CBPP prior to generation of specific antibodies, or in a late chronic stage when few animals remain seropositive. The presence of \textit{M. mycoides} SC varies with the stage of disease and it may be difficult to isolate from chronic lesions, especially where antibiotics have been used. This means that negative isolation results are not conclusive (OIE, 2010).

The causative organism can be isolated for identification from pieces of the lung, nasal swabs, trans-tracheal washes or pleural fluid. The specimen should be cultured within 48 hours of collection (OIE, 2004). The presence of specific antibodies coming from serological investigations should be treated as a sign of infection until such results are confirmed to be positive by follow up investigations such as culture and pathology (Bashiruddin \textit{et al.}, 1994; OIE, 2010).

A number of serological tests have been reported (Le Goff and Thiaucourt, 1998). The period preceding 1997 saw the use of complement fixation test (CFT) as the principal test
in the campaign for the eradication of CBPP. With the disease prevalence declining in subsequent years, the number of false positive results became critically high and so was its specificity (Regalla, 1995). In 2004, the World Organization on animal health adopted competitive ELISA (c-ELISA) as an alternative to the previously recommended complement fixation test (OIE, 2004; OIE, 2010). Use of the CFT results in a very low number of false negative results (Le Goff and Thiacourt, 1998; Bruderer et al., 2002). On the contrary, enzyme linked immunosorbent assay (ELISA) has been reported to be more specific especially in CBPP free areas (Amanfu et al., 1998). The test is easy to standardize (Amanfu et al., 2000). It is dependent on ability to reduce monoclonal antibody binding. The c-ELISA uses a mouse monoclonal antibody IgG1 that recognizes an epitope of *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony localized on a protein with an apparent molecular weight of 80kDa (Le Goff and Thiacourt, 1998; Gaurivaud et al., 2004).

A rapid latex agglutination test (LAT) for field CBPP screening was reported in 1999. It detects antibodies in serum or whole blood and takes two minutes (Ayling et al., 1999). A polymerase chain reaction (PCR) for the identification of *M. mycoides* SC was first reported in 1994 (Bashiruddin et al., 1994). Real-time PCR assays have since been developed (Lorenzon et al., 2008; Fitzmaurice et al., 2008; Vilei and Frey, 2010). A novel multiplex PCR assay for detection of CBPP has also been tested (Schnee et. al., 2011). The polymerase chain reaction (PCR) is a sensitive, specific, rapid and relatively easy method to use. It has become the primary tool for identification of *Mycoplasma mycoides* subsp. *mycoides* biotype Small Colony (OIE, 2010). In spite of the
shortcomings, serological diagnosis is more suitable for Africa for large scale monitoring of CBPP (Nicholas et al., 2000).

2.1.7 Prevention, control and treatment of contagious bovine pleuropneumonia

Hammond and Branagan (1965) give a detailed account of the historical approach to CBPP control in East Africa. The report starts around 1912 when the disease began to spread from the Laikipia District of Kenya, where it had been confined in preceding decades. In those days, quarantine of infected areas formed the basis of control. The colonial era and the subsequent two decades saw great progress in the control of CBPP in Africa. The greater part of western, southern and eastern Africa, were cleared of CBPP by slaughter and movement control (Egwu et al., 1996). When vaccines of moderate duration of immunity became available, CBPP control programmes became more inclined toward frequent vaccination and movement control. In the 1960’s and 1970’s, sustained research on CBPP in Kenya, Chad and other African countries as well as a massive international CBPP eradication campaign dumped ‘Joint Project 16’, resulted in the disappearance of clinical disease from most parts of the African continent (FAO, 2002).

In Kenya, a control policy of mass vaccination in enzoonotic areas, field testing of reactors and rigid movement control ultimately wiped out the outbreaks in the western part of the country (Kane, 1975; Wanyoike et al., 2004). Kenya has since 1901 always established CBPP zones (not mapped) based on the disease situation or disease outbreak which are constantly revised (Kariuki, 1971). According to the Ministry of livestock and
fisheries development (1978), four CBPP zones were in place before 1975 (Table 1.1). In the 1970’s, two east African neighbors, Kenya and Uganda had rid themselves off of CBPP (Egwu et al., 1996). However, during the 1980’s, CBPP reappeared in both countries (FAO, 1992; FAO, 1993). Hard economic times saw the funding available for public veterinary services decline in the 1980’s and 1990’s. This grossly affected the control programmes and surveillance (Windsor, 2000). Cattle movement restriction as a tool in CBPP control received a rapidly declining acceptance among the pastoralists (Masiga and Domenech, 1995).

Table 2.2 Zones established in Kenya for CBPP control before 1975 (Ministry of Livestock and Fisheries Development (MLFD), 1978)

<table>
<thead>
<tr>
<th>Zone</th>
<th>State</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Infected or severely threatened</td>
<td>The entire North &amp; Eastern districts; Turkana Marsabit, Mandera, Wajir, Isiolo, Garissa and Tana river</td>
</tr>
<tr>
<td>II</td>
<td>Under threat but no disease</td>
<td>West pokot, Baringo, Samburu, Kitui, Kilifi and Maasai land</td>
</tr>
<tr>
<td>III</td>
<td>Clean areas</td>
<td>The rest of the country, mainly central and Western highlands and part of the coast</td>
</tr>
</tbody>
</table>

In the 1970’s, two east African neighbors, Kenya and Uganda had rid themselves off of CBPP (Egwu et al., 1996). However, during the 1980’s, CBPP reappeared in both countries (FAO, 1992; FAO, 1993). Hard economic times saw the funding available for public veterinary services decline in the 1980’s and 1990’s. This grossly affected the control programmes and surveillance (Windsor, 2000). Cattle movement restriction as a tool in CBPP control received a rapidly declining acceptance among the pastoralists (Masiga and Domenech, 1995).
Vaccine coverage in eastern, central and western Africa declined in 1999 following the closure of the Pan-African rinderpest campaign (PARC) which ensured routine administration of rinderpest and CBPP vaccines.

Between the year 2006 and 2008, OIE reported contagious pleuropneumonia outbreaks in a record 21 African countries (OIE, 2009). Kenya in particular has seen an upsurge of the disease over the past few years (FAO, 2004). Outbreaks were experienced in Maasailand which had been CBPP free between 1968 and 1986 and as a result of cattle movement, the neighbouring Kitui, Mwingi, Machakos, Makueni, Nakuru and Thika Districts were now threatened (Wanyoike et al., 2004). Although CBPP has been endemic in the north and north-east of Kenya, it has since 1990 spread to the southern areas bordering Tanzania such as Narok District and the Nyanza region as well as to Tanzania where it had been absent for more than 30 years (Masiga et al., 1996). In 1992, other outbreaks appeared in the northwest of Tanzania as a result of which the disease spread to Uganda. The disease was introduced into southern Tanzania in 1994 (Masiga et al., 1996). The zonation for CBPP control in Kenya was revised in 2003 (GOK, 2003).

The surveillance of any animal disease requires not only a method of diagnosis but also the knowledge of the conditions for its transmission as well as an efficient system for notification and for sounding an alarm (Blancou, 1996). The potential methods for controlling CBPP include diagnosis, administration of antibiotics, quarantine measures and vaccination. Mycoplasmas are naturally resistant to beta lactam antibiotics (the penicillin group), essentially because they lack a cell wall which is what penicillin
targets (Nicolet, 1996). Ayling et al. (2005) carried out in vitro studies and found antibiotics targeting protein and nucleic synthesis to be effective. Other researchers also suggest that antibiotics such as tetracyclines can be used for treatment of CBPP (Yaya et al., 2004; Niang et al., 2007). It is important to note that antibiotic use is not recommended although routine administration is largely practiced (Amanfu, 2006; Hubschle et al., 2006). Pastoralist communities have been known to widely rely on chemotherapy (Twinamasiko, 2004; Mariner et al., 2006a; Mariner et al., 2006b).

