MOLECULAR CHARACTERIZATION OF *MYCOPLASMA BOVIS* AND *MYCOPLASMA MYCOIDES* SUBSPECIES *MYCOIDES* FROM SLAUGHTERED CATTLE IN ADAMAWA AND TARABA STATES, NORTH-EASTERN NIGERIA

BY

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NOVEMBER, 2019

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P14VTPM9002

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

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NOVEMBER, 2019

DECLARATION

I declare that the work in this thesis entitled: "**Molecular characterization of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides* from slaughtered cattle in Adamawa and Taraba States, North-Eastern Nigeria**", has been performed by me in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, under the supervision of Prof. C. N. Kwanashie, Dr. J. Adamu and Prof. L. Allam. The information derived from literature have been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for the award of degree or diploma at any university.

Markus Isa FRANCIS …… ……..

Signature Date

CERTIFICATION

This thesis entitled, "MOLECULAR CHARACTERIZATION OF *MYCOPLASMA BOVIS* AND *MYCOPLASMA MYCOIDES* SUBSPECIES *MYCOIDES* FROM SLAUGHTERED CATTLE IN ADAMAWA AND TARABA STATES, NORTH-EASTERN NIGERIA" by **Markus Isa FRANCIS** meets the regulations governing the award of the degree of **Doctor of Philosophy** in Veterinary Microbiology of the Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

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DEDICATION

I dedicate this thesis to God Almighty for His love over my life; my parents **Mr and Mrs Francis Shamaki** for their encouragement; my beloved wife **Mrs Agnes Francis** for her love, encouragement, prayer, support through all the years; and my children **Kedwalya Manasseh Francis** and **Nyampimuya Michal Francis** who often had to give up some of the pleasures of life as I followed a dream.

ACKNOWLEDGEMENTS

I sincerely want to acknowledge God Almighty the Giver and Sustainer of life for seeing me through my Ph.D Programme. He gave me good health, knowledge, wisdom, and strength in the course of this programme. I cannot forget His divine and miraculous provision of my basic needs as well as the funds needed to carry out this research. I thank Him for giving me the grace to contribute to the benefit of mankind.

From the bottom of my heart, I sincerely appreciate the warmth and liberality of my supervisors: Prof. C. N. Kwanashie, Dr. J. Adamu and Prof. L. Allam for accepting me as their student and for the constant support, dedication, constructive criticisms, advice, and providing directions during the research and writing of this thesis as well as encouragement during the work. Your corrections and inputs have made this work of professional standard. I am indeed indebted to you, my team of supervisors. The effort of Prof. M. A Raji at the initial stage of this research work is highly acknowledged.

I would like to thank the National Veterinary Research Institute (NVRI) Vom for giving me bench space for primary isolation of samples and storage of my isolates. Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM) "G. Caporale", Teramo, Italy for offering me free bench space. Italian Ministry of Health, for financing the molecular aspect of this research. My employer, Adamawa State Government for giving me study leave.

I cannot forget the love, care, support and prayers offered on my behalf from my parents Mr. and Mrs. Francis Shamaki, your financial and moral support throughout my educational pursuit is highly recognized and appreciated. May God keep and lengthen your days on earth. To my siblings, Paulina, Zachary, John, Rhoda, Safiratu, thank you for standing with me even at toughest of times. I cannot forget the warm love and care of my darling wife Agnes Francis and children Kedwalya Manasseh Francis and Nyampimuya Michal Francis, for their encouragement, understanding, and support and being there despite tight schedules.

I want to acknowledge the warmth and concerns of the Head, Department of Veterinary Microbiology, Dr J. Adamu. May the Lord reward you. In the same vein, I wish to appreciate the moral support offered me from academic staff of the Faculty, worth mentioning are Profs. J. K. P. Kwaga, C. A Kudi, H. M Kazeem, S. Adamu, B. Mohammed, S. B. Oladele, J. O. Ayo, A. K. B. Sackey, L. B. Tekdek, S. U. Abdullahi and. S. O. Okaiyeto, Drs. J. S. Dalis, P. H. Mamman, and J. S. Sambo.

My acknowledgement will not be complete if mention is not made of my friends, colleagues and class/school mates. Their encouragements and assistance in no small measure contributed to my graduation. These include, Drs. Joel Japhet, Emmanuel T. Vandi, Joshua Shallangwa, David Oshadu, Caleb S. Kilyobas, Japhet Daniel, Francis Ejeh, Jasini Musa, Peter Ehizibolo, Emmanuel Ngbede, Adam Mohammed, Kabiru H. Bakura, Lt. (Dr) David Pindar among others. Mention must also be made of Obadiah John, Victor Adiel, Othniel Ishaku, Innocent Demuel, Philip Daniel, Godiya Daniel,

John Gundiri, Hezron Luka, Benjamin Christopher, Cletus Williams, your prayers/ time spent with me especially during laboratory phase of my work is highly appreciated. Mr/Mrs Philip H. Jalo, Rev/Mrs Jonathan Jampeo, Mr/Mrs Parisa H. Piya, Mr/Mrs Dennis Dakare, Dr/Mrs Markus Susari, Mr/Mrs Christopher Barnabas, Engr/Mrs Joshua Pogu, I am grateful for your advice and encouragement to me at various points.

Thanks to staff of Mycoplasma Laboratory of Bacterial Research Division, National Veterinary Research Institute Vom for your enormous support and time spared during the laboratory phase of my work. I acknowledge your personal assistance, Mr. Sunday Kigbu, Maichibi Mugla, Drs. Paul Ankeli, Livinus Ikpa thanks for your cooperation. Also to members of staff of the Department of Veterinary Pathology and Microbiology, ABU, Zaria, Mr Albert, Mal Dodo, Mr Bitrus (late). I appreciate your support and assistance. I am grateful to you.

I wish also to thank Dr. Massimo Scacchia, the Head of OIE Reference Laboratory for Contagious Bovine Pleuropneumonia (CBPP) at Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", Teramo, Italy. The technical efforts of Drs. Flavio Sacchini, Katiuscia Zilli, Massimiliano Orsini, Massimo Ancora, Cesare Camma, Marta Di Federico and Ivanka Krasteva for accepting, recovering and purifying the isolates as well as carrying out the identification, sequencing and analysis of the sequenced data. Their technical skills, ideas, determination, and commitment made this work worthwhile.

Lastly, I wish to sincerely appreciate the entire members of Unity Chapel Adasolid Estate Jimeta/Yola, for standing with me and the spiritual support from the Pastors and everyone is highly appreciated. Thank you very much.

ABSTRACT

Mycoplasma bovis (*M. bovis*) and *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) are the two most important pathogenic species of mycoplasmas of cattle. This study was aimed to isolate, identify and confirm *M. bovis* and *Mmm* by culture and PCR as well as to characterize the genomes of identified *Mmm* isolates from cattle in Adamawa and Taraba States, Nigeria. A total of 480 samples of lung tissues (180), pleural fluid (180), nasal swab (60) and ear swab (60) were collected from 190 heads of cattle at slaughter in two abattoirs, namely; Yola Modern Abattoir and Jalingo Abbatoir in Adamawa and Taraba States respectively. Samples were processed based on standard laboratory protocols. Identification and confirmation were done using standard techniques. Genomic DNA was extracted using Maxwell 16 Tissue/cell DNA purