# CHARACTERISATION OF THE *Mycoplasma mycoides* subspecies *mycoides* Small Colony FROM KAZUNGULA DISTRICT OF ZAMBIA, A CONTAGIOUS BOVINE PLEUROPNEUMONIA OUTBREAK AREA



BY

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**Thesis submitted to the University of Zambia in fulfilment of the requirements of the degree of Master of Science in Veterinary Microbiology**



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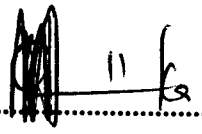
## DECLARATION

I **Geoffrey Munkombwe Muuka** declare that the work presented in this dissertation was done by myself and has not been presented to this or any other University for award of any degree.

**Signature:**.....  .....

**Date:**.....12/08/2011.....

## DEDICATION

I dedicate this work to my Father, Mr Peter Munankopa Munkombwe for his support and desire to see me attain higher education, my late Mother, Lily Buumba Namakobo for believing in me, my wife Nkweto, my son Muuka jnr and my daughters Buumba and Mulenga, for their love and for being there for me.

## APPROVAL

This thesis of **GEOFFREY MUNKOMBWE MUUKA** is approved as fulfilling the requirements for the award of the Degree of Master of Science in Veterinary Microbiology of the University of Zambia

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## ABSTRACT

This study was conducted to characterise the *Mycoplasma mycoides* subspecies *mycoides* Small Colony (*Mmm*SC) variant in the Contagious Bovine Pleuropneumonia (CBPP)new outbreak area of Kazungula District of Southern Province of Zambia. Kazungula is the southern most in Southern province and borders the Western Province, a known CBPP endemic area.

During the study it was observed that 186 cattle (23.3%, CI 20.5 to 26.5) out of 797 examined at the farms showed overt clinical signs of CBPP. Samples were collected from carcases showing pathognomonic lesions of CBPP at slaughter of the observed 186 animalswhich were arbitrally categorised in to onset, acute and chronic depending on the predominating lesions seen onpost-mortem. Using this type of categorisation, 19 (36.5%, CI 24.0 to 51.1%), 11 (21.3%, CI 11.6 to 35.1) and 22 (42.3%, CI 29.0 to 56.7) of onset, acute and chronic lesions respectively were observed. A total of 80 samples were collected from the main study area using the purposive sampling design. Additionally, 10 samples using the same method were collected from Western and North-western Provinces for comparative purposes on the results obtained. It was demonstrated during the study that *Mycoplasma mycoides* subsp. *mycoides* Small Colony (*Mmm*SC) could be isolated from the pathological tissues using the PPLO conventional media containing Horse serum and the non conventional media containing donkey blood agar. The isolated colonies were confirmed to be *Mmm*SC using Polymerase Chain Reaction. Antibiotic susceptibility of the studied isolates generally showed that the *Mmm*SC were susceptible to growth inhibition effects of nitrofurantoin,tetracycline, gentamycin, erythromycin, co-trimoxazole and tylosin except one from Kazungula district which resisted all the antibiotic effects other than that of tylosin.

The isolates were further characterised using Restriction Enzyme Analysis (REA) and the isolates studied did not show any polymorphic differences and a conclusion inferred that the *Mmm*SC causing CBPP in the study area could have a common originality. Furthermore, the study confirmed that the disease outbreak was from Western Province as isolates from Western and Southern Provinces were similar apart from one isolate from Kazungula which showed antibiotic resistance to the antibiotics studied except tylosin.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| BRE | Barotse Royal Establishment |
| bp | Base pairs |
| C | Cytosine |
| CAP | Cyclic Adenosine Phosphate |
| CBPP | Contagious Bovine Pleuropneumonia |
| CFT | Complement Fixation Test |
| DNA | Deoxyribonucleic Acid |
| dNTPs | Deoxynucleotide TriPhosphates |
| FAO | Food and Agricultural Organisation |
| FMD | Foot and Mouth Disease |
| G | Guanine |
| GDP | Gross Domestic Product |
| Kb | Kilobases |
| Km | Kilometre |
| LC | Large Colony |
| MIC | Minimum Inhibitory Concentration |
| Ml | Millilitres |

| | |
|---|---|
| MMC | Minimum Mycoplasmacidal Concentration |
| MmmSC | *Mycoplasma mycoides* Subspecies *mycoides* Small Colony |
| NALEIC | National Livestock Epidemiology and Information Centre |
| OIE | Office International des Epizooties |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulsed Fragment Gel Electrophoresis |
| PPLO | Pleuropneumonia Like Organisms |
| REA | Restriction Enzyme Analysis |
| RFLP | Restriction Fragment Length Polymorphism |
| rRNA | ribosomal Ribonucleic Acid |
| SADC | Southern African Development Community |
| µm | Micromoles |
| °C | Degrees Celsius |

# CHAPTER ONE

## INTRODUCTION

The Agriculture sector in Zambia contributes about 18 percent of the Gross Domestic Products (GDP) since the early 1990s. Agriculture provides a livelihood to about 16 percent of the economically active individuals in Zambia (FAO 2006). It also remains by far the main opportunity for income and employment for women who comprise 65 percent of the rural population. Livestock production is an important socio-economic activity in Zambia and contributes about 35 percent of the total agriculture production (Anon 2006a). In Zambia, livestock production is broadly categorised into commercial and traditional sectors. Commercial livestock is predominantly reared around Lusaka, Southern and Central Provinces while a large concentration of traditional cattle is raised in Western, Southern, Central and Eastern Provinces. The main livestock produced are cattle, goats, pigs and poultry.

Zambia has an estimated cattle population of about 2.9 million of which 84 percent is under the traditional sector (Anon 2006a). The traditional livestock sector is dominated by the indigenous people, who keep cattle on communal land held in trust by local chiefs (Perry *et al.*, 1984). The local Zebu and Sanga are the predominant cattle breed types that are reared under extensive grazing system. Livestock keeping is a major occupation in these communities and serves as a source of income, food and social security investment (Perry *et al.*, 1984). Animals are usually kept for milk and beef, although the majority of farmers rarely slaughter animals for beef. Most of the farmers graze their cattle on communal grazing areas where their cattle frequently mix during grazing and at watering points. In the plains of

Western Province and the Kafue flats of Southern Province, farmers practise transhumance grazing where, in the dry season, cattle are moved to graze communally on plains which have adequate pasture all year round leading to mixing of cattle from different areas. These practises have been implicated in the spread of disease in the traditional livestock sector (Muma *et al.*, 2009). However, it has been observed that the traditional livestock sector is characterised by numerous production and management problems which condensates in reduced productivity. These include low off-take rates, diseases such theileriosis, Foot and Mouth disease (FMD) and Hemorrhagic Septicaemia. Recently Contagious Bovine Pleuropneumonia (CBPP) outbreaks have adversely affected the livelihood of most people who depend on cattle for survival (Muma *et al.*, 2009).

Contagious Bovine Pleuropneumonia (CBPP) is a bacterial disease caused by *Mycoplasma mycoides* subsp.*mycoides* Small Colony (*MmmSC*). It is a disease of economic importance because of the huge financial losses resulting from mortalities, especially during disease outbreaks. The disease also affects local and international trade due to animal movement restrictions and additional financial losses may be incurred during eradication campaigns as evidenced in the Ngamiland, Botswana outbreak control in 1995 (Amanfu *et al.*, 2000; Roeder *et al.*, 1999; Masiga *et al.*, 1996). Thus CBPP has serious implications for food security and people's livelihoods in affected countries and is a major constraint to cattle production in Africa (Amanfu 2009). The socio-security impact of CBPP eradication was seen in Botswana where it was postulated that the economic recovery of households whose cattle was slaughtered during depopulation would take a prolonged period (Mullins *et al.*, 2000). In Zambia, CBPP is a major contributor to cattle mortalities, especially in Western province and it also affected cattle during the outbreak of 2006 in Southern province (Muma *et al.*, 2009; Anon 2007a).

Since the 1980s, CBPP has become the major infectious disease affecting livestock in Africa, and the number of countries with CBPP-infected animals rose from fifteen in the late 1970s to twenty six in 2002 (OIE 2005; Nicholas *et al.,* 2000). In the Southern African Development Community (SADC) region, CBPP affects Angola, Namibia, Tanzania and Zambia (SADC 2009). Figure 1 shows the spatial distribution of CBPP in the SADC region as at the end of 2009. It is reported to be the fifth most widely distributed trans-boundary disease in the SADC region (SADC 2009) after Foot and Mouth Disease (FMD), African Swine Fever (ASF), Newcastle (ND) and Rabies. The potential of the disease spreading to the unaffected countries is noted by the proximity to the borders of affected countries. Comparatively, the number of reported cases of CBPP reduced in 2009 as compared to 2008 (Table 1). A total of sixty four outbreaks of CBPP were reported in four member states in the SADC region in 2009 compared to 94 that were observed in 2008 (SADC 2009). According to the SADC (2009) report, 77 percent of the outbreaks were suspected to be from endemic foci.

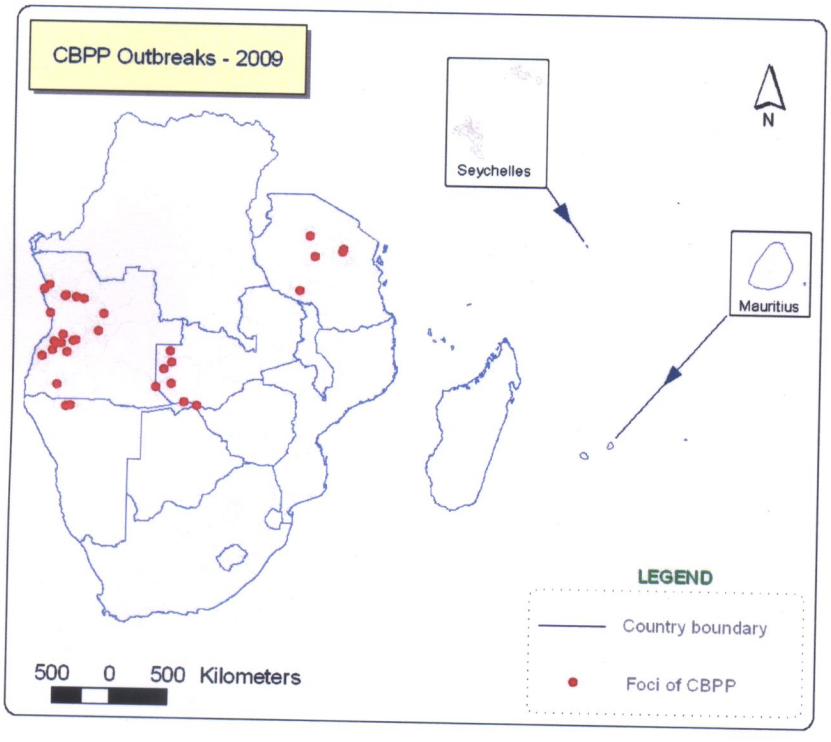


Figure 1: Areas of Reported CBPP cases for 2009 in SADC Region (SADC 2009)

Table 1: CBPP cases as seen in the SADC region in 2008 and 2009. (Source: SADC ANIMAL HEALTH YEARBOOK 2009)

| Details | Year | |
|---|---|---|
| | 2008 | 2009 |
| Number of Countries affected | 4 | 4 |
| Number of Outbreaks | 94 | 64 |
| Number of Cases | 1,909 | 604 |
| Number of Deaths | 738 | 112 |
| Mean Outbreaks/Country | 24 | 16 |
| Mean Cases/Outbreak | 20 | 9 |

CBPP was first reported in Zambia, in the then Barotseland, in 1915 and was eradicated in 1946. Then a second outbreak was recorded in Western Province in 1969, which was eradicated in 1973 (Akafekwa 1975; Anon 1974; Revell 1973). In early 1997, a third outbreak was recorded and the focus was eradicated in 1998 (Anon 1998). The fourth outbreak occurred in 2001 following the heightened civil strife in Angola in the late 1990s. The war is resulted into the influx of refugees into Zambia with most of them coming with their cattle. These cattle mingled with those of the local community and are thought to have transmitted *Mmm*SC to the local cattle (Anon 2002; FAO 2001). This outbreak could not be contained and it led to the spread of the disease from Shangombo, the initial focus to affect the whole of Western Province and in May 2002, the disease was reported in Zambezi district of North-Western Province (Anon 2003; FAO 2003).

On the Northern front of the country, CBPP was detected in Nakonde district in 1998 (Anon 1998) and the last case of the disease was detected in 2001. The threat of the disease transmission from Tanzania to Zambia was realised earlier in 1995 after it spread from Northern Tanzania to the Southern Districts of Ulanga and Kilombero districts in Morogoro region, an area closer to the Zambian border (Anon 2007b; Msami *et al.*, 2001). Contributing factors to the trend of CBPP spread and perpetuation are thought to be and include, breakdown in veterinary services, increased and unrestricted animal movement and lack of efficacious vaccine (FAO 2004). Thus, the current CBPP situation in the country can be described as endemic to the whole of Western Province and some parts of Northwestern Province. In the Southern Province the first case of CBPP was recorded in Kazungula district in March 2004 although a serious outbreak of the disease in the district was only reported in 2006 (Anon 2006b; FAO 2006; Anon 2004). The rest of the province had not reported any cases of CBPP. In Kazungula District, the disease assumed an epidemic nature with

numerous outbreaks. In Zambia, CBPP is now the number one priority livestock disease (FAO 2006). In view of the above, it was considered necessary that more detailed work be conducted to ascertain the characteristic of the *Mmm*SC that was afflicting cattle in Kazungula District of Southern Province. Furthermore the study was to compare the isolated *Mmm*SC both genetically and phenotypically with other isolates from other areas in the country and the vaccine strain T1/44.

The main objective of the study was;

To characterise the *Mycoplasma mycoides* subspecies *mycoides* Small Colony Variant (*Mmm*SC) from Kazungula District of Zambia, a new Contagious Bovine Pleuropneumonia outbreak area.

The specific objectives of this study therefore, were:

a) To establish the degree of genotypic polymorphism that exists within *Mmm*SC strains isolated from Kazungula District of Zambia and compare them with those from Western and Northwestern Provinces using Restriction Enzyme Length Polymorphism (RFLP).

b) To determine the antimicrobial susceptibility of the *Mmm*SC isolates from Kazungula district and other areas within the country.

c) To determine any phenotypic differences exhibited by the isolates.

**STUDY JUSTIFICATION**

Recent sporadic CBPP outbreaks in Kazungula District of Southern Province are believed to have spread from Sesheke District of Western Province. Southern Province is an area which is considered as a major cattle producing area of the country and thus this outbreak posed a major threat to the industry in the country.

