MOLECULAR CHARACTERIZATION OF *MYCOPLASMAMYCOIDES*SUBSP. *MYCOIDES* FROMCATTLE AND KNOWLEDGE, ATTITUDE AND PRACTICES OF HERDSMEN IN THREE SOUTH EASTERN STATES OF NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY IN FOOD ANIMAL MEDICINE

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ZARIA, NIGERIA

NOVEMBER, 2021.

DECLARATION

I declare that the work in this Thesis entitled "**Molecular Characterization of *Mycoplasmamycoides*subsp.*mycoides*fromCattle and Knowledge, Attitude and Practices of Herdsmen in Three South Eastern States ofNigeria"** has been performed by me in the Department of Veterinary Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis has been previously presented for another degree, diploma or certificate at any institution.

Kingsley Chineto ANYIKA	----------	----------
Name of student	Signature	Date

CERTIFICATION

This thesis entitled "MOLECULAR CHARACTERIZATION OF M*YCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* FROM CATTLE AND KNOWLEDGE, ATTITUDE AND PRACTICES OF HERDSMEN IN THREE SOUTH EASTERN STATES OF NIGERIA" by Kingsley Chineto ANYIKA meets the regulations governing the award of doctor of philosophy ofAhmadu Bello University, Zaria and is approved for its contribution to scientific knowledge and literary presentation.

Prof. S.O Okaiyeto…… ……
Chairman, Supervisory Committee Signature Date

Prof. A. K.B. Sackey…… ……...
Member, Supervisory Committee Signature Date

Prof. C.N.Kwanashie…… ……....
Member, Supervisory Committee Signature Date

Prof. C.A. Kudi……..... …….
Head, Department of Veterinary Medicine Signature Date

Prof. Sani A. Abdullahi…… …….....
Dean, School of Postgraduate Studies Signature Date

DEDICATION

This Thesis is dedicated to Almighty Jesus, for his grace throughout the course of my study at Ahmadu Bello University, Zaria. My beloved parents, Late Igwe Dim A.C Anyika and Lolo LateciaAnyika for all the prayers and support.To my lovely wife and kids, Naetochukwu, Chidera and Chimamanda.

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ABSTRACT

Contagious Bovine Pleuropneumonia (CBPP) is a trans-boundary animal disease (TAD) and, endemic in Nigeria. It is caused by *Mycoplasma mycoides*subsp. *mycoides* (Mmm). The study was aimed at confirming the presence of Mmmusing serological, cultural and molecular methods in three selected South Eastern (SE) states of Nigeria (Anambra, Enugu and Imo). Cattle (n= 20) suspected of having CBPP were clinically examined at different Fulani sedentary settlements in the study area and clinical signs recorded.A total of 438 sera samples were collected from 42 cattle herds in the different Fulani sedentary settlements within the study area and screened by competitive-ELISA technique. The samples were collected between December 2019 and April 2020. The blood samples (5mls) were collected through the jugular veins using sterile 18G needle and 10mls syringe. The blood was allowed to stand in a slanting position for 3 hours for the separation of serum from the cellular component of the blood after which the serum was collected in a labeled sample bottle.Furthermore, a total of 90 samples (65 lungs and 25 pleural fluids) were also collected for isolation and molecular characterization of Mmmfrom cattle at slaughter in the abattoirs from the three selected South Eastern states. The lungs samples were collected using sterile blades while the pleural fluid was collected using sterile needle.The PCR was performed according to specific protocols. The production of a band size equivalent to 1.1kbp by the isolates was confirmatory for *Mycoplasma mycoides* subsp.*mycoides*.The PCR confirmed isolates were further characterized using Multi-locus Sequence Analysis (MLSA). The locus; LocPG-0001 was sequenced with the corresponding primers. The sequences were assembled and aligned using BioEdit^{tm} software.The obtained sequences

were also compared with the vaccine reference strain (T1/44) using ClustalW^{tm} software and polymorphism was observed and documented. The knowledge, attitude and awareness of pastoralist towards CBPP were also assessed using questionnaires in the three selected South Eastern states. A total of ninety (90) questionnaires were administered to herdsmen in the three South Eastern states (35 in Anambra, 30 in Enugu and 25 in Imo state). It was interpreted in Huasa language. The clinical signs observed were; fever, extented neck and abducted forelimbs, rapid and difficult breathing, coughing especially when exercised, mucoid nasal discharge, emaciation, recumbency and lethargy in the affected animals. Gross lesions such as; pleural fluid accumulation, inflamed lungs, lung adhesion to thoracic wall and presence of sequestrum were observed and documented at different abattoirs in the three selected south eastern states. Two hundred and sixty (260) sera samples were positive for CBPP using c-ELISA, giving an overall prevalence of 59.4% (260/438). Anambra state recorded a sero-prevalence rate of 50.7%(74/146), Imo state 63% (92/146) while Enugu state recorded a sero-prevalence rate of 64.3% (94/146). *Mycoplasma mycoides*subsp. *mycoides* antibodies were detected in all the three selected states. Twenty five (25) out of the ninety (90) samples (Lung and pleural fluid) were positive for Mmmon pleuro-pneumonia like organism (PPLO) agar, giving an isolation rate of 27.7% while only twenty one (21) of the isolates were further confirmed to be Mmm using conventional polymerase chain reaction (PCR).Three different Allelic numbers were identified using MLSA; Allele number 1, 8 and 9. Allele 8 and 9, being new findings. There were differences between the sequences of the field isolates and the reference vaccine strain (T1/44). Result showed high awareness (94%) of CBPP among livestock owners within the three selected South East states. Eighty eight percent (88%) of the respondent admitted using antibiotics for the management of the disease in their herd. This study confirms the presence of CBPP in the

study area and it is the first time such study is undertaken in the region using this techniques.The isolates were characterized into 3 allelic numbers. There were differences in the sequences of the field isolates and the vaccine reference strain T1/44. The molecular tools used in this study may be useful in the characterization of other isolates and can also be used for epidemiological tracing of Mmmduring outbreak. It can also serve as base line data for future studies in this region.

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LIST OF ABBREVIATIONS

ABU Ahmadu Bello University

AbAntibody

Ag Antigen

AGID Agar gel Immunodiffusion Test

AU African Union

CBPP Contagious Bovine Pleuropneumonia

CCPP Contagious CaprinePleuropneumonia

c-ELISA Competitive Enzyme-linked Immunosorbent Assay

CFT Complement Fixation Test

CPS Capsular Polysaccharides

DNA Deoxyribonucleic acid

ELISA Enzyme-linked Immunosorbent Assay

EMPRES Emergency and Prevention System

EU European Union

FAO Food and Agriculture Organizations

FAT Flourescent Antibody Test

FMD Foot and Mouth Disease

G Gauge

IAEA International Atomic Energy Agency

IBAR Inter African Bureau for Animal Resources

IBT Immunoblotting Test

IFAT Immunoflourescent Antibody Test

IgA Immunoglobulin A

IgGImmunoglobullin G

IgMImmunoglobullin M

IHC Immunohistochemistry

ISCOM Immune Stimulating Complexes

IU International Units

JP-28 Joint Project-28

kDakilodalton

LAT Latex Agglutination Test

LGA Local Government Area

Mabs Monoclonal Antibodies

MCCP *Mycoplasma Capri subsp. capripneumonia*

MLMillilitres

MLSA Multi-locus Sequence Analysis

MLST Multi-Locus Sequence Typing

MmmLC*Mycoplasma mycoides* subsp. *mycoides* Large Colony

MmmSC*Mycoplasma mycoides*subsp.*mycoides* Small Colony

NADIS National Animal Disease Information System

NVMA Nigerian Veterinary Medical Association

NVRI National Veterinary Research Institute

^{o}CDegree Celsius

OIE Office Internationale des Epizootics.

PANVAC Pan-African Veterinary Vaccine Center

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reactions

PPLO Pleuropneumnia-like Organisms

RNA Ribonucleic acids

rRNAribosomal Ribonucleic acids

SAT Slide Agglutination Test

SNP: Single Nucleotide Polymorphism

TADs Trans boundary Animal Disease

TMB 3,3^{1}5 Tetramethylbenzidine

µl Microlitres

VNTR Variable-Number Tandem Repeat

> Greater than

< Less than

% Percentage

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Contagious Bovine Pleuropneumonia (CBPP) is a highly contagious disease of cattle caused by *Mycoplasma mycoides* subsp. *mycoides* (Mmm) (Campbell, 2015). Based on its transmissibility and economic impacts, CBPP is categorized as a trans-boundaryanimal disease (TAD) and has thus been included as one of the bacterial diseases in the OIE list A diseases (OIE, 1997; Litamoi *et al.,* 2004). CBPP is characterized by a relatively long incubation period and highly inconsistent clinical course (Litamoi *et al.,* 2004). Clinical signs of the disease include fever, dyspnea, mucoid nasal discharge, extension of neck and abduction of forelimbs, cough and nasal discharges (Campbell, 2015). In adult cattle, most infections are limited to the respiratory tract, while in calves the disease usually presents as arthritis (Provost *et al.,* 1987).

The disease is considered to be the most economically important cattle disease in Africa causing greater losses in cattle than any other disease (OIE, 2003). With the eradication of Rinderpest, CBPP has become the major infectious disease of livestock on the African continent (OIE, 2003). Eradication of CBPP from both U.S and Australia was dependent on strict control of cattle movement, herd scale slaughter and financial compensation to owners. This practice has become difficult in Nigeria due to several factors such as; poor implementation of the test and slaughter policy, unrestricted movement of nomads across state boundaries which made accurate monitoring of the disease difficult (Nwankpa *et al.,* 2003). Several authors have documented the outbreaks, prevalence, and the economic importance of the disease in Nigeria (Osiyemi, 1981; Nwanta and Umoh, 1992; Egwu *et*

al., 1996; Jasini *et al.,* 2016). The disease was present in most sub-Saharan countries and had not only re-infected countries like Uganda and Kenya but has also infected countries like Tanzania (1990) , Botswana (1995) and Rwanda (1995) which had been previously declared CBPP free (Nicholas and Bashiruddin, 1995; OIE, 1997).

Control of CBPP in Nigeria was probably achieved in the late 1960; this was largely ascribed to mass vaccination, well organized disease reporting, efficient laboratory diagnosis, effective quarantine, and strict control of cattle movement. Unfortunately, this effort did not last long as the disease re-emerged few years later probably from chronic carriers of the disease (Lungers), from one of the neighboring countries (Nwanta and Umoh, 1992). In spite of an eradication campaign re-launched in 1970, outbreaks of CBPP rose rapidly from 1986 onwards to a peak in 1989 where over 10,000 cattle were affected (Nwanta and Umoh, 1992). Worried by the situation, the meeting of the FAO/OIE/OAU IBAR Consultative Group on Contagious Bovine Pleuropneumonia was convened for the first time in over 25 years, in June 1998, to discuss the deteriorating situation of the disease in Africa (FAO, 2000).

In Africa, where the application of the stamping out policy for the eradication of CBPP has not been feasible, the control of the disease has relied heavily on vaccination using vaccine strains T1/44 or its streptomycin resistant derivative T1-SR (Litamoi *et al.,* 2004).

National Veterinary Research Institute (NVRI) Vom has been producing CBPP vaccine (T1/44) in Nigeria since 1985, the recommended minimum age of vaccination of cattle is six month and the immunity usually lasts for 12 months (NVRI, 2007). However, this vaccine has some draw backs such as; short duration of efficacy, swellings at site of injection and cold chain dependence (Obi, 1997; Thiaucourt *et al.,* 2006).

1.2 Statement of the Research Problem

Contagious Bovine Pleuropneumonia (CBPP) is a highly contagious and fatal respiratory disease primarily of cattle (Thiaucourt *et al.,* 2006).The disease is endemic in Africa where it accounts for high economic losses and is a limiting factor in cattle production. It is included among the Office International des Epizooties (OIE) diseases requiring official notification, leading to banning of countries involved, from participation in international cattle trade (OIE, 2003). It is a disease of major economic importance in Nigeria because of its severe respiratory symptoms, protracted course and endemicity in the cattle rearing northern parts of the country, which has led to considerable loss in productivity that translates to heavy financial losses (Fayomi and Aliyu 1997; Aliyu *et al.,* 2003). Contagious bovine pleuropneumonia causes NGN1.307 billion losses to Nigeria as a whole with the Sudan Savanna zone accounting for 24 % of all losses, the Northern Guinea Savanna for 68 %, the sub-humid zone 4 % and the humid zone for 5 % (Fadiga *et al.,* 2013). A five year retrospective study at Mubi, Adamawa state by Halle *et al.,* (1998) reported that 238 infected cattle lungs were condemned due to CBPP out of 43, 810 cattle slaughtered (0.54%) and the worth of organs condemned was estimated at NGN 28,580. Onu (2004) reported an abattoir based retrospective study at the Sokoto Metropolitan abattoir between 1990 and 1994; in which, out of 16, 211 cattle slaughtered, 3, 338 (2.1%) had their lungs condemned as a result of CBPP and the worth of the condemned lungs was estimated at NGN 237, 780. Annual losses of up to €30.1 million due to CBPP were estimated in 12 endemic sub-Saharan African countries (Tambi *et al.,* 2006).

There has been increase in the movement of cattle from the northern region to the southern region of the country by herdsmen in search of grazing fields (OIE, 2009). Consequently, there is an increasing the spread of this disease within the southern region.

Furthermore, the inability of serological tests to discriminate between natural and vaccinal exposures in animal has led to a greater reliance on post-mortem examination of lung lesions for monitoring and surveillance of CBPP (Aliyu *et al*., 2000). Despite several vaccination campaigns against the disease, CBPP has persisted in Nigeria. This could be due to new strains of Mmm circulating within the country. The establishment of the true prevalence rates of CBPP in infected countries is an important prerequisite to mounting a successful control programme of the disease (FAO, 2004).

1.3 Justification for the Study

Contagious bovine pleuropneumonia is an economically important disease, this emphasizes the need for an upto date data on the prevalence of the infection, if prompt and effective control measures are to be instituted. Presently, in Nigeria, the extent and pattern of CBPP in South East Nigeria is largely unknown, as many studies have centered within the Northern region of the country (Nwanta and Umoh, 1992; Nwankpa, 2008; Okaiyeto *et al.,* 2011; Jasini *et al.*, 2016; Ikpa *et al.,* 2020). The South east region of Nigeria is estimated to have a cattle population of 4.5 million, there is an apparent successful settlement of pastoralist within the Southern region of Nigeria in the past two decades with thousands of zebu cattle (Blench, 1994) invariably leading to an increase in their population and movement within the region. This is largely due to several factors such as: changes in the ecological conditions of the region, drastic reduction in the incidence of tsetse fly infestation, a vector that helps in the transmission of animal Trypanosomosis(Blench, 1994). It is therefore important to ascertain and document the true status of CBPP within this region in an attempt to control the disease in Nigeria.

There is the need to also determine the possibility of other circulating strains of Mmm within the south east region and compare them with the vaccine strains. This can be used as an efficient tool to determine the origin of any outbreak in the country and also in designing control strategies. A number of publications and expert group meetings have reported on the inability of the current vaccine (T1/44) to control the disease in Africa due to its short duration of immunity conferred to the animals (Anon, 2000; Nicholas *et al.*, 2000). Several studies have also established different strains within northern Nigeria (Nwankpa, 2008; Jasini *et al*, 2016). Nwankpa, (2008) isolated seven (7) different strains of Mmm within 12 Northern States of Nigeria. Folashade, (2014) reported differences in the band size between the isolated field strain and vaccine strains using restriction fragment length polymorphism technique.

There is also paucity of information on the status of this disease within the South Eastern States of Nigeria. Therefore, data generated from this research will serve as baseline data for future studies and will informcontrol within these States.

1.4 Aim of the Study

To isolate and molecularly characterize *Mycoplasma mycoides* subsp. *mycoides* from cattle in three South Eastern states of Nigeria.

1.5 Objectives of the Study

i. To examine and reaffirm clinical signs observed in suspected CBPP infected cattle in three South Eastern states of Nigeria.
ii. To determine the seroprevalence of *Mycoplasma mycoides* subsp. *Mycoides* in three South Eastern states of Nigeria using competitive ELISA.

iii. To culture and isolate *Mycoplasma mycoides* subsp. *mycoides* from cattle in three South Eastern states of Nigeria using cultural methods.

iv. Determine the presence of the aetiological agent of CBPP (*Mycoplasma mycoides* subsp.*mycoides*) from cattle in three South Eastern states of Nigeria using Conventional PCR.

v. Characterize the isolates of *Mycoplasma mycoides* subsp. *mycoides,*from the three South Eastern states of Nigeria using Multi-locus sequence Analysis (MLSA).

vi. To compare the sequence of*Mycoplasma mycoides* subsp. *mycoides* field strains from the three South Eastern states of Nigeria with the vaccine strain (T1/44) sequence using ClusterW sequence alignment.

vii. To determine the knowledge, attitude and practices of pastoralists in three South Eastern states of Nigeria towards CBPP using structured questionnaire.