The potential use of antibiotics in the control of CBPP has been discussed by the CBPP consultative group of the Food Agricultural Organization (FAO, 2007). Indeed, both in vivo and in vitro studies have demonstrated the usefulness for treating CBPP (Twinamasiko et al., 2004; Hubschle et al., 2006). The problem with antibiotic use is that antibiotics do not completely eliminate MmmSC colonization and results in: carriers that can infect susceptible cattle (Yaya et al., 2004; Niang et al., 2007), resistant mycoplasma strains and antibiotic residues in human food (Wesonga and Thiacourt, 2000). Quarantine based on detection of infected animals can be used to limit the spread of CBPP, but livestock movement makes implementation difficult (Wesonga, personal communication). Although slaughter of all infected herds led to eradication of CBPP from both Europe in the beginning of the 20th century and from Botswana in 1995, it is not considered a reasonable method for controlling the disease in Africa (Dedieu, 2008; Jores et al., 2008). Currently CBPP control in Kenya is based on a system of Zones reviewed in 2003 (GOK, 2003; Wanyoike et al., 2004).
Table 2.3 Zones established in Kenya for CBPP control in 2003 (GOK, 2003).

<table>
<thead>
<tr>
<th>Zone</th>
<th>State</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Clean zone (Prevention)</td>
<td>Costal Central and Western highlands.</td>
</tr>
<tr>
<td>II</td>
<td>Surveillance Zone (Neighbors clean zone)</td>
<td>Ukambani (Machakos, Mwingi, Kitui, Makueni) and Samburu district</td>
</tr>
<tr>
<td>III</td>
<td>Buffer Zone (Vaccination)</td>
<td>Northern part of Eastern province (Marsabit and Isiolo districts) Northern part of Narok District and Districts neighbouring the surveillance Zone</td>
</tr>
<tr>
<td>III</td>
<td>Infected Zone</td>
<td>The Southern part of Narok District &amp; other pastoral Districts like Turkana, West Pokot, Garissa, Moyale, Ijara and Lamu. Also, the Eastern half of Tana River District, Kajiado &amp; Trans Mara District</td>
</tr>
</tbody>
</table>

2.2 Contagious bovine pleuropneumonia vaccines

In the year 1852, Louis Willems of Belgium established the ways of inserting infected material in cattle in order to protect them. He was the first to publish a detailed account of the procedure (Willems, 1852; Egwu et al., 1996). African pastoralist, however, practiced a form of vaccination long before the discovery of preventive inoculation by Louis Willems (Blancou, 1996). The practice varied in form from South Africa where a thread saturated with pleuropneumonic lymph was inserted under the skin at the tip of the tail, to
Kenya and Mauritania where a lung fragment was subcutaneously inserted at the bridge of the nose (Curasson, 1936; Bizimana, 1994). In some countries the inoculum was first treated before administration. Louis Willem’s published his findings following the experimental establishment that subcutaneous inoculation of CBPP infective ‘lymph’ into areas of thick connective tissue in cattle protected them against contact challenge (Provost et al., 1987). The procedure, however, often caused fatal inflammatory reactions at the site of inoculum administration. The reactions also referred to as Willems’ reactions very often led to the loss of the tail (Blancou, 1996; Mbulu et al., 2004).

Vaccination against CBPP is mainly done with an empirically attenuated strain (Sheriff and Piercy, 1952; OIE, 2008) although the Willem’s method using live cells of *Mycoplasma mycoides* subsp. *mycoides* Small Colony biotype (MmmSC) for subcutaneous vaccination is still widely practiced in most African countries (Provost et al., 1987). The vaccines used in Africa over the years have been based on strains KH₃J (first isolated in Juba in Southern Sudan) and strain T₁ a 1951 Tanzanian isolate, (Karst, 1971; Howard and Taylor, 1985) which are used in their live forms as liquid or as lyophilized preparations. The use of KH₃J vaccine (passaged 88 times before its use in making vaccines) was however discouraged by the CBPP consultative group (Litamoi and Seck, 1999) because it confers poor immunity (Litamoi et al., 2007).

The T₁ strain isolated in Tanzania in 1951 was then serially passaged in embryonated eggs up to the tenth passage and used extensively in East Africa in CBPP vaccine campaigns during the 1960’s. However, due to unfavorable reactions, probably allergies
to egg proteins, the use of the avianised vaccine was discontinued (Tulasne et al., 1996). The strain was further passaged in chicken embryo up to the 44th passage (Davies et al., 1968). The broth vaccine was later replaced with a freeze dried live attenuated *M. mycoides* SC T144 vaccine (OIE, 2008a; OIE, 2008b). Strain T144 was subjected by CITRAD-EMVT to three passages in media containing increasing concentrations of streptomycin giving rise to the variant T1 SR (Provost et al., 1987). It was due to the strain T1 SR’s ability to grow in the presence of streptomycin that it was combined with the streptomycin containing rinderpest vaccine culture medium in the production of combined CBPP–rinderpest vaccine (Provost et al., 1987).

The occurrence of CBPP in T1 SR vaccinated herds during the 1995 outbreak in Botswana triggered doubts on immugenicity of the T1 SR parental stock (Masupu et al., 1997). The use of T1 SR was later stopped for its low potency and efficacy (Tulasne et al., 1996; Thiacourt et al., 2004). In 1996, the Food and Agriculture Organization recommended the exclusive use of attenuated live *M. mycoides* SC T144, a streptomycin variant of the vaccine strain T1 (OIE, 2008a). There are records to show that inactivated vaccines have been tested in the laboratory (Gray et al., 1986; Nicholas et al., 2004). Trials for completely new vaccine have been carried out in the recent past. These include the subunit vaccine based on the capsular polysaccharide of *M. mycoides* SC which proved unprotective to the mouse model (Waite and March, 2002) and a subunit vaccine based on the immunogenic lipoprotein LppQ which seemed to exacerbate the CBPP symptoms in vaccinated animals compared to control animals (Dedieu, 2008). A saponin inactivated whole cell vaccine (Nicholas et al., 2004) as well as immunostimulating complex
(ISCOM) formulation (Hubschle et al., 2003) have also been evaluated.

The eradication of CBPP from Botswana by stamping out policies in recent times is a significant success story (Masupu et al., 1997; Amanfu et al., 1998). The financial input was as would be expected quite enormous, certainly beyond the reach of many African countries. Current vaccines include T₁ SR and T₁44, a naturally mild strain isolated in 1951 by Sheriff and Piercy in Tanzania (Yaya et al., 1999; Wesonga and Thiacourt, 2000). Strain T₁ 44 has been shown to confer protection for approximately 1 year (Hudson, 1971; Egwu, 1996).

Vaccines are behind the CBPP eradication success stories in Africa. It is the vaccine’s safety and efficacy that protects the healthy herds (Mcleod and Rushton, 2007). However, both T₁ 44 and T₁ SR vaccines have been found to be unable to control the disease in the continent (Masiga and Domenech, 1995; Nicholas et al., 2000). This has been attributed to low vaccine efficacy (Kusiluka and Sudi, 2003; March, 2004). This poor efficacy is attributed by some scientists (Roeder, 1998; Thiacourt et al., 2004) to poorly maintained cold chains. A study done by March et al. (1999) indicates that these two attenuated vaccines’ poor efficacy is aggravated by apparent regression of manufacturing standards in Africa over the past decade. He cites problems in existing protocols; the need for buffering the growth medium so that neutral pH can be maintained, thereby enabling attainment of a minimum level of $10^8$ viable organisms per dose of vaccine, and the need for a pH indicator in the vaccine so that deleterious acidification would be visibly obvious and ineffective vaccine could be discarded and the need to substitute the use of 1
molar MgSO$_4$ vaccine reconstitution (March et al., 2002).

2.2.1 Contagious bovine pleuropneumonia vaccine titers

The T$_1$ 44 vaccine efficacy is not in doubt considering its successful application in Namibia (Banhare, 2001). March et al. (1999) suggests that the major factor behind poor vaccine efficacy is likely to be sub-optimal bacterial titres. These findings are consistent with those previously made by Rweyemamu et al. (1995). The Office International des Epizooties (OIE, 2008b; OIE, 2010) requirements stipulate that the minimum titer per vaccine dose must be at least 10$^7$ viable mycoplasmas. Due to the conditions under which the vaccines are transported, the organization recommended that production laboratories maintain titres of 10$^8$ mycoplasmas for every dose supplied (OIE, 1992). While doses of 10$^7$ and 10$^9$ of the T$_1$ strain have been shown to give similar protection rates of 63 to 80% (Gilbert and Windsor, 1971), lower doses of 10$^5$ resulted in very low protection.

March (2004) suggests that reduction in vaccine pH during culture growth makes it difficult to achieve and maintain effective titres. Besides excessive heat (greater than 43°C), the pH of the growth medium is the principal factor which affects mycoplasma viability (Gourlay and MacLeod, 1966; Windsor, 1978). Current vaccine media like the gourlay media and media F$_{66}$ (Gourlay, 1964; Provost et al., 1970) are poorly buffered which is the main reason for the fall in pH during MmmSC which translates to loss of culture viability. To avoid unacceptable loss of titer, the vaccine has to be used within 2 hours (Provost et al., 1987).
The growth medium used to produce the successful V5 broth vaccine did not contain glucose unlike current vaccines media and as a result, the pH did not fall below neutrality. The result was that vaccines were highly stable for relatively long periods at high ambient temperatures (at least 1 month at 37°C) (Turner et al., 1935; Hudson, 1968). Waite and March (2001) recommended the use of a buffer system based upon Hydroxyethyl Piperazine Ethanesulfonic Acid (HEPES) if glucose is to be kept in the growth medium. The buffer causes an increase in the final titre and boosts culture survival by maintaining a neutral pH during the growth and stationary phases (March et al., 2002).

2.2.2 Inclusion of pH indicators in CBPP vaccines

Although high ambient temperatures can evidently lead to MmmSC cell death, cultures remain stable at 37°C for several weeks as long as a neutral pH is maintained (Turner et al., 1935) and the culture is protected from direct sunlight (Hudson, 1968). It is not until the temperature is above 42°C that rapid cell death occurs. Therefore, as long as the culture pH is maintained above pH 6, vaccines should be stable for several days in the field (without a fall in titre). March (2004) suggests the incorporation of a simple pH indicator, such as phenol red to the culture medium and/or reconstitution fluid to monitor changes in pH. A printed pH chart attached to the vial will then provide a visual cut off point below which a vaccine should not be used.

2.2.3 Changes in CBPP vaccine reconstitution procedure

All the laboratories wishing to produce CBPP vaccine must obtain the T₁44 seed from
the Pan-African Vaccine Centre (PANVAC) which also certifies the quality of the vaccine (OIE, 2005b). However, the high instability in current vaccines can be attributed to the OIE recommended reconstitution procedure for CBPP vaccines. The procedure involves the use of a 1M MgSO$_4$ solution (Anonymous, 2001). The re-suspension of freeze dried CBPP vaccines in 1M MgSO$_4$ makes the vaccine even more acidic due to precipitation of the phosphate buffer component present in all current vaccine media (March et al., 2002). The acidic pH rapidly leads to mycoplasma death and vaccine inactivation. Not only does the MgSO$_4$ solution cause a drop in pH, but the culture pH is lower to begin with due to the presence of the reducing sugar (glucose) and the use of the T$_{44}$ and T$_{SR}$ which are highly acidifying strains.

The cumulative effect of all of these changes is a large reduction in pH and reduction in stability for ‘modern’ vaccines when compared to pre-1970 vaccine formulations (March, 2004). In contrast, when a vaccine culture is reconstituted in buffered saline it remains stable for many days at 37°C. In 1M MgSO$_4$ the titres drop by 6 log$_{10}$ over an 8 hour period (March et al., 2002).

The confidence scientists continue to have in the T$_{44}$ vaccine efficacy stems from such success stories as those of its application in Namibia (Banhare, 2001). Studies have been ongoing to improve the vaccine. March et al. (1999a) in his studies came up with recommendations that led to the formulation referred to in this thesis as the modified T$_{44}$ vaccine. Thus far, several studies have been done on various vaccines used to control CBPP. A number of researchers have reported on the efficacy of T$_{44}$ at different months following vaccination. Although the efficacy of the modified T$_{44}$ vaccine at 3
months following vaccination has been documented (Jores et al., 2008), there is so far, no published work on the efficacy of the modified T₄44 vaccine formulation for a longer duration. This research sought to establish the efficacy of the T₄44 vaccine prepared in both modified and conventional media at 16 months post vaccination.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Kenya Agricultural Research Institute (KARI) in Muguga, Kenya.

3.2 Sample size

Forty four local male zebu cattle aged 2-3 years were purchased from Kakamega, a CBPP
free zone after testing negative for CBPP on a rapid field slide agglutination test (SAT). So far no good small animal model is available for CBPP studies (Dedieu et al., 2009). Animals testing negative for CBPP on CFT and c-ELISA were then transported to KARI –Muguga, where they were allowed a period of 4 weeks to adapt to the new environment. During this period, the cattle were also drenched with an anti-helminth and vaccinated against foot and mouth disease (FMD), black quarter and anthrax. The cattle were grazed during the day and confined in a paddock at night until the time of vaccination.

3.3 Study design
Randomized experimental design was used. The 44 cattle were randomly divided in to three groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cattle</th>
<th>Treatment to be given</th>
<th>Nature of Vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>Conventional vaccine</td>
<td>Has phenol red pH indicator &amp; HEPES buffer</td>
</tr>
</tbody>
</table>
3.4 Estimation of T₄44 vaccine titre

Two Vaccine vials (a conventional vaccine vial and a modified vaccine vial) were purchased from the Veterinary Vaccine Production Centre (VVPC) in Nairobi at a cost of 2750 Kenya shillings each. Each vaccine had 50 doses of $10^8$ Mycoplasmas per dose. Briefly, 10 ml of diluent was transferred into vaccine vial (normal saline as used in the field for current vaccine and Phosphate buffered saline (PBS) for modified vaccine). A glass pipette was used to draw out the contents of conventional vaccine vial into 3 universal bottles. One of these vaccine portions (in universal bottles) was stored at 4°C, another at 25°C and the other at 37°C. The same was done with the modified vaccine.

To carry out a 10-fold dilution series from $10^{-1}$ to $10^{-10}$ of each vaccine, 0.3 ml of diluted vaccine were added to a bijou bottles containing 2.7 ml of the Gourlay media with phenol red. These ten fold dilutions were specifically done for each of the 3 portions (stored at 4°C, 25°C, and 37°C). Repeat 10-fold dilutions were done after 1, 2, 4, 8, 12, 24, 48, 72, and 168 hours. Each time 10-fold dilutions were done (in bijou bottles) the contents of the bijous were microtitrated (in microtitre plates) to measure the vaccine titres by colour changing units (Litamoi et al., 1996). To do this, 100 µl of Gourlay medium was dispensed into each well of columns 1 to 10 of the microtitration plate (column 11 was skipped, but 200 µl was dispensed into each well of column 12).
A multi-channel pipette was used to draw and add 100 µl of vaccine dilution of $10^{-10}$ into each well of row H from columns 1 to 10. A hundred microlitres of vaccine dilution $10^{-9}$ was added into each well of row G from columns 1 to 10. This process was repeated for each dilutions towards row A ($10^{-3}$), the lowest dilution of the bottles to be used in microtitration. Bottle dilution $10^{-1}$ and $10^{-2}$ were not used. With the aid of a plate reading mirror the cultures were examined daily for evidence of growth over the 10 day period. Growth was indicated by change of colour of the medium in the wells from pink to yellow. A dilution where only some of the wells registered growth was considered the end point. The titration in the microtitre plates went from $10^{-3}$ to $10^{-10}$, so the $10^{10}$ titres are not the end point.

After microtitration, 0.1 ml (a drop) of each of the dilutions in the 4 ml bijou bottle of vaccine reconstitution was flooded onto the gourlay medium agar plates in duplicates using aseptic techniques. The agar plates were allowed to dry before they were sealed (hermetic sealing). The remainder of the bijou bottle contents and the agar plates were incubated at $37^\circ C$ and examined daily for 10 days.

Spearman-Karber Formula was used to calculate the viable mycoplasma in the cultures (Litamoi et al., 1996).

\[
\text{Log}_{10} \text{ Median Dose} = (X_0 - (d/2) + d(\sum r_i/n_i))
\]
$X_0 = \log_{10}$ 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

$r_i =$ 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 starts at dilution $X_0$.  

The end points were 50% and were expressed as colour changing units (ccu $\log_{10}$) and the geometric means of tests given as titers.

### 3.5 Vaccination and observation

Using a list of random numbers, the animals were divided into 3 groups; both group 1 and 3 had 15 cattle each while group 2 had 14 cattle (Table 3.1). The animals were bled for pre-vaccination samples. Two of the groups were vaccinated; one with conventional T$_{144}$ vaccine and the other with T$_{144}$ vaccine prepared in modified media. The third group (the control group) was injected with phosphate buffered saline (PBS). The modified vaccine had been reconstituted in normal saline and cultured in media buffered with Hydroxyethyl Piperazine Ethanesulfonic Acid (HEPES). It also contained an indicator, phenol red. The conventional vaccine had been reconstituted in 1M Magnesium sulphate and cultured in gourlay media without an indicator.

### 3.6 Preparation of *Mycoplasma mycoides mycoides* SC infection culture

The inoculum was prepared from the primary seed (stored at passage 2). A 5 ml aliquot
of frozen culture was allowed to thaw for 30 min in a water bath at 37°C. Subculture in duplicate 10-fold dilutions were made in bottles containing pre-warmed Gourlay broth. Twenty microlitres of each dilution was plated on agar plates. Microtitration was also done and the plates incubated at 37 °C. Serial dilutions of these were prepared each day in bijoul bottles and incubated at 37 °C. Larger volumes of the 10 fold dilutions were prepared and scaled up for 3 consecutive days in 500 ml culture bottles holding a total of 1.8 litres of infection culture. At every stage of up-scaling, the cultures were incubated for 48 hours at 37°C. Serial dilutions were made every day from day 0 in bijou bottles. Growth was monitored daily based on turbidity and colour change, and appearance of filaments.

3.7 Challenge of experimental animals

The protection of the vaccinated cattle, sixteen months post vaccination was assessed by challenging with pure culture of a Kenyan field isolate of Mycoplasma mycoides small colony identified by growth inhibition and Polymerase chain reaction (CITRAD-EMTV, 1992). Challenge of the 44 animals was done endobronchially in the confinement of a crush. Using a bronchoscope, (VFS-2A, Swiss Precision, USA) 60 ml of MmmSC culture containing $3 \times 10^8$ cfu of MmmSC was introduced into the trachea at a concentration of $10^8 /\text{ml}$, which was a pure culture of the pathogenic B237 strain. The bronchoscope was passed through the nostrils to the larynx and down to the tracheal bifurcation and kept in this position until completion of intubation. Sixty millilitres of MmmSC using a syringe, followed by 15 ml of 1.5% agar suspended in distilled water and 35 ml of phosphate buffered saline (PBS) to flush down all the material to the target site. Rectal temperature
was taken daily at 8.30 am. The cattle were observed for clinical signs between 9.00 am and 3.30 pm daily, the time during which they were grazed. They were confined in a paddock at night.

3.7.1 Clinical examination of the challenged animals

Rectal temperature of the cattle was recorded daily at 8.00 am following challenge for the whole period of the trial. The animals were also observed for other clinical signs such as cough and nasal discharge. Cattle that showed a temperature of 39.5°C were considered to have fever. Where the 39.5°C temperature occurred for 10 consecutive days, the animal was considered to have clinical CBPP.

3.7.2 Collection and preparation of samples

All the animals were routinely bled once a week following vaccination. Samples of blood were taken from the jugular vein into 10 milliliter vacutainer tubes containing alserver solution. It was 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.

3.7.3 Examination of sera

All sera were analysed using two tests; the complement fixation test (CFT) of Campbell and Turner, 1953 with some modifications and competitive enzyme linked immunosorbed assay (c-ELISA) test, with an aim to analyse the same sera twice using c-ELISA and CFT to determine the best serological test for monitoring response to CBPP vaccines and for diagnosis. The complement fixing MmmSC antigen, sheep red blood cells (SRBC), positive control sera and buffer used in the CFT were prepared locally.
while the complement (C’) and hemolytic system (HS) were commercially obtained from CIRAD-EMVT, France.

### 3.7.3.1 Complement Fixation Test (CFT)

Campell and Turner complement fixation test (CFT) is based on whole cell antigens (Campbell and Turner, 1953). Briefly, the procedure started off with the preparation of the antigen. Two litres of tryptose broth were inoculated with T₁ strain *Mycoides* and incubated at 37 °C for 7 days. The culture was centrifuged at 10,000 *g* for 20 minutes. The packed organisms were resuspended in 20 ml of 0.85% NaCl and mixed vigorously after adding some sterile glass beads. The preparation was then autoclaved for 10 min at 15 1bs pressure and allowed to cool. Thereafter, it was agitated in an ‘atomix’ (Measuring and scientific equipment, London, England) for 5 minutes in order to achieve an even suspension. To preserve this suspension and make it isotonic, 0.05 g of phenol and 0.085g NaCl was added to each 10 ml. The antigen suspension was stored at 4 °C for 6 weeks, with shaking at weekly intervals, to allow the antigen to stabilize.

Sheep were bled into a graduated bottle containing 50 ml alsever’s solution at the ratio of 1:1 (alserver’s solution:sheep blood). The blood was then stored at 4 °C and used within 7 days. On the day of the test, the blood was spun for 10 minutes and the liquid part decanted. The cells were then washed three times with 9 volumes of veronal buffer with calcium and magnesium (VCM). This was one volume of pelleted red blood cells to 9 volumes of VCM. The red blood cell pellet was reconstituted to make a 6% suspension. Separately, haemolytic serum was diluted and an equal amount then added to the 6% red
blood cells. It was then left on the bench (under gentle agitation) for at least 30 minutes to allow sensitisation of the red blood cells. At the same time, the test and control serum were transferred to a test plate to which 25 µl of diluted antigen (whole cell *M. mycoides* SC) was subsequently added. To the 50 µl of mixture, 25 µl of diluted pre-titrated complement from normal guinea pig serum was added followed by incubation at 37 °C for 30 minutes with gentle shaking. Twenty five microlitres of the sensitized sheep red blood cells were added before another 30 minute incubation at 37 °C. This incubation involved shaking, vigorous enough not to allow cells to settle at the bottom of the wells. The plates were then kept at 4°C overnight then results read the next day for complement fixation.

If a serum sample contained antibodies to *M. mycoides* SC, then those antibodies bound the antigen and activated the compliment, which in turn was consumed during lysis of the *M. mycoides* SC cells. Once the haemolytic system (consisting of sheep red blood cells (SRBC) and guinea pig serum) was added, guinea pig antibodies bound the SRBC but there would be no complement left and therefore no haemolysis was observed. The classification of the results depended on the size of the SRBC button:

a) 4: complete fixation (All the SRBCs settle at the bottom of the well and clear supernatant is seen).

b) 3: almost complete fixation (very slight haemolysis)

c) 2: Partial fixation (partial haemolysis)

d) 1: very slight fixation (almost complete haemolysis)

e) 0: no fixation (complete haemolysis)
The interpretation of the results was done according to the OIE recommended procedure. At 1:10 dilutions, samples with a reading of 3 or 4 were considered positive and were retitrated to get the end point titre while those with readings of 2, 1 and 0 were considered negative.

3.7.3.2 Competitive enzyme linked immunosorbent assay (c-ELISA)

The competitive ELISA test published in 1998 (Le Goff and Thiacourt, 1998) is based on whole cell antigens in combination with a monoclonal antibody. Following the instructions given in the manufacturers manual (CIRAD-EMVT; FAO World reference centre for CBPP) a commercial c-ELISA test kit was used. Briefly, it consisted of 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 (HRP), Tetramethyl Benzidine (TMB) substrate, controls (Positive and negative control sera, conjugate control and monoclonal control) and other reagents.

In brief, 1/10 veronal buffer diluted serum samples were added to U-bottom shaped well microtitre plates. An equal amount of monoclonal antibody diluted according to manufacturer’s recommendations was then added to the wells. A hundred microlitres of the mixture was then transferred to the antigen pre-coated test plate. This was then incubated at 37 °C for 1 hour under gentle agitation. Then a HRP-conjugated secondary antibody targeting the MAb was added. The plate was incubated for another 30 minutes at 37°C then washed. A calourimetric substrate (Tetramethyl Benzidine) was added and the optical densities (OD) read at a wavelength of 450 nm. Where the serum sample was
CBPP positive, there was reduced binding of the monoclonal antibody and ultimately a reduced colour reaction.

The results were expressed as percentage inhibition (PI).

\[
PI = \left( \frac{OD_{Cm} - OD_{Test}}{OD_{Cm} - OD_{Cc}} \right) \times 100
\]

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. Animals with a PI value between 45% and 55% were considered weakly positive. Those with titers above 55 were regarded strongly positive. However, a PI value of 50% or more was considered positive.

3.7.4 Postmortem analysis and necropsy

A veterinary pathologist at the Kenya Agricultural Research Institute (KARI) was engaged at this point. Cattle showing fever continuously for 10 days were killed (in extremis) on day 11 of fever while cattle that did not succumb to disease were killed 45 days post challenge. Forty five days were chosen on the basis of being the incubation period for CBPP. Post mortem investigations were preceded by stunning and exsangination of the animals. Slaughtering of the animals was carried out at random without prior knowledge to which group the animal belonged. After the animals’ death blood for serum was collected in vacutainer tubes, the gross pathological changes of both lungs were examined for such pneumonic changes as, marbled appearance, sequestration,
pleuritis and/or the presence of a large amount of pleural fluid and adhesion of lungs to the rib cage (Egwu et al., 1996; Masiga et al., 1996; EMPRES, 2002). Where present, the pleural fluid was aspirated into a 10 ml syringe and immediately stored in a cool box for laboratory examination. The size of lung lesions (diameter in cm) was recorded.

Pieces of lung from an area between the lesion and the grossly normal area were cut and placed in sterile polythene bags, these polythene bags were placed in a cool box and transported to the laboratory where they were processed and cultured for isolation of *Mycoplasma*. Lesion scoring was carried out to determine severity of the disease in individual animals using the method of Hudson and Turner (1963). The scale was used to quantitate and intergrate both autopsy and bacteriological findings which are the main criteria of infection and susceptibility. The pathology score was calculated using a score allocated on the basis of whether adhesions (either fibrous or pleural adhesions) or lesions were present, a factor determined by the average diameter of the lesion and a score allocated whenever mycoplasma was isolated from the lung lesion. The presence of only encapsulated, resolving or fibrous lesions or the presence of pleura adhesions only were rated 1; if other types of lesions were present, namely consolidated, acute, necrotic or sequestrated, these lesions were rated 2; In addition, if *MmmSC* is isolated, 2 was added. This initial score was then multiplied by a factor depending on average diameter of the lesions: 1 (if the lesion size is under 5 cm), 2 (if it is over 5 and under 20 cm) and 3 (if it is over 20 cm). Hence, the maximum pathology score was (2+2)3=12. The mean pathology score of each of the three groups of cattle were obtained (Hudson and Turner, 1963).
The mean pathology scores of vaccinated cattle were divided by the mean pathology score of the control group. The results were subtracted from one and multiplied by a hundred to give the efficacy (percentage protection) of the vaccine (Hudson and Turner, 1963).

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

3.7.5 Mycoplasma isolation from lungs of CBPP infected animals

For isolation of Mycoplasma, small pieces of lung tissue were cultured in 4 ml bijou bottles containing gourlay media and incubated at 37 °C. Twenty four hours later, 1 ml of the supernatants was sub-cultured in $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions also in bijou bottles. A 0.2 ml volume of the serially diluted cultures were plated on agar plates and incubated at 37 °C under humid conditions. The 0.8 ml culture that remained was incubated at 37 °C. The incubation of both the plated and the bijou bottles took 10 days during which a daily inspection was done to monitor growth. Growth was confirmed by the change in colour, from pink to yellow or the presence of filaments in the bijous, or microcolonies in the agar plates.

3.7.6 Data analysis

The Paired t-test, the analysis of variance (ANOVA) and the Tukey test were used in the analysis of the data collected. This was done with a computer software package, Sigma XL. Comparisons between vaccine titers, temperature effects and pathology outcomes were the aspects of data analyzed. The student t-test statistic was used to analyze titres of the vaccine cultures and for analysis of data based on pathology. To determine whether
there were differences in the *in-vivo* serological data from the three groups, analysis of variance (ANOVA) was done, followed by the Tukey test. A $P$ value $< 0.05$ was considered statistically significant.

**CHAPTER FOUR: RESULTS**

4.1 T144 vaccine titrations

4.1.1 Vaccine titres at different storage durations

The two vaccines showed differences in titres at different times of titration. At one hour following storage at 4 °C, 25 °C and 37 °C, the titres were $10^{7.5}$, $10^3$ and $10^{7.2}$ for the conventional vaccine cultures (Figure 4.1a). During the same period of storage, the modified vaccine showed a uniform titre of $10^{10}$ (Figure 4.1b) at 4 °C, 25 °C and 37°C. The higher titres of the modified vaccine were maintained up to 72 hours.
For the first 12 hours following reconstitution, the modified vaccine culture showed higher titres than the conventional vaccine. The mean titre was $10^{8.8}$ and $10^{4.2}$ for the modified and conventional vaccine respectively. Between the 24 and 168 hours, the mean titres for the modified and the conventional vaccines were $10^{9.2}$ and $10^{4.6}$ respectively. For the 168 hours of storage, the modified T_1 44 vaccine titers were higher than those of the conventional T_1 44 vaccine titers (Figure 4.1a and 4.1b). Statistically, the difference between the two vaccines for the 168 hours was found to be significant ($t = 2.623$, df = 24, $p < 0.05$).

Figure 4.1a. *Mycoplasma* titres of the Conventional vaccine incubated at different temperatures.
Figure 4.1b. Mycoplasma titres of the Modified vaccine incubated at different temperatures. At 37 °C the vaccine maintained titres above 10^{10}.

4.1.2 Fluctuations in mean vaccine titer at selected storage temperatures

At 4 °C and 37 °C storage temperatures, the titre fluctuations in the conventional vaccine were much greater in magnitude as well as more frequent than those of the modified vaccine (Figure 4.1a and b). For instance, the conventional vaccine culture stored at 4°C dropped in titre from 10^{7.5} at hour 1 to 10^{2.9} at hour 2, it then rose suddenly to 10^{6.7} within the next two hours after which it declined to a minimum of 10^{3.4}, only to rise to 10^{7.4} at the 24^{th} hour. There was a repeated rise and fall in titre of the conventional vaccine at 37 °C until it reached the highest titre of 10^{10} before it dropped to 10^{3} on the 2^{nd} day. On the contrary, the titres of the modified vaccine cultures either did not fluctuate at all or fluctuated minimally in all the incubation temperatures. It is important to note that the in vivo protection results were not affected by the titre fluctuations since the diluted vaccine
used contained the recommended dose, 3.0 x 10^7 live mycoplasmas per millilitre.

4.1.3 Effect of storage temperature on vaccine titer

Besides causing titer fluctuations in some vaccine cultures, storage temperature variation also affected the average titre values of the vaccine cultures (Figure 4.1c, d and e). At the storage temperature of 4°C (Four degrees Celsius is the recommended temperature at which vaccine is administered (on ice) in the field) the conventional vaccine titres were low (Figure 4.1c).

![Figure 4.1c. The titres of the conventional and the modified T₄44 vaccines stored at 4°C](image-url)
Bars show mean titre ± SD. The conventional vaccine titres were significantly lower than those of the modified vaccine (P < 0.05).

Both the modified and the conventional vaccine had their lowest average titres at 25 °C.
Figure 4.1d. Titres of the conventional and modified T,44 stored on the laboratory bench. Room temperature is taken to be 25 °C. Bars show mean titre ± SD. The modified vaccine titres were however significantly higher than those of the conventional vaccine (P < 0.05).

Higher titers were also noted in the modified vaccine at the extreme storage temperature of 37 °C.
Figure 4.1e. Titres of the conventional and modified T44 stored at 37 °C. Bars show mean titre ± SD. The difference between the two vaccine was significant at 37 °C (P < 0.05).

At different storage temperatures, titer differences were observed among the conventional vaccine cultures (Figure 4.1a). At 37 °C, the vaccine culture registered higher titres than at 4 °C, however, the difference was not significant (t = 1.216, df = 7, P > 0.05). Titres of the same vaccine culture stored at 4 °C were significantly different from those stored at and 25 °C (t = 3.065, df = 7, P < 0.05). A significant difference was also noted between the titres of the conventional vaccine culture stored at 25 °C and that stored at 37 °C (t = 2.506, df = 8, P < 0.05).

Similarly the titers of the modified vaccine stored 37 °C were higher than those of the culture stored at 25 °C and the difference was significant (t = 4.096, df = 7, P < 0.05). Although the titres stored at 37 °C were higher than those stored at 4 °C, the difference was not significant (t = 1.814, df = 7, P > 0.05). The mycoplasma content in the vaccine stored at 4°C was higher than that of the culture stored at 25 °C, the difference was however not significant (t = 2.013, df = 7, P > 0.05).

4.2 Clinical manifestations of CBPP in cattle following challenge

Temperature records were maintained from day 0 to 45 post challenge. The onset of fever (39.5 °C and above) was on day 4 following intubation and was observed in 21/44 animal as follows: Three out of 15 animals in the conventional vaccine group, five out of 14
animals in the modified vaccine group and 13 out of 15 animals in the control group. The normal body temperature of cattle is 37 °C.

Some animals extended their necks and/or appeared generally dull while resting (Plate 4.1).

Plate 4.1 A zebu bull after challenge with *M. mycoides* SC. The animal is showing clinical signs of CBPP, namely; extended neck and dullness.
Other clinical manifestations of CBPP observed included, a cough (five cattle; two controls, two animals vaccinated with the conventional vaccine and one animal vaccinated with the modified vaccine), laboured breathing which was observed in three cattle, all from the control group (Table 4.1).

**Table 4.1 Clinical symptoms and proportions (numbers) of cattle per group**

<table>
<thead>
<tr>
<th>Cattle group</th>
<th>Fever</th>
<th>Labored breathing</th>
<th>Cough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1(n=15)</td>
<td>20% (3/15)</td>
<td>0% (0/15)</td>
<td>13.3% (2/15)</td>
</tr>
<tr>
<td>(Conventional Vaccine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2(n=14)</td>
<td>35.7% (5/14)</td>
<td>0% (0/14)</td>
<td>7.14 % (1/14)</td>
</tr>
<tr>
<td>(Modified Vaccine)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3(n=15)</td>
<td>86.7% (13/15)</td>
<td>20% (3/15)</td>
<td>13.3% (2/15)</td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There was no significant difference in the number of cattle that developed fever in the group vaccinated with the modified vaccine and that vaccinated with the conventional vaccine (p > 0.05)
4.3 Immunoassay results

4.3.1 Complement fixation test results

Complement fixation test (CFT) results showed that none of the pre-vaccination samples were positive. Four post vaccination samples from an animal vaccinated with the conventional vaccine were found to be positive at dilution 1/10 (Table 4.2). A single sample from one of the control animals also tested positive by CFT at dilution 1/10. Analysis of post challenge samples by CFT showed that 13 out of 29 vaccinated cattle were positive. These were, 5 out of 15 cattle vaccinated with conventional vaccine and 8 out of 14 vaccinated with the modified vaccine. Seven of the control animals were also found to be positive. Thirty five percent of the 20 cattle found to be positive by CFT after challenge did not show clinical signs. The percentage of positive samples and the actual number of the samples were tabulated alongside the serological test applied complement fixation test (CFT) titres produced in response to challenge were as high as 1:320.

The percentage of samples that showed presence of antibodies is shown in the table 4.2 alongside the actual number of samples.
Table 4.2 The number and percentage of serologically positive samples following vaccination and challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre-vaccination</th>
<th>Post-vaccination</th>
<th>Post-challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFT</td>
<td>c-ELISA</td>
<td>CFT</td>
</tr>
<tr>
<td>1 (n=15) (Convent. Vaccine)</td>
<td>0% (0/85)</td>
<td>0% (0/85)</td>
<td>0.25% (4/1596)</td>
</tr>
<tr>
<td>2 (n=14) (Modified Vaccine)</td>
<td>0% (0/85)</td>
<td>0% (0/85)</td>
<td>0% (0/1596)</td>
</tr>
</tbody>
</table>
There was no significant difference between the two vaccinated groups ($q = 1.19$, $P > 0.05$).

### 4.3.2 Competitive ELISA results

The competitive ELISA indicated that no animal was positive prior to vaccination. Following vaccination, the titers in the vaccinated group increased substantially. Cattle vaccinated with the conventional vaccine had higher titers than the control unvaccinated group (Figure 4.3a). Similarly, the group treated with the modified vaccine posted much higher titers than the control group. The modified vaccine registered higher titers than the conventional vaccine following vaccination. Computation with one way analysis of variance (ANOVA) indicated that there was a significant difference in the reactions of the three groups ($F = 3.84$, df = 3, $P < 0.05$). The Tukey test was the post ANOVA test of choice. There was no significant difference between the two vaccinated groups ($q = 1.19$, $P > 0.05$).
P > 0.05). The vaccinated group was however significantly different from the control group (q = 4.78, P < 0.05).

The mean percentage inhibition (calculated from the optical density) for the two groups after vaccination was between 34% and 47%. Fourteen out of 15 of these were cattle vaccinated with the conventional vaccine and 11 out of 14 vaccinated with the modified vaccine. Among the positive animals, ten out of 14 (71.4%) vaccinated with the conventional vaccine and seven out of 11 (63.6%) cattle vaccinated with the modified vaccine were strongly positive. The percentages of samples that showed presence of antibodies are shown in Table 4.2 alongside the actual number of samples. High titres of 62% and above were recorded in 7 vaccinated animals. Such animals had large quantities of MmmSC antibodies.
Figure 4.3a. The mean antibody levels by c-ELISA in cattle after vaccination with the Conventional and the Modified vaccines. The controls received phosphate bovine saline instead of a vaccine. PI is the percentage inhibition calculated from the optical densities in the c-ELISA test plate. There was no significant difference between the two vaccinated groups (q = 1.19, P > 0.05).

Post challenge data indicated that 43 out of 44 challenged cattle cattle were seropositive.
The percentage inhibitions calculated from the optical densities were on average above 60%. The three groups were found to be significantly different (F= 32.21; df = 14; P< 0.05). The difference between the titres of the two vaccinated groups was not significant (q = 1.15, P > 0.05). The vaccinated cattle, however, registered significantly higher titers than the control group (q = 5.78, P < 0.05).

![Graph showing Mean weekly antibody levels as measured by c-ELISA in vaccinated cattle after challenge.](image)

**Figure 4.3b.** Mean weekly antibody levels as measured by c-ELISA in vaccinated cattle after challenge. The difference between the two vaccinated groups was not significant (q = 1.15, P > 0.05).

### 4.3.3 Comparison of the CFT and the c-ELISA test results
A total of nineteen cattle tested positive by the two tests following challenge. By the end of week one, 5 out of these 19 cattle tested positive by c-ELISA and by end of week two, all the 19 cattle had tested positive by c-ELISA. By CFT, none of the 19 cattle had detectable antibodies by the end of week one, 13 out of the 19 cattle turned positive by the end of week two. The remaining 6 cattle tested positive by CFT after the end of the second week. (Table 4.3).

Table 4.3 The 19 cattle that tested positive by both CFT and c-ELISA after challenge.

<table>
<thead>
<tr>
<th>Test</th>
<th>Number of Positive Cattle / The week after challenge when antibodies were detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 1</td>
</tr>
<tr>
<td>CFT</td>
<td>0/19</td>
</tr>
<tr>
<td>c-ELISA</td>
<td>5/19</td>
</tr>
</tbody>
</table>

The Table shows that the two tests detected antibody presence at different times in the same animal.
4.4 Pathology and lesion scores

4.4.1 Clinical signs, lung condition and pleuritis

The experiment was designed so that cattle showing fever consecutively would be sacrificed on the 11th day of fever before they reached the terminal stage of disease. Using this criteria, a total of 21 cattle (47.7%) were killed after showing fever for 11 days while the remaining 23 (52.3%) were sacrificed at 45 days post challenge. Post-mortem examination showed gross pathological lesions characteristic of CBPP. These included consolidation of the lung parenchyma, pleuritis and well-developed sequestra (Plate 4.2). Sequestra were characterized by a fibrous capsule surrounding lobules of lung tissue. The lesions were either unilateral or bilateral. In some cases, the pleural cavity contained copious amounts of fluid (up to 5 litres) of yellowish-coloured clear fluid with fibrinous flecks.
Plate 4.2 Cross section of lung tissue from a bull challenged with *MmmSC*, showing the diameter of a sequestra on the lung tissue (arrow). The lung was obtained from one of the cattle in the control group. The controls had significant higher pathology score than the group given the conventional vaccine ($P < 0.05$).

Other lesions included fibrous adhesions of the parietal and visceral pleurae in long standing cases. Pleuritis was characterized by a shiny surface with thickening of pleura or adhesions (Plate 4.3). Consolidation was a characteristic of CBPP with both red (Plate 4.4) and grey hepatization.
Plate 4.3 The thoracic cavity of one of the cattle challenged with *MmmSC*. There were adhesions between the parietal pleura and the visceral pleura (see arrows). The lung could not be detached without the application of force. The lung was obtained from one of the cattle vaccinated with the conventional vaccine. The mean pathology scores of the two vaccinated groups were not significantly different ($P > 0.05$).
Plate 4.4 A cut surface of lung tissue from one of the cattle challenged with *Mmm*SC showing Red hepatization (single arrow) and normal part of the lung (double arrow). The lung was obtained from one of the cattle vaccinated with the modified vaccine. The mean pathology scores of the two vaccinated groups were not significantly different (P > 0.05).

Large lesions were particularly present in the control animals. Clinical signs were absent in half (50%) of the serologically positive vaccinated cattle (by the CFT test) compared to only 14% of the control animals. Analysis of pathological lesions was carried out using the student t-test. The mean pathology scores of the two vaccinated groups were not significantly different (t = 0.841, df = 13, P > 0.05). The controls had significant higher pathology score than the group given the conventional vaccine (t = 2.71, df = 13, P <
Similarly, the controls had significantly higher pathology score than the group given the modified vaccine ($t = 2.6, df = 13, P < 0.05$).

### 4.4.2 Isolation of bacteria from infected tissue

*Mycoplasma mycoides* subspecies *mycoides* biotype small colony was isolated from the lungs of 31 out of the 44 CBPP challenged animals. These were; 11 out of 15 cattle (73%), 9 out of 14 cattle (64%) and 11 out of 15 cattle (73%) in the groups vaccinated with the conventional vaccine, the modified vaccine and the control groups respectively.

Fourteen of the 31 (45%) cattle with *Mycoplasma mycoides* had no visible lesions.

### 4.4.3 Scoring of lesions by Hudson and Turner scoring system (1963)

Twenty five out of the 44 (57%) animals had lung lesions (Table 4.4). Sixteen of these had a high pathology score between 8 and 12. Twelve out of the 25 (48%) animals that developed lesions were vaccinated. These were six from each of groups 1 and 2. It was noted that the vaccinated group had fewer and smaller lesions as compared to the control group, hence, lower mean pathology scores. The mean pathology scores of the animals vaccinated with the conventional and the modified T1:44 vaccine were 3.4 and 3.9 respectively. The cattle were killed after 11 days of fever or 45 days following infection and therefore the observed lesions were not terminal.

### 4.5 Protection rates

The protection rate for the conventional and that of the modified vaccine was 61.8% and 56.1% respectively (Table 4.4).
Table 4.4 The protection rates of the conventional and the modified T₄ 44 vaccine 16 months post vaccination

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>No. of cattle</th>
<th>No. of cattle with lesions</th>
<th>Pathology Score Mean</th>
<th>Protection Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional vaccine</td>
<td>15</td>
<td>6</td>
<td>3.4</td>
<td>61.8</td>
</tr>
<tr>
<td>Modified vaccine</td>
<td>14</td>
<td>6</td>
<td>3.9</td>
<td>56.1</td>
</tr>
<tr>
<td>Negative control</td>
<td>15</td>
<td>13</td>
<td>8.9</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER FIVE: DISCUSSION

5.1 Variations and fluctuations in vaccine titers

The fluctuations demonstrated by the conventional vaccine cultures in this study are not a new phenomenon, and neither is the absence of fluctuations when modified media is used. A study by Waite and March (2001) reported that the modified media stabilizes the MmmSC in culture. Due to the high stability of the modified T144 vaccine under extreme temperatures (37 °C), its application in the field is expected to be without decline in viability or fluctuations in titer. The titer fluctuation recorded for both vaccines at 25 °C (on the bench where temperature was presumed to be 25 °C), are probably as a result of environmental temperature fluctuations.

5.2 Clinical signs of contagious bovine pleuropneumonia

Among the clinical observations was fever recorded between day 4 and 20 following challenge. Documented findings by Hudson and Turner (1963) reported fever after three weeks while separately, Wesonga and Thiacourt (2000) observed fever between 40-57 days after challenge. In the current study, fourteen out of the 21 cattle showing fever, registered the febrile temperatures between days 7 and 20 following infection. This would therefore be the appropriate time to pick a donor for a challenge by contact experiment. Laboured breathing and coughing has been reported in previous studies (Provost et al., 1987; FAO, 2002). It has been reported that a high percentage of animals infected with the disease fail to produce detectable clinical signs even in cattle where subsequent postmortem results indicate the presence of lesions. It is for this reason that
some researchers have recommended the combining of clinical examination with post-mortem and laboratory culture findings as well as serological tests in diagnosis (FAO, 2002).

5.3 Humoral responses in experimental animals

In the post vaccination period, the CFT test detected only one positive animal in spite of the alternative serological tests detecting a substantial number. Hubschle et al. (2003) made similar observations following infection of animals with immunostimulating complexes (ISCOM) vaccine. Previous studies have indicated that serology cannot be used exclusively to determine the effect of vaccination. According to Hudson (1968), studies based on the V₅ strain indicated that the response to vaccination varied between different cattle groups. Similar observations were made by Hudson (1965) in a study based on the highly attenuated KH₃J strain where he noted that though there was a minimum serological response from the use of an immunizing dose of the vaccine, the protection was not comparable with that of the more virulent V₅ strain.

In the current study, twelve out of the 22 CFT positive cattle had CBPP lesions from which *M. mycoides* was isolated. According to Kusiluka and Sudi (2003), antibody titers are highly individualized often bearing no relationship to the severity of the lesions. Gilbert and Windsor (1971) suggested that with the T₁ strain, in a given cattle population, protection is related to the presence of antibodies on a group basis as detected by CFT.

Turner (1954) was among the earlier scientists who reported on the inability of
serological tests to identify cattle in the latent phase of CBPP (the time between infection and seroconversion). He noted that this period ranged between seven weeks to 200 days. In the current study, a big number of control animals took long to respond to challenge and when they did, the increase in antibody titres was sudden. Naïve animals may not show response by CFT at week 4 (Wesonga, personal communication). Nicholas et al. (2004) made similar observations during trials for a saponin inactivated whole cell vaccine. From the CFT data collected in that trial, naïve animals took as long as 3 months to seroconvert.

The complement fixation test (CFT) and the competitive ELISA test seem to detect antibodies against MmmSC animals at different stages of infection. For instance none of the cattle was positive by CFT one week after challenge. This was in spite of 23 cattle being positive by c-ELISA at the same period. According to some researchers CFT identifies as CBPP positive only a small number of cattle during the early stages of the disease. Some of the cattle began showing CBPP antibodies a few days prior to their being killed (45 days after challenge). There is a possibility that by using the complement fixation test exclusively, the immune response could have been missed in some clinically ill cattle. In an experiment where cattle were allowed to live longer with CBPP the complement fixation test was found to detect almost 100% of infected herds (OIE, 2008b).

The c-ELISA test detected as positive a total of 43 cattle while the CFT registered only 20 cases. This is consistent with finding made previously by researchers such as
Campbell and Turner (1953) and Bellini et al. (1998) who describe the complement fixation test as a very specific test, with a very low number of false positives.

Dohoo et al. (2003) explains that in interpreting the CFT and C-ELISA results, either of two approaches can be used. A parallel interpretation means a sample is taken to be positive if at least one of the two tests shows it to be so. In serial interpretation however, a sample is not considered positive unless it tests positive by the two tests (Dohoo et al., 2003). Seroconversion studies in areas where vaccination has been carried out have indicated a poor agreement between the complement fixation test and the competitive ELISA test (Matua et al., 2006). Amanfu et al. (2000) attributes the poor incompatibility of the two tests to their detecting different isotypes of antibodies. According to Bruderer et al. (2002) the c-ELISA measures IgG titres because it has a higher complement fixation ability (Goldsby et al. 2003), while CFT measures IgM titres.

While 19 cattle were found to have antibodies by both the complement fixation test and competitive ELISA, some of the cattle previously shown to have circulating antibodies by CFT were no longer being detected by the CFT test. According to OIE (2008b) the sensitivity of the complement fixation test falls after the acute phase of CBPP. Unlike with the complement fixation test, any animal that tested positive by c-ELISA continued to be positive by the same test until it was killed. Similar observations have been made in earlier studies (Le Goff and Thiacourt, 1998), where, the LppQ-ELISA gave positive results for a longer time following infection compared to the complement fixation test.
5.4 Pathology and *Mycoplasma mycoides* in post mortem samples

The isolation of *M. mycoides* from lungs of animals with no macroscopic lesions has been reported previously (Gourlay, 1964; Davies *et al.*, 1968). It was noted that, of the animals testing positive by CFT, clinical signs were more common among the non vaccinated group than they were among the vaccinated animals. Sixty two (13 out of 21) percent of the cattle with fever were unvaccinated. This may be attributed to more or larger lesions in the unvaccinated group.

5.5 The Protection rate of the modified and the conventional T144 vaccines

Contagious bovine pleuropneumonia occurred in some vaccinated cattle (12 out of 29). Vaccination does not confer absolute protection (100%) on all subjects. Hudson and Turner (1963) made a similar observation. The percentage protection conferred by the conventional and the modified vaccine was found to be 61.8% and 56.1% respectively. Earlier studies indicated the percentage protection by the T144 vaccine to be between 66 and 78% for experimental animals challenged 12 to 15 months post vaccination (Gilbert *et al.*, 1970; Masiga and Windsor, 1978; Wesonga and Thiacourt, 2000).

Wesonga and Thiacourt (2000) reported a 59% protection rate for cattle challenged 3 months after vaccination. A secondary vaccination was done in this study. Similarly Mariner *et al.* (2006a and b) estimated efficacy of T144 vaccine to lie between 50-80% while Thiacourt *et al.* (2004) pegged it at 40-60%. However, a much lower protection rate of 30% was reported in a study conducted by Yaya *et al.* (1999) in Cameroon where
animals were challenged 6 months after vaccination.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

a) The modified \( T_144 \) vaccine has been demonstrated in this study, to provide protection to cattle 16 months after vaccination

b) The conventional and modified \( T_144 \) vaccine were both efficacious at 16 months post vaccination, with the latter giving a 56.1% protection rate and the former giving a 61.8% protection.

6.2 Recommendations

a) The two \( T_144 \) formulations confer sustained protection to cattle against CBPP. This study recommends the continued use of the conventional vaccine.

b) For the purpose of determining the efficacy of CBPP vaccines, the two immunoassays, CFT and c-ELISA need to be used together since they compliment each other. Clinical findings and postmortem findings should also be used together with serology findings, if the diagnosis is to be considered accurate.

c) This study recommends the application of the modified vaccine in the field especially where refrigeration facilities are not available. Field studies are recommended to take advantage of the observed stability of the modified vaccine after which the vaccine can be recommended for field application.
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AU/IBAR-IAEA Consultative group on Contagious bovine Pleuropneumonia 12-14 November 2003, Rome, Italy.


subsp *mycoides* SC through experimental infection in cattle. *Veterinary research* 10: 1186-1888.


APPENDIXES

Appendix Ia: Mycoplasma titre/ml of T1 44 vaccine prepared in conventional media and titrated at intervals between 0 hour and 168 hours when stored at various temperatures.

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>4 °C</th>
<th>25 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hours)</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>$10^{7.5}$</td>
<td>$10^{3.0}$</td>
<td>$10^{7.2}$</td>
</tr>
<tr>
<td>2</td>
<td>$10^{2.9}$</td>
<td>$10^{2.6}$</td>
<td>$10^{3.7}$</td>
</tr>
<tr>
<td>4</td>
<td>$10^{6.7}$</td>
<td>$10^{3.3}$</td>
<td>$10^{7.7}$</td>
</tr>
<tr>
<td>8</td>
<td>$10^{3.4}$</td>
<td>$10^{3.1}$</td>
<td>$10^{2.9}$</td>
</tr>
<tr>
<td>12</td>
<td>$10^{4.0}$</td>
<td>$10^{2.9}$</td>
<td>$10^{5.6}$</td>
</tr>
<tr>
<td>24</td>
<td>$10^{7.4}$</td>
<td>$10^{5.1}$</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>48</td>
<td>$10^{2.6}$</td>
<td>$10^{0.9}$</td>
<td>$10^{3}$</td>
</tr>
<tr>
<td>72</td>
<td>$10^{5.5}$</td>
<td>$10^{1.9}$</td>
<td>$10^{3}$</td>
</tr>
<tr>
<td>168</td>
<td>$10^{6.5}$</td>
<td>contamination</td>
<td>All negative</td>
</tr>
</tbody>
</table>
Appendix Ib: Mycoplasma titre/ml of T, 44 vaccine prepared in modified media titrated at intervals between 0 hour and 168 hours when stored at various temperatures.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>4 °C</th>
<th>25 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>2</td>
<td>$10^{10}$</td>
<td>$10^{5}$</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>4</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
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<td>8</td>
<td>$10^{10}$</td>
<td>$10^{7.6}$</td>
<td>$10^{10}$</td>
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<td>12</td>
<td>$10^{7.5}$</td>
<td>$10^{5.6}$</td>
<td>$10^{10}$</td>
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<td>24</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
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<td>48</td>
<td>$10^{10}$</td>
<td>$10^{7.5}$</td>
<td>$10^{10}$</td>
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<tr>
<td>72</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
<td>$10^{10}$</td>
</tr>
<tr>
<td>168</td>
<td>$10^{6.9}$</td>
<td>contamination</td>
<td>All negative</td>
</tr>
</tbody>
</table>

With regard to how the two vaccines compared at individual the same storage temperature, titres of the modified vaccine in vitro were higher at 4 °C, 25 °C and 37 °C than those of the conventional vaccine.
## Appendix II: Hudson and Turner pathology scores of vaccinates and control animals

<table>
<thead>
<tr>
<th>Animal No.</th>
<th>Highest CFT titer</th>
<th>Clinical Outcome</th>
<th>Lesion dimensions.</th>
<th>Score for presence of lesions (Lesion present = 2 scores)</th>
<th>Score for weather or not MmmSC was isolated</th>
<th>H &amp; T Score Factor determined by lesion size</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>No lesion</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>21</td>
<td></td>
<td>16×12</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>508</td>
<td></td>
<td>No lesion</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>512</td>
<td>fever</td>
<td>No lesion</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>528</td>
<td></td>
<td>No lesion</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>533</td>
<td></td>
<td>No lesion</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>538</td>
<td>160</td>
<td>fever</td>
<td>32×28</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
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