Characterisation of the isolates from this new outbreak area and determining their sensitivity to antibiotics would give an accurate picture of the sources and the relationship of the *Mmm*SC causing CBPP in Kazungula District of Southern Province with those from Western and Northwestern Provinces. This would assist the Government in determining the best strategy for control and eradication of the disease from the country. The data would create a base for molecular epidemiology of CBPP in Zambia and increase the ease with which such information can be accessed by the National Livestock Epidemiological and Information Centre (NALEIC) of the Department of Veterinary and Livestock Development (DVLD) for decision making in disease control measures.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. General Overview of Contagious Bovine Pleuropneumonia (CBPP)

Contagious Bovine Pleuropneumonia (CBPP) is a highly contagious disease of cattle and water buffaloes whose importance in causing mass mortality of cattle was recognised as far back as the eighteenth century (Fisher 2003). It used to be the only bacterial disease among the many viral diseases that belonged to the now defunct Office International des Epizooties (OIE) list A of notifiable diseases because of its high potential for contagiousness irrespective of national borders (OIE 2008). Between 2008 and 2009 a total of twenty three countries worldwide reported cases of CBPP and of these the majority were in sub Saharan Africa where a total of twenty two countries are endemic for the disease (OIE 2009). According to the OIE (2009), the situation of CBPP in the Middle East is not well known, while in Asia, CBPP was suspected in Malaysia in 2008 although it was not confirmed. Now that rinderpest is largely controlled in Africa, CBPP remains the most important disease in tropical Africa causing great economic losses including mortality, loss of weight, reduced working ability, reduced fertility and indirect costs due to control programmes (Masiga *et al.*, 1996).

It is known that CBPP was endemic in the Eastern Italian Alps up to the sixteenth century and had spread throughout Europe by the eighteenth century (Bellini *et al.*, 1998). The origins of the disease are not clearly understood and according to Fisher (2003), CBPP was identified as a separate disease in Switzerland in the eighteenth century. It is postulated that CBPP caused death to a lot of cattle only second to rinderpest and that veterinarians at the time did not have a solution for the suffering farmers (Fisher 1986).

Historically, CBPP was a disease of Europe, North America and Asia. Between 1830 and 1860, it is reported that CBPP became established on all major land masses apart from South America (Worboys 1992). According to studies by Fisher (2003), introduction of CBPP in Europe was inevitable with the revival of cattle trade after the end of the French wars, with an estimated 40,000 cattle moving annually across its borders from all over the world in the 1830s. It is claimed that the Netherlands had become "the hot-bed of pleuropneumonia" by the mid century, a source of infection for the British Isles, North America and South Africa (Koolmess *et al.,* 1993). It was eradicated from the United States, Canada and most of Europe in the nineteenth century prior to the identification of the causative agent through clinical diagnosis, movement control and slaughter of suspected cases (Provost *et al.*, 1987). The impetus towards eradication in Western Europe came from other developments notably the proof of validity of the slaughter policy which seemingly worked well in the eradication of rinderpest. This was extended to CBPP eradication and in its implementation it was backed with rigorous quarantine (Fisher 1980).

Although CBPP was eradicated from most of Europe in the nineteenth century, incidence and cost of control of the disease was nevertheless highly variable due to differences in the impact of the disease in different areas. It was worst and costly where cattle density and turn over were highest (Huygelen 1997). Although not all cattle were infected, death rates were high and it was estimated that 30 percent of herds died, even when the disease had become enzootic. Another 30 percent suffered a severe depreciation in value. This was the most serious problem faced by the European cattle producers in an otherwise favourable market at the time. The depreciation of value was as a result of the apathy towards cattle trade that developed resulting in some farmers deciding to get rid of their herds at very low prices.

Despite being eradicated from most of Europe in the nineteenth century, CBPP re-emerged in Spain, France and Italy in the 1980s and the source of these outbreaks was not known (Poumarat *et al.*, 1995). Since 1999, CBPP has never been reported in Europe (OIE 2009).

CBPP is believed to have been present in East and West Africa prior to the colonial era although the exact origin is not known. According to Hudson (1971) however, CBPP originated from Europe and was disseminated to other continents by trade in animals. The spread of CBPP into Southern Africa from Europe in 1854 and its subsequent spread as far as Angola is documented (Windsor 2000). Progress was made in controlling CBPP in Africa during the colonial era and the first two decades following independence of most African countries (Mariner *et al.*, 2006a). Large parts of Southern, Western and Eastern Africa were cleared using slaughter and movement control which later incorporated testing strategies based on the complement fixation test. A few spots remained on the continent where the disease was still present and causing cattle deaths. The majority of these spots were the war ravaged parts of the continent (OIE 2005).

In the 1980s and 1990s, economic crises engulfed many nations of Africa and funding available for public veterinary services declined. As a consequence of this, CBPP surveillance and control programmes were dramatically curtailed (Windsor 2000). Additionally, public empowerment and the recognition of the pervasive negative effects of movement control on pastoral livelihoods decreased the acceptability of this tool as a control option (Masiga *et al.*, 1996). This resulted in the spread of the disease from the few spots that had not eradicated the disease. In Southern Africa, the long standing focus of CBPP in Angola spread to affect Namibia and Zambia (FAO 2001). The only country in sub Saharan Africa which successfully eradicated CBPP is Botswana which in 1995 eradicated the disease

through the implementation of the stump out policy and this resulted in the slaughter of 320,000 cattle (Amanfu *et al.*, 2000).

### 2.2. Classification of *Mycoplasma mycoides* subspecies *mycoides* Small Colony

*Mycoplasma mycoides* subtype *mycoides* Small Colony Biotype (*Mmm*SC), is a bacteria that belongs to the class Mollicutes, whose members lack a cell wall and are currently known to be the smallest self-replicating organisms on the planet (Razin 1992). *Mycoplasmas* have been classified in the phylum *Firmicutes*, the order *Mycoplasmatales* and the family *Mycoplasmataceae*. Phylogenic classification has grouped *Mmm*SC together with five other closely related and highly pathogenic *Mycoplasmas* into *M. mycoides* cluster of the spiroplasma group (Westburg *et al.*, 2004; Johansson *et al.*, 1996; Wiesburg *et al.*, 1989). The other members in this group are *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma capricolum* subsp. *capripneumoniae*, *Mycoplasma mycoides* subsp. *capri*, *Mycoplasma mycoides* subsp.*mycoides* Large Colony and species strain PG50. However, in the study by Heldtander *et al* (1998) and Wiesburg *et al* (1989), *Mycoplasma putrefaciens* and related saprophytic species *M. cottwei* and *M. yeatsii* are included in the phylogenic *Mycoplasma mycoides* cluster within the *Spiroplasma* group.

Further studies of the *M. mycoides* cluster have led to the differentiations within the cluster based on the sequence analysis of two genes of ribosomal Ribonucleic Acid (rRNA) operons of the 16S rRNA. The *Mycoplasma mycoides* cluster could be differentiated into a group, *Mycoplasma mycoides* consisting of *M. mycoides* subspecies *mycoides* Small Colony (SC), *M. mycoides* subspecies *mycoides* Large Colony (LC) and *M. mycoides* subspecies *capri*. The other one, the *Mycoplasma capricolum* group consisting of *M. capricolum* subspecies

*capricolum, M. capricolum* subspecies *capripneumoniae* and *Mycoplasma* species strain PG50 (Johansson *et al.*, 1996).

The *Mycoplasma* cluster was also inferred on the basis of a set of concatenated sequences from five house keeping genes namely *fusA, glpQ, gyrB, lepA* and *rpoB* (Manson-Silvan *et al.*, 2007) where two sub-clusters within the *Mycoplasma* cluster were distinguished. These were the *M. mycoides* and the *M. capricolum* sub-clusters. The *M. mycoides* subcluster consisted of *M. mycoides* subspecies *mycoides* Small Colony (SC), *M. mycoides* subspecies *mycoides* Large Colony (LC) and *M. mycoides* subspecies *capri.* The *M. capricolum* sub-cluster consisted of *M. capricolum* subspecies *capricolum, M. capricolum* subspecies *capripneumoniae* and *Mycoplasma* species bovine group 7 of leach, a group of strains that still remains unassigned. In this work Manson-Silvan *et al* (2007) managed to construct reliable phylogenic trees that assigned phylogenic positions to all members of the *M. mycoides* cluster and related species. Although *Mmm*SC can also be distinguished by its very high pathogenicity from the other members of closely related members of the *Mycoplasma mycoides* cluster, which are mostly of only low epidemiological importance, the *Mmm*SC are phenotypically and antigenically very similar to most of these ( Pilo *et al.*,2007).

### 2.3. Epidemiology of CBPP

The dynamics of CBPP transmission vary widely between livestock production systems (Mariner *et al.*, 2006b). It was shown in the study by Mariner *et al* (2006b) that the homogenous model indicates that the critical community size for the persistence of CBPP falls within the typical herd sizes for pastoral communities in East Africa. This herd size would be one with as few as five cattle to as many as over hundred cattle which is compatible

with the situation in most African regions including Southern Africa and Zambia in particular.

The disease is spread mostly through contact of susceptible cattle with carriers which could either be incubating the *Mmm*SC and apparently healthy, clinically sick animals with overt clinical signs of CBPP or recovering cattle with or without clinical signs of the disease (Thiaucourt *et al.*, 2004) although wind-borne and indirect transmission cannot be excluded (Regalla *et al.*, 1996). The role of *Mmm*SC which has been found in other exudates and body fluid other than those from the respiratory system such as in spermatozole is not known in transmission of CBPP (Goncalves 1994). Although sheep and goats can become infected with *Mmm*SC, it is postulated that a CBPP outbreak cannot be caused by the transmission of the bacteria from these animals to cattle. (Kusilika *et al.*, 2000; Srivastava *et al.*, 2000; Brandaõ 1995). Formites have never been linked to the spread of the disease because the bacteria cannot survive in the environment (OIE 2008). Some studies have shown that the vaccine strain of the *Mmm*SC, T1/44 when introduced into cattle through the endotracheal route could cause clinical disease in some animals which eventually became infective (Huebschle *et al.*, 2004). However, in the field, these vaccines are inoculated into the cattle via the subcutaneous route and thus the probability of causing CBPP spread via this route is unknown. In some instances some post-vaccinal reactions occur with adverse health effect on the cattle. Such reactions have taken the form of local reactions at the inoculation site (known as "Willem reactions") and also previously with tail tip falls (Thiaucourt *et al.*, 2004). Animals usually recover if they are treated with antibiotics (Sori 2005; Revell 1973) over a period of three days (Hubshle *et al.*, 2006). During the course of the disease caused by vaccinations, it was demonstrated that such infected cattle could spread the disease to other susceptible cattle (Thomson 2005). The probability of recovered cattle previously infected

with CBPP due to vaccinations to infect other susceptible cattle is unknown although studies by Hubschle *et al* (2004) have shown that *Mmm*SC infected cattle when treated with antimicrobials had reduced chances of transmitting CBPP to susceptible cattle. The identity of the T1/44 vaccine strain can be readily verified by using a specific Polymerase Chain Reaction (PCR) detection technique (Lorenzon *et al.*, 2008). This PCR identifies a 700-base pair long amplicon which is unique only to the T1/44 vaccine strain and does not amplify any other *Mmm*SC strains.

According to Masiga *et al* (1996), the epidemiology of CBPP in Africa is defined by a number of factors. These include; cattle being the only species affected, with no reservoir in wild animals. Furthermore clinical cases or chronic carriers are the usual sources of infection through direct contact, and finally cattle movements playing a very important role in the maintenance and extension of the disease.

### 2.4. Molecular Biology of *Mycoplasmas*

*Mycoplasma* species generally possess a relatively small genome of 0.5-1.38 megabases, which results in drastically reduced biosynthetic capabilities and explains their dependence on the host (Razin *et al.,* 1992). Genomic sizes of *Mycoplasmas* are variable not only within the same genus but even among the strains of the same species (Razin *et al.*, 1998). One of the reasons for this variability is the frequent occurrence in mollicute genomes of repetitive elements, consisting of segments of protein genes differing in sizes and number, or insertion sequence (IS) elements.

### 2.4.1. Genetic characteristics of *Mmm*SC

The genome of *Mmm*SC type strain PG1™ has been sequenced to map all the genes and to facilitate further studies regarding the cell function of the organisms and the disease it causes, CBPP (Westberg *et al.,* 2004). The study by Westberg *et al* (2004) showed that the genome of *Mmm*SC is characterised by a single circular chromosome of 1,211,703 base pairs with the lowest Guanine (G) and Cystocin (C) content (24 mole percent) and the highest density of insertion sequences (13 percent of the genome size) of all sequenced bacterial genomes. Consequently, many intergenic regions have a higher Arginine (A) and Thymine (T) content than the coding regions, reaching values as high as eighty to ninety percent mole. It was postulated during the study by Westberg *et al* (2004) that anomalies in the GC-skew pattern and the presence of large repetitive sequences were indicative of a high genomic plasticity. It was also stated by Razin *et al* (1998) that the variable G and C content of coding regions within the *Mmm*SC genome has phylogenic relevance, indicating the highly conserved nature of the rRNA and tRNA genes.

The development of Polymerase Chain Reaction (PCR) enabled rapid diagnosis and differentiation of the *Mycoplasma* 'cluster' group from all other *Mycoplasmas* (Hotzel *et al.*, 1996). This scheme involved, a set of primers derived from the CAP-21 genomic regions of the members of the cluster. Nested PCR involving the cluster specific amplification at the first stage and group specific amplification using internal primers at the second stage was shown to be applicable for identification of all six groups forming the cluster. Different PCRs have been developed for specific diagnosis and characterisation of the CBPP causing agent (Bashirrudin *et al* 1994; Dedieu *et al.*, 1994). These reactions improved and accelerated the diagnosis of CBPP in cattle.

As demonstrated by Dedieu *et al* (1994), the PCR used two different assays, a control PCR which identified the pathogen as a member of the mycoides cluster and the one which was specific for the *Mmm*SC. The control PCR targeted a 460 base pair sequence which is found in all *mycoides* cluster strains while the *Mmm*SC specific assay targeted a 275 base pair sequence unique to *Mmm*SC.

It was demonstrated by March *et al* (2000) that a strain denoted M375 which was isolated in Botswana had significantly different phenotypic characters from the ideal that are usually seen in *Mmm*SC. These included altered morphology, reduced capsular polysaccharide production, high sensitivity to *Mmm*SC rabbit hyperimmune antisera *in vitro*, and unique polymorphism following hybridisation. When the genetic characteristics of the isolate were examined using the *IS1634* hybridisation, it showed that it did not have the usual band which appears in the related strains in the region. This suggested that deletion had occurred and it was postulated that this would be the cause of the unique phenotypic characters seen.

### 2.4.2. Restriction Enzyme Fragment Length Polymorphism of *Mmm*SC (RFLP)

Studies to demonstrate the differences in the genomic make up of *Mmm*SC strains have been done using restriction enzyme activity (Poumarat *et al.*, 1995). This is based on DNA extraction, digestion with a restriction enzyme and analysis of the fragments obtained by electrophoretic migration in an agarose gel. The number of fragments obtained depends on the enzyme used and the ability of such an enzyme to find an appropriate cleavage site in the sequence being analysed (Thiaucourt *et al.*, 1998). It is therefore possible to identify a point mutation in *Mmm*SC strains being observed because this may result in a modification of the number of bands obtained. In the works by Dedieu *et al* (1994), three internal restriction sites

were identified which could be used to identify the *Mmm*SC from a group of members of the *Mycoplasma* cluster.

Further works by Bashirrudin *et al* (1994), the PCR was based on the use of two sets of primers which initially identified the *Mmm*SC as part of the *mycoides* 'cluster' and this was then identified specifically as *Mmm*SC by the use of Restriction enzymes *AnsI* which produced patterns with two fragments unlike the other members of the cluster that gave three fragments. Although African strains are very similar, Restriction Fragment Length Polymorphisms (RFLPs) have shown variations in the banding patterns (Lorenzon *et al.*, 2003; Thiaucourt *et al.*, 1998). Thiaucourt *et al* (1998) showed using restriction enzyme analysis that the African strains could be grouped according to the geographical origins. In this study, Thiaucourt *et al* (1998) observed that the groupings were independent of the date of isolation of the strains, thus it could be linked to the common source of such identical strains.

During the examination of the *Mmm*SC from the outbreak areas in Tanzania using the Amplified Fragment Length Polymorphism (AFLP) and Pulsed-field Gel Electrophoresis (PFGE), it was shown that the outbreaks should have been caused by a single epidemic clone (Kusilika *et al.*, 2000). The strains from other regions however had different patterns showing the diversity of the African strains. Phenotypic differences exhibited among *Mmm*SC might be a result of the antigenic drift. As observed by the March *et al* (2000), strain M375 isolated from Botswana displayed numerous and significant phenotypic differences from both contemporary field isolates, older field strains and vaccine strains. This raises the potential that antigenic drift may have occurred among newer field isolates.

## 2.5. Growth requirements and culture characteristics of *Mmm*SC

*Mycoplasmas* have lost many genes and gene complexes involved in the metabolism of amino acids and co-factors, energy metabolism, synthesis of cell wall components, signal transmission and gene expression (Zakharov *et al.*, 2009). This entails that most of these molecules necessary for metabolism have to be provided in the environment for *Mmm*SC to grow. Nutritionally, the mollicutes are an extremely fastidious group of organisms, being dependant on their host for a large variety of organic nutrients such as vitamins, nucleic acid precursors, amino acids, fatty acids and lipids (Bashirrudin and Nicholas 1995). The large number of amino acids required by *Mmm*SC reflects the limited synthetic capability of the organism. It will not grow in media lacking the preformed bases uracil, cystocine, thymine and guanine, which are required for nucleic acid synthesis. Sterol, which makes up some 20 percent of the membrane lipids, is required by *Mmm*SC and in the media it is provided as cholesterol. *Mmm*SC is referred to as fermentative because it produces acid from anaerobic glucose metabolism.

Most mollicutes including *Mmm*SC are facultative anaerobes, growing well in both anaerobic and aerobic environments. *Mmm*SC normal growths have been seen to occur at pH 7.0 to pH 8.0, the optimum being pH 7.4; while a decrease in pH to less than 6.5 cause cessation of growth and rapid death of cells (Hayflick *et al.*, 1965). Windsor *et al* (1998) demonstrated that it was a drop in pH rather than a loss of nutrients during the growth that was the cause of bacterial death following entry into the stationary phase of growth of *Mmm*SC. They usually grow well in sealed liquid broth cultures, especially if the broth level is a few inches deep to allow for oxygen or air gradient. Gentle but not vigorous aeration increases the growth rate and yield of *Mmm*SC.

*Mmm*SC on semi-solid medium grows down into the agar (Hayflick *et al.*, 1965). Some growths also occur over the surface around the central embedded area, giving rise to a lighter zone. Thus a characteristic "fried egg" appearance is produced. This characteristic growth of *Mmm*SC in which the central part of the colony is embedded in the agar, and the requirement for the moist environment, have dictated the use of a softer agar than that used in routine bacteriology (Meloni *et al.*, 1980; Hayflick and Chanock., 1965). The colonies of *Mmm*SC are usually uniform in size although a unique "atypical" colony M375 was isolated in Tanzania and Botswana which showed the colonies of *Mmm*SC to be of varying sizes (March *et al.*, 2000). Although this growth pattern was noted to be unique in the growth of *Mmm*SC, Meloni *et al* (1980) stated that colony size and morphology were not useful parameters for the characterisation of *Mycoplasma* species because these properties vary within the same species and are influenced by many factors, e.g. the degree of hydration at the agar surface, the inoculum sizes, the agar concentration and gel strength. In the broth, the distinctive feature of *Mmm*SC is the production of a moderate turbidity with a whitish deposit at the bottom of the culture vessel (Sori *et al.*, 2005). This feature has been used as an additional parameter to determine the growth of *Mmm*SC. In actively growing cultures, *Mmm*SC is filamentous, because of nuclear division that precedes cytoplasmic division. At the end of growth however, short beaded filaments predominate and ultimately only coccoid bodies are seen. *Mmm*SC reproduce by binary fission, but cytoplasmic division frequently may lag behind genomic replication, resulting in formation of multicellular filaments (Gourlay *et al.*, 1968).

## 2.6. Antimicrobial susceptibility of *Mmm*SC

As in other bacteria, the phenotype of *Mmm*SC can be linked to the genotypic characters of the organisms (Plessis *et al.*, 2009). Antibiotic resistance is one of the phenotypic

characteristics that bacteria may express. According to studies by Lee *et al* (1987), four different ways of acquiring bacterial resistance have been identified. These are efflux mechanism, enzymatic modification of the drug, alteration in the drug target site or reduced permeability. Antibiotic resistance that results from altered cellular physiology and structure can be acquired by genetic mutation or acquisition of new genes via genetic transfer. In the study by Schwartz and Perlman (1971), it was noted that drug resistance in *Mycoplasmas* is mainly due to reduced uptake rather than specific degradation of the antibiotic.

*Mycoplasmas* are characterised by high mutation frequencies (Nicholas 2004) than conventional bacteria due to the high number of insertion sequences that they acquire. Recently, the availability of full sequenced mollicute genomes show the limited amount of genetic information dedicated to DNA repair system. It has been shown in other bacteria that the lack of the DNA repair system like the 'mut' gene is associated to a mutator phenotype (Razin *et al.,* 1998). Thus a link could be hypothesised between high mutation rates and drug resistance among the *Mycoplasmas* as it has been found for other bacteria. Like all other *Mycoplasma* strains, *Mmm*SC can easily acquire resistance, which is correlated to a mutation at the 16S RNA level (Thiaucourt *et al.*, 1998). Additionally, many *Mycoplasmas* are known to undergo high frequency genetic variations during growth which lead to observable phenotypic changes (Frey *et al.,* 1995). While surface antigenic variations are caused by a special set of genes which undergo spontaneous mutations in periodically coding regions, Insertion Sequences (*IS*) present at multiple copies may produce chromosomal rearrangements causing other phenotypic changes (Costas *et al.,* 1987) thus this may also affect the ability to respond to antimicrobials or acquire resistance.

Different bacterial species and indeed different *Mycoplasma* species respond differently to antimicrobials and have different Minimum Inhibition Concentration (MIC) values. Thirumalachar (1973) noted that many *Mycoplasma* species and their strains are inhibited by specific groups of antibiotics, but almost all of them were resistant to penicillin, bacitracin, polymixins and sulphonamides. Al Momani *et al* (2006) demonstrated that one of the members of the closely related '*mycoides* cluster' that causes contagious agalactia syndrome in small ruminants responded differently to antimicrobials *in vitro* even though some isolates came from the same animal.

In the study by Ayling *et al* (2005), however a range of Minimum Inhibitory Concentration (MIC) values for a number of antimicrobials used *in vitro* against *Mmm*SC were produced. It was noted that the *in vitro* MIC values of *Mmm*SC isolates reaction to different antimicrobials were not significantly different. However, there were significant differences in the values between *Mmm*SC and *M. bovis*. The data that was obtained would be useful in selecting potentially effective antimicrobials against *Mmm*SC the causative agent for CBPP and also provided a baseline of MIC values to monitor and assess development of antimicrobial resistance.

The difference demonstrated in the MIC values of *Mmm*SC and *M. bovis* would be caused by the inherent differences in the two organisms. The genome sizes are different with *Mmm*SC having a genome size of 1.14 Mega base pairs and *M. bovis* with 961+/-18.9 kb. It was also noted using molecular typing methods by Frey *et al* (1995) and Yaya *et al* (2008) that *Mmm*SC was genomically stable with little variances while the variability of *M. bovis* was noted to be very high by McAuliffe *et al* (2007). Furthermore, presence of variable surface

antigens and the ability to incorporate insertion elements into the genome may also affect the two organisms' ability to respond to antimicrobials or acquire resistance.

According to Lee *et al* (1987), strains of *Mmm*SC T1 that were resistant to some antibiotics were isolated. These single step mutants were shown to have mutated from the parent strain which initially did not exhibit signs of resistance to antimicrobial effects. The MIC of tetracycline in single step resistant mutants was found to be 0.6μg/ml being tenfold higher that the parent strain and on further investigation, it was seen that it could not be increased. During the study by Lee *et al* (1987), it was observed that the *Mmm*SC strain had only a two fold increase in resistance to chloramphenicol in single step mutants. The frequency of resistant mutants varied with the antibiotics and was between $4x10^{-6}$ and $2x10^{-8}$ per generation. The mutation rate to antibiotic resistance to streptomycin, novobiocin, erythromycin and tylosin was between $3x10^{-8}$ and $5x10^{-9}$ per cell per generation. In this work, Lee *et al* (1987) disputed the earlier speculation by Razin (1985) that there may be a higher mutation rate, and thus a more rapid evolution in *Mycoplasmas* than in other bacterial groups. *Mycoplasmas* are inherently resistant to those antibiotics that target the bacterial cell wall such as penicillin (Lee *et al.,* 1987). However, all other antimicrobials that have other modes of action other than the cell wall which is absent in *Mmm*SC and are unable to inhibit or minimise growth suggest resistance of *Mmm*SC to such antimicrobials (Thomas *et al.*, 2002). Although the *in vitro* effectiveness of antimicrobials indicates the ability of such antibiotics to work against bacteria, it is known that *in vivo* antimicrobial activity does not always match in *vitro* effectiveness, although the compound showing little or no activity *in vitro* is unlikely to be effective in aiding the body's defence to eradicate the organisms (Ayling *et al.*, 2000).

*Mmm*SC isolation or detection using PCR from lesions of cattle previously treated with mycoplasmicidal or mycoplasmastatic drugs is very difficult or not possible in most cases (Hubschle *et al.*, 2004). As shown by Hubschle *et al* (2004) the sequestra that were observed after treatment with dinofloxacin were mostly sterile with no *Mmm*SC found either by cultural methods or by PCR. This was also supported by later studies done by Nicholas *et al* (2005) which showed that the concentration of *Mmm*SC reduces after treatment of infected cattle with oxytetracycline. This indicates the vulnerability of *Mmm*SC to various antimicrobials available for use in cattle.

### 2.7. Clinical signs of CBPP

Approximately 50 percent of cattle exposed to CBPP develop clinical signs following a variable incubation period (McGuire *et al.*, 1996). The incubation period is not clearly defined and has been reported to be very variable with some variance of five to two hundred and seven days recorded (Regalla *et al.*, 1996). Mariner *et al* (2006a) reported estimates ranging from four to six weeks while others found individual cases of up to three months (Thiaucourt *et al.*, 2004). Thiaucourt *et al* (2004) stated that the quantity of the infecting dose affects the course of the disease because when animals are exposed to massive aerosol infection, the incubation period may only be 15 to 35 days. It is therefore clear that the degree of infectiousness of cattle differs markedly during the course of the disease although the minimum dose required to infect susceptible cattle is unknown (Newton and Norris 2000). Because of the long incubation period, it takes a long time for the disease outbreak to be recognised through visibly sick cattle and pathognomonic lesions at post-mortem. This may lead to a large number of animals becoming infected and the disease could have spread over long distances from the original focus of infection if there is relatively free movement and mixing of cattle (Wood 1983).

There is considerable variation in severity of signs observed in cattle affected by CBPP, ranging from hyperacute through acute to chronic and subclinical forms (Regalla *et al.*, 1996). Not all cattle infected with *Mmm*SC will go through the same patho-physiology in the development of the disease (FAO 1987). In case of the hyperacute form, involving at least 10 percent of the infected cattle, animals die suddenly without accompanying lesions. This usually occurs during the onset of the disease in a herd. In the case of the disease course with overt clinical signs, the early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy. Animals show dullness, anorexia, irregular rumination with moderate fever and may show signs of respiratory disease such as laboured breathing, an intermittent dry honking cough and sometimes a nasal discharge. As the disease progresses into the acute stage, the fever goes up to between 40°C and 42°C, and the animal prostrates with difficulty of movement. In milking cows, there is a sudden drop in the milk yield while pregnant cows and heifers may abort. As the typical lung lesions develop, signs become more pronounced with increased frequency of coughing and the animal becomes prostrate or stands with the back arched, head extended and elbows abducted. Any activity that demands high respiratory efficiency may lead to the animal dropping dead. In calves the predominant sign is arthritis due to the localisation of the causative agent in the joint (FAO 1987). The subacute form occurs in 40 to 50 percent of the infected cattle and the clinical signs resemble those of the acute stage although they are less severe but intermittent fever is very pronounced (FAO 1987). The chronic form is the natural evolution of both the acute and subacute forms although some animals may progress directly into this form. The clinical signs regress but the animals may still have intermittent fever and may lose weight (Thiaucourt *et al.*, 2004; FAO 1987).

## 2.8. CBPP in Zambia

The first recorded CBPP outbreak occurred in 1915 at a place called Nalikalako near Lealui (Wood 1983; Anon 1981). This outbreak was introduced from Angola by oxen which were requisitioned from various villages in the flood plain by British officers involved in the Anglo-Portuguese joint boundary commission which demarcated the Angolan-Barotse border in 1914 to 1915. At the time of the outbreak, there were no veterinary staff in the Barotseland protectorate and the control measures were left in the hands of the untrained colonial officers who together with Indunas organised a traditional inoculation programme. This involved placing an infected lung tissue on the cut slit in apparently healthy cattle. The results were horrendous and abandoned by 1920 and the cattle left to their fate (Wood 1983).

Suggestions of renewal of vaccinations were rejected by cattle owners in 1923. Although no cattle census was allowed by the Barotse Royal Establishment (BRE) until 1930, various estimates agree that the loss due to CBPP was in the order of 280,000 cattle reducing from a population of 350,000 in 1913 to 72,000 by 1926 (Wood 1983). Accordingly, measures were put in place to control and eradicate the disease during the first outbreak. These included the following:

a) Construction of the Barotse-Namwala cattle cordon in 1933 at a cost of £60,000 and this was to be manned by the Police all along its length.

b) Construction of the Angolan-Barotse cordon line in 1935. This was done as a result of the Foot and Mouth Disease (FMD) outbreak in Angola.

c) Vaccination of all cattle in the Protectorate from 1938 until 1944.

d) Compulsory sale of cattle in order to remove "lungers" from 1943.

Following these measures, the spread of the disease was halted and the incidence reduced to minimum levels allowing the eradication process to commence. Normal cattle trade from the protectorate was able to resume by 1947 and the Barotse- Namwala cordon was disbanded in the same year (Anon 1981).

The second recorded outbreak had a first incidence of suspected CBPP in September 1969 amongst a herd in Senanga District of Western Province (Wood 1983; Anon 1973a). This case was ignored especially after the results from the local laboratory indicated that the samples were negative for the *Mmm*SC on culture. The outbreak happened exactly 2 years after disbanding of the cordon line by the Government of the newly independent state of the Republic of Zambia (Akafekwa 1975; Anon 1973a; Shaw 1972; Wood 1983). After disbanding of the cordon line, there was an increased traffic of cattle across the border from Angola. This included cattle of the refugees from the hinterland of Angola who were running away from the civil strife where it was not possible to organise activities to combat cattle diseases such as CBPP (Anon 1970b). In April 1970, the disease broke out in Nalukanda area about 16km south of Shangombo and by May 1970, one hundred out of four hundred and thirty eight cattle that had shown clinical signs of CBPP had died. The government at that time implemented measures to control and eradicate CBPP between 1970 and 1973 and these involved;

a) Creation of a task force comprising the Barotse Royal Establishment (BRE), the government veterinary services, the police and other government ministries. The main duties among the many for this task force was to mobilise financial and material resources needed to implement the recommended control measures and also to mobilise the community to participate in the government organised activities directed towards CBPP eradication.

b) Restriction of cattle movement by issuing a Statutory Instrument on 8th May 1970. This led to establishing a controlled area within which no cattle movement from village to village was allowed and also an infected area was demarcated within which no cattle movement out of the area was allowed. This was a 30 km wide zone bordering Angola from the Southern Lueti River to Sinjembela. Along the boundary of this area, a cordon was established with 154 guards posted every 1.6 km along its 254 km length. On 3rd November 1970, the whole of Western Province was put under quarantine (Anon 1970a).

c) Branding of cattle with a letter "Z" within the identified zone to facilitate their identification. This measure led to branding of a total of 134,468 cattle within the zone.

d) Compulsory slaughter with compensation to the owners of the cattle commenced thereafter. At that time, 30 to 40 percent of the slaughtered cattle were active CBPP cases and 95 percent had signs of the disease. It is reported that the compensation funds were actually higher than the then prevailing market price of cattle in the area at the time (Anon 1973a). This was done to encourage as many people as possible to volunteer to have their cattle slaughtered so that the disease could be brought under control. Despite such a measure, the resistance was quite high with the owners of the cattle demanding that their cattle be treated like in all other diseases such as Foot and Mouth Disease (FMD) or anthrax where the cattle were usually vaccinated or treated respectively. The intensity of the slaughtering reduced by August 1970 after it was realised that the disease had spread beyond the cordoned area. In December 1970, the slaughters were completely stopped and by then 3, 828 cattle had been slaughtered.

e) Introduction of vaccinations to control the spread of CBPP led to the testing of the most appropriate vaccine and on 4th February 1971, vaccinations using the recommended vaccine, T1 lyophilised vaccine commenced. During the year, a total of 420,430 cattle were vaccinated starting with areas of active disease (Revell 1973; Anon 1971). Vaccinations

brought the active disease under control within 3 to 4 months with the exception of Mongu where there was high resistance to vaccination and also in other areas such as Liangati, Liliachi, Lukona and Kaunga mashi due to incomplete vaccination coverage. The second round of vaccination commenced in December 1971 up to the middle of 1972 (Anon 1972). There was little resistance during this round except in Sesheke which had experienced high mortalities due to reactions to the vaccine in the first round. A total of 310,596 cattle were vaccinated during the period.

f) When the disease was brought to a relatively low level, the eradication campaign was launched. This involved testing all cattle using Complement Fixation Test (CFT) in areas which had suffered from the disease and slaughtering with compensation of all reactors. Such a campaign had to wait until the disease was well under control in order to prevent excessive slaughtering and compensation costs.

By the end of 1973, the only area remaining for testing was the area between the border and the cordon line. This area was tested in 1974 and 1975 and thereafter the cattle within this zone were then to be vaccinated against CBPP at least once a year and it would also be subjected to serological testing once a year (Anon 1974).

Generally the incidents of CBPP during the period up to 1996 were limited to a few sporadic outbreaks which were quickly eliminated through slaughter and compensation. In total seven outbreaks were recorded between 1975 and 1981 in areas both within the buffer zone and outside the buffer zone (Anon 1998; Wood 1983). The buffer zone was an area extending 10 km in width from the national boundary between Angola and Zambia into Zambia demarcated along its boundaries by cattle barriers.

After the change of government in 1991, economic reforms were implemented by the government which did not spare the veterinary department. This led to reduced funding to the department leading to the declaration of cordon guards redundant, freezing of recruitment of professionals and other allied critical staff. The vaccination of cattle within the cordon area was halted and the cordon line was vandalised leaving the border between the CBPP endemic Angola and Western Province very porous (Anon 2002). As a result of the increased unchecked movement across the border of the two countries CBPP, was detected in February/March 1997, in the buffer zone at Sinjembela and Likulushitu. This focus was traced to illegally moved oxen from Angola into Zambia (Anon 1998). By June 1997, nineteen kraals out of one hundred and two in the area were affected and clinical cases and deaths were reported. The focal area was quarantined and all the positive herds were slaughtered resulting in the slaughter of 1,568 cattle. Additionally, all the cattle at risk were vaccinated. By the end of 1998, this focus was eradicated and no cases were reported thereafter (Anon 1999).

Suspected CBPP broke out again in December 2000 at Lueti in Mambolomoka area of Shangombo district, an area between the Buffer zone and the free zone and was confirmed in January 2001 (Figure 2).

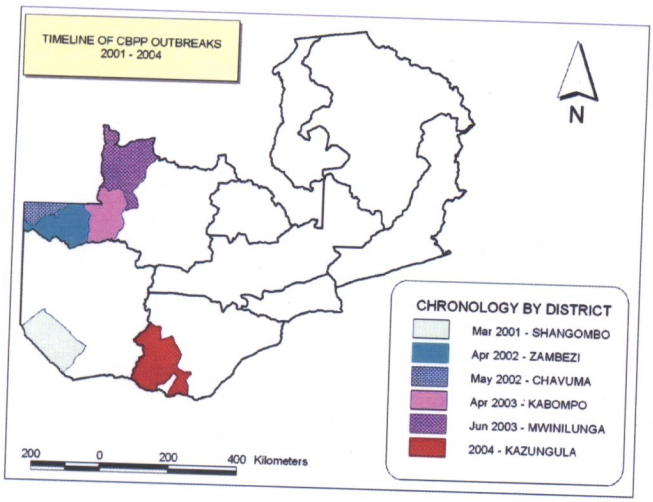


Figure 2: Map showing the chronological outbreaks of CBPP in districts between 2001 and 2004

At the time of the outbreak, the department did not have funds to contain this outbreak. As a result enforcement of quarantine measures became extremely difficult (Anon 2003). This outbreak was attributed to a mass movement of refugees from Angola which was witnessed in early 2000 at the height of the civil strife in their country. These came with their livestock which was initially impounded and destroyed (approximately 1,967 cattle) but it was later discovered that some of the cattle had been integrated in the herds of the local people. At the time of the outbreak, the cordon line had been vandalised and was no longer effective to combat cross border cattle movement, neither were there cordon guards to enforce movement controls into the free zone (Anon 2002).

Funding to the department was so erratic and inadequate such that resources to procure the vaccine were inadequate and the little vaccine that was procured was only enough to cover a small proportion of cattle in the province. By October 2003, the first case of CBPP was recorded in Sesheke district (Anon 2004; FAO 2004) and by January 2004, the disease had spread to reach Magumwi at the border between Sesheke and Kazungula districts. By 2005 the whole of Western Province was considered infected (Anon 2006b).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1. Study Area/Population

The study focused on cattle with suspected CBPP within the confines of Kazungula District, an area bordering Western Province to the west, Kalomo to the north, Zimbabwe to the south and south east, Namibia and Botswana to the south-west (Figure 3). The district lies in the most Southern part of the country, located by coordinates 17°05′S to 17°45′S and 25°38′E to 25°59′E along the Zambezi basin. The altitude of the area is 981 metres above sea level. Kazungula has a semi arid-steppe type of climate and generally receives on average lower rainfall compared to the rest of the country (average of 740mm per year, country average 1,200mm). The average maximum temperature per year is 34.8°C making it the hottest in the country, while the minimum temperature per year is 7.1°C (Anon 2010).

In this area of study the cattle population was estimated at 46,500 (Anon 2007a) and all at risk of contracting the disease. The system of animal management is pastoral with animals from individual kraals mixing with those from the other kraals. Additionally samples were collected from abattoirs and slaughter slabs in Western and Northwestern Provinces.

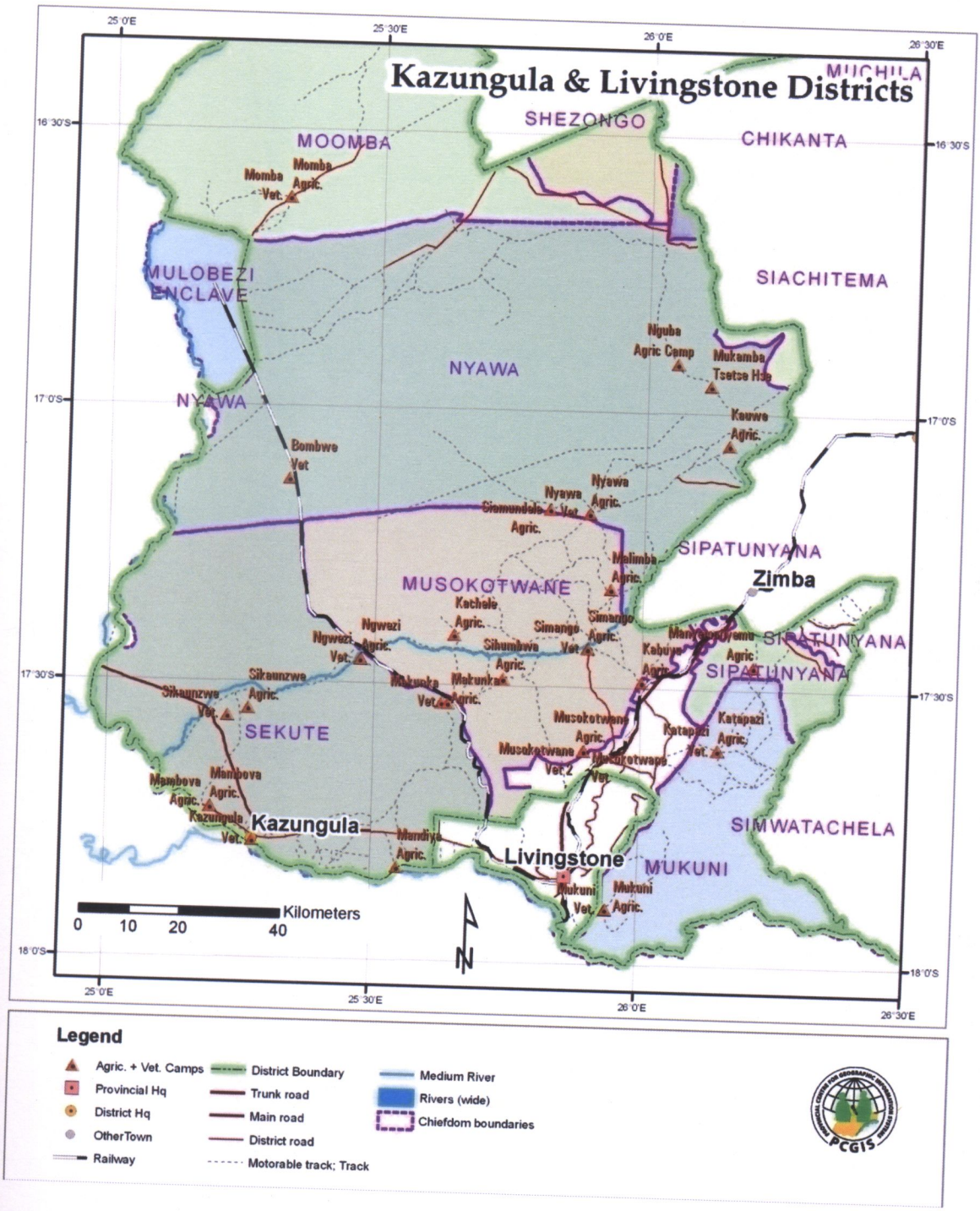


Figure 3: Map of Kazungula District, where the study was conducted

### 3.2. Study Design

A purposive (biased) sampling design was employed, where the selection parameter was cattle with suspected CBPP. These animals were identified from herds that had at least one animal positive with the Complement Fixation Test (CFT) and were earmarked for slaughter. Additionally in contact animals were also targeted for clinical examination even if on serology such herds were negative for *Mmm*SC antibodies. Records of such cattle selected were then cross checked with the initial sampling records for the serology. They were then examined clinically before slaughter to observe CBPP clinical signs. The animals were then followed to the abattoir where they were slaughtered and samples collected. Using this method, a total of 80 samples were collected from within the confines of Kazungula District. Additionally fifteen samples each from Northwestern and Western Provinces were collected using the same method. The study was conducted over a two year period from 2008 to 2010.

### 3.3. Clinical Examination

The identified herds with at least one positive animal on serology were observed for CBPP clinical signs. The observation involved visiting the individual herds once and clinically examining cattle within these herds and looking for CBPP associated signs. Clinical signs considered were fever, dyspnoea, exercise intolerance, polypnoea cough, nasal discharges, standing with head and neck extended and legs widely placed and abduction of the elbows; and when moving, those animals that lagged behind. Additionally weight loss of cattle in the observed herds was investigated. In calves, signs of swollen joints were investigated. The findings in each herd were then recorded. The herds were also re-examined just before slaughter at the abattoir. The records included the number observed with clinical signs related to CBPP, sex of clinically sick and age.

## 3.4. Sample Collection

### 3.4.1. Sampling

Cattle identified for inclusion in the study were followed and upon slaughter post-mortem was conducted with the keen interest to organs that are usually associated with CBPP lesions. These included the lungs, mediastinal lymph nodes, kidneys and pleural fluids. The tissues with CBPP pathognomonic lesions were then identified and the stage of CBPP determined as either onset, acute or chronic. The classification criteria as earlier described by Nicholas *et al* (1996) was adopted but with slight modifications. Briefly, onset stage of the disease was one with lesions ranging from early pneumonic lung, enlarged mediastinal lymph nodes without pleural fluids and without the lung adhering to the pleura. The acute stage was classified as a stage with lungs which superficially had serous fluid distended interlobular connective tissue. These un-collapsing lungs on the cut surface had a marbled appearance showing lobules at varying stages of hepatisation and were adhering to the pleura. Additionally there was copious pleural fluid with yellow or yellowish-grey fibrin coatings resembling an omelette in the pleural cavity. The lymph nodes in this stage were greatly enlarged and oedematous. The chronic stage of classification were those with lungs that ranged from non-collapsing lungs to those with areas of the parenchyma showing signs of recovery but with one or numerous sequestra (localised areas of necrotic and liquefied parenchyma surrounded by thick fibrous tissue) and rarely with pleural fluid. These sequestra which mostly were deep sited could only be detected by palpation and incision. Adhesion connected the thickened visceral and parietal pleura.