1.6 Research Questions

i. Are there new clinical signs of CBPP in cattle in the three South Eastern states of Nigeria?

ii. What is the seroprevalence of M*ycoplasma mycoides subsp. mycoides* of cattle in the three South Eastern states of Nigeria?

iii. What is the isolation rate of M*ycoplasma mycoides subsp. mycoides* of cattle in the three South Eastern states of Nigeria?

iv. Are Mycoplasma *mycoides subsp. mycoides* strains present in in the three South Eastern states of Nigeria?

v. What are the circulating strains of M*ycoplasma mycoides subsp. mycoides* in the three South Eastern states of Nigeria?

vi. Are there differences between the isolated M*ycoplasma mycoides subsp. mycoides* field strains and the vaccine strains (T1/44) in the three South Eastern states of Nigeria?

vii. What is the level of knowledge, attitude and practices of pastoralist towards CBPP in the three South Eastern states of Nigeria?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Biology of Mycoplasma

Mycoplasma is a trivial name for a group of microorganisms which belong to the Class Mollicutes and Order *Mycoplasmatales* (Maniloff, 1980; Woese, 1980). It is an extra-cellular pathogen that lives in close association with the host cells (Woese, 1980). They appear to have evolved from Gram-positive bacteria by a process of degenerative evolution towards genome reduction and the loss of a cell wall (Woese, 1980). Their genomes range from 580 kb for *Mycoplasma genitalium* (Fraser *et al.*, 1995) to 1358 kb for *Mycoplasma penetrans* (Sasaki *et al.*, 2002), while that of *M. mycoides* subsp. *mycoides* has a size of 1211 kb (Westberg *et al.*, 2004). This minimal genome has led the *Mycoplasma* species to radically economize genetic resources and biosynthetic capacities, and adapt to an obligate parasitic lifestyle (Razin, 1997).The Family *Mycoplasmataceae* contains two genera, *Mycoplasma* (urea negative) and *Ureaplasma* (urea positive) (Nicolet, 1996). "Mycoplasma cluster" consist of five closely related mycoplasmas that are referred to as: *Mycoplasma mycoides* subsp. *Mycoides* (Mmm), *Mycoplasma capricolum* subsp. *Capricolum* (Mcc), *Mycoplasma capricolum* subsp. *Capripneumoniae* (Mccp), *Mycoplasma mycoides* subsp. *Capri* (Mmc) and *Mycoplasma leachii* (Ml). *Mycoplasma leachii* is a group of strains that had remained unassigned until recently when a modification of the cluster taxonomy was proposed based on both phenotypic and recent phylogenetic studies (Manso-Silva *et al.*, 2009). The designation *Mycoplasma mycoides* subsp. *mycoides* Large Colony (MmmLC) was discarded and the corresponding LC isolates are now considered to be an additional serovar of Mmc (Manso-Silva *et al.*, 2009). Despite their small genome size, Mycoplasmas cause a wide range of disease in both humans and animals (Westberg *et al.*, 2004). The

primary habitats of human and animal *Mycoplasmas* are the mucous surfaces of the respiratory and urinogenital tracts, the eyes, alimentary canal, mammary glands and joints (Westberg *et al.*, 2004). *Mycoplasma mycoides* subsp. *Mycoides* is the etiological agent of contagious bovine pleuropneumonia (CBPP), a severe infectious disease of cattle (Food and Agriculture Organization, 2003).

2.1.1 Taxonomy tree:

Domain: Bacteria

Phylum: Firmicutes

Class: Mollicutes

Order: Mycoplasmatales

Family: Mycoplasmataceae

Genus: Mycoplasma

Species: *Mycoplasma mycoides* subsp. *mycoides*

(Adapted from Songer and Post, 2005).

2.2 Contagious Bovine Pleuropneumonia (CBPP).

2.2.1 Definition

Contagious bovine pleuropnuemonia (CBPP) is a highly contagious disease of cattle that is caused by *Mycoplasma mycoides* subsp. *mycoides* (Mmm) (Provost *et al.*, 1987; Yaya *et al.*, 2008).Clinically, the disease occurs as hyper-acute, acute, sub-acute or chronic (Provost *et al.*, 1987). Clinical signs include; anorexia, fever, dyspnoea, polypnoea, cough and nasal discharges (Egwu *et al*., 1996; Mbulu *et al.*, 2004; Radostits *et al.*, 2003). The disease is spread through direct contact with cough droplets and is worsened by crowding of animals (Provost *et al.*, 1987).

2.2.2 Host specificity of Contagious Bovine Pleuropneumonia

Under natural conditions, CBPP affects cattle and allied animals including buffalo, yak, bison and reindeer (Hutyra *et al.*, 1938). Study in Australia showed that buffaloes could be infected by artificial means but did not spread CBPP to in-contact buffaloes (Newton, 1992). However, Santini *et al.* (1992) documented pulmonary lesions and isolated *Mmm* from sero positive buffaloes which had been in contact with CBPP-affected cattle in Italy. They concluded that buffaloes were susceptible but at a low level and recommended further research to clarify the role of buffaloes as a reservoir of infection for cattle. Small ruminants, in particular goats, have also been shown to harbor Mmm (Hudson, 1971).

2.2.3 Historic perspective of CBPP

CBPP seems to have existed in the ancient world according to early classical writings (Provost *et al.*, 1987). It was then referred to as *polmonera* by Gallo in Italy and was confined to the Alps and Pyrenees in the 16th Century. It reached nearly world-wide distribution in the 19th century through cattle trade and was eradicated from most continents

by stamping out policies. Early descriptions of the disease were found in writings from Gallo in Italy (1550) and also from Testienne in France (1554). *Mycoplasma mycoides* subsp. *mycoides*, the infectious agent of CBPP, was first isolated and described by Nocard and Roux in 1898.

"The spread of CBPP throughout Europe started at the end of the18th century and culminated in the middle of the 19th century. All European countries became infected: Northern France 1922, Belgium 1828, Holland and Prussia 1830, Schleswig Holstein 1841, Sweden 1847, Norway. The first cases seen in Spain in 1846 in Barcelona region and later on in 1864 in central Spain"(Thiaucour*et al.,* 2000b).The disease was introduced into the United States of America, Asia, Australia and Japan in the 19th and 20th century through importation of cattle from Europe. In Africa, South Africa became infected in 1854 from either Britain (Hutyra *et al.,* 1938) or the Netherlands (Provost *et al.*, 1987).Thereafter, the disease spread to other countries in southern Africa. In Namibia, the disease was first recorded in 1856 and is reported to have caused large scale cattle losses for the next 40 years (Hutyra *et al.,* 1938). The rapid spread of the disease was aided by people trying to flee with their animals from the disease, wars and the use of trekking cattle for freight. Laws aimed at controlling the disease were promulgated in 1885 and CBPP became a notifiable disease and has remained so since 1887 (Masiga *et al.,* 1996). Vaccination, cattle movement control, and good extension services greatly helped in the reduction of the disease and by 1904 only minor outbreaks were reported (Masiga *et al.*, 1996).

From Australia CBPP spread to New Zealand, India, China, Mongolia, Korea, Hong Kong and Japan in the late 19th Century and early 20th Century (Laak, 1992). CBPP was eradicated in Britain in 1898 using a stamping out method (Laak, 1992).

Zimbabwe eradicated CBPP in 1904, South Africa in 1924 (FAO, 2000). Most countries in southern Africa eradicated CBPP by the end of 1939 (Masiga *et al.,* 1996; Amanfu *et al.,* 1998).

2.2.3.1 CBPP in Africa

CBPP is endemic in parts of Africa (OIE, 2009; (Nicholas, 2004)).CBPP has spread to so many territories in Africa, and threatens to spread further, in a continent with growing human population but dwindling meat supply (FAO, 2000).By the end of 1999, the disease was present in at least 27 countries in equatorial, central and southern Africa although it is difficult to be certain due to the discrepancy between official and non-official reports (Nicholas *et.al.* 2000). The two main CBPP infection foci in west and central Africa are the Inner Delta area of Niger and the Lake Chad area (FAO, 2000).

In Africa, Contagious Bovine Pleuropneumonia (CBPP) is present in at least 29countries and the disease was said to have made its initial entry into Africa in 1854, when an infected bull was introduced to Mossel Bay, South Africa, from the Netherlands (Nicholas, 2004). In the 1960s and 1970s, consistent research on CBPP in countries like Nigeria, Kenya, Chad and other African countries, together with a massive international campaign code-named Joint Project 16 (JP 16) resulted in the eradication of the disease in most African countries. However, factors such as poor economy and poor/inadequate veterinary services, the disease re-emerged in the early 1990s. The disease is endemic in much of West Africa and in the greater horn of Africa, Angola, Tanzania, Northern Namibia and Zambia. Seasonal cattle movements by pastoralists resulted in the spread of the disease in 1990 from Kenya, where the disease has been endemic for some time, into the Ngorogoro crater in Tanzania, which had been free of CBPP for 30 years.

Presently, the disease is absent in some southern African countries such as Botswana, Lesotho, South Africa, Swaziland and Zimbabwe, and parts of Namibia and Zambia (OIE, 2009).

2.2.3.2 CBPP in Nigeria

CBPP is endemic in Nigeria and is spread through unrestricted movement of cattle, seasonal migration and transhumance activities (Aliyu *et al*., 2000; Ajuwape *et al*., 2004). Outbreaks of the disease still occurs in the country particularly in the northern region where three-quarter of the country's 16.3 million cattle are located (Foluso, 2004, PACE, 2004, Ikhatua, 2011).

The first outbreak of the disease in Nigeria was documented in 1924 according to reliable records available; this was the year when laboratory facilities for vaccine production were carried out in the National Veterinary Research Institute, Vom (Foluso, 2004). Between 1924 and 1960 an average of 200 outbreaks of CBPP were recorded each year and mostly in Borno and Kano states (Foluso, 2004). Most cattle in Nigeria are owned by nomadic Fulanis who move over long distances with their animals and consequently spreading the disease. During the 1970s up to 1990s, the socio economic and cultural setting of the producers/herdsmen changed. Trans-human and trade cattle movement from North to the Southern states of Nigeria became more rapid due to adoption of vehicular transportation and consequently established large scale settlements of herdsmen within the South western states and South eastern stated of Nigeria (Foluso, 2004). All these changes impacted tremendously on the livestock disease profile in the country, particularly CBPP (Foluso, 2004). The disease therefore had a gradual spread beyond the traditional far North to the Southern States. As at today, the whole country is considered endemic (Foluso, 2004).

In Nigeria, CBPP was regarded as extinct in 1965 following ten years of mass vaccination, well organized disease reporting, laboratory diagnosis, quarantine, slaughter policy, and strict control of cattle movements (Anon, 1975a; Chima*et al.*,1999). However, this did not last long as the disease re-emerged a few years later perhaps from bordering countries of Niger, Chad, and Cameroon and also the outbreak of Nigeria civil war and the consequent breakdown of necessary surveillance measures (Nwanta and Umoh, 1992).In spite of an eradication campaign re-launched in 1970, outbreaks rose rapidly from 1986 onwards to a peak in 1989 where over 10,000 cattle were affected (Nwanta and Umoh, 1992).

2.2.4 Mechanism for the predilection of *Mycoplasma* in the lungs of animals.

The attachment process of *M. pneumonia* and *M. gallisepticum* are involved in the initial and critical event in host parasite interaction with a receptor site on the external membrane surface of the host cell (Collier, 1979). They have specialized attachment organelles called the tip or bleb and are concentrated at one site of these Mycoplasmal cells (Collier, 1979). These attachment organelles adhere with the aid of Sialic acid residues on the host cells, but there is evidence that other components of the cell membrane may have a role in this process (Collier, 1979). The intimate association between Mycoplsma and the ciliated epithelial cells of the host enables Mycoplasmas to avoid clearance by the mucocilliary escalators (Hu *et al.,* 1977).

Adherence of *M. pneumonia* to a host cell (usually lungs) is the initiating events for pneumonic disease and related symptoms. The specialized organelles are a bipolar, electron dense and elongated cell extension that facilitates motility and adherence to host cells (Hu *et al.,* 1977).

A number of protein are known to contribute to the formation and functionality of the attachment organelles, including the accessory proteins HMW1-HMW5, P30, P56 and P90 that confers structure and adhesion support (Hu *et al.,* 1977). P1, P30 and P116 are involved directly in attachment. This network of proteins participates not only in the adhesion but also motility (Hu *et al.*, 1977).

2.2.5 Mechanism of action of Mmm

To be pathogenic, *Mycoplasma mycoides* subsp. *mycoides* possesses particular mechanisms to adhere to the specific host tissue, to evade the host's immune defense, to enable persistence and dissemination in the infected animal, and to cause inflammation and disease signs through cyto-toxicity (March *et al.,* 1999). The loss of any of these mechanisms can lead to attenuation or loss of virulence of the organism March *et al.* (1999). Below are some of the mechanisms:

2.2.5.1 Capsular Polysaccharide

An important pathogenicity factor in Mmmis the capsular polysaccharide (CPS), earlier known as galactan (March *et al*., 1999). The capsular polysaccharide, consist of 6-*O*-β-d-galactofuranosyl-d-galactose (Plackett *et al* 1964). It was shown to increase the virulence of the strongly attenuated *M. mycoides* subsp. *Mycoides* vaccine strain KH_3J when added to the inoculum prior to experimental infections of cattle (Hudson *et al.,* 1967). Buttery *et al.,* (1976) observed that intravenous injection of galactan from *M. mycoides* subsp. *mycoides* to calves produced the following clinical signs; transient apnoea, increased pulmonary arterial pressure and pulmonary oedema, showing that capsular polysaccharide of this species has a direct cytopathic effect and seems to lead to the contraction of blood vessels,

which may initiate thrombosis. The effect of capsular polysaccharide on virulence was also shown by growth inhibition tests, which primarily measure serum resistance, and by a mouse infection model, which mainly measures the capacity of the *Mycoplasma* strain to cause bacteraemia. March *et al.,* (1999) observed that a strain of *M. mycoides* subsp. *Mycoides which* produced low amounts of capsular polysaccharide was much more sensitive to growth inhibiting antisera than strains that produced larger amounts of polysaccharide. Similarly, the same strains that produced large amounts of capsular polysaccharide also generated a significantly longer duration of bacteraemia in a mouse infection assay than the strain that produced little capsular polysaccharide (March and Brodlie, 2000). Therefore, capsular polysaccharide seems to play a role in the capacity of persistence and dissemination of *Mycoplasma mycoides* subsp. *mycoides* in the infected host.

2.2.5.2 Surface proteins

Lipoproteins have been shown to play a role as triggers in mechanisms of pathogenicity of *Mmm*, since they are known to have a central role in interactions between mycoplasmas and eukaryotic cells, particularly with respect to adhesion (Calcutt *et al.,* 1999). More so, lipoproteins have been reported to stimulate the release of pro-inflammatory cytokines (Calcutt *et al.*, 1999). Lipoproteins are usually strongly antigenic proteins, so they are also considered to be valuable targets for specific and sensitive sero-diagnosis. Presently, a few lipoproteins from *M. mycoides* subsp. *mycoides* have been characterized in detail. Most of them are major antigens and are readily detected in the serum of infected cattle on immunoblots (Calcutt *et al.,* 1999). Lipoproteins such as LppA is strongly conserved

among mycoplasmas of the "*M. mycoides* cluster" and it is expressed by highly virulent *Mycoplasma* species as well as by non-pathogenic or low-pathogenic species (Calcutt *et al.*, 1999). LppB is found only in strains belonging to the African/Australian cluster, but is not found in strains isolated from the re-emerging European outbreaks in 1980–1999. LppB is, however, also present in other mycoplasmas of the "*M. mycoides* cluster", in particular, in the bovine pathogen *Mycoplasma* sp. bovine group 7 (Vilei and Abdo, 2000). Another lipoprotein, LppC, which is expressed by all *M. mycoides* subsp. *mycoides* strains, is also found in other, less pathogenic *Mycoplasma* species (Abdo *et al.*, 2000; Pilo *et al.*, 2007).

2.2.5.3 Adhesion factors

Adhesins play a vital role in the pathogenesis of most micro-organisms (Razin *et al.*, 1998). Since mycoplasmas do not seem to secrete toxins that could act over long distances, adhesion is particularly important in mycoplasmal virulence (Razin *et al.*, 1998). Adhesion plays a central role in the intimate interactions of pathogenic mycoplasmas with mammalian cells for long periods and is assumed to trigger a cascade of signals, which are transduced to the host cell and induce inflammation (Razin *et al.*, 1998). Adhesins also seem to be responsible for host specificity and tissue tropism. Several adhesins have been identified and documented in various *Mycoplasma* species (Fleury *et al.*, 2002; Razin *et al.*, 1998), but specific adhesins have not yet been demonstrated in *M. mycoides* subsp. *mycoides*, but can be postulated to occur from the above considerations.

2.2.5.4 Toxic metabolic pathway products

Oxygen uptake and H_2O_2 production were identified as major characteristics in fermentative *Mycoplasma* species and were expected to influence the virulence of

pathogenic mycoplasmas (Miles *et al.*, 1991). However, it was later discovered that *M. mycoides* subsp. *mycoides* strains isolated from the re-emerging European outbreaks of CBPP in 1980–1999 produced much less H_2O_2 when grown in the presence of glycerol than strains isolated in the African and Australian continents and it was therefore suggested that a glycerophosphate oxidase could represent a significant virulence factor of *M. mycoides* subsp. *mycoides* (Houshaymi *et al.*, 1997; Rice *et al.*, 2001).

2.2.5.5 Surface protein.

A variable surface protein, designated Vmm, has been discovered in *M. mycoides* subsp. *mycoides* (Persson *et al.*, 2002). Vmm is a 16-kDa protein, specific to this *Mycoplasma* species. It is expressed by nearly all strains analyzed, where it has shown a reversible ON–OFF phase variation at a high frequency per cell generation. This variation is regulated at the transcriptional level by dinucleotide insertions or deletions in a repetitive region of the promoter (Persson *et al.*, 2002). Genes resembling the V*mm* gene were also found in other species of mycoplasma, but analogous Vmm-like proteins in these species could not be detected with a specific monoclonal antibody directed to Vmm of *M. mycoides* subsp. *mycoides* SC. The function of Vmm is currently unknown, but repeating elements in variable membrane proteins of mycoplasmas have been suggested to increase the pathogen's ability to adhere to host cells and to evade the host immune response (Persson *et al.*, 2002).

2.2.6 Spread of Contagious Bovine Pleuropneumonia

Spread of CBPP occurs through direct and repeated contacts between infected and susceptible cattle especially through expectorations of coughing (Nicholas and Bashidurin, 1995). Shedding of organisms from the respiratory tract has been shown to occur before the onset of detectable serological responses in experimentally infected animals (Bashiruddin *et al.,* 1994; Miserez *et al.,* 1997; Frey *et al.,* 1998). The positive findings from the swab samples taken in Portugal, showed the presence of Mmmin nasal secretions of infected cattle in the field and therefore confirms, the respiratory shedding of the organisms as a mode of dispersion. Involvement of chronic carriers in the spread of the infection has been suggested by several authors (Egwu *et al.,* 1996; Mahoney, 1954; Martel *et al.,* 1985) but it is still being debated (Windsor and Masiga, 1977). Risk factors for its spread include crowding of animals and use of common grasslands and watering places (Provost *et al.,* 1987). In Africa, between-zone or country spread essentially is related to cattle movements caused by trade, transhumance and social troubles (Egwu *et al.,* 1996; Roeder and Rweyemamu, 1995). Chronic carriers are often suspected to cause field outbreaks and endemic situations in cattle populations (Dedieu *et al.,* 1996; Egwu *et al.,* 1996), although this hypothesis remains unproven. For example, experimental studies done by Windsor and Masiga (1977) did not observe any disease transmission after challenging healthy animals with chronic carriers. The authors therefore concluded that carriers play only an occasional role in the epidemiology of CBPP.

2.2.7 Clinical signs of CBPP

CBPP is characterized by severe coughing, weakness, emaciation and sometimes fever (Provost *et al.,* 1987).The disease symptoms varies from hyper acute, acute, sub-acute and

chronic forms. Calves up to six months normally develop arthritis and show lameness from swollen, hot and painful limb joints (Egwu, *et. al.,* 1996; Perssone*t al.,* 2002).

2.2.7.1 Hyper-acute form

The clinical signs observed in the hyper-acute form are greatly accelerated. The pathological signs are usually characteristic with marked pleural adhesion accompanied by exudative pericarditis (Provost *et al.*, 1987). Affected animals may die within a few days of exhibiting classical respiratory signs (Provost *et al.*, 1987).

2.2.7.2 Acute form

The early stages of CBPP are indistinguishable from any severe pneumonia with pleurisy (Scudamore, 1995; Provost *et al.*, 1987). Animals show dullness, anorexia, and irregular to cessation of rumination, fever and sometimes show signs of respiratory distress (Provost *et al.*, 1987).Coughing is usually persistent and is slight or dry, sometimes body temperature rises from 40 to 42°C and the animal prostrates with difficulty of movement (Scudamore, 1995; Provost *et al.*, 1987). As the lung lesions develops, the clinical signs become more pronounced with increased frequency of coughing and the animal may stand with back arched, head extended and elbows abducted (Provost *et al.,* 1987). Because the pleurisy is so painful, it is rare for animals to go off their feet until just before death (Provost *et al.*, 1987). While classical respiratory signs may be present in calves, CBPP agent often localizes in the joints with attendant arthritis and involvement of tendons (Martel *et al.*, 1985). Complications accompanying this disease in calves may also include valvular endocarditis and myocarditis (Martel *et al.*, 1985). Calves are more resistant to CBPP than adults (Curasson, 1942; Provost *et al.*, 1987).

2.2.7.3 Chronic forms

In the chronic form of CBPP, clinical signs may appear as slight cough, only noticeable when the animal is exercised (Regalla, 1984). CBPP in Europe, unlike that caused in Africa where mortality rates are typically 10-70% in epizootics, is characterized by low morbidity and low or non-existent mortality with the majority of infected cattle showing chronic lesions; this is characteristic of endemic disease (Regalla *et al.*, 1994). These differences are perhaps due to the fact that European cattle are healthier, better fed, subjected to less physical stress and are often permanently housed throughout the year (Nicholas and Palmer, 1994). In Italy during the early 1990s, less than 5% of cattle in an infected herd showed clinical signs of CBPP (Guadagnini *et al.*, 1991). The use of antibiotics and anti-inflammatory drugs may also help to mask clinical signs and to accelerate the formation of chronic lesions.

2.2.8 Gross lesions of Contagious Bovine Pleuropneumonia

The gross lesions of CBPP are mostly confined to the thoracic cavity and lungs, and lesions are usually unilateral (Egwu *et al.*, 1996; Nunes *et al.*, 1990). In a study of 566 CBPP-affected lungs in Portugal, Nunes *et al.*, 1990, showed 95% of lesions to be unilateral which is in contrasts with infections caused by *Pasteurella haemolytica*, where both lungs are usually affected. In a study in Nigeria, 95% of lesions were also restricted to a single lung (Egwu *et al.*, 1996). The diaphragmatic lobe was observed to be more commonly affected than the apical lobes (Nunes *et al.*, 1990). The thoracic cavity of affected animals may contain many liters of clear yellowish brown fluid containing some pieces of fibrin (Laak, 1992). Distension of the Interlobular septa of the affected lung with amber-coloured fluid surrounding the distended lymphatics is usually observed (Hudson, 1971). Consolidation of

the lungs with typical marbled appearance, sometimes accompanied by adhesion of the parietal and visceral surfaces is also characteristic. In chronic cases, the sequestra is the main lesion type and consists of necrotic material surrounded by afibrotic capsule ranging from 10 to 100 mm in diameter (Martel *et al.,*1983; Santini *et al.,* 1992). Necrotic foci have been reported in the kidneys of acutely infected cattle (Hudson, 1971; Provost *et al.,* 1987). Scuamore, 1976, isolated *Mmm* from the kidneys and urine of cattle affected with CBPP.

2.2.9 Differential Diagnosis of Contagious Bovine Pleuropneumonia

There are several differential diagnoses of CBPP as listed below:

2.2.9.1 Pneumonic pasteurellosis or 'shipping fever'

This is the main differential for CBPP. It is caused by *Mannheimia haemolytica* and less commonly *Pasteurella multocida* which at necropsy is characterized by serofibrinous pleural exudate and fibrinous bronchopneumonia (OIE, 2009).

2.2.9.2 Foot and mouth disease (FMD)

This is a viral disease that can be confused with CBPP due to clinical signs like salivation, lameness and fever. However, in FMD there are blisters inside the mouth, excessive secretion of stringy and drooling saliva, blisters on the feet along with swelling of the testicles in mature male cattle (OIE, 2009).

2.2.9.3Haemorrhagic septicaemia (HS)

The lung lesions seen in animals that survive the longest can appear very similar to the marbling lesion of CBPP. There may be yellow fluid in the chest and the affected lung may adhere to the inside of the rib cage. Thus, in the individual case distinguishing between HS

and CBPP can be difficult. This is a very acute disease and most affected animals die within 6 to 72 hours after the onset of clinical signs. Buffaloes are particularly susceptible. Oedema of the throat and neck to the brisket is often very pronounced (Gordon, 2005).

2.2.9.4 Bacterial or viral broncho-pneumonia

Clinical signs of certain bacterial or viral broncho-pneumonia may resemble closely those of acute CBPP. Post mortem examination usually shows both lungs to be affected, fibrinous exudate may be present but not to the same extent as in CBPP. While dark, solid areas of lung may be seen, these are usually restricted to the anterior lobes (not the diaphragmatic lobe as in CBPP) and marbled lungs are not often seen (OIE, 2009; Radostits *et al.*, 2003).

2.2.9.5Abscesses

Abscesses can be mistaken for sequestra. When cut open the contents of abscesses are seen to be offensive smelling, liquid purulent material, which is absent in sequestra (OIE, 2009).

2.2.9.6 Tuberculosis

Tubercular nodules can superficially resemble sequestra but they are degenerative cheese like lesions, sometimes calcified. The lung tissue is destroyed. The same lesions are also seen in lymph nodes in the chest. The capsule of the tubercular nodules is not well defined when compared to that of sequestra. There is no marbling and adhesions of the lung to the rib cage (Abubakar *et al.*, 2011).

2.2.9.7 Theilerosis (East Coast Fever)

Clinical signs such as coughing, nasal discharges and diarrhea are observed in Theilorosis. Affected cattle also show general enlargement of superficial lymph nodes. The lungs may contain clear liquid which is also present in the chest cavity. Neither pneumonia and nor inflammation of the pleural are present as in the case of CBPP (OIE, 2009).

2.2.9 Laboratory diagnosis of Contagious Bovine Pleuropneumonia

The isolation and growth of Mmm is very important for the diagnosis and confirmation of outbreaks of CBPP (OIE, 2009). Furthermore, it is also a requirement of the OIE for countries wishing to declare freedom from CBPP under the recommended standards for epidemiological surveillance systems for the disease (OIE, 2009). Nasal swabs, bronchoalveolar lavage and blood may be taken from live animals and tissue samples from edge of pulmonary lesions, broncho-pulmonary lymph nodes, pleural fluid, and joint fluid from calves may be taken at postmortem(OIE, 1997).

2.2.9.1Growth, isolation and transport media

Mycoplasmas are dependent on their host for a wide variety of organic nutrient such as vitamins, amino acids, fatty acids and lipids. Furthermore, *Mmm* will not grow in media lacking the preformed bases such as uracil and thymine that it requires for nucleic acid synthesis (Razin, 1978).

Mycoplasma mycoides subsp. *mycoides* is facultatively anaerobic, growing well in both anaerobic and aerobic environments at a pH of 7.6-7.8 (Nicholas and Baker, 1998). It usually grows well in sealed liquid broth cultures, especially if the broth level is a few

inches deep to allow for an oxygen or air gradient. Gentle aeration increases the growth rate and yield of *M. m. mycoides* (Rodwell and Mitchell, 1979). Mmm is not intrinsically difficult to grow but requires a fully functioning bacteriological laboratory with access to specialist mycoplasma media. Many Mycoplasma media have been described which enhances the growth of *M. m. mycoides* (Hudson, 1971; Freundt, 1983; Nicholas and Baker, 1998). A number of commercially prepared media for the isolation and identification of mycoplasmas of veterinary importance are also available, for example, Mycoplasma Experience, Reigate, UK. Isolation media for *M. m. mycoides* also traditionally serve as transport media and are based on conventional growth media with the addition of inhibitors such as ampicillin, bacitracin, penicillin G, polymyxin B, and thallium acetate to stop the growth of cell-walled bacteria.

2.2.9.2Biochemical tests for Mmm.

There are series of biochemical tests standardized by Aluotto *et al.* (1970) for the detection of *Mmm*. The strains may be identified by the fermentation of glucose, the reduction of tetrazolium aerobically and anaerobically, and the digestion of casein; they do not hydrolyse arginine, liquify coagulated serum, produce film and spots, and have no phospatase activity (Aluotto *et al.* (1970).

Final identification of mycoplasmas is usually achieved by growth inhibition (GI) and/or immunofluorescence (IF) tests which are carried out on agar with specific antiserum (Aluotto *et al.*, (1970). The tests are relatively specific; they can identify the two subspecies of *M. mycoides* but cannot distinguish between SC and *M. m. Capri.* Recently, a nitroblue tetrazolium (NBT) reduction technique has been described for the detection of substrate

metabolism by washed cell suspensions and may be suitable for use in routine laboratories to differentiate *M. m. capri*, and Mmm strains (Miles and Agbanyim, 1998).

A medium was developed by Bashiruddin *et al.*, (1999) and Bashiruddin and Windsor (1998) in which *M. m. mycoides* colonies were coloured red due to tetrazolium reduction. Using clinical material and isolated mycoplasmas to inoculate plates, *Mycoplasma mycoides capri* colonies were dark red after 3 days, whereas *Mycoplasma mycoides subsp. mycoides* colonies were much lighter in color and only became dark red after approximately 7 days.

This medium may be useful in the primary isolation of *M. m. mycoides* from clinical material, enabling immediate identification of colonies for subsequent testing by standard and molecular methods (Bashiruddin *et al.*, 1999c; Bashiruddin and Windsor, 1998).The results of substrate oxidation studies have also led to the development of rapid tests for the utilization of maltose and glycerol by members of the *M. mycoides* cluster. The inability to use maltose differentiates *M. m. mycoides* strains from other *M. mycoides* strains (Rice *et al.*, 2000a).

In summary, *Mycoplasma mycoides* subsp. *mycoides* is sensitive to digitonin, does not produce 'film spots', ferment glucose, reduces tetrazolium salts, does not hydolze arginine, has no phosphatase activity and has no or weak proteolytic properties (Aluotto *et al.*,1970).

2.2.10 Serological tests for Contagious Bovine Pleuropneumonia

Serodiagnosis plays an important role in the survey and control programs of Contagious Bovine Pleuropneumonia (Le Goff and Thiaucourt, 1998). Generally, serological methods have been proven useful for the detection of outbreaks of CBPP and they have had an important role in successful CBPP eradication campaigns in several countries (Newton, 1992). Some of these serological methods include, the serum agglutination slide test (SAST) (Turner and Etheridge, 1963), the Complement Fixation Test (CFT) (Campbell and Turner, 1953; Gambles, 1956), and the detection of circulating antigen by Agar Gel Immuno-Diffusion (AGID) (Griffin, 1965; Shifrin, 1967). However, some of these techniques had drawbacks, for example, an Indirect ELISA based on the systematic, genetic, biochemical and antigenic analysis of surface exposed lipoproteins of *Mycoplasm mycoides subsp. mycoides* seemed very promising but was not specific enough (Le Goff and Lefevre, 1989).

There are two OIE approved serological tests for the diagnosis of CBPP, the complement fixation test (CFT) and the competitive ELISA (OIE, 2009).

2.2.10.1 Complement fixation test (CFT)

The CFT is specific for the diagnosis of CBPP but lacks sensitivity (Thiaucourt and Bolske, 1996). With a positive result being any reaction at 1/10 or higher, CFT is also far from robust. In a thorough examination of CFT in which over 33,000 sera from healthy herds were tested between 1991-1994 in Italy (Bellini *et al.* 1998) reported that CFT was 98% specific while its sensitivity, based on nearly 600 cattle with specific lesions from 11 infected herds, was only 64%. Isolation of the causative mycoplasma from affected animals

was even more insensitive at 54%. This therefore means that by using CFT as a screening test, some CBPP affected cattle, in the early or later stages of infection may be missed.

2.2.10.2 Competitive ELISA

A *c*-ELISA has been developed by Le Goff and Thiaucourt, (1998) and validation tests have been performed in Africa (Thiaucourt,1999). This test uses a mouse monoclonal antibody IgG1 that recognizes an epitope localized on a protein with an apparent molecular weight of 80 kDa. This test has considerable advantages in terms of ease of testing and standardization of results, the c-ELISA has sensitivity levels similar to the CFT. This *c*-ELISA kit now comes as a ready to use kit that contains all the necessary reagents including pre-coated plates kept in sealed aluminium foil (Le-Goff and Thiaucourt, 1998). The kit has been especially designed to be robust and offers a good repeatability. The substrate has also been modified and is now Tetramethyl Benzidine (TMB) in a liquid buffer. The reading of the result is at450nm (Le-Goff and Thiaucourt, 1998). It is usually not possible to use this technique for assessing vaccination efficiency as vaccination with strains such as T1/44 or T1-sr does not always induce detectable antibody responses (Le-Goff and Thiaucourt, 1998). However, as post vaccinal antibodies do not persist after 3months, CFT or c-ELISA can be satisfactorily used for the detection of natural infections even in areas where vaccination is used (Le-Goff and Thiaucourt, 1998).

2.2.11 Antigen detection systems for Contagious Bovine Pleuropneumonia

Rodriguez *et al.* (1996) reported a monoclonal antibody-based sandwich ELISA that could detect as little as 10^5 CFU/ml of both *M. mycoides* biotypes within two hours. Sensitivity of this test could be improved significantly by incubating samples for 48 hours. However, this

test could not distinguish between the *M. m. mycoides* and *M. m capri*, but combination of pathological and serological information from affected animals with this test could prove very useful.

Immunocytochemistry (ICC) is also another technique increasingly used to confirm the diagnosis of CBPP particularly where the causative organism, *M. m. mycoides*, is not recoverable (often following long transport distances), where the animal has died of acute disease or where serology is not possible or unclear (Scanziani *et al.*, 1997). However, the sensitivity of ICC using polyclonal serum can be low and non-specific results frequently occur (Bashiruddin *et al.*, 1999a).

Latex Agglutination Test (LAT), which gives results in less than two minutes, using sera or whole blood, was developed for screening cattle in the field (Ayling *et al.*, 1999a). This test uses a "specific" polysaccharide antigen extracted from the *Mmm*SC capsule, which is then bound to latex beads. This test has been evaluated using CBPP negative sera from England and CBPP positive sera from Africa, Portugal and Italy. Sensitivity was comparable to the internationally recognized complement fixation test, but is far simpler and more rapid to perform. This test may have great potential in parts of Africa where there are great distances between the outbreaks, usually in nomadic herds, and diagnostic laboratories enabling control measures to be implemented rapidly. Studies have been conducted using this method in Nigeria by Okaiyeto *et al.*, (2011).

2.2.12 Molecular Diagnosis of CBPP

The advent of molecular biology has greatly enhanced the capability to detect and identify species, to classify and characterize strains and to assess the genetic diversity of

populations. (Bashiruddin *et al.,* 1994; Thiaucourt, *et al.,* 2000a). The following are some of the molecular methods employed in the diagnosis of CBPP:

2.2.12.1 Polymerase Chain Reaction (PCR)

Generally, species within the *Mycoplasma mycoides* cluster share many immunological, biochemical and genetic properties, which result in major problems for diagnostic laboratories in the identification of field strains (Cottew *et al.,* 1987; Persson, 2002). However, the advent of advanced diagnostic methods based on PCR has been developed for the rapid detection, identification and differentiation of members of the *M. mycoides* cluster and the specific identification of *Mmm*(Bashiruddin *et al.,* 1994; Dedieu *et al.,* 1994; Miles *et al.,* 2006).

The use of PCR to detect *Mycoplasma* species from various clinical samples has demonstrated a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture- based diagnostic methodologies (Bashiruddin *et al.,* 1994).

A number of PCR assays have been developed including real-time PCRs that are highly sensitive and specific and less susceptible to contamination than earlier PCRs.

PCR has the advantage of being a fast, specific, and very sensitive technique. Most of the diagnostic PCR systems which are used today(Bashiruddin *et al.,* 1994; Dedieu *et al.,* 1994; Miles *et al.,* 2006) are designed to target the CAP-21 gene, whose gene product has an unknown function and known genes (Bashiruddin *et al.,* 1994b; Dedieu *et al.,* 1994; Rodriguez *et al.,* 1997; Persson *et al.,* 1999). With many of these tests, confirmation of the presence of *Mmm* or the production of the expected amplification product was possible by the digestion of the product with specific restriction enzymes. In some cases the standard

detection of PCR products by agarose gel electrophoresis was replaced with enhanced methods which improved the sensitivity and allowed some degree of automation (Bashiruddin *et al.*, 1999a; Persson *et al.*, 1999). These tests have been used for the detection and identification of *M. m. mycoides* from culture and clinical materials for example nasal mucous, pleural fluid, tissue from lung, lymph node, kidney, spleen, and semen from bovines (Bashiruddin *et al.*, 1994; 1994b; Nicholas *et al.*, 1994; Bashiruddin *et al.*, 1999a); and from the milk and respiratory tract of small ruminants (Brandao, 1995; Kusiluka *et al.*, 2000; Srivastava *et al.*, 2000).

Miles *et al*. (2006) developed PCR assays that will detect *Mycoplasma mycoides subsp. mycoides* and differentiate European and African/Australian isolates. More recent technological advances have resulted in the use of real-time PCR assays. Vilei and Frey (2010)described a TaqMan real-time PCR which they designed specifically to target the LppQ gene, which may be useful if a LppQ devoid vaccine was successfully developed. Schnee *et al.* (2011) described a novel multiplex real-time PCR which they demonstrated to be specific and sensitive when assessed using experimentally infected cattle.

The polymerase chain reaction (PCR) has been shown by others to be an extremely sensitive and specific technique capable of amplification factors between $10^5$and 10^6, and it has been used in the laboratory for the diagnosis of CBPP (Maes *et al.*, 1990).

2.2.12.2 Multi-locus Sequence Analysis

Multi-locus sequence typing/analysis (MLST/MLSA) is a technique in molecular biology for the typing of multiple loci of an organism (Maiden, 2006; Yaya *et al.*, 2008). MLSA uses several genes within the genome, which are analyzed for unique sequences. It is a nucleotide sequence-based approach for the unambiguous characterization of prokaryotes

via the Internet, which directly characterizes DNA sequence variations in a set of housekeeping genes and evaluates relationships between strains based on their unique allelic profiles or sequences (Maiden, 2006). The different sequences present within a bacterial specie are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). Molecular typing has been useful in determining the population structure and evolution of bacterial pathogens. MLST was first developed as a typing tool but based on sequence data from housekeeping genes that are not prone to horizontal gene transfer (Yaya *et al.,* 2008).While amplification of the specie-specific PCR products can result in a direct identification of the organisms, Multi-locus sequence analysis can be used to discriminate between strains (Maiden, 2006). *Mycoplasma mycoides* subsp. *mycoides* strains were presumed very homogeneous not until 1995, when various molecular techniques e.g. enzymatic restriction of whole DNA or Southern blotting proved it otherwise (Lorenzon *et al.*, 2003).

Multi-locus sequence analyses of specific genomic fragments have been successfully used for the typing of some *Mycoplasma species*. For example, the case of *M. genitalium*, the MG309 gene sequence was proven stable in sequential urine samples obtained from single patients for at least five weeks and may be valuable candidates for further typing studies (Ma and Martin,2004). Also, Multi-locus sequence analysis was demonstrated for a genomic fragment of 2400 bp of *M. capricolum* subp. *Capripneumoniae* (Lorenzon *et al.*, 2002). Nucleotide variations in this specific fragment were used to determine the geographical distribution of different strains. However, some exceptions have been reported where a number of housekeeping genes showed too few differences to be useful for typing (Dumke *et al.,* 2003; Manning *et al.,* 2003). For these cases, carefully selected species specific genes may be used instead.

Several studies have been carried out on *Mycoplasma mycoides* subsp. *Mycoides* using MLSA, for example: Lorenzon *et al.*, 2003 carried out a technique based on multi-locus sequence analysis" (MLSA) and identified 15 different allelic profiles within a representative number of Mmmstrains and also allowed a clear distinction between European, south-western African and sub-Saharan strains. Similarly, studies by Yaya *et al.*, 2008, identified three main groups Mmmwith 31 different allelic profiles amongst 51 strain and strain variant. It also showed a link between a European strain isolated in 1967and those found in southern Africa and Australia.

2.3 Disease Course of CBPP

The incubation period of CBPP in natural infection is usually between 3 to 8 weeks and may even be longer (Baker, 1998; Radostitis *et al.*, 2003). Turner and Campbell (1937) reported a range of 29-58 days and Provost *et al.*, (1987)observed 20 to 40 days for the incubation period. In experimental infections, Regalla *et al.*, (1994) reported disease symptoms appearing in cattle 40 days after contact with inoculated animals.

When control animals (cattle) were placed in contact with naturally infected cattle from an outbreak in Namibia, sero-conversion was seen after 6 weeks and rose rapidly in the next two weeks by which time 40% of contacts had died (Nicholas*et al.*, 2004).

2.4 Morbidity and Mortality of CBPP

Morbidity and mortality rates of CBPP show variation. In endemic regions, the disease may only affect 5–10 percent of cattle in a herd. However, in naïve herds or animals kept in close proximity with CBPP infected cattle, morbidity rates can be much higher. Mortality is most often seen in acute forms and mortality rates can be as high as 50 percent. Factors

such as *Mycoplasma mycoides mycoides* strain, breed of cattle, animal's nutritional status, affects the morbidity and mortality of the disease. Animals that recover from CBPP become fully immune (Masiga *et al.,* 1996)

2.5 Control and Prevention of CBPP

The options for control of contagious bovine pleuropneumonia (CBPP) include the following: Restriction of cattle movement and quarantine, stamping out policy, test and slaughter, treatment and vaccination (Yaya *et al.,* 2008).

2.5.1 Treatment

Mycoplasmas have no cell walls and are resistant to penicillin and related antimicrobials (Yaya *et al.,* 2008). Other Mycoplasma species such as *M. bovis* are sensitive in-vitro to enrofloxacin and lincomycin but there is no information available on the antimicrobial susceptibility of M. mycoides subsp. mycoides. The use of antimicrobials is discouraged in both Europe and Africa (Egwu *et al.,* 1996). However, antibiotic treatment against CBPP is widely used in pastoralist communities (Msami *et al.,* 2001; Mariner *et al.,* 2006). It is not part of any official control strategy due to suspicion that its use could facilitate development of sequestra, increase the number of carrier animals along with the increase development of resistant strains, and mask the occurrence of clinical disease making diagnosis difficult, which may contribute to unrecognized infections and CBPP transmission (Provost *et al.,* 1987).

At a meeting of international experts organized by the FAO in 2003, it was recommended that chemotherapy be reconsidered for CBPP control despite the fact that the effectiveness of treatment has not been adequately studied. However both in-vivo and in-vitro studies demonstrating usefulness of antibiotics for treating CBPP have been reported (Ayling *et al.,*

2000a; Yaya *et al.,* 2004; Hubschle *et al.,* 2004). In an in-vitro experiment, tilmicosin, danofloxacn, oxytetracyclines, florfenicol and spectinomycin were found to be effective against a variety of strains of Mmm isolated from CBPP cases that had occurred in Africa and Europe (Ayling *et al.,* 2000a).

A study was carried out in Namibia, where it was demonstrated that naïve animals kept in-contact with danofloxacin treated animals that had CBPP had significantly fewer lesions, were less likely to die and to develop clinical disease than naïve animals kept in-contact with untreated animals with CBPP (Niang *et al.,* 2007). In the same study, Mmmwas isolated from a limited number of the in-contact controls kept with the treated animals suggesting low spread of infection (Hubschle *et al.,* 2004). Similarly, In a different experiment, Oxytetracycline long acting (TLA) antibiotics was shown to be effective in limiting clinical severity of CBPP but ineffective in the prevention of persistence of viable Mmm in treated animals, consequently, the direct effect of tetracycline on the individual is positive showing less clinical damage, but there are fear of an indirect negative effect on the population, such as masking of clinical signs leading to transmission and spreading of the disease (Niang *et al.,* 2007; Yaya *et al.,* 2004).

2.5.2 Vaccination

In Africa, CBPP is majorly controlled through a vaccination policy that uses variant strains of *Mycoplasma mycoides* subsp *mycoides* biotype SC, called T1/44 or T1sr (Thiaucourt *et al.,* 2006).The minimum vaccinating dose for the $T_1$44 vaccine was estimated to be 10^7 cfu by Gilbert and Windsor (1971).This vaccine has some drawbacks such as: It suffers from a short duration of efficacy, adverse reactions and cold chain dependence (Thiaucourt *et al.,* 1996). Endobronchial inoculation of the vaccine has been shown to lead to the disease

(Mbulu *et al.*, 2004). Furthermore, there has been suspicion that this vaccine and its variants have lost efficacy during the past 10 years and that the vaccination campaigns of 1995 and 1996 did not provide expected levels of protection (Thiaucourt *et al.*, 1996). The low potency of the T1/SR vaccine that was in use during the initial outbreaks of CBPP in 1990s frustrated both the farmers and livestock experts (Masiga *et al.*, 1996; Tulsane *et al.*, 1996). Various postulations were put forward to explain vaccine failure such as: loss of immunogenicity, increased virulence of outbreaks, lack of maintenance of cold chain, expertise of the vaccinating personnel and insufficient vaccine titres (Egwu *et al.*, 1996; March, 2004). It was therefore, recommended that improved buffering of the vaccine would likely improve the viable dose of the vaccine (March, 2004).

The challenge with the use of CBPP vaccines are: Firstly, the efficacy of the vaccine itself and secondly, the efficient conduct of the vaccination campaign (Thiaucourt*et al.*, 2006). Mycoplasmas are extremely heat labile and as a result, current freeze dried vaccines for the prevention of CBPP must be maintained under cold storage until used in order to preserve the viability and potency of the immunizing organisms (Litamoi *et al.*, 2004, Hudson, 1968). Apart from the alleged low potency, the effectiveness of the CBPP vaccines might have been reduced by inefficient cold chain. During the vaccination campaigns of the past, the vaccines were stored in paraffin-fuelled refrigerators which were unreliable because they often failed to attain optimal cooling temperature. The low potency of the vaccine could have resulted also from the use of harmful diluents such as chlorinated or tap water or mishandling of the reconstituted vaccine by exposing the vaccine to light and high temperatures for a long time (Karst, 1972).In Africa, where the application of the stamping out policy of eradication is difficult and impracticable, the control of CBPP has relied on

preventive immune-prophylaxis using live attenuated cultures of the causative agent (Thiaurcourt *et al.*, 2000b). CBPP vaccination is the method that is currently in use in most African countries employing the vaccine strains T1/44 or its streptomycin resistant derivative T1-SR (Litamoi *et al.*, 2004). The CBPP T1-44 vaccine that is currently recommended for use in endemic areas of Africa confers immunity for 1 year (Tulasne *et al.*, 1996). These vaccines though have been used quite successfully in the past for the control of CBPP in Australia, Nigeria and East Africa (Brown *et al.*, 1965, Hudson 1968).The T1/44 is noted for post vaccinal reactions while the T1 SR induces a shorter period of immunity than the T144 (Heubschle *et al.*, 2002). In Nigeria, the T1/44 vaccine is currently in use for the control of the disease. It is produced by the National Veterinary Research Institute Vom (NVRI, 2007).

2.6 Economic Importance of CBPP

The economic effects of CBPP can be enormous, resulting in heavy losses in cattle populations. In Britain 187,000 cattle died of CBPP in 1860 (Hutyra *et al.*, 1938). In the Netherlands nearly 65,000 cattle died of CBPP between the years 1833-1850 (Laak, 1992). Over 100,000 cattle died within two years of the introduction of CBPP into South Africa (Trichard *et al.*, 1989). In the early 1860s when the disease spread rapidly throughout Australia, it behaved as a virulent epidemic with losses of up to 75% of animals in an affected herd amounting to 1.4 million head.

Contagious Bovine Pleuropneumonia (CBPP) is a highly contagious disease. It is still considered to be the most economically important cattle disease in Africa, causing greater losses in cattle than any other disease after Rinderpest (OIE, 1997).The economic importance of contagious bovine pleuropneumonia (CBPP), especially losses due to the

chronic disease, is difficult to assess. Losses include mortality, loss of weight, reduced working ability, reduced fertility, reduced growth rate, and losses caused by control program (due to vaccination campaigns, quarantine, and restrictions on cattle trade). The direct losses result from mortality, reduced milk yield vaccination campaign cost, disease surveillance and research programs. The indirect losses are mainly due to the chronic nature of the disease and this includes loss of weight and working ability, delayed marketing, reduced fertility (Egwu *et al.,* 1996).

The annual economic loss due to CBPP in the Northern states was then estimated at NGN 498 million (Fayomi and Aliyu 1997). A five year retrospective study at Mubi, Adamawa state by Halle *et al.*, (1998) reported that 238 infected cattle lungs were condemned due to CBPP out of 43, 810 cattle slaughtered (0.54%) and the worth of organs condemned was estimated at NGN 28,580. Annual losses of up to €30.1 million due to CBPP were estimated in 12 endemic sub-Saharan African countries (Tambi *et al.,* 2006). Contagious bovine pleuropneumonia causes NGN1.307 billion losses to Nigeria as a whole with the Sudan Savanna zone accounting for 24 % of all losses, the Northern Guinea Savanna for 68%, the sub-humid zone 4 % and the humid zone for 5 % (Fadiga *et al.,* 2013). The Botswana veterinary authorities in 1995 decided to depopulate the whole district of Ngamiland, slaughtering 220, 000 cattle. Compensation alone was estimated at $30 million, this was successful in eradicating the disease in Botswana being officially declared free of CBPP in the year 2000 (Fadiga *et al.,* 2013). Tanzania had outbreaks of CBPP between the year 1990 and 1995, which resulted in the deaths of 14,000 cattle valued at over $1million (Fadiga *et al.,* 2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in three selected South East states of Nigeria (Anambra, Enugu and Imo states). The states were selected based on cattle population and

established cattle market. South-east Nigeria is one of the six geopolitical zones in the country. The region consists of Anambra, Enugu, Imo, Ebonyi and Abia states. Anambra state lies between latitude 5° 32’ and 6° 45’ N and longitude 6° 43’ and 7° 22’ E; Enugu state lies between latitude 5° 27’and 6° 33’ N and longitude 6° 28’ and 7° 32’ E and Imo state is located between latitude 4° 45’ and 7° and 15’ N and longitude 6° 50’ and 7°25’ E. The south eastern region of Nigeria has an estimated cattle population of 4.5 million from the total 16.3 million estimated cattle population in Nigeria (Ikhatua, 2011).

3.2 Sample Size

Sample size was determined using the formula by Thrusfield (2009) for simple random method at 95% confidence interval (CI) and 5% absolute precision using the prevalence of 10.65% (Olabode *et al.*, 2013).

$$N = \frac{Z^2 \times P \times (q)}{d^2}$$

Where:

N = sample size for the study

Z = standard normal deviation for 95% confidence level (1.96)

p = expected prevalence of 10.65%

q = 1- p

d = desired absolute precision (5% or 0.05).

$$\frac{1.96^2 \times 0.1065 \times (1-0.1065)}{(0.05)^2}$$

146 Samples /State

A total of 438samples were collected from the three selected south eastern states.

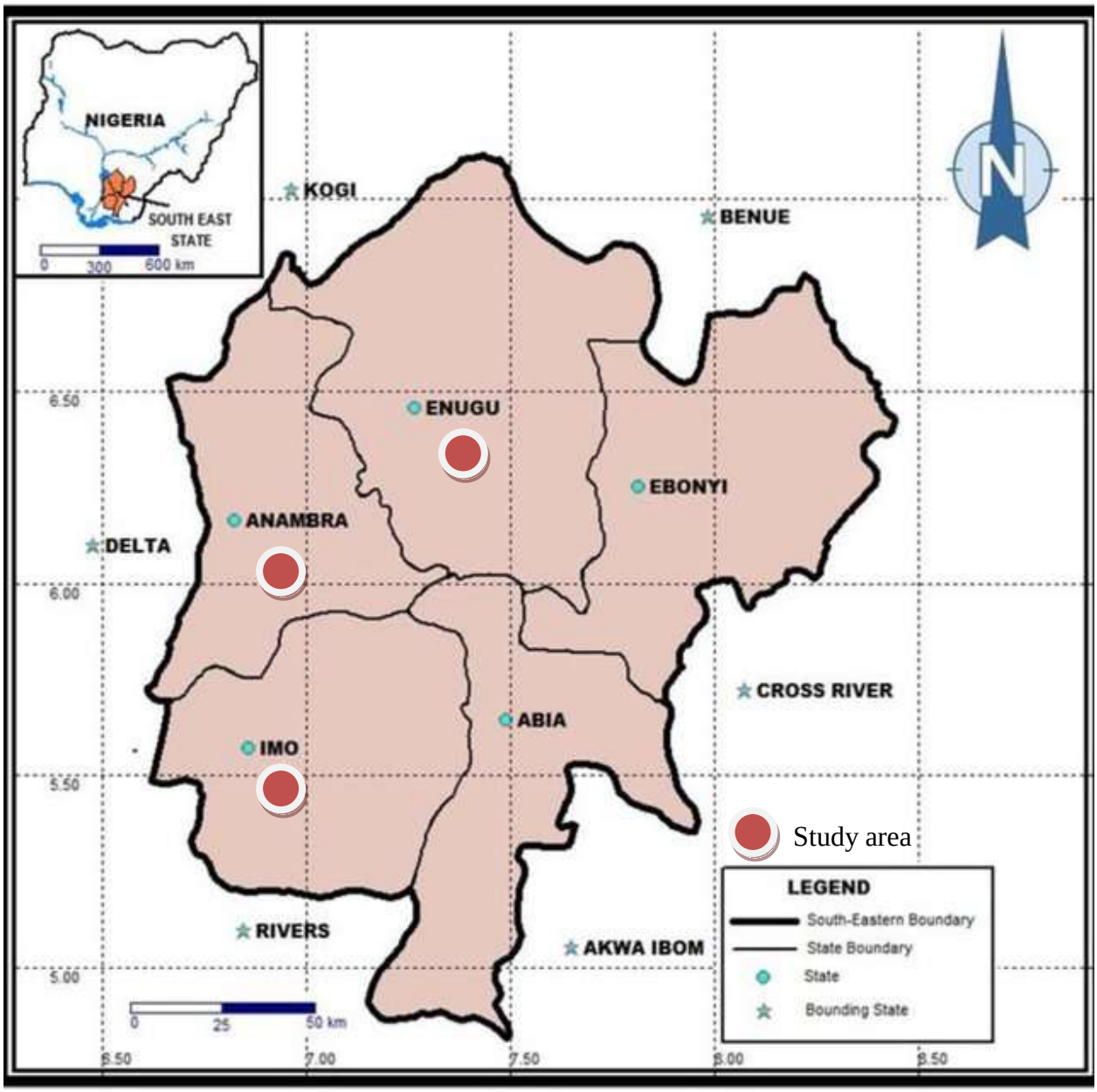

Figure 1: Map of Nigeria showing the three selected south east states sampled

Source: www.researchgate.net.

Retrieved: 3-07-2020.

3.3 Study Design and Sampling Method

This was a cross sectional studies. Snowball sampling method was used in this research. Fulani sedentary settlements sampled were selected based on information by participating pastoralist in identifying other sedentary herds within the study area. Cattle were physically examined and clinical signs observed were recorded at the different Fulani herds visited in the three selected south east states. Vital parameters were taken (respiratory rate, pulse rates and temperature). Rectal temperature was taken using clinical thermometer; respiratory rates were taken through the use of stethoscope. Gross lesions were observed and documented in CBPP infected cattle at slaughter in the different abattoirs in the study area.

The cattle to be sampled at the abattoir were selected using a purposive sampling method. Briefly, tissue samples (lungs) and pleural fluids were collected from apparently sick cattle seen to show clinical signs/ gross lesions suggestive of CBPP before and after slaughter at the abattoir (emaciation, marbled appearance of the lungs and accumulation of pleural fluid within the thoracic cavity).

Blood samples were collected from cattle that were not vaccinated against CBPP within six (6) months as at the time of sampling from the different Fulani sedentary settlements in the study area. The age and sex of cattle sampled were also documented.

3.4 Sample Collection and Processing

The samples (blood, lung tissues and pleural fluids) were collected between December 2019 and April 2020. Blood samples (5mls) were aseptically collected through the jugular vein of each animal using sterile 18G needle and 10ml syringe. The blood samples were allowed to stand in a slanting position for 3 hours to allow for separation of the serum from

the cellular components of the blood. The serum was then poured into labeled sample bottles and packed in ice.

The lung samples were collected using sterile blades while the pleural fluids were collected using sterile 18G syringes. Subsequently, the samples collected at post mortem (lungs and pleural fluid) were put in sterile sample bottles and transported in CBPP transport medium (PPLO broth).

All the samples collected (serum, lung samples and pleural fluid) were all properly labeled and transported to the CBPP laboratory of the National Veterinary Research Institute Vom, Plateau state, where they were stored at -20°C until further use.

3.5 Serological Examination Using Competitive ELISA

The serological analysis was performed using IDEXX CBPP Antibody test kit with batch number P05410/10 (CIRAD, France) according to manufacturer's instruction. Briefly, the serum test samples were pre-mixed with a specific monoclonal antibody Mab 117/5 in a separate plate called the 'pre-plate' and the content of the pre-plate was then transferred into the pre-coated micro-plate lysed with *Mycoplasma mycoides* subsp. *mycoides* antigen and incubated for 1 hour at 37°C under gentle agitation. The unbound materials were washed twice with phosphate-buffered saline (300µl) and an anti-mouse conjugate concentrate (100µl) was then added to each well. This was again incubated for 30minutes at 37°C under gentle agitation, avoiding the desiccation of the plates. After incubation, the unbound materials were again washed three times with phosphate-buffered saline (300µl). Subsequently, 100µl of an enzyme substrate, $33^{1}55^{1}$Tetramethylbenzidine (TMB) was then added to each well and incubated for 20minutes at 37°C. Finally, the reaction was stopped

by adding 100µl of a stop solution (Sulphuric acid) into each well. The optical densities of the reactions were then read at 450nm using Multiskan EX (Lab-systems). Samples were considered negative if the percentage inhibition was below 50% and positive, if above 50%.

3.6 Culture and Isolation of *Mycoplasma* Species

The lung tissues and pleural fluids collected from the suspected CBPP cattle from the abattoirs were cultured in growth media according to protocols by the OIE (2014). Briefly, the suspected lung tissue and pleural fluid were incubated in Pleuropneumonia like organism (PPLO) broth for at least 48 hours at 37 °C under aerobic condition. After incubation, 20 µl of the overnight broth was added to 180 µl of PPLO broth and a four ten-fold serial dilution (10^{-1} to 10^{-4}) was made and finally sub-cultured onto PPLO agar. The agar plates were incubated for at least 72 hours at 37 °C and 5% CO_2 and monitored for growth. The presence of small to medium sized colonies with the characteristic 'fried egg' appearance under the stereomicroscope at ×35 magnifications was considered presumptive of *Mycoplasma mycoides mycoides*.

3.7 Confirmation of Mycoplasma mycoides subsp. mycoides isolates Using Conventional Polymerase Chain Reaction

All the positive *Mycoplasma* isolates obtained from culture on growth medium (PPLO agar) were subjected to conventional PCR test for the detection of Mmmaccording to protocols by Miles *et al.,* (2006).

3.7.1 Extraction of DNA

DNA was extracted from a 3 ml Mmm broth culture using QlAamp® DNA Mini kit according to manufacturer's instructions. Lyophilized T1/44 vaccine (NVRI, Vom Nigeria) was used as the positive control for this study. Briefly, 20µl of Proteinase K was pipetted into 1.5ml micro-centrifuge tube after which 200 µl of the sample (broth culture) was added. Subsequently, 200 µl Buffer AL (Tris-HCl, Potassium chloride and Magnesium chloride) was then added and mixed thoroughly by vortexing for 15seconds. The mixture was incubated at 56 ^{o}C for 10 min. Thereafter, 200µl ethanol was added and mixed thoroughly. The mixture was centrifuged at 6000 x g for 1 minute. The resultant pellets were then washed twice in Buffer AW1 and AW2 ((Tris-HCl, Potassium chloride and Magnesium chloride)) respectively. Finally, 200 µl of Buffer AE was added and incubated at room temperature for 1 minute and centrifuged at 8000×g for 1 minute to elute the DNA. Thereafter, 2.5µl of the DNA extract was used as the template for all the reactions

3.7.2 Specific PCR Assay for the identification of *Mycoplasma mycoides* subsp. *mycoides*

All the PCR reactions were carried out in a final volume of 25µl, which contained dH_2O, 5x FIREPol® master mix (12mM MgCl, 1 mM dNTP mix, FIREPol® DNA polymerase and 1µl IS1296F: Primer (5' to 3'): CTA AAG AGC TTG GAG TTC AGT G and 1µl R (all) (sequence 3' to 5'): CCA GCT CAACCA GCT CCA G (Miles *et al.*, 2006).

3.7.3 Amplification of DNA and agarose gel electrophoresis

DNA amplification was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France). Thermal cycling consisted of an initial denaturation step at 95°C for

5 minutes, followed by 32 cycles of denaturation at 95°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute and 20 seconds. The final extension step was maintained at 72°C for 5 minutes. The PCR product was then ran on 1.5% agarose gel impregnated with ethidium bromide (0.5ug/ml) at 130 volts for 30minutes and the DNA migration was viewed under UV light and photographs taken. The production of a band equivalent to 1.1 kbp and at the same distance with positive control was considered confirmatory for Mmm.

3.8 Molecular Characterization of Mmm Isolates Using Multi-locus Sequence Analysis

The amplicons were stored in PCR tubes and transported to Macrogen Europe B.V, The Netherlands for sequencing.

The PCR confirmed isolates were characterized using Multi-Locus Sequence Analysis (MLSA) as described by Yaya *et al.*, (2008). MLSA was performed using the locus: Loc-PG1-0001(non-coding region). The selection of the locus was informed by the ability to differentiate the strains from West Africa (Yaya *et al.*, 2008). Briefly, PCR was performed using GeneAmp® PCR system 2720 (Perkin Elmer, Courtaboeuf, France) in 50µl reaction mix. Samples were amplified with the following primers: Loc-PG1-0001-F 5'AACAAAAGAGATCTTAAATCACACTTTA 3' and Loc-PG1-0001-R 3' CCTCTTGTTTAACTTCTAGATCAGAAT 5'. Thermal cycling consisted of an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at72°C for 90 seconds. The final extension step was maintained at 72°C for 7 minutes. The PCR amplification products were then analyzed by electrophoresis through 1.5% agarose gel (QA-Agarose, MP

Biomedicas, IllKirch, France) at 100V and visualized after staining with Ethidium bromide on a UV transilluminator. Samples with relevant features with the expected base pair of 538bp were sent to Macrogen Europe B.V, The Netherlands for sequencing.

The sequences obtained from each corresponding forward and reverse primers were assembled using BioEdit™. The extremities corresponding to a single strand sequence or showing aberrant features was trimmed and a consensus sequences was obtained. The sequences obtained from different strains for the selected locus were aligned using ClustalW (BioEdit™). Subsequently, the sequences obtained were compared to those previously obtained on 51 strains of *Mmm* (Yaya *et al.,* 2008). If the feature of a strain corresponds to one of the strains in the work of Yaya *et al.*, 2008, its allele number was assigned to the strain. Otherwise, a new allele number was assigned to it. At the end, each strain was characterized using an allelic number.

3.9Allelic Sequence Comparison of the Field Isolates with the Vaccine Strain

Sequences obtained from the field isolates were compared with the Vaccine strain reference sequence (T1/44) from the Genbank. The sequences were aligned using ClustalW Multiple sequence analysis (BioEdit™). Points of mutation between the field isolates and vaccine strain were recorded.

3.10 Determination of the knowledge, attitude and practices of Pastoralists towards CBPP using Questionnaires

A total of Ninety open ended questionnaires were used for this study. Thirty five (35) in Enugu state, thirty (30) in Anambra state and twenty five (25) in Imo state. They were

administered to pastoralists and cattle farm owners who consented to the study. The questionnaires were interpreted in Hausa languages to the respondents. The data collected comprised demographic information which included sex, age and breed of cattle in their herds, awareness of the existence of CBPP and its associated clinical signs, as well as information on previous and recent suspected outbreaks of the disease in their herds. The questionnaire also focused on the source(s) of vaccines, the vaccination programmes practiced and personnel used for vaccination. Information was also obtained on the use of antibiotics for treatment of CBPP by pastoralist and cattle farm owners.

3.11 Data Analyses

The data obtained were presented in tables. Descriptive analysis was used to analyze data obtained from the questionnaires administered to the pastoralists. Chi-square test using SPSS Version 20 for windows was used to determine any significant difference in the prevalence rate of the disease based on sex and age of cattle. Values of $P \leq 0.05$ were considered significant at 95% confidence Interval. Prevalence rate was calculated using the formula:

$$\text{Prevalence} = \frac{\text{Number of positive samples}}{\text{Total samples analyzed}} \times 100$$

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical Signs observed in CBPP suspected cattle in the three South Eastern States

Of the four hundred and thirty eight (438) cattle from which blood samples were collected from the different sedentary Fulani herds in the three selected south east states of Nigeria, twenty (Anambra 5, Enugu 10 and Imo 5) cattle were suspected of having CBPP and were examined physically. Vital parameters were also taken (Table 4.1). The mean respiratory rates of suspected CBPP infected cattle examined was 48 ± 1 cycles per minute while the mean average pulse rate was 70 ± 1 beats per minute and the mean rectal temperature of the 20 suspected CBPP infected cattle was 39.6ºC ± 0.1 (Table 4.1). Clinical signs observed in the 20 suspected CBPP infected cattle are as follow: Slight to severe cough especially when exercised was seen in 50% (10/20).Congested mucus membrane in 12 (60%).Furthermore, Mucoid nasal discharges was observed in 5(25%) of the infected animals while 9(45%) had extended neck with abduction of the forelimbs. Fifteen (75%) out of the 20 suspected CBPP cattle were emaciated with associated lethargy.

4.2 Gross Lesions of CBPP Observed in Cattle at the Abattoir in the Three South Eastern States, Nigeria

Gross lesions were observed and documented from the ninety (90) CBPP infected cattle at the different abattoirs in the three selected south east states (Plates I-III). These includes;Lung adhesion to the thoracic wall (Plate I), collection of up to 8 litres of pale to yellow coloured fluid (Pleural fluid) in the thoracic cavity (Plate II) and marbled appearance of lung tissue when cut open (Plate III). The diaphragmatic lobes were usually affected by the disease with the other lung lobes being normal.

Table 4.1: Mean vital parameters of suspected CBPP infected cattle (n = 20) from sedentary herds (n = 41) in the three South Eastern states, Nigeria

Vital Parameters	Mean Values	Normal range
Respiratory Rate	48cpm ± 1	15-30cpm
Pulse rate	70bpm ± 1	60-80bpm
Rectal temperature	39.6 ºC± 0.1	37.5-38.2 ºC

Adapted from: Robertshaw, D. (2004).Temperature regulation and Thermal environment.

Key;

ºC: degree Celsius

bpm: beats per minute

cpm: cycles per minute

Table 4.2: Clinical signs observed from suspected CBPP infected cattle at Fulani settlements in the three South Eastern states, Nigeria

Observed clinical signs	Number of animals examined per state (Number positive)			Total (%)
	Anambra	Enugu	Imo	

Cough	5(4)	10(8)	5(3)	15(75)
Congested mucus membrane	5(3)	10(7)	5(2)	12(60)
Rapid laboured breathing	5(4)	10(8)	5(3)	15(75)
Mucoid nasal discharges	5(1)	10(2)	5(2)	5(25)
Extended neck and abducted forelimbs	5(2)	10(4)	5(3)	9(45)
Lethargy	5(3)	10(7)	5(4)	14(70)
Emaciation	5(3)	10(8)	5(4)	15(75)

Table 4.3: Gross lesions of CBPP observed in Cattle slaughtered (n= 90) at the abattoir in the three South Eastern states of Nigeria

Gross lesions observed per state	Number of lesions observed per state

Anambra state		
Inflamed lungs		15
Pleural fluid accumulation	15	
Sequestrum formation		4
Lung adhesion to thoracic wall		6
Enugu state		
Inflamed lungs		10
Pleural fluid accumulation		10
Sequestrum formation		2
Lung adhesion to thoracic wall		8
Imo state		
Inflamed lungs		18
Lung adhesion to thoracic wall		2
Total		90

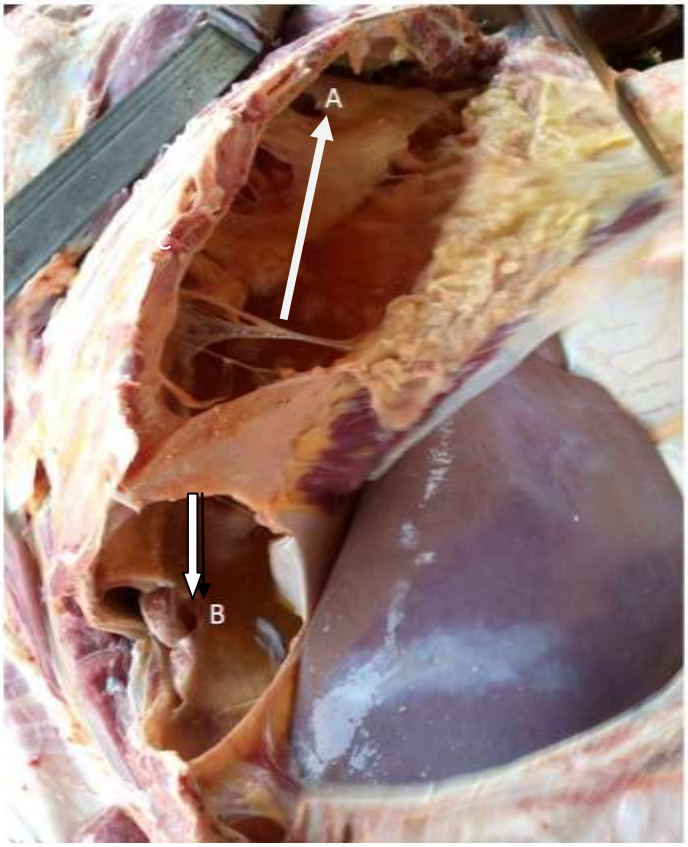

Plate I: Photograph showing lung adhesion (arrows) to thoracic wall (C) of a CBPP infected cow at slaughter

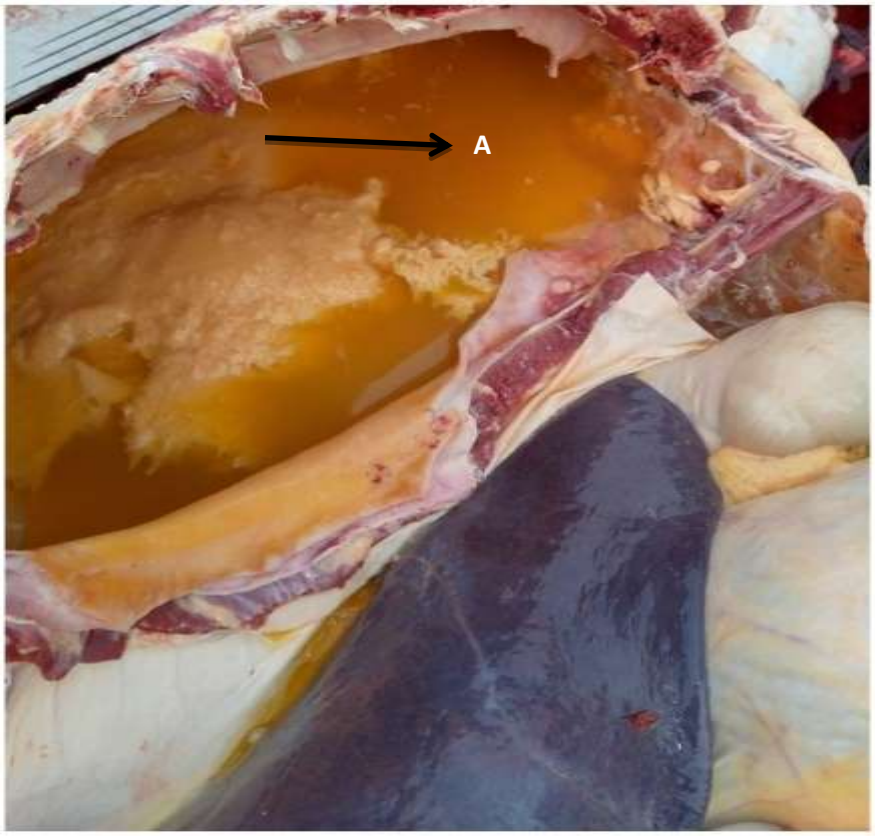

Plate II: Photograph showing presence of collected pleural fluid (arrow) in the thoracic cavity of CBPP infected cow at slaughter in the abattoir.

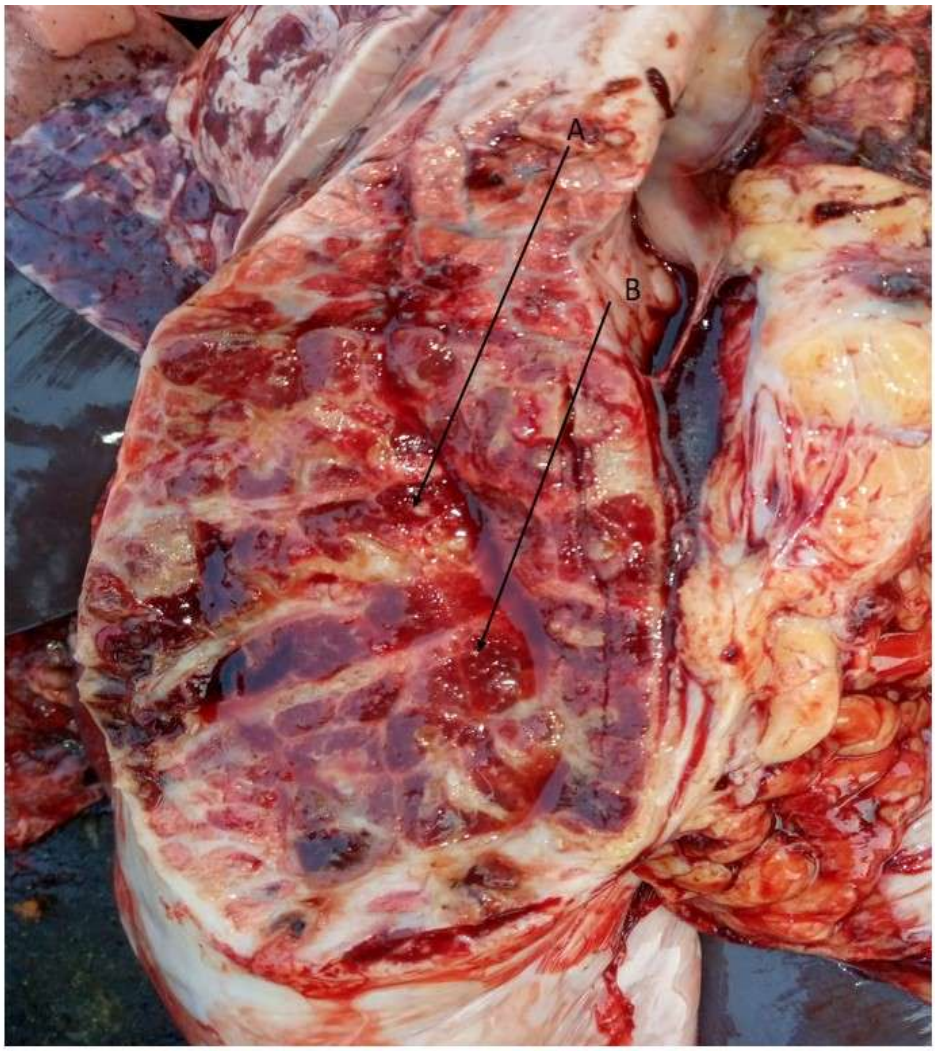

Plate III: Incised section of diaphragmatic lung lobe of CBPP infected cow at post mortem showing (arrows) thickening of the interlobular septa (marbled appearance).

4.3 Sero-prevalence of CBPP in Cattle using *c*-ELISA in the Three South East States, Nigeria.

Of the total of 438 sera samples collected from the different Fulani sedentary settlements, 260 were positive for CBPP using c-ELISA, giving an overall prevalence of 59.4% (Table 4.4).Out of the 146 sera samples collected from each state, 74 (50.7%) were positive for *Mycoplasma mycoides* subsp. *mycoides* antibodies using c-ELISA in Anambra State, 92 (63%) were positive in Imo state and 94(64.3%) were positive for *Mycoplasma mycoides* subsp. *mycoides* antibodies in Enugu state (Table 4.4). Based on sex, 19 (19%) out of a total of 100 Bulls sampled for CBPP antibody in the three selected south east states were positive for CBPP, while 241 (71.3%) cows out of 338 sampled were positive (Table 4.5). This wasstatistically significant($P \leq 0.05$). Similarly, there was also significant difference ($P \leq 0.05$) observed in the sero-prevalence of CBPP based on age of cattle. Adult cattle had higher infection rate (68.4%) than calves less than 1 year old (18.75%) (Table 4.6).

4.4 Culture and Isolation of *Mycoplasma mycoides* subsp. *mycoides*

Ninety (90) samples namely pleural fluid (25) and lung tissues (65) were collected from suspected CBPP infected cattle in the abattoirs from the three selected south east states. The 25 pleural fluids were from Enugu state (10), Anambra state (15) and none from Imo state. The 65 lung tissues were from Enugu state (20), Anambra state (25) and Imo state (20). On being cultured on PPLO agar and based on the colonial morphology of “fried egg appearance” with either dense centres or raised centres with nipple like appearance (Plate V &VI), 25 (27.7%) were considered positive for *Mycoplasma mycoides* subsp. *mycoides* in the three selected south east states. These consist of 10 pleural fluids and 4 lung tissues

from Anambra state. Seven (7) pleural fluids and 4 lung tissues from Enugu state.None from the 20 lung tissues in Imo State.(Table 4.7).

Table 4.4: Sero-prevalence of Mmm antibodies using c-ELISA in the three South Eastern states of Nigeria.

Selected States	Number of cattle sampled	Number of cattle positive by c-ELISA (%)

Anambra	146	74 (50.7)
Enugu	146	94 (64.3)
Imo	146	92 (63)
Total	438	260 (59.4)

Table 4.5: Sero-prevalence of Mmm antibodies based on sex of cattle in the three South Eastern states of Nigeria

State	Sex

	Male		Female	
	Number sampled	Number positive (%)	Number sampled	Number positive (%)
Anambra	32	6(18.7)	114	68 (59.6)
Enugu	40	6(15)	106	88(83)
Imo	28	7(25)	118	85(72)
Total	100	19(19%)	338	241(71%)

P value: 0.0001; X^2: 87.5; OR: 0.0941; 95% CI: 0.0054-0.1641

Table 4.6: Sero-prevalence of Mmm antibodies based on Age of Cattle in the Three South Eastern States of Nigeria

State	AGE

	≤ 1year		> 1 year	
	Number of cattle sampled	Number positive (%)	Number of cattle sampled	Number positive (%)
Anambra	25	5(20%)	121	69(57%)
Enugu	30	6(20%)	116	88(75.8%)
Imo	25	4(16%)	121	88(72.7%)
Total	80	15(18.7%)	358	245(68.4%)

P Value: 0.0001; X^2; 66.91; df: 1; OR: 0.1064; 95% CI: 0.05817- 0.1948

Table 4.7: Number of *Mycoplasma mycoides* subsp. *mycoides* positive on pleuropneumonia-like organism (PPLO) agar from the three South Eastern states of Nigeria

State	Number of samples		Number positive on PPLO agar		
	Lung Tissue	Pleural Fluid	Lung tissue	Pleural fluid	Total (%)
Anambra	25	15	4	10	14(35%)

Enugu	20	10	4	7	11 (36.6%)
Imo	20	0	-		-
Total	65	25			25 (27.7%)

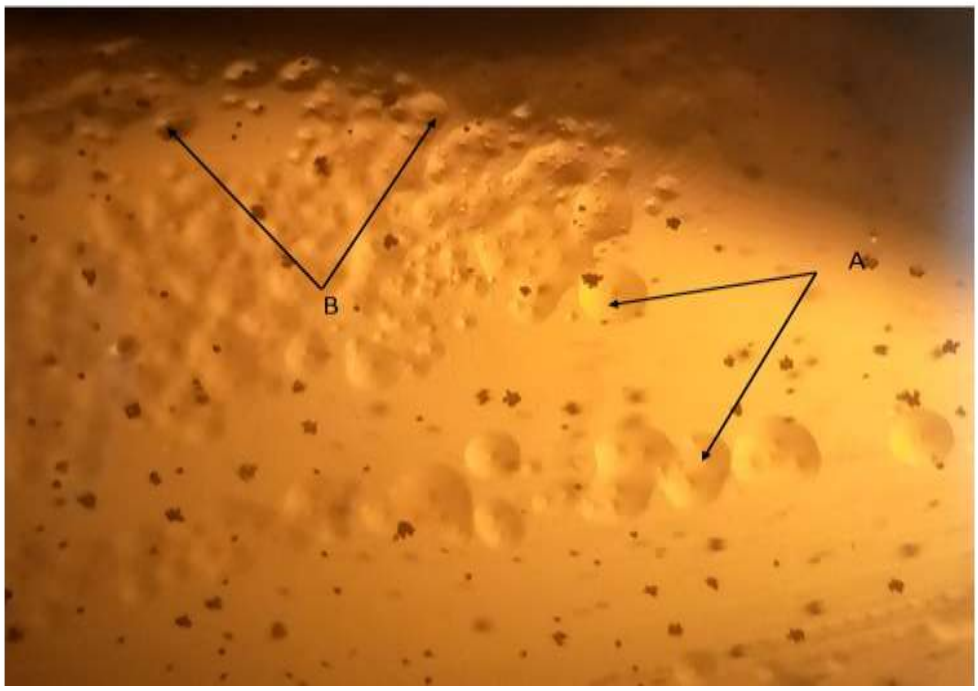

Plate IV: photograph showing *Mycoplasma mycoides* subsp. *mycoides* colonies (A&B) on Pleuro-pneumonia like organism (PPLO) agar under a stereomiscrope (x4.5 magnification)

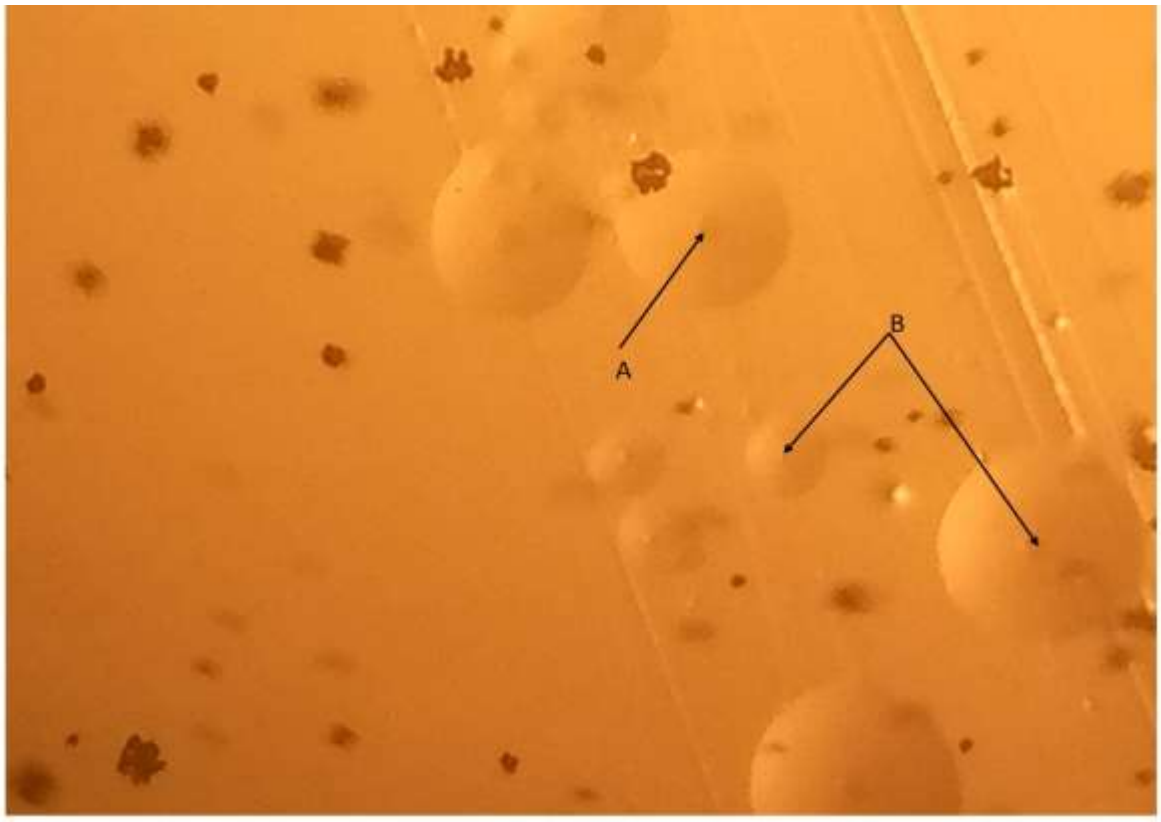

Plate V: Photograph showing *Mycoplasma mycoides* subsp. *mycoides* colonies (raised with nipple like appearance at the centre (A&B) on Pleuro-pneumonia like organism (PPLO) agar under a stereomiscrope (x4.5 magnification)

4.5 Identification and confirmation of *Mycoplasma mycoides* subsp. *mycoides* isolates by conventional Polymerase Chain Reaction

Twenty one (21) out of the twenty five (25) *Mycoplasma mycoides* subsp *mycoides* isolates cultured on PPLO agar were confirmed to be *Mycoplasma mycoides* subsp. *mycoides* by conventional Polymerase Chain Reaction. Production of a band equivalent to 1.1kbp and at the same distance with T1/44 positive control confirms Mmm (Plates VI & VII).

4.6 Multi-locus Sequence Analysis on Loc-PG1-0001.

Twenty one (21) of the PCR positive*Mycoplasma mycoides* subsp *mycoides* isolates were of the expected band size for the selected locus, Loc-PG1-0001as indicated by the production of a band size equivalent to 538bp (plate VIII). The sequences were deposited in the Genbank with the accession numbers MW487814 toMW487834 (Appendix 2).

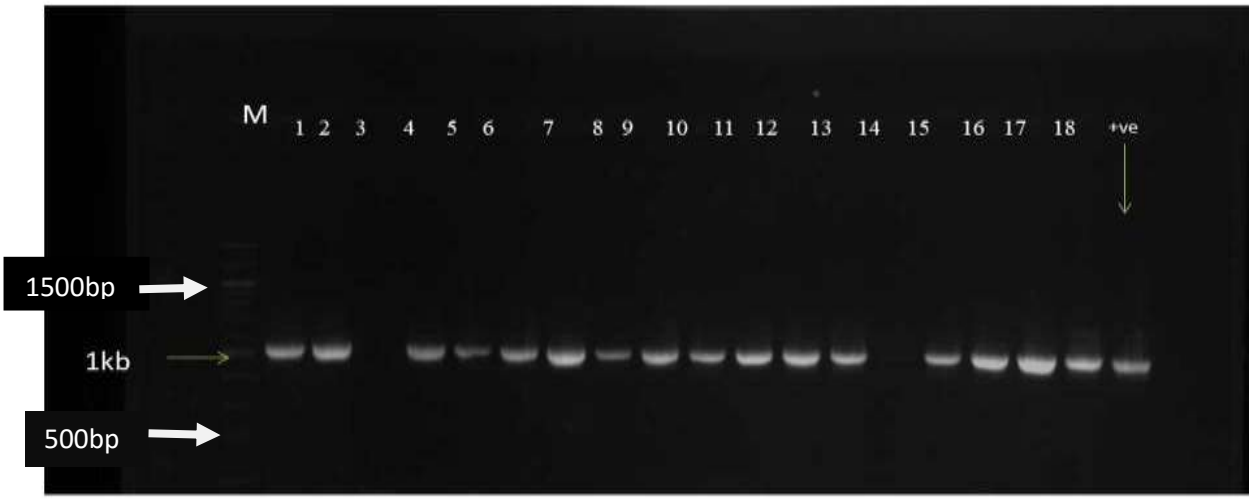

Plate VI:Mycoplasma mycoides subsp mycoides specific PCR showing 1.1kbp: lane M is the molecular marker (100), lanes 1-18 positive isolates, lane +ve for positive control (T1/44).

Plate VII:Mycoplasma mycoides subsp mycoidesspecific PCR showing 1.1kbp: lane M is the molecular marker (100bp), lane 19-21 positive isolates, lane +ve for positive control (T1/44).

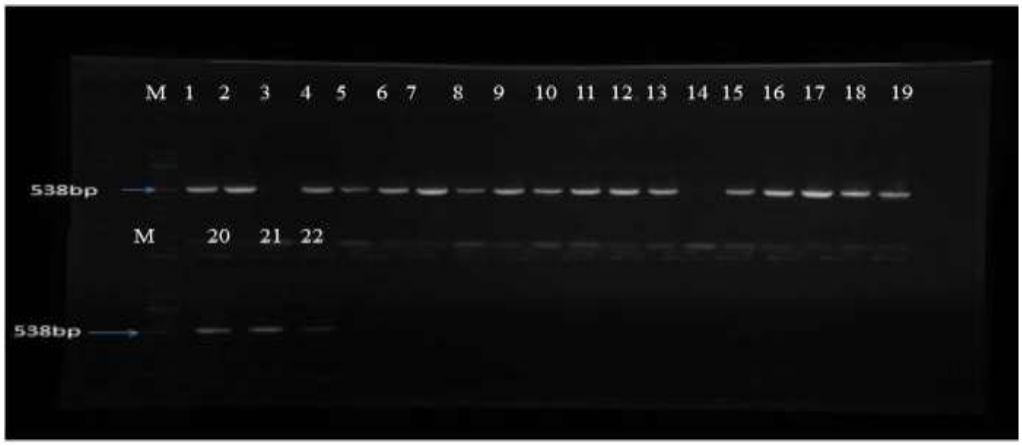

Plate VIII: PCR confirmed isolates of Mycoplasma mycoides subsp mycoides prior to sequencing with the expected band size of 538bp for the locus: Loc PG-0001. Lane M

4.6.1 Alleles defined in non-coding sequences Loc-PG1-0001

Three allelic numbers were identified on the locus Loc-PG-0001 (allele 1, 8 and 9) (Plate X and XI). There was no point mutation at position 1523 on the PG1 reference genome. All the isolates bore A (Plate X) (Allele 1). However, there were point mutations on two positions: position 1525 and 1751 on the PG1 reference genome. Isolate A8 had A at position 1525 of the PG1 genome while the others bore T (Plate X) (Allele 8). Similarly, isolate A3 had A at position 1751 while the other isolates bore T (Plate XI) (Allele 9). Allele 1 was found in both Anambra and Enugu states while Allele 8 and 9 were only found in Anambra state. Two Allele; 8 and 9 are new findings as they were not observed on the 51 strains previously described by Yaya *et al.,* (2008). Therefore, a new allelic numbers (allele number 8 and 9) were assigned to them.

4.6.2 Allelic sequence comparison of field isolates with the vaccine strain (T1/44).

After alignment of the field isolates sequences with the vaccine strain sequence (T1/44), points of mutation were observed at two positions: positions 1525 and 1750 (Plate XII). Isolates A3 and A8 bore A instead of T at position 1525 and 1750 respectively. All other isolates were identical with the vaccine (T1/44) at Loc-PG-0001 (Plate XII).

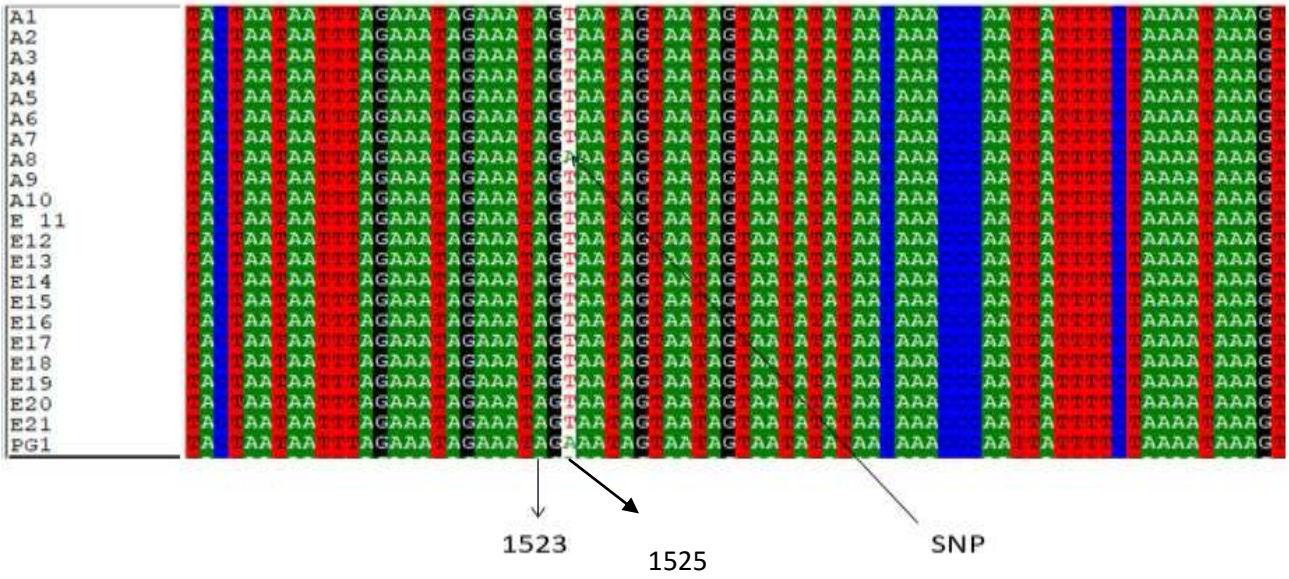

Plate IX: Alignment of sequences on locus Loc-PG1-0001: polymorphism at position 1525 of PG1 reference strain. Isolate A8 bore A while the other isolates bore T**.**

Key:

SNP: Single Nucleotide Polymorphism

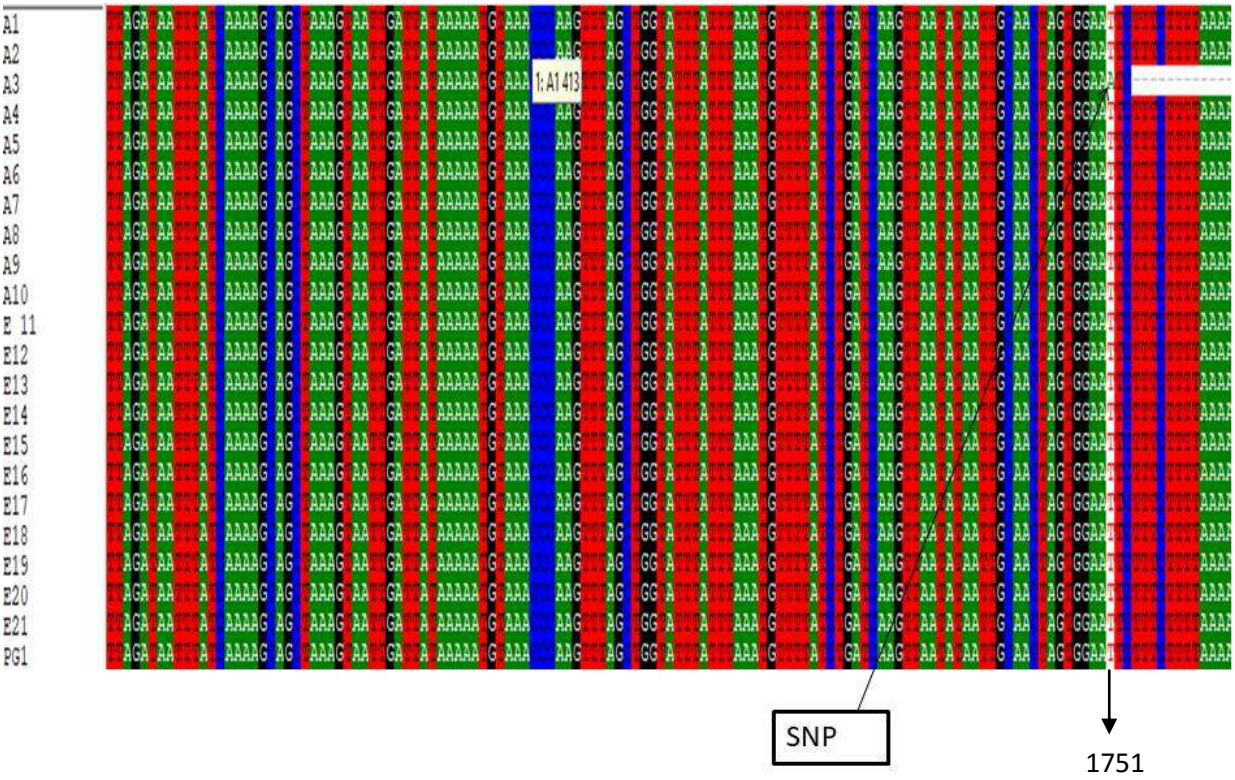

Plate X: Alignment of sequences on locus Loc-PG1-0001: polymorphism at position 1751 of PG1 reference strain. Isolate A3 had A while the other isolates bore T.

Key:

SNP: Single Nucleotide Polymorphism

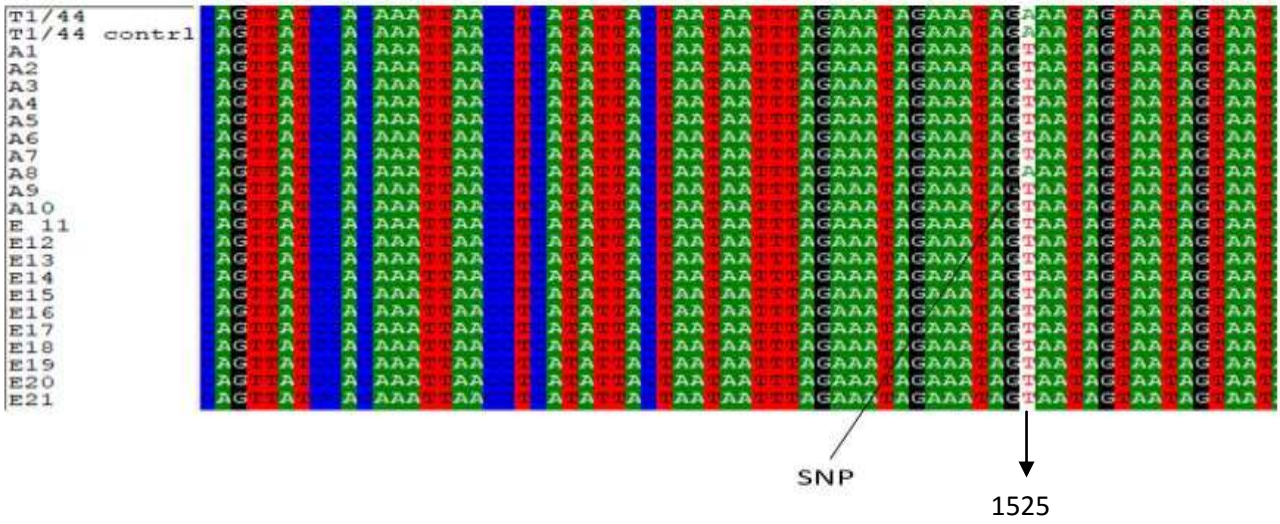

Plate XI: Alignment of sequence on the vaccine reference strain (T1/44): polymorphism at position 1525 of T1/44 reference strain. Isolate A8 had A while the other isolates bore T.

Key

SNP: Single Nucleotide Polymorphism

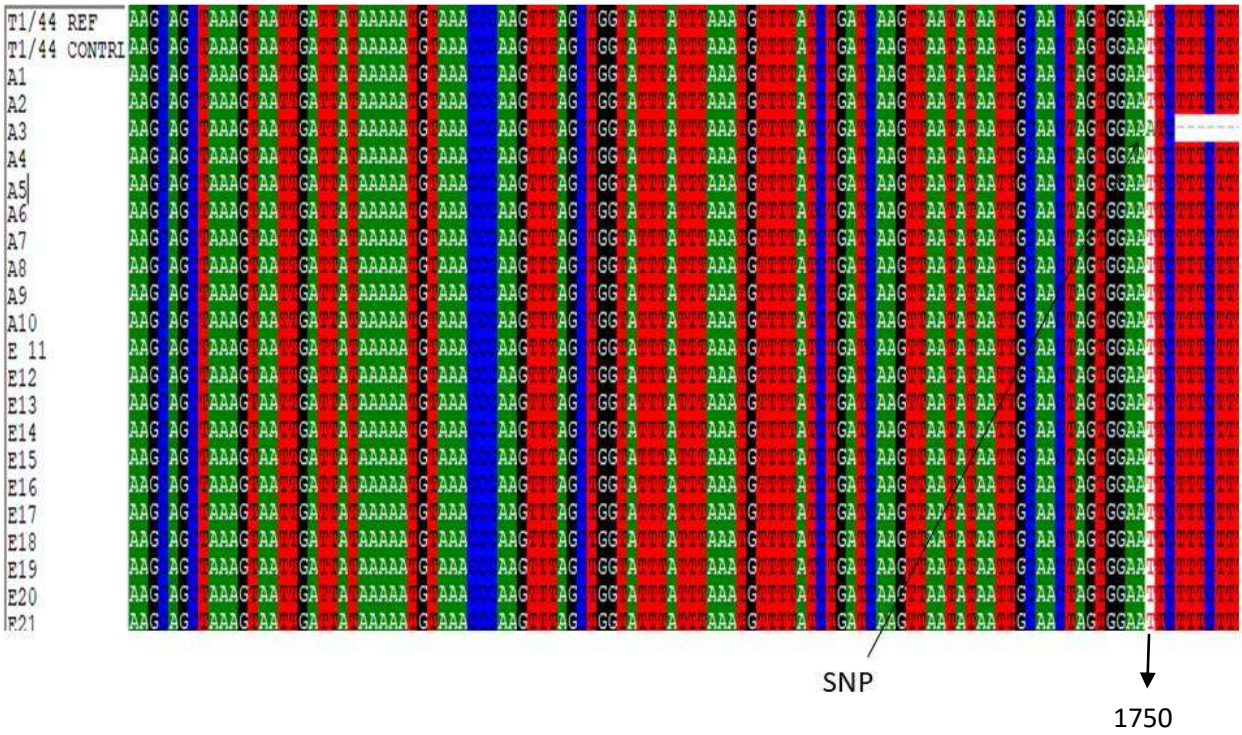

Plate XII: Alignment of sequence of vaccine strain T1/44: polymorphism at position 1750 of T1/44 reference strain. Isolate A3 bore A while the other isolates bore T.

Key:

SNP: Single Nucleotide Polymorphism

4.6.3 Awareness and Practices of Pastoralist toward CBPP in the Three South East States

Of the 90 respondents in the three selected south east states (35 in Anambra, 30 in Enugu and 25 in Imo state), 6(6%) were literate (3 in Anambra, 1 in Enugu and 2 in Imo state), with primary school education,Eighty five (94%) of the 90 respondents were aware of the existence of Contagious Bovine Pleuropneumonia (33 in Anambra, 28 in Enugu and 24 in

Imo state). Ten (11%) of the pastoralist reported previous cases of suspected CBPP outbreak in their herd (3 in Anambra, 4 in Enugu and 3 in Imo state), while 5(5%) reported loss of cattle in their herd due to CBPP (Table 4.8). Also, 80 (88%) of the respondents within the three selected states usually vaccinate their cattle with CBPP vaccine (30 from Anambra state, 28 from Enugu state and 22 from Imo state) while 10(11%) do not vaccinate at all (5 from Anambra state, 2 from Enugu state and 3 from Imo state) (Table 4.9).

Similarly, 80 (88%) of the respondents purchased their vaccine from open market while 10(11%) of the respondents purchased from National Veterinary Research Institute (Table 4.9). Seventy five (83%) of the respondents vaccinated against CBPP by themselves (29 from Anambra state, 26 from Enugu state and 20 from Imo state), 5(5%) employ the services of animal health workers (2 from Anambra state, 2 from Enugu state and 1 from Imo state) while 10 (11%) use drug vendors for vaccination (4 from Anambra state, 2 from Enugu state and 4 from Imo state) (Table 4.10). Similarly, 88(97%) of the respondents usually treat their animals with suspected case of CBPP using antibiotics (33 in Anambra, 30 in Enugu and 25 in Imo state). Also 88 (97%) of the respondents admitted using combinations of antibiotics (Oxytetracycline 20% and Tylosin tartrate), for the treatment of CBPP in their herd (33 in Anambra, 29 in Enugu and 25 in Imo state) (Table 4.11).

Table 4.8. Literacy and awareness level of respondents with regards to CBPP in the three south eastern states of Nigeria.

States	No Respondents	Level of literacy	CBPP Awareness level	Report on Recent Outbreaks of CBPP	Reported Losses in their herd
Anambra	35	3(8%)	33(94%)	3(8%)	2(5%)
Enugu	30	1(3%)	28(93%)	4(13%)	2(6%)

Imo	25	2(8%)	24(96%)	3(12%)	1(4%)
Total	90	6(6%)	85(94%)	10(11%)	5(5%)

Table 4.9: Vaccination against CBPP and sources of vaccines used by respondents, in the threeSouth eastern states of Nigeria.

Selected States against CBPP	No. of Respondents	Vaccinate	Do not Vaccinate	Sources of CBPP Vaccines	
		Open market (%)	NVRI (%)		
Anambra	35	30(85%)	5(16%)	30(85%)	5(14%)
Enugu	30	28(93%)	2(6%)	28(93%)	2(6%)
Imo	25	22(88%)	3(12%)	22(88%)	3(12%)
Total	90	80(88%)	10(11%)	80(88%)	10(11%)

Table 4.10: Personnel Used for Vaccination against CBPP by Respondents in the Three South Eastern States of Nigeria.

Selected States	No. of Respondents	Personnel used for CBPP vaccination		
		Self	Animal health workers	Drug Vendors
Anambra	35	29(82%)	2(5%)	4(11%)
Enugu	30	26(86%)	2(6%)	2(6%)
Imo	25	20(80%)	1(4%)	4(16%)
Total	90	75(83%)	5(5%)	10(11%)

Table 4.11: Management practices for CBPP among pastoralist in the three south east states of Nigeria.

Selected States	No. of Respondents	Method of management		Number of Antibiotics used for treatment	
		Treatment with antibiotics	Cull	Single	Multiple
Anambra	35	33(94%)	2(6%)	2(6%)	33(94%)
Enugu	30	30(100%)	0(0%)	1(3%)	29(97%)
Imo	25	25(100%)	0(0%)	0(0%)	25(100%)
Total	90	88(98%)	2(2%)	3(3%)	87(97%)

CHAPTER FIVE

5.0 DISCUSSION

The mean rectal temperature of the suspected CBPP infected cattle in the three selected south eastern state was 39.6ºC ± 0.1. This high rectal temperature recorded was an indication of pyrexia. This may be due to an acute CBPP infection, as the immune system of the cattle was triggered to eliminate the pathogen from the system. Pyrexia is part of the body's innate immune system, which helps in fighting microbial pathogens (Campbell, 2015). This finding is similar to report of Thiaucourt *et al.* (1996), who reported increase in rectal temperature of cattle with acute CBPP, also work done by Niang *et al.* (2007), recorded a mean rectal temperature of 40ºC in CBPP experimentally infected animal. Similarly, the observed respiratory rate in this study was 48 ± 1cpm. This high respiratory rate may be caused by difficulty in breathing by the affected animals due to the lesions in the respiratory system in CBPP infection such as accumulation of pleural fluid and inflammation of the lungs. This finding is in agreement with studies by Niang *et al.,* (2007), who recorded high average respiratory rate of 55cpm in CBPP experimentally infected cattle. The clinical signs observed in this study; congested ocular mucus membrane,

extension of the neck and abduction of forelimbs, coughing especially when exercised, rapid difficult breathing, lethargy and emaciation in suspected CBPP infected cattle in this study are consistent with reports by several authors (Martel *et al.,* 1985; Nunes *et al.,* 1990; Laak *et al.,* 1992; Thiaucourt *et al.,* 1996; Niang *et al.,* 2007). Congested ocular mucus membrane observed in some of the infected cattle maybe as a result of septicaemia due to the presence of the organism in circulation. Similarly, extension of the neck and abduction of the forelimbs are among the cardinal signs of CBPP. This is due to difficulty in breathing by the infected animal, largely caused by inflammation of the lungs and pleural fluid accumulation. Consequently, the infected animal assumes such characteristic stance in order to allow the chest to expand maximally, and open its mouth to ease breathing. Furthermore, coughing especially when exercised as observed in the suspected CBPP infected cattle in the study area could be due toaccumulation of pleural fluid in the thoracic cavity which can cause blockage of air passages thereby triggering cough in the infected animals. Cough is one of the cardinal signs of CBPP especially at the acute and chronic stages of the disease, it's an attempt by the system to clear the air ways (Thiaucourt *et al.,* 1996). Lethargy and difficulty in climbing elevations during grazing was also observed in some of the suspected animals in the study area, this may be due to the pressure exerted on the thoracic region where most of the lesions associated with this condition are localized. Consequently, infected animals may experience pain during movement thereby leading to difficulty in walking or climbing elevated surfaces during grazing. Emaciation was also observed in some of the suspected CBPP infected cattle. This may be largely due to poor feeding associated with the difficulty in grazing due to the pain experienced by infected animals. Infected cattle become anorexic and are unable to graze.

The gross lesions (lung adhesion to thoracic wall, pleural fluid collection in the thoracic cavity, marbled appearance of the lungs and sequestrum formation) observed at post mortem in this study are also in agreement with several reports by different authors (Nunes *et al.,* 1990; Laak *et al.,* 1992; Thiaucourt *et al.,* 1996). For instance, all the lesions observed in the lungs were unilateral and confined to the thoracic cavity; as only one lung lobe (diaphragmatic lobe) was seen to be affected. This is similar to findings by Nunes *et al.,* 1990, who also reported that out of 566 CBPP affected lungs sampled in Portugal, 95% of them were unilaterally affected. Similarly, the accumulation of up to 10 litres of pleural fluid in the thoracic cavity of CBPP infected cattle is also in agreement with reports by Thiaucourt *et al.*, (1996) and Niang *et al.,* (2007). The accumulation of pleural fluid is usually as a result of inflammation of the lung alveoli and uptake of alveolar fluid into tissue spaces. More so, the lung hepatization (marbled appearance of the lungs) observed in this study is largely due to the distension of the interlobular septa of the lungs with fluid caused by inflammatory reactions. This fluid separates the lung lobules thereby giving the marble appearance of the affected lungs when cut open. Furthermore, the sequestrum observed in some of the CBPP infected cattle in this study may be an indication of a chronic case. Usually, in chronic or advanced CBPP, a sequestrum is formed as an immune response to engulf and eliminate the pathogen. It usually consists of necrotic lung parenchyma surrounded by a fibrous capsule (Trichard *et al.,* 1989; Satini *et al.*, 1992).

The overall seroprevalence of CBPP using c-ELISA in the study area was 59.4%. This high prevalence recorded in the study area may be attributed to several factors such as; breakdown in veterinary services within this region (personal experience). This has adversely affected the quality of veterinary services within this region and as such most of

the herdsmen/cattle owners resort to self-help in the management of the disease. Similarly, indiscriminate use of antibiotics by herdsmen/pastoralist for the treatment of CBPP as reported by Egwu *et al.* (2012) could also account for the high prevalence recorded in the study. Indiscriminate use of these antibiotics could also lead to the establishment of chronic carriers of the disease, thereby facilitating the spread of the disease to susceptible animals within the herd. In Nigeria, "a live with the disease" attitude has prevailed over the past five years; farmers rarely report cases of CBPP but resort to treatment with antibiotics like any other bacterial disease (Chima *et al.,* 2001). Furthermore, lack of access to quality and well preserved CBPP vaccines by herdsmen in the study area could also account for the high prevalence of CBPP recorded in this study, as most of them purchase CBPP vaccines from the open markets rather than NVRI outstations within the region. This agrees with findings by Ishaya, (2014), who reported high patronage of CBPP vaccine from open market by pastoralists. Similarly, improper vaccination practises under-taken by the herdsmen could also attribute to the high seroprevalence of CBPP recorded in this study, as some of the herdsmen engage in the act of mixing different vaccine types within the same bottle before vaccinating their cattle. This practice could affect the potency of the vaccine due to under dosage of the vaccine and wrong route of vaccination. The high seroprevalence of CBPP recorded in the study area, could also be because apparently healthy animals not showing clinical signs of the disease were sampled. Consequently, some infected animals within the incubatory stage of the disease and also those apparently recovered from CBPP (lungers) would have been sampled. The high prevalence of CBPP recorded in this region may indicate the endemicity of the disease in South East Nigeria. This is in agreement with the annual report by OIE (2009) that CBPP is gradually spreading across Nigerian states from the Northern Region to other regions because of constant migration of cattle herders. It is

noteworthy to state that this is the first time such study was undertaken within the south eastern region of Nigeria using this method. Most of the cases recorded in this study are likely due to natural infection as vaccination history (cattle not vaccinated against CBPP within 3 months) was taken before sample collection. More so, this technique is capable of detecting field infections even in areas where vaccination is practised as antibodies produced when T1/44 vaccine is administered to animals do not persist for more than 3 months (Le Goff and Thiaucourt, 1998). The high prevalence observed in this study is similar to reports by Olorunshola *et al.* (2020), who recorded a high prevalence rate of 56.2% in the Middle-Belt region of Nigeria. Nwankpa (2008), also reported high prevalence of CBPP in some Northern states of Nigeria such as; Bauchi State with 32.4%, Gombe State (50.5%) and Taraba State (40%) CBPP seroprevalence rate. Similarly, Danbirni *et al* (2010) reported a high CBPP seroprevalence of 47% in Kaduna State. This may be attributed to the endemicity of CBPP within the Northern region of the country where most of the cattle population is located (Egwu *et al.,* 1996; Nwankpa, 2008). However, this finding is in contrast with those of Olabode *et al*. (2013) who reported a lower CBPP prevalence of 10.65% in Kwara State and Jasini *et al.*, (2015) who also reported CBPP prevalence rate of 0.63% in Borno state. This lower prevalence may be attributed to the absence of infection at the point of sampling and the smaller number of sera samples collected for screening of *Mmm* antibodies. There was higher prevalence ($P \leq 0.05$) of CBPP observed in cows compared to bulls in this study. This finding is in agreement with reports by Nwankpa (2008) and Ishaya (2014) who both reported higher prevalence in cows than bulls. However, this is not in agreement with works by Ankeli *et al.* (2017) who reported higher prevalence of CBPP in bulls than cows. This high prevalence could be because fewer bulls are usually kept in the herd than the cows. Most of

the cattle owners fatten and sell most of the bulls in the herd so as to prevent competition during breeding. It could also be due to the fact that the female animals stay longer within the herd, thereby increasing the chances of repeated exposure to the disease.Similarly, there was higher seroprevalence ($P \leq 0.05$) observed in cattle above 1 year of age as compared to those below 1 year. The higher prevalence observed, could be because of the higher number of adult cattle sampled within the study region.

The overall Mmmisolation rate in this study was 27.7%. This high isolation rate could be attributed to the fact that purposive sampling method was used for this study. Only apparently sick animals showing classical clinical signs and gross lesions suggestive of CBPP at slaughter were sampled. Pleural fluid accumulation is one of the characteristic of CBPP and serves as the best medium for the isolation of *Mycoplasma mycoides* subsp. *mycoides* (Thiaucourt *et al*., 2006). Anambra state recorded the highest isolation rate of Mmm. Possibly due to the fact that more animals showing classical gross lesions of CBPP at slaughter were sampled in Anambra state (n= 35) followed by Enugu State (n =30). There was no isolate from Imo state. This could be because most of the cattle sampled were apparently healthy, showing no classical signs of CBPP at slaughter. The isolation rate of 27.7% in this study is higher than that reported by Jasini *et al.,* (2016), who recorded an overall isolation rate of 3.33% in North eastern Nigeria. Similarly, Ankeli *et al.* (2017), recorded 1.81% in Plateau state and Nick Nwakpa, (2008) who recorded 6.27% within Northern Nigeria. Ikpa *et al.* (2020) also recorded a 4% isolation rate of Mmm in Nasarawa State, Nigeria.

Out of the total of 25Mmmisolates based on growth on PPLO agar, 21 isolates were confirmed to be *Mycoplasmamycoides* subsp. *mycoides* by conventional polymerase chain

reaction (PCR) using the protocol by Miles *et al.* (2006). The primer targets the house keeping genes (*CAP- 21* gene) of Mmm, which is a conserved gene that is present in all the Mycoplasma species. The PCR amplification of the isolates yielded molecular size of 1.1kbp specific for Mmm. This is in agreement with findings by Miles *et al.,* 2006. Generally, species within the *Mycoplasma mycoides* cluster share many immunological, biochemical and genetic properties, which result in major problems for diagnostic laboratories in the identification of field strains (Cottew *et al.,* 1987; Persson, 2002). Perhaps, some of the positive isolates on PPLO agar were *Mycoplasma* species within the *Mycoplasma* cluster as the isolates were not subjected to biochemical tests. The use of PCR to detect *Mycoplasma* species from various clinical samples has demonstrated a higher efficiency, specificity, and sensitivity for laboratory diagnosis when compared with conventional culture- based diagnostic methodologies (Bashiruddin *et al.,* 1994). PCR has the advantage of being a fast, specific, and very sensitive technique (Bashiruddin *et al.,* 1994; Dedieu *et al.,* 1994; Miles *et al.,* 2006).

Furthermore, the isolates in this study were characterized using Multi-locus sequence analysis (MLSA). Three allelic numbers were obtained in this study (Allele 1, 8 and 9). Allele 1 was found in both Anambra state and Enugu state while Allele number 8 and 9 were identified only in Anambra State. The isolate in this study, Allele 1, was earlier reported by Nwakpa, (2008) in Northern Nigeria in Kaduna and Taraba States. This could be possible, as most cattle slaughtered in south eastern region of Nigeria are brought from the Northern parts of the country (Egwu *et al.,* 1996). From this study, we realized that most of the cattle slaughtered at the abattoirs were brought in from the Northern parts of the country such as Taraba, Kaduna and Gombe states. This could explain why Allele 1 earlier

identified by Nwakpa, (2008) in Northern Nigeria was also found in the south eastern region. However, Allele 8 and 9 are new strains ofMmm that have not been earlier reported in both the works of Yaya *et al.* (2008) and Nwakpa, (2008). This could be new strains of *Mmm* circulating within the south eastern region of Nigeria. The finding of two new allele which have not been described before in the previous works of Nwakpa, (2008) and Yaya *et al.*, (2008) is a significant observation in this study. These new Allele numbers (1, 8 and 9) identified in this study, may be under profile A strains according to groupings by Yaya *et al*, (2008), which established thirty one (31) allelic profile using eight (8) loci. These profiles are divided into seven groups: A, B, C, D, E, F and G. Profile A had the largest number of strains with thirteen (13) profiles and are from African origin especially from West Africa.

The observed points of mutation between the field isolates and the vaccine reference strain (T1/44)in this study, may account for the in-effectiveness of the vaccine (T1/44) to protect cattle from field infections as reported by Yaya *et al.* (1999), Thiaucourt *et al.*, (2000a) Thiaucourt *et al.* (2006). For instance, isolate A3 from Anambra state bore A at position 1560 of the T1/44 reference strain while other isolates bore T. Perhaps, this change in Nucleotide could account for failure of the vaccine to confer maximum immunity against CBPP. Also, Folashade (2015), reported difference between the vaccine strain and field strain using restriction fragment length polymorphism. Similarly, Nwakpa, (2008) also reported differences between the sequences of the field isolates and the vaccine strain (T1/44), and observed points of mutation among some of the field isolates from Northern Nigeria and the vaccine strain T1/44. The efficacy of CBPP vaccine in protecting cattle against infection has been widely studied (Yaya *et al.*, 1999; Thiaucourt *et al.*, 2000a;

Wesonga and Thiaucourt, 2000). Some of these studies have indicated the inability of the T1/44 vaccine to effectively protect cattle against CBPP (March *et al.*, 2002).

This study established 94% awareness of CBPP by pastoralist in the study area. This could be as a result of the endemic nature of the disease and the frequent outbreak and spread of the disease within different herds in the region. Most of the cattle herds sampled within this region share the same watering source, thereby increasing the spread of the disease. This finding is similar to report by Ishaya *et al.*, 2014, who recorded 88.9% awareness rate of CBPP in Kaduna State and Tambuwal *et al.*, (2011a) who also recorded an awareness level of 65% in two north western states of Sokoto and Kano. Similarly, 88% of the respondents usually vaccinate their animals with CBPP vaccine, while 11% do not vaccinate. 83% of the respondent vaccinates their animals against CBPP by themselves. This high percentage of vaccination coverage could be as a result of the high awareness of the disease within the region and its economic implication; these pastoralists therefore, attempt to protect their animals by vaccination. However, wrong vaccination practices such as, administration of improperly preserved vaccines, administration through wrong route, inadequate dose of vaccine and mixing of different types of vaccines in one bottle before vaccination can affect the potency of the vaccine thereby affecting the overall efficiency of the vaccination. More so, 88% of the respondents within the study area purchased CBPP vaccine from the open market. This could be as a result of inadequate / un-availability of CBPP vaccines within the different veterinary outstations in the region and this could further facilitate the spread of the disease within the region as improperly preserved/expired vaccines can be sold to them at the open market.

Furthermore, 97% of pastoralists in this study usually treat their CBPP infected animals with antibiotic. Perhaps, an attempt to cure the disease by the pastoralist and this is due to the little knowledge of the negative effects of indiscriminate antibiotics use in the management of CBPP. Several studies have reported that CBPP infected animals treated with antibiotics may result in the development of lungers, thereby facilitating the spread of the disease (Huebschle *et al.*, 2004; Yaya *et al.*, 2004; Ayling *et al.*, 2007) This result is similar to findings by Ishaya *et al.*, (2014), who observed that 76% of CBPP outbreak in Kaduna state were treated with antibiotics. Similarly, Huebschle *et al.*, (2004) also reported 69% of the farmers in Ethiopia admitted treating CBPP infected animals with antibiotics.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The following conclusions were obtained from this study;

i. Clinical signs of suspected CBPP infected animals were; fever, congested ocular mucus membrane, cough, extended neck and abduction of forelimbs, rapid laboured breathing, lethargy, emaciation and gross lesions; lung consolidation, pleural fluid accumulation, lung adhesion to the thoracic wall of infected cattle.

ii. The over-all sero-prevalence of CBPP using c-ELISAin the three selected south east state of Nigeria was 59.4%

iii. The isolation rate of *Mycoplasma mycoides* subsp. *mycoides* on PPLO agar within the three selected south east states of Nigeria was 27.7%

iv. Twenty one (21) isolates from the three selected South-east states of Nigeria were confirmed to be *Mycoplasma mycoides* subsp. *mycoides* using Polymerase Chain Reaction.

v. The Mmm isolates were characterized into three allelic number; 1, 8 and 9 using Multi-locus sequence analysis on Loc-PG-0001.

vi. Points of mutation were observed between the field isolates in the study area and the vaccine strain T1/44.

vii. There was high awareness (94%) of CBPP by the Livestock owners in the three selected south east states of Nigeria.

6.2 Recommendations

The following recommendations are hereby made:

i. Similar studies should be extended to other parts of the country so as to identify the circulating strains of *Mycoplasmamycoides* subsp. *mycoides* in Nigeria.

ii. Emphasis should be placed on yearly vaccination campaign against CBPP within the south eastern states of Nigeria

iii. Veterinary outstations within the south east region should be provided with adequate CBPP vaccines to enable veterinary health workers have access to quality vaccine for effective vaccination campaign in the study area.

iv. Public awareness/education on the negative effects of the use of antibiotics in the treatment of the disease should be organized routinely within the study area

v. Further genotypic studies should be conducted to ascertain the implication of the observed polymorphism between the field *Mycoplasma mycoides* subsp. *mycoides* isolates and the vaccine strain vis a viz the efficacy of the vaccines to confer solid immunity.

6.3 Contribution to Knowledge

1. This study, for the first time, to the best of our knowledge, established the seroprevalence of CBPP in South Eastern Nigeria. This can be used as baseline data for future studies on CBPP in the region.
2. *Mycoplasma mycoides* subsp. *mycoides* from south east Nigeria, was characterized into three allelic numbers. Allele 1, 8 and 9.
3. This study established differences at position 1525 and 1750 of the reference T1/44 vaccine strain sequences and the field strains of *Mycoplasma mycoides* subsp. *mycoides* in south east Nigeria.

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APPENDICES

Appendix 1:

STRUCTURED QUESTIONNAIRE FOR PASTORALIST ON CONTAGIOUS BOVINE PLEUROPNEUMONIA (CBPP) IN THREE SELECTED SOUTH EAST STATES OF NIGERIA.

DEMOGRAPHY

1. Name of respondent (optional)……
2. State: Anambra ( ), Enugu ( ), Imo ( ).
3. Occupation: Butcher ( ), Livestock owner ( ), Pastoralist ( ).
4. Sex: Male ( ) Female ( ).
5. Level of education: Primary ( ), Secondary ( ), Tertiary ( ), Non ( ).
6. Breed of cattle…….

SECTION II

1. Are u aware of CBPP? Yes ( ) or No ( ).
2. If yes, what are the clinical signs you observed?

   a…….

   b…….

   c…….

   d…….
3. Have you had any outbreak of CBPP? Yes ( ) or No ( ).
4. If yes in 3, how often do you encounter CBPP? Often ( ), Occasional ( ), Never ( ).
5. If yes in 3, what do you do about it? Treat ( ), Cull ( ), Vaccinate ( ), Report ( )
6. What drug do you use in treatment? Antibiotics ( ) Others ( )
7. If yes in 6 above, how many? Single (), Multiple ().
8. How do you source your vaccine? Open market ( ), Drug store ( ), NVRI ( ).
9. How often do you vaccinate? Annually ( ), Quarterly ( ), During Outbreak ( ).
10. Personnel used for vaccination/treatment? Self ( ), Veterinarian ( ), Quacks ( ).
11. What is the source of water for the cattle? Pond ( ), Rivers ( ), Well ( ) Borehole ( ).
12. What is the local name of the disease…….

Appendix 2: Gene bank Accession Numbers

The sequences were successfully deposited in the Gene Bank with the under-listed accession numbers:

BankIt2418453 seq1	MW487814
BankIt2418453 seq2	MW487815
BankIt2418453 seq3	MW487816
BankIt2418453 seq4	MW487817
BankIt2418453 seq5	MW487818
BankIt2418453 seq6	MW487819
BankIt2418453 seq7	MW487820
BankIt2418453 seq8	MW487821
BankIt2418453 seq9	MW487822
BankIt2418453 seq10	MW487823
BankIt2418453 seq11	MW487824
BankIt2418453 seq12	MW487825
BankIt2418453 seq13	MW487826
BankIt2418453 seq14	MW487827
BankIt2418453 seq15	MW487828
BankIt2418453 seq16	MW487829
BankIt2418453 seq17	MW487830
BankIt2418453 seq18	MW487831
BankIt2418453 seq19	MW487832

BankIt2418453 seq20 MW487833
BankIt2418453 seq21 MW487834