### 3.4.2. Packaging of Samples

Tissues with lesions associated with CBPP were aseptically placed into sample bags making sure that they included the diseased region and part of the normal tissue. Details of the animal identity and the owner were labelled clearly on the sample package. Thoracic exudates associated with CBPP were collected aseptically into sterile 5ml vials and labelled accordingly like the tissue samples. The samples were then frozen at -18°C at the district veterinary offices in readiness for transportation to the laboratory. The samples were transported to the laboratory in cooler boxes and were then frozen again to -18°C while waiting to be worked on.

## 3.5. Isolation of *Mycoplasma mycoides* subsp. *mycoides* Small Colony

### 3.5.1. Preparation of Horse and Donkey Serum

Horse and Donkey sera were obtained from blood of respective animal hosts. 250mls of blood was collected into sterile bottles from the jugular veins, allowed to clot and then centrifuged at 1500 *x g* for 10 minutes. Serum was pipetted and stored in sterile bottles at refrigeration temperature.

### 3.5.2. Preparation of supplement

Supplements were prepared by mixing 100µg/ml of Penicillin G, 10% ultra-filtrated yeast extract, 0.025% thalium acetate, 20% ultra-filtrated horse serum and 0.02% of DNA. The pH was adjusted to 7.6-7.8 using 1M NaOH and finally sterilized by ultra-filtration through a 0.22µm filter.

### 3.5.3. Media Preparation

Preparation of PPLO broth was done according to manufacturer's instructions (Surrey, London, UK) and sterilized by autoclaving, then cooled to 40°C before serum and other supplements were added in the proportion described above. PPLO broth powder base was dissolved in 350ml of distilled water by heating and agitating. The resultant solution was then autoclaved at 121°C for 15 minutes. The solution was cooled to 40°C and then added 2.5 ml of Penicillin G, 40 ml ultra filtered sterile yeast extract, 0.25 g thallium acetate, and 100 ml ultra filtered sterile Horse serum and 0.02 g of DNA. The pH of the solution was then adjusted to between 7.6 and 7.8 using 1 Molar of Sodium Hydroxide solution. The solution was then agitated to dissolve the constituents properly and then it was ultra-filtrated with a 0.22 μm filter. Then 10mls of the filtered medium was each poured into sterile test tubes.

#### 3.5.3.1. PPLO serum broth with supplement

Depending on the need, PPLO broth with horse or donkey serum was prepared and 10mls dispensed in sterile test tubes; and were used for enrichment of the inoculum.

#### 3.5.3.2. PPLO serum agar with supplement

For PPLO agar with horse or donkey serum, the preparation was as for PPLO broth except that instead of the broth, the agar base was used. The media was dispensed into petri dishes and allowed to solidify and was employed for sub-culturing from the enrichment cultures and for colony characterisation.

#### 3.5.3.3 Preparation of donkey blood agar

Donkey blood agar was prepared as earlier described by Thigpen *et al* (1983). Briefly, 40 g of blood agar base suspended in 100 ml of distilled water. It was then heated to boil in order to

dissolve the powder after which it was sterilised at 121°C for 15 minutes and then allowed to cool to 50°C and then 10 ml of ultra-filtrated donkey blood was added to the preparation. Then 15 ml was dispensed into each sterile petri dish. The media was then allowed to solidify.

#### 3.5.3.4. PPLO serum agar without supplement for drug susceptibility testing

PPLO was prepared exactly the same way as described earlier with horse serum but without adding any Penicillin G.

### 3.5.4 Inoculation and isolation

#### 3.5.4.1 Pre-enrichment culture

The tissue samples were minced with a sterile scalpel blade in a sterile petri-dish containing 0.8 ml of the PPLO broth earlier prepared. Then 0.5 ml of the resultant supernatant from the homogenous fluid generated by the mincing was then put in each of the test tubes, each sample added in duplicates in the tubes. For the pleural fluid, 2 ml was pipetted directly from the sample into the broth medium. The test tubes containing the samples were then incubated at 37°C, one of the tubes of each sample in aerobic and anaerobic conditions and were then observed daily for opacity. The tubes that showed opacity were then sub-cultured on to the agar plates.

#### 3.5.4.2 Isolation of suspect *Mmm*SC

The plates were examined daily over a period of 10 days and the characteristic growth pattern of *Mmm*SC observed.

### 3.5.5 Antimicrobial susceptibility testing

The PPLO agar plates prepared without any Penicillin G were flooded with 0.5 ml of the homogenate growth (3 days old) from the broth, allowed to dry in the incubator for 30 minutes and then various antibiotic discs applied. The discs were applied at equal distance apart. The discs that were applied were those of nitrofurantoin, gentamycin, tylosin, tetracycline, co-trimoxazole and erythromycin. The plates were then incubated in a 5 percent carbon dioxide environment for 7 to 10 days. Antibiotic susceptibility was tested by checking the diameter of the zone of inhibition and anything above 0.1mm was regarded susceptible.

## 3.6. Genomic characterisation of *Mmm*SC

### 3.6.1. Extraction of genomic DNA

Single colonies of each of the organisms that grew on the plates were sub-cultured into 2 ml PPLO broth as earlier described and these were incubated in 5 percent carbon dioxide for 3 to 4 days. Cells were then harvested aseptically by centrifugation at 10,000 *x g* for 5 minutes at 4°C and washed once in phosphate-buffered saline.

*Mycoplasma* cells were then lysed by addition of 1 ml 2 percent (w/v) SDS in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 100 µl RNase. The solution was then incubated at 37°C for 1 hour and then 70 µl of Proteinase K was added and again incubated at 37°C for 1 hour. The mixture was then extracted with an equal volume of phenol/chloroform and DNA precipitated from the aqueous phase with 2 volumes of ethanol. The DNA was then re-dissolved in 100 µl TE and stored at -20°C ready for use.

### 3.6.2. Amplification of genomic DNA

Oligonucleotides that identify the CAP-21 region of the genomic region were used for the specific identification of *Mmm*SC (Dedieu *et al.,* 1994) were obtained commercially. The forward primer used had the sequence 5'CTGATTATGATGACAGTGGTCA'3 and the reverse primer sequence was 5'ATACTTCTGTTCTAGTAATATG'3. The amplification was performed in a DNA thermal cycler Gene Amp 9600 (Applied Biosystems) as described by Kusilika *et al* (2000), Bashiruddin *et al* (1994), Dedieu *et al* (1994) and Thiaucourt *et al* (2008). Briefly, a 50 µl reaction PCR was performed composed of 1 µl Taq® Polymerase, 2.5µl of a 20 X concentrated reaction buffer (Tris-HCL 1M. pH 9.0; $(NH_4)_2SO_4$ 0.4M), 1.5µl 50mM $MgCl_2$, 0.3µM of each primer, 200µM of each dNTP, 5µl sample and 15 µl de-ionised water.

The PCR was run for 30 cycles starting with a single denaturing step at 94°C for 2 minutes and ending with a final 5 minute extension step at 72°C. Normal cycles consisted of 30 seconds denaturing at 94°C, annealing at 56°C for 60 seconds and extension at 72°C for 90 seconds. A total of 5 µl of each of the PCR products and the λ/*Hind*III fragments of size 0.5 kbp DNA markers which were used were then electrophoresed through a 2 percent agarose gel and then stained with ethidium bromide and the PCR products were visualised by ultraviolet transillumination.

### 3.6.3. Digestion of Amplification products by restriction enzymes

A total of 7 µl of each of the PCR product was pipetted in to a 50 µl reaction tube. To this was added 4 µl of the 10 X specific enzyme buffer (Takara Laboratories), 7 µl distilled water and 2 µl of each of the Restriction enzymes, *Eco*RI, *Hind*III, *Pst*I, *Bam*HI and *Xba*I (Takara Laboratories). The reaction tubes were then closed tightly and the contents mixed thoroughly. The mixture was then incubated at 37°C for 24 hours in a dry heat block water bath. After 24

hours, the tubes were put on ice for 5 minutes and then 10 µl of the mixture from the individual tubes was mixed with 5 µl of loading buffer. Electrophoresis was then carried out at 110 V, 30 minutes using 2 percent (w/v) agarose gel and bands visualised by ultraviolet fluorescence after staining with ethidium bromide.

### 3.7.0. Statistical Analysis

The 95% confidence intervals for prevalence rates in the study areas were computed in excel according to Daniel (1991).

# CHAPTER FOUR

# RESULTS

## 4.1. Herds Inspections

At total of 63 herds with 797 heads of cattle were examined for clinical signs of CBPP. Of these examined 439 were females above eight months old, 241 were males above eight months old while 117 were calves of up to seven months old. The examinations were conducted from a total of eight veterinary camps as shown in Table 2 below. A total of 59 cattle were examined in Momba, 118 in Kazungula central, 203 in Makunka, 312 in Nyawa, 32 in Bombwe, 18 in Simoonga, 39 in Simango and 16 in Musokotwane veterinary camps.

During the study, it was observed that, a total of 186 cattle (23.3%, CI 20.5 to 26.5) showed overt clinical signs of CBPP which included coughing, laboured breathing, extension of the neck, abduction of the forelimbs and in some cases loss of weight. When walked, some cattle were distinctly remaining behind the group. The total number of cattle observed with clinical signs in relation to the total number examined in each of the camps was 36 (61.0%, CI 47.4 to 73.2) in Momba, 44 (37.3%, CI 28.7 to 46.7) in Kazungula, 28 (13.8%, CI 9.5 to 19.5) in Makunka, 58 (18.6%, CI 14.5 to 23.5) in Nyawa, 5 (15.6%, CI 5.9 to 33.5) in Bombwe, 5 (27.8%, CI 10.7 to 53.6) in Simoonga, 7 (18.0%, CI 8.1 to 34.1) in Simango and 3 (18.8%, CI 5.0 to 46.3) in Musokotwane. The total number of females showing clinical signs of CBPP out of the total number of cattle with signs of the disease upon inspection was 67 (36.0%, CI 29.2 to 43.4). These were distributed as; 12 (17.9%, CI 9.9 to 29.6) in Momba, 17 (25.4%, CI 15.9 to 37.7) in Kazungula, 9 (13.4%, CI 6.7 to 24.5) in Makunka, 21 (31.3%, CI 20.9 to 44.0) in Nyawa, 3 (4.5%, CI 1.2 to 13.4) in Bombwe, 1 (1.50%, CI 0.1 to 9.1) in Simoonga

and 4 (6.0%, CI 1.9 to 15.4) in Simango. No cow or heifer was showing any clinical signs in Musokotwane. The total number of males that showed CBPP clinical signs on observations in relation to the total number of cattle with clinical signs were 119 (64.0%, CI 56.6 to 70.8) with the distribution as; 24 (20.2%, CI 13.1 to 28.7) in Momba, 27 (22.7%, CI 15.8 to 31.5) in Kazungula, 19 (16.0%, CI 10.1 to 24.1) in Makunka, 37 (31.1%, CI 23.1 to 40.3) in Nyawa, 2 (1.7%, CI 0.3 to 6.5) in Bombwe, 4 (3.4%, CI 1.1 to 9.0) in Simoonga, 3 (2.5%, CI 0.7 to 7.7) in Simango and 3 (2.5%, CI 0.7 to 7.7) in Musokotwane (Table 2). During the study, none of the inspected calves exhibited obvious clinical signs associated with CBPP.

### 4.2. Post-mortem

Post-mortem of cattle suspected to be infected with the disease under investigation revealed lesions typical of CBPP. It was observed that the infections of individual cattle could be classified as onset, acute and chronic. Although arbitrally classified into these categories, it was noted that the lesion features overlapped between the identified categories and thus could only be classified as earlier described by the most prominent feature in the lesions seen. Using this categorisation, 19 (36.5%, CI 24.0 to 51.1), 11(21.3%, CI 11.5 to 35.1) and 22 (42.3%, CI 29.0 to 56.7) of onset, acute and chronic lesions respectively were observed as shown in Table 3.

Table 2: Distribution of cattle observed during inspection

| **Veterinary Camp** | **No. Observed** | **Number with clinical signs (%; CI)** | **Number of Females with Clinical signs (%; CI)** | **Number of Males with Clinical signs (%; CI)** |
|---|---|---|---|---|
| Momba | 59 | 36 (61.0%, CI 47.4 to 73.2) | 12 (17.91%, CI 9.9 to 29.6)) | 24 (20.2%, CI 13.1 to 28.7) |
| Kazungula | 118 | 44 (37.3%, CI 28.7 to 46.7) | 17 (25.4%, CI 15.9 to 37.7) | 27 (22.7%, CI 15.7 to 31.5) |
| Makunka | 203 | 28 (13.8%, CI 9.5 to 19.5) | 9 (13.4%, CI 6.7 to 24.5) | 19 (16.0%, CI 10.1 to 24.1) |
| Nyawa | 312 | 58 (18.6%, CI 14.5 to 23.5) | 21 (31.34%, CI 20.9 to 44.0) | 37 (31.1%, CI 23.1 to 40.3) |
| Bombwe | 32 | 5 (15.6%, CI 5.9 to 33.6) | 3 (4.5%, CI 1.2 to 13.4) | 2 (1.7%, CI 0.3 to 6.5) |
| Simonga | 18 | 5 (27.8%, CI 10.7 to 53.6) | 1 (1.5%, CI 0.1 to 9.1) | 4 (3.4%, CI 1.1 to 8.9) |
| Simango | 39 | 7 (18.0%, CI 8.1 to 34.1) | 4 (6.0% CI 1.9 to 15.4) | 3 (2.5%, CI 0.7 to 7.7) |
| Musokotwane | 16 | 3 (18.8%, CI 5.0 to 46.3) | 0 (0.0%, CI 0.0 to 6.8) | 3 (100.0%, CI 30.9 to 100.0) |
| Total | 797 | 186 (23.3%, CI 20.5 to 26.5) | 67 (36.0%, CI 29.2 to 43.4) | 119 (64.0%, CI 56.6 to 70.8) |

Table 3: Lesions observed as per categorization

| Category of Lesion | Number Observed (n) | Percent % (CI) |
|---|---|---|
| Onset | 19 | 36.5 (CI 24.0 to 51.1) |
| Acute | 11 | 21.2 (CI 11.5 to 35.1) |
| Chronic | 22 | 42.3 (CI 29.0 to 56.7) |
| Total | 52 | 100.0 (CI 91.4 to 100.0) |

The pathological lesions seen were prominently unilateral involving only one side of the lung. From the study it was apparent that the disease could affect either lung and there was no evidence of any prominent side being affected more than the other. It was also seen that in most cases, it was the diaphragmatic lobe that was affected more than any other lobe of the lung. As shown in Figure 4 below, the onset stage of infection could only be seen as pneumonic lungs with non-specific CBPP lesions. However the lesions could be inferred to be due to CBPP due to the majority of the lesions that were found with CBPP pathognomonic lesions in the acute and chronic stages of infection.

The acute stage of infection (Figures 5 and 6) distinctly showed lung lesions that are associated with CBPP. The lungs did not collapse and the interlobular septa on the cut surface were visibly distended. The affected lung parenchyma showed various stages of grey and red hepatisation (Figure 5). The lung surface was covered by a yellow or yellowish-grey fibrin coating (Figure 6). One distinct feature of the acute stage of infection noticed was the availability of copious volumes of yellow (straw like) pleural fluid (figure 7) in the pleural cavity. This fluid could be as much as 10 litres in quantity and had distinctive clots on exposure to the air

Lungs in the chronic stage of infection showed the majority of the parenchyma to be normal. The distinctive feature in this stage of infection was the presence of sequestra of various diameters occurring singly or multiple in the affected lung. The affected lungs in most of the cases in this stage of infection were firmly adhering to the parietal pleura by fibrous connective tissue. As shown in Figure 8, the external surface of the lung had the normal texture. On palpation though, a distinctive hard area within the lung parenchyma could be felt. On excision as shown in Figure 9, the interlobular septa of the lungs showed distinctively distended lobes although the lung parenchyma looked normal. The sequestra when closely examined were seen to be areas of dead tissue surrounded by thick fibrous tissue. As shown in Figure 10, the contents of the sequestra were usually a yellow caseous material and this was only a small area of the lung with the majority of the parenchyma looking normal.

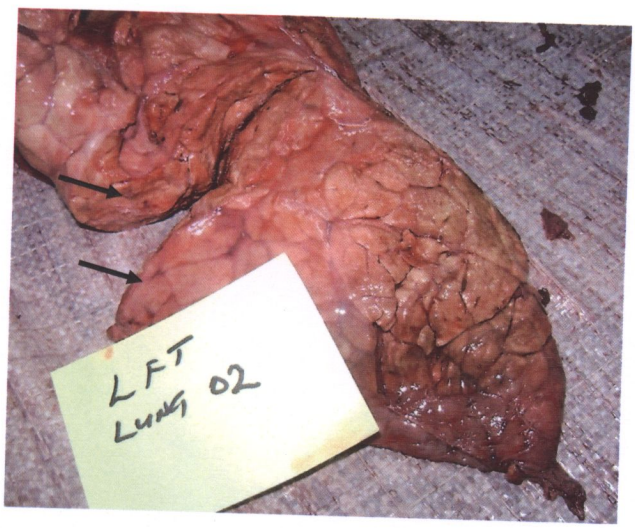


Figure 4: A Lung showing early pneumonic signs in the Onset category of CBPP. Arrows show the enlarged interlobular septa.

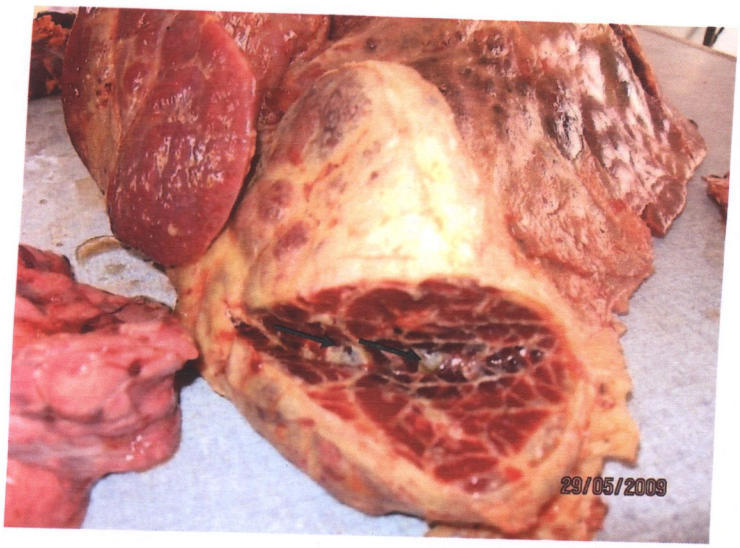


Figure 5: Typical lesions of the lung in the acute stage of infection (Sample No. 21). Arrows show the typical marbled appearance with grey areas of necrotic tissue.

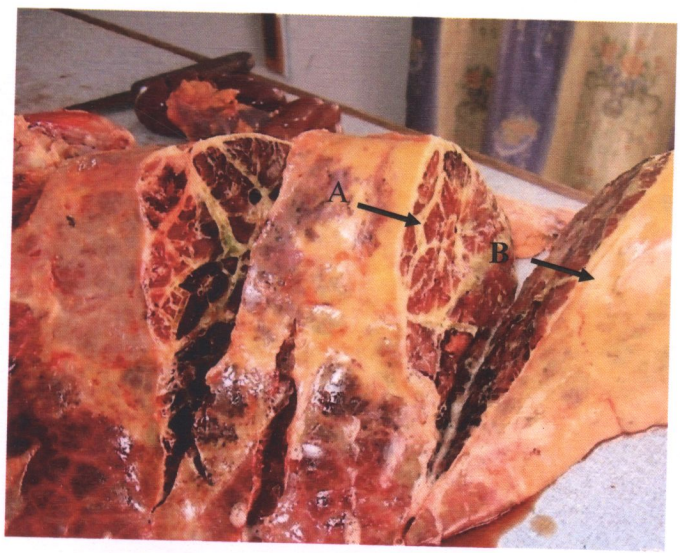


Figure 6: The lung sectioned to show the typical thickened interlobular septa and the pseudomembrane in the acute stage of infection. Arrow A the thickened interlobular septa and Arrow B the pseudomebrane.

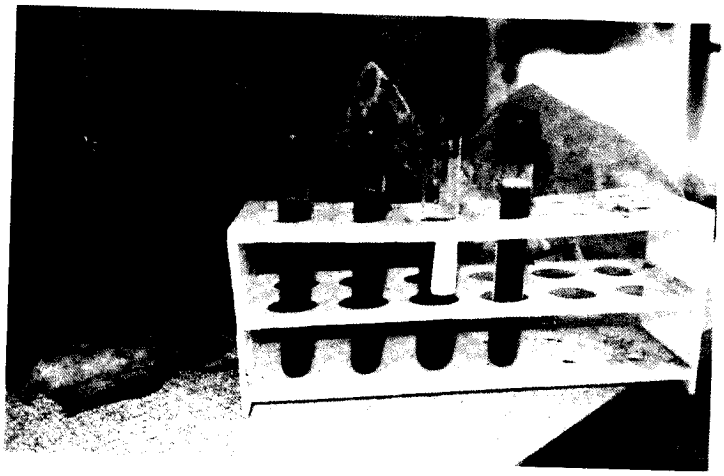

Figure 7: The characteristic yellow (straw-coloured) pleura fluid (Arrow) found in the acute stage of infection in CBPP.

Figure 8: The External appearance of the Sequestra (Arrow) although most of the lung looks normal.

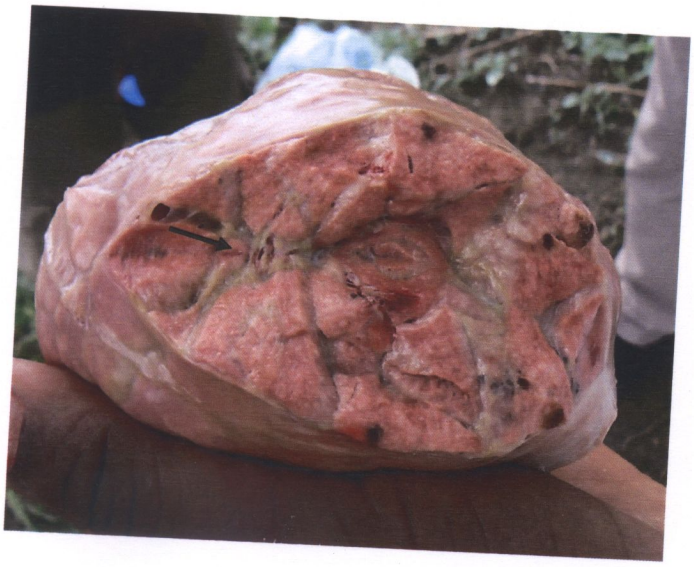

Figure 9: A sectioned area of a lung in Figure 8, showing the interlobular septa discernibly thickened (Arrow) although most of the lung tissue looks normal.

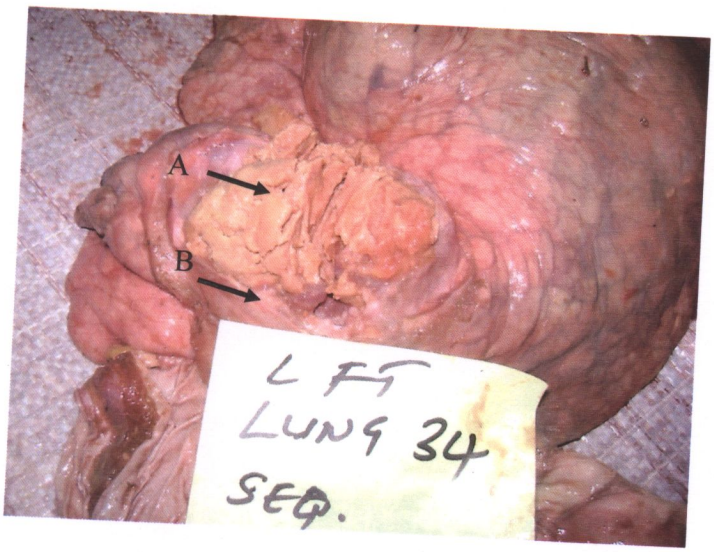


Figure 10: Sequestra opened to show the caseous yellowish contents (Arrow A) and the surrounding fibrous tissue (Arrow B) while the lung tissue looks generally normal.

### 4.3. Culture and isolation of *Mmm*SC

The broth tubes seeded with samples were observed to develop a homogenous cloudiness which commenced 2 day after inoculation. These had a silky, fragile filament which disappeared on agitation. With increasing days of culturing, a uniform opacity developed

with swirls when tapped at the bottom of the tube. These swirls could be observed by holding the tubes containing the broth in the direction of light and could be seen to move from the bottom of the tube upwards in a characteristic circular pattern.

On solid media, growth was seen starting at 4 days after inoculation. As shown in figures 11 and 12, the colonies were seen to be small and had a homogenous size. On examination under a light microscope at a magnification of 32 x, they were transparent, flat and had a typical "fried egg" appearance which is synonymous with the growth of *Mmm*SC. The edges of the colonies were rough. No significant colony morphology differences could be observed in the growth pattern of the vaccine strain and all the other isolates on culture. It was observed as shown in figure 12 and 14 that the colonies grew more fastidiously when grown in anaerobic conditions than when grown in aerobic conditions. It was however noted as can be demonstrated in the two figures (Figures 11 and 12) that it was difficult to observe the colony morphology of *Mmm*SC on blood agar as the colonies were not very clear especially those grown anaerobically on blood agar.

The isolates from the PPLO horse serum containing plates grown in both aerobic and anaerobic conditions showed that the colonies were of uniform size both at high and low colony densities. It was however seen that colonies noticeably grew better and quicker in an environment with 5 percent carbon dioxide than in aerobic conditions. It was also very evident for all the strains grown that the colonies grew much better after initially being grown in the broth in both aerobic and anaerobic conditions and then plating them on to the agar plates. The growth of the colonies on donkey blood agar was noticeably slower than those grown on PPLO agar and the resultant colonies were also much smaller in size.

Isolation of the *Mmm*SC was attempted from all the categories of the lesions seen at the abattoir. It was observed that it was more probable to isolate *Mmm*SC from lesions in the acute stage of infection than any of the other categories. This was as shown in Table 4 below.

Table 4: *Mmm*SC isolates from the different categories of lesions and different medium

| Category of Lesion | Total investigated | Total Isolated Aerobic Conditions | Total Isolated Anaerobic Conditions | Medium used | | |
|---|---|---|---|---|---|---|
| | | | | PPLO with Horse Serum | PPLO with Donkey Serum | Donkey Blood Agar |
| Onset | 19 | 13 | 15 | 13 | 10 | 9 |
| Acute | 11 | 9 | 8 | 9 | 8 | 6 |
| Chronic | 22 | 12 | 9 | 11 | 8 | 4 |
| Total | 52 | 34 | 32 | 34 | 26 | 19 |

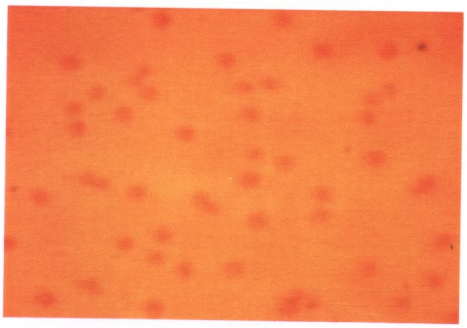

Figure 11: Colony morphology of the T1/44 vaccine strain after aerobic growth (7 days)

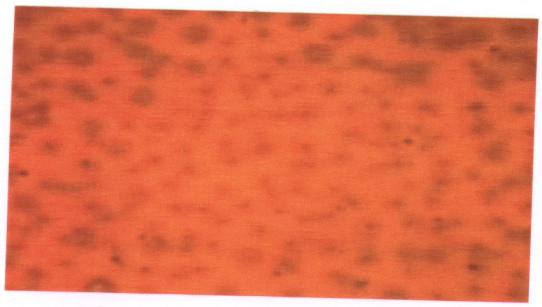

Figure 12: The same T1/44 vaccine strain as in Figure 11 above grown in anaerobic conditions shows a more fastidious growth than with aerobic conditions with the density of the colonies higher

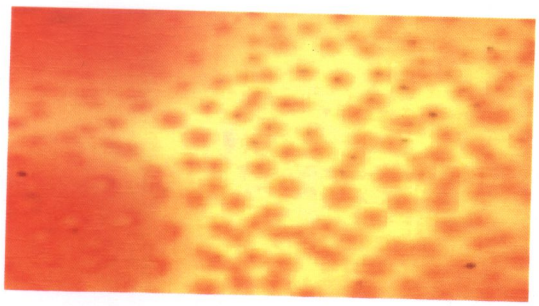

Figure 13: Isolates from sample 13 grown in aerobic conditions (7 days)

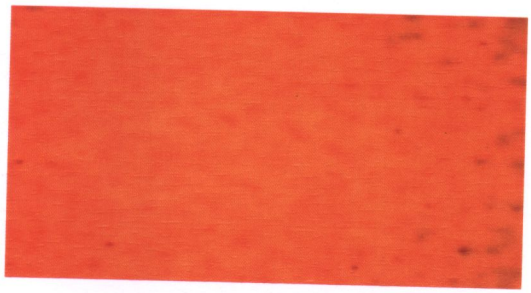

Figure 14: The same colonies as in Figure 13 as obtained in anaerobic conditions showing high density (7 days)

### 4.4. Antibiotic susceptibility

From four days (4) of incubation on wards, the plates were examined and the degree of growth inhibition noted. It was very clearly observed that most *Mmm*SC isolates were sensitive to nitrofurantoin, tetracycline and gentamycin. They were not sensitive to

erythromycin and co-trimoxazole. It was also seen that there was no difference in the degree of sensitivity whether the isolates were grown in aerobic or anaerobic conditions. During the study, it was observed that sample 23 was resistant to all antimicrobial effects except that of tylosin.

Table 5: Isolates showing Antimicrobial susceptibility with the zones of inhibition

| | | Growth-Inhibition Zone in mm | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Nitrofurantoin | | Tetracycline | | Gentamycin | | Erythromycin | | Co-trimoxazole | | Tylosin | |
| Aeration | | An | Ae | An | Ae | An | Ae | An | Ae | An | Ae | An | Ae |
| Source of Sample | Sample ID | | | | | | | | | | | | |
| Vaccine | T1/44 | 24 | 19 | 8 | 6 | 10 | 9 | 3 | 2 | 2 | 2 | 21 | 19 |
| Kazungula | 23 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 17 | 16 |
| Kazungula | 32 | 12 | 14 | 5 | 3 | 11 | 16 | 9 | 14 | 4 | 5 | 13 | 16 |
| Sikongo | 14 | 14 | 13 | 2 | 4 | 7 | 10 | 7 | 14 | 5 | 4 | 6 | 9 |
| Kazungula | 3 | 15 | 11 | 9 | 13 | 0 | 0 | 3 | 6 | 0 | 0 | 14 | 10 |
| Mufumbwe | 43 | 14 | 12 | 11 | 15 | 15 | 9 | 2 | 3 | 20 | 21 | 19 | 23 |

Key: An- Anaerobic incubation in 5% $CO_2$; Ae- Aerobic incubation

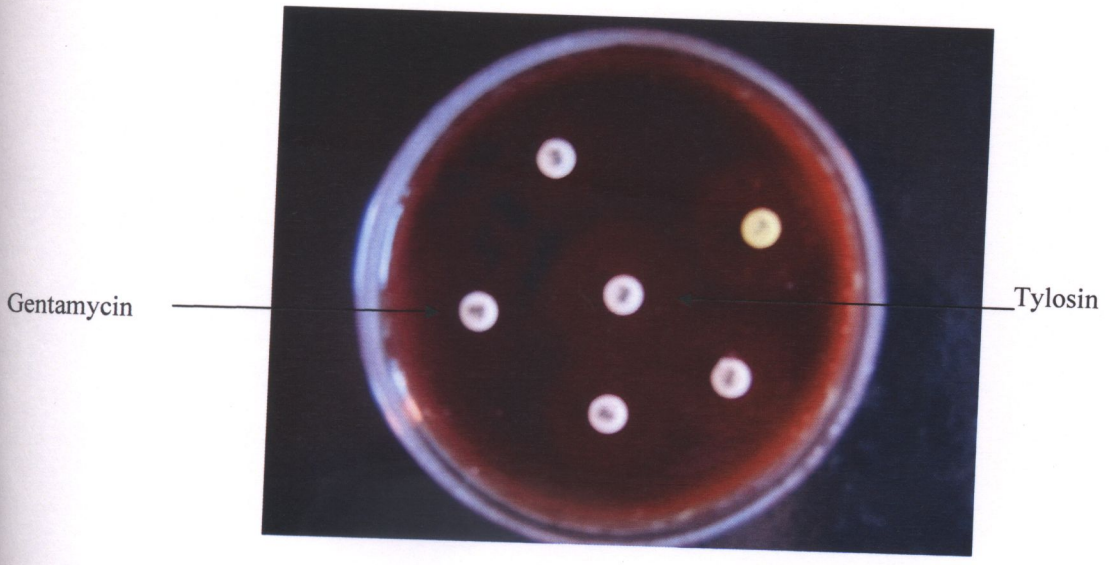


Figure 15: Antibiotic sensitivity of sample No. 3 clearly showing zones of inhibition to growth by Tylosin, Nitrofurantoin, Erythromycin and Tetracycline while co-trimoxazole and Gentamycin did not show any discernible clear zones of inhibition.

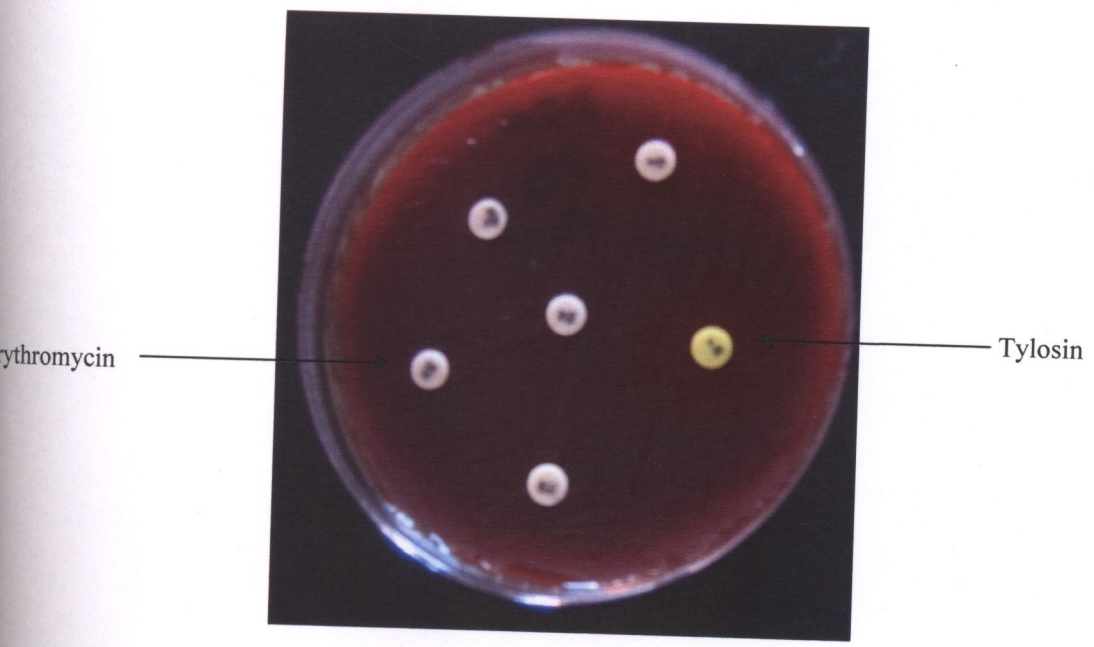


Figure 16: Sample 23 showing susceptibility to Tylosin with a clear zone inhibition while it resisted all other antibiotic effects

## 4.5. Genomic characterisation of *Mmm*SC

### 4.5.1. *Mmm*SC DNA amplification

The oligonucleotides used in the amplification of the *Mmm*SC were designed to identify a DNA fragment of 275 base pairs in size. Using these primers, a single band of the expected size was obtained as shown in Figure 17 and 18. It was noted that the band sizes for the positive control was the same size as that of samples under investigation as illustrated in Figure 17. The bands obtained from direct pathological tissues were not very distinct as compared to bands from colonies. This was mainly due to DNA smearing on the gel from the direct tissues. Spectophotometric data however showed that the PCR products obtained were of the same size from the DNA of colonies from the cultures of the pathological lesions and that from the colonies of tissues. The results also showed that even if some pathological tissues had distinct lesions suggestive of CBPP, no DNA was detected by PCR directly from the tissues or colonies.

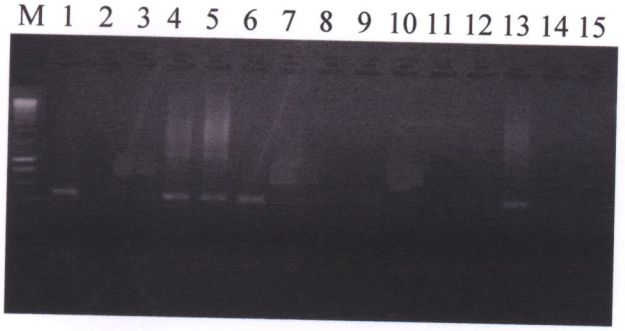


Figure 17: Detection of *Mycoplasma mycoides mycoides subspecies* SC directly from suspected clinical specimens using the *Mmm*SC specific primers. A 275 bp amplicon was amplified. M is the molecular weight marker, 1 is the positive control, 2 and 14 are negative controls with 4 as the vaccine isolate and 5, 6 from Kazungula, 3,7,8,9,10 and 14 are samples from pathological lesions that did not amplify, 12 from Northwestern province and 13 from Western province.

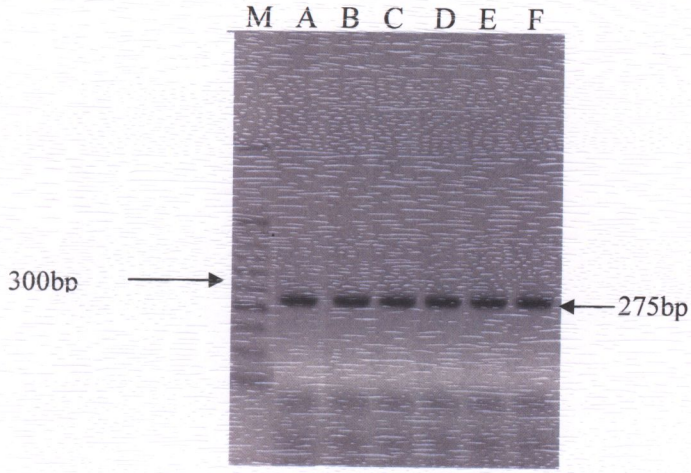


Figure 18: Isolated colony isolates subjected to PCR, exhibiting a 275 bp expected amplicon. M is the molecular weight marker, A is a positive control, B is a vaccine strain of T1/44, C and D are isolates from Kazungula, while E and F are from Northwestern and Western provinces respectively.

### 4.5.2. Restriction Enzyme treatment of *Mmm*SC isolates

During restriction enzyme analysis, it was seen that restriction enzymes *Eco*RI, *Hind*III and *Pst*I did not show any activity with the PCR products after electrophoresis. With *Xba*I, it was seen to result in many smaller fragments which mostly smeared the gel and were difficulty to analyse after electrophoresis. The results from restriction enzyme activity of *Bam*HI resulted into two bands obtained from the PCR product upon electrophoresis. These bands were of size 64bp and 173bp. The bands in the two size categories were very prominent although there was smearing with the excess DNA that was visible as shown in figure 19. The bands obtained were of the same size and pattern whether the samples were from the vaccine strain or the field isolates. It was also seen that the pattern of the bands was the same whether the isolates were from Western, Northwestern or Southern Provinces.

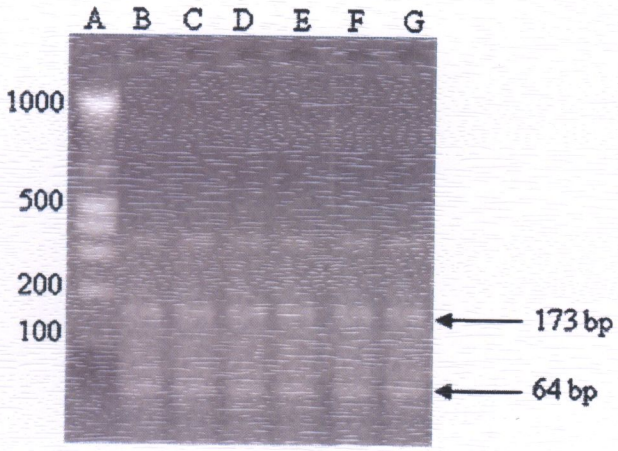


Figure 19: Restriction Enzyme profiles with *Bam*HI *o*btained showing approximately 64 bp and 173 bp bands. Lane A is the molecular ladder while B is the T1/44 strain, C, D, E are from Kazungula Southern province, F and G from Western and North western provinces respectively.

# CHAPTER FIVE

# DISCUSSION

## 5.1. Herd Inspection

The clinical signs observed were consistent with CBPP findings as stated by FAO (1987) and Regalla *et al* (1996). These signs included laboured breathing, extension of the neck, abduction of the forelimbs and a typical dry honking cough. It was noticed that most of the cattle that showed these signs of respiratory distress also had their nasal orifice dilated with a mucoid discharge. Some cattle exhibited signs of loss of condition with marked weight loss and a rough coat.

It was generally observed that more males 119 (64.0%, CI 56.6 to 70.8) showed clinical signs than females 67 (36.0%, CI 29.2 to 43.4) during the study. This could be attributed to the use of males in draught power as they are more likely to interact with cattle from other locations in areas such as the market centres, health centres and other community gatherings like marriages, funerals and traditional ceremonies to name a few. However, this could not apply to individual kraals since it was observed that there were more females showing clinical signs than males. This could however be attributed to the high number of females in a kraal compared to males.

During the study no cattle below the age of eight months showed any clinical signs associated with CBPP despite the numerous adult cattle showing such signs. The signs that were expected to be seen in calves found in these CBPP afflicted herds in addition to the respiratory distress signs, was the swelling of the carpal joints which has been described

previously (FAO 1987). This could be attributed to the husbandry practises where the calves are usually separated from the dams most of the day and are only allowed to suckle from the mothers over a brief period of the day especially during milking. During this brief interaction, the infective *Mmm*SC discharged by the dams could be insufficient to cause CBPP in the calves. This observation is important as it shows that for CBPP transmission to occur there should be a minimum interactive period within which CBPP infected cattle can transmit infective doses of *Mmm*SC to susceptible cattle (Thiaucourt *et al*, 1998).

The lack of clinical signs in the calves could also be attributed to the passive immunity that the calves acquire. It was shown in studies by Stone (1970) that calves usually acquire antibodies specific for CBPP after ingestion of colostrum from the dams that were experimentally infected with CBPP. The extent of protection against CBPP infection of such calves with this acquired immunity is subject to further investigation.

**5.2. Post-mortems**

A total of 115 carcases from the 63 herds that were inspected for clinical signs of CBPP were examined at post-mortem for probable lesions associated with CBPP. Of these carcases examined, 52 (45.2%, CI 36.0 to 54.8) were amongst those that had initially been seen to have signs that are associated with CBPP during herd inspection while 63 (54.8%, CI 45.3 to 64.0) were amongst those that had not shown any clinical signs of CBPP. From the results it was observed that lesions pathognomonic to CBPP were identified in both the cattle showing clinical signs of CBPP on herd inspection and those that did not show clinical signs of CBPP on inspection. The observation agree with the earlier findings of Nicholas *et al* (1996), Regalla *et al* (1996) and Provost *et al* (1987) who showed that CBPP lesions could also be

found in cattle which did not show any clinical signs associated with the disease on physical examination.

In this study, an additional category of onset was assigned to lesions with predominating signs of general pneumonia. Using this basis of categorisation, 19 (36.5%, CI 24.0 to 51.1) of the examined carcases were categorised in this group and generally conformed to the description of carcases with early pneumonic lung and enlarged mediastinal lymph nodes. It is difficult to assign this group exclusively due to the generality of the signs that can be found in any severe pneumonia caused by various different diseases in cattle. However, in an outbreak situation where the majority of cattle are dying from suspected CBPP, cases of early severe non specific pneumonia can be inferred to be due to CBPP and these should be a basis for further investigation from the samples collected from such cases.

As observed in earlier works by Masiga *et al* (1996), Nicholas *et al* (1996) and Martel *et al* (1983) the acute category of CBPP lesions during post-mortem can be classified as those with a striking feature of large volumes of yellow (straw coloured) fluid containing clots in the thoracic cavity. The predominant gross change is consolidation, or thickening, of individual lobules that become encased in markedly widened interlobular septa, resulting in the characteristic marbled appearance. The lesions invariably affect one lung and its pleura only and are rarely found in both lungs. The lymph nodes of the thoracic cavity are generally enlarged and oedematous. In this study, classical lesions as described by earlier workers (Masiga *et al,* 1996; Nicholas *et al,* 1996; Martel *et al,* 1983) were demonstrated. During the study, 11 (21.2 %, CI 11.5 to 35.1) of the lesions seen were in the acute category with typical lesions. The acute category of lesions in CBPP infections are pathognomonic of the disease and are usually a basis of gross diagnosis of the disease in the field.

The findings of the study showed that there various intermediate stages between the acute lesions and the fully formed sequestra. The sequestra seen during the study were of various diameters with the largest being 8 cm. Some of the sequestra observed were small and deeply sited and could only be identified by careful palpation. The adhesions connected the thickened visceral and the parietal pleura. The chronic stage of infection as described by earlier workers is characterised by the formation of a sequestra which is usually an infarct delineated from surrounding parenchyma by granulation tissue/fibrous capsule (FAO 1987). The diameters of these sequestra usually vary from 2 to 25 cm. The contents of the sequestra are usually necrotic and liquefied parenchyma. Adhesions between the lung lobes and between lungs and the chest wall are commonly found, while pleura fluid is rarely found. In this study, 22 (42.3%, CI 29.0 to 56.7) of the carcases observed were in the chronic category. The findings of this study are in general agreement with earlier studies by Nicholas *et al* (1996) who showed that lesions in the chronic stage of infection consisted of sequestra of various diameters up to 15 cm. The sequestra in the chronic stage of CBPP can easily be missed during meat inspection.

Because the lesions in CBPP are so characteristic, slaughter house monitoring is a powerful tool which should be used in detecting the introduction and spread of CBPP. However, it should be noted that for this to be useful, the personnel that conduct the slaughter house inspections must be well trained to detect even the smallest of the deep sited lesions that can easily be missed. According to a SADC report (SADC 2009), the source of infection for 77 percent of the outbreaks of CBPP was suspected to be endemic foci, and all the outbreaks were confirmed by clinical pathology or post-mortem. This entails that the diagnostic

capabilities of staff in the field should be competent enough to diagnose all cases of CBPP whether in the onset, acute or chronic stages.

### 5.3. *Mycoplasma mycoides* subspecies *mycoides* SC Morphology

*Mmm*SC was isolated from lungs, pleural fluid, and lymph nodes collected from post-mortem of cattle in the study area. The confirmatory diagnosis was done by PCR of the isolates. Using the 20 percent horse enriched PPLO broth, it was seen that it became turbid after four days of incubation, while that from 20 percent donkey serum took six to seven days. When an aliquot of the broth was plated on 20 percent horse serum enriched PPLO agar, the isolates obtained were homogenous in size and had a typical "fried egg" appearance at high magnification using an inverted microscope. The results obtained were identical whether the samples were from the field isolates or from the vaccine strains. The growth of the colonies on the plates was observed after six days of incubation onwards.

The *Mmm*SC also grew on 20 percent rich donkey PPLO media and was seen just like in 20 percent horse serum enriched PPLO media, to be of homogenous size and typical "fried egg" morphology. When compared to the duration before the first colonies could be seen, it was noted that the colonies grown on PPLO horse serum enriched medium appeared earlier by a day than those grown on either Donkey serum or those grown on Donkey blood agar. As noted by Thigpen *et al* (1983), *Mmm*SC grown on blood agar grew relatively slow as compared to *Mmm*LC under similar conditions. Thus when grown on media devoid of horse serum *Mmm*SC indeed grows very slowly as demonstrated by this study. On 20 percent defribrinated donkey blood agar, the colonies grew smaller than in the horse and donkey serum enriched PPLO media although they showed to be of homogenous colony size with

typical fried egg appearance. It was also seen that the rate of growth using this media was very slow when compared to either horse serum or donkey serum containing media.

As shown by earlier studies of Gizaw (2004), March *et al* (2000) and Thigpen *et al* (1983) the growth characters observed during this study are typical of the *Mmm*SC growth. Although in the study of Thigpen *et al* (1983), it was shown in this study that the isolation of *Mmm*SC in 5 percent defribrinated sheep blood agar was difficult, it was possible to isolate the organism using 20 percent defribrinated donkey blood agar although the growth was slow and the organisms did not grow to the same size as in horse or donkey enriched PPLO.

From this study it was observed that isolation of *Mmm*SC from field samples is easier with the use of *Mycoplasma* growth supporting media enriched with horse serum than with sera from donkeys. However, it was demonstrated that in time of difficulty in obtaining horse serum, donkey serum could be used to obtain the same results within the same period. This study has demonstrated that even if the growth requirements of *Mmm*SC are complex (OIE 2009), it is possible to isolate the bacteria from pathological samples using simple media such as blood agar. The isolated bacteria can then be subjected to specific confirmatory tests such as PCR or immunological tests such as the growth inhibition test which is a very specific confirmatory test for *Mmm*SC (OIE 2009).

### 5.4. Antibiotic Susceptibility

The *in vitro* antibiotic susceptibility of *Mmm*SC to a selected number of antimicrobials was determined during the study. It was observed that all the *Mmm*SC were susceptible to nitrofurantoin, tetracycline, gentamycin, erythromycin, co-trimoxazole and tylosin except the isolate from sample number 23, which was seen to resist all the antibiotics except tylosin.

From the antibiogram, it is clear that comparatively, tylosin inhibited the growth of *Mmm*SC markedly than any other antibiotic investigated with an average minimum zone of inhibition of 7.5 mm and the maximum zone of inhibition of 21 mm. It was also evident that co-trimoxazole had the least inhibitory effects on the isolates studied. In terms of isolate susceptibility, sample 43 showed that it was inhibited more than any other sample with comparatively larger zones of inhibition by most of the antimicrobials studied than any of the other isolates.

The antimicrobial profile of *Mmm*SC has never been investigated in Zambia. It is known that in Zambia, laws are not strictly followed on antibiotic use and purchase of drugs from retailers; as such antibiotics have frequently been used in prophylactic and treatment of most cattle diseases by farmers themselves (Pandey and Sharma 1994). The uncontrolled use of antibiotics in the treatment of cattle diseases including CBPP in Africa is postulated to be high (FAO 2006). Although the policy of the Zambian government is to prohibit the use of antibiotics in CBPP confirmed cases (Anon 2007b), the probability of antibiotic use due to the uncontrolled availability of antibiotics in the markets is high. This has led to the concern that antibiotics will inevitably lead to the development of bacterial resistance to most drugs. Although several reports of antimicrobial resistance by *Mycoplasma* species have been documented (Suzuki *et al.*, 2006; Francoz *et al.*, 2005; Ayling *et al.*, 2000), there is no documented report of field *Mmm*SC isolates that are resistant to antimicrobials. It is interesting therefore that in this study; one of the isolates studied resisted the inhibitory effects of some antibiotics. Whether this resistance invariably indicates the proliferation of some antibiotic resistant strains, is a subject for further investigation. Although the in *vivo* antimicrobial activity does not always match *in vitro* effectiveness, a compound showing little or no activity *in vitro* is unlikely to be effective in aiding the body's defences in

eradication of the organisms. Thus even if the antimicrobials studied were not subjected to *in vivo* tests to investigate their effectiveness against *Mmm*SC, it is postulated that these would have the same effect to these isolates. The purpose of the quantitative studies of antimicrobials is to assist the veterinarian in choosing an effective antimicrobial to control or prevent disease. In the case of these antimicrobials studied it is clear therefore that only tylosin, tetracycline, erythromycin and nitrofurantoin would be recommended for use in our environment to treat or control CBPP although these should be subjected to further *in vivo* investigation.

The study examined the inhibitory effects of antimicrobials at the concentration of the manufacturer and thus did not look at the Minimum Inhibitory Concentration (MIC) of these antimicrobials. This could have assisted in determining the minimum concentration at which each of the studied drugs could inhibit the growth of *Mmm*SC (Ayling *et al.,* 2000) and also check whether they are within the ranges determined by Ayling *et al* (2000). This in turn would help in determining the antimicrobials that would lose the ability to inhibit the growth of *Mmm*SC quickly.

## 5.5. Genomic characterisation of *Mmm*SC

### 5.5.1. *Mmm*SC DNA amplification

The observations during the study indicate the amplification of *Mmm*SC DNA from the samples examined. These results are consistent with findings of Taylor *et al* (1992), Dedieu *et al* (1994), Bashiruddin *et al* (1994) and Hotzel *et al* (1996) whose works helped in the development of rapid, specific and sensitive PCR assays used in the laboratory for the detection of *Mmm*SC, the causative agent of CBPP.

This study showed that a 275 base pair long product was obtained on examination of the amplicon under ultraviolet illumination. From the study, it was seen that even if the sensitivity of PCR is said to be high (Dedieu *et al.*, 1994) it will not demonstrate the presence of the DNA of the *Mmm*SC in all the lungs, pleural fluid or broncho-pulmonary lymph nodes even if such samples had pathognomonic lesions in the tissues examined. This agrees with the studies of Poumarat *et al* (2004), who in their study to assess PCR for routine identification of species of *Mycoplasma mycoides* cluster in ruminants found that the sensitivity was low when a single test to identify *Mmm*SC was employed creating some doubts and ambiguous reactions. Further, Stakenborg (2005) stated that at times amplification of PCR products from pathological tissues suspected to be of CBPP fails because these tissues contain inhibitors that inhibit the amplification of the PCR product. This could explain the non amplification from some pathological samples despite the tissues showing obvious pathognomonic lesions for CBPP.

In this study, the PCR amplicons obtained from pathological tissues and those from colonies were of the same size. However, the bands from the colonies were significantly clearer than those directly from tissues. This was due to smearing experienced when the PCR was conducted directly from the samples. The smearing was due to the excess amount of unused DNA during the reaction with the primers specific for *Mmm*SC. This therefore should be taken into consideration whenever PCR is to be conducted directly from the pathological tissues when testing for *Mmm*SC in suspected CBPP cases.

### 5.5.2. Restriction Enzyme Analysis

Restriction Enzyme Analysis (REA) showed that the restriction products obtained from the enzyme *Bam*HI were of two sizes, 64bp and 173bp. This enzyme which identifies the 5' GGATC 3' and 3' CCTAG 5'pattern in the *Mmm*SC amplified cleaves the amplicon at such recognised sites and in this case it is hypothesised that it cleaved the amplicon at three sites with 38bp, 64bp and 173bp size of products obtained. The 38bp product could not be visualised by electrophoretic analysis due to the smaller size of the product.

The size and pattern of the products of enzyme restriction activity with *Bam*HI were seen to be the same despite the diverse origin of the samples analysed during the study. The method used also did not detect any difference in the size and pattern of the products whether the samples was from the vaccine strain or isolates from pathological samples. In the study by Poumarat *et al* (1995), *Pst*I and *Bam*HI were used to show that *Mmm*SC strains from different regions had different patterns by REA. In this study no difference could be observed by the method used, probably due to the shorter PCR product (275bp) which was subjected to REA and thus could not demonstrate the variability of the isolates studied. It is also probable that the part of the product subjected to REA did not contain any variability of the *Mmm*SC isolates studied.

The restriction enzymes *Eco*RI, *Hind*III and *Xba*I did not show any restriction activity on the PCR product. This is because *Eco*RI, *Hind*III and *Xba*I do not have a cleavage site within the PCR product that was subjected to restriction enzyme activity. This finding is in conformity with the information provided through NEBCUTTER (Roberts *et al.*, 2003) which showed that these enzymes did not have a cleavage site in the PCR product that was being considered. They were however used during the study in order to act as controls.

# CHAPTER SIX

## RECOMMENDATIONS AND FURTHER INVESTIGATIONS

Based on the findings of this study it is recommended that;

1) More detailed studiesbe conducted to characterize the *Mmm*SC strains prevailing in Zambia using more sensitive methods such as Multi-locus sequence Analysis or Repeat Tandem analysis that will elucidate the finer differences in the strains from the country.

2) Further investigations on the isolate showing resistance to antibioticsbe conducted and carry out detailed characterization assays to confirm the genotypic polymorphism of such an isolate and determine whether the isolate entails the emergence of a new biotype of *Mmm*SC in Zambia.

3) There is need to isolate *Mmm*SC from reported outbreaks, store and characterize them to act as reference material in future and compare them with the strains that will be prevailing at that time.

4) In view of the observations made concerning the non-infection of calves despite the fact that they interact with infected dams during suckling, it is recommended that further detailed investigations be conducted to ascertain the degree of protection conferred to calves through colostrum.

5) Considering the fact that vaccinations have been used to control CBPP in Zambia for a long time yet the disease has spread to affect other areas, studies need to be conducted to evaluate the impact of vaccination as a tool for controlling CBPP and also evaluate the efficacy of the vaccines used in the local environment.

6) The study of CBPP in Zambia has never been undertaken and it is the recommendation of this study that more diverse studies of the disease be done so that a pool of detailed scientific information is made available to decision makers in the country and the world at large.

7) The spread of CBPP into Western Province of Zambia was implicated to have been mostly due to movement of cattle from Angola at the height of the civil war in that country. In view of the cessation of the hostilities in Angola and thus stoppage of mass movement of refugees, there is need to carry out a detailed study to ascertain the best way of eradicating CBPP from Zambia without further re-infection from Angola due to illegal movements.

8) Traditional practices are known to encourage the spread and perpetuation of disease such as CBPP in Africa and Zambia is not an exception. There is therefore need to study how these practices can be harnessed so that they can instead contribute to the fight against such diseases.

# CHAPTER SEVEN

## CONCLUSION

1) The study successfully detected *Mmm*SC using PCR for the first time in Zambia and characterized the isolates from pathological lesions of cattle.

2) The REA method used in this study did not show any significant differences in the characteristics of the isolates from the different parts of the country and that of the vaccine strain.

3) The isolates examined in this study suggest the common originality of the *Mmm*SC that affects cattle in Western Province and this same isolate could have later spread to Kazungula district of Southern Province in 2007.

4) The antibiotic sensitivity of the isolates did not show any signs of antibiotic resistance except one isolate from Kazungula District which showed signs of antibiotic resistance to all antibiotics except tylosin.

5) Unconventional methods of *Mmm*SC isolation using cheap and readily available media such as donkey blood agar was used to isolate the bacteria and diagnosis inferred from such.

6) Although anaerobic cultivation of *Mmm*SC on blood agar yields undistinguishable colonies to those of aerobic cultivation, the colonies are usually not clearly discernible mostly due to the chemical reactions of the blood leading to a dark background.

7) The CBPP lesions at postmortem can be categorized distinctly between acute and chronic lesions depending on the predominating stage of the lesions although most of the time the two types will be found in the same animal.

8) The isolation of *Mmm*SC is not dependent on the category of the lesions seen whether onset, acute or chronic.

9) Even though pathognomonic lesions of CBPP are observed in cattle, *Mmm*SC cannot always be isolated from such lesions and also PCR will not always lead to the observation of the expected amplicon. Thus the diagnosis of CBPP during an outbreak period will rely on a number of factors such as; the history, clinical signs and finally diagnostic procedures.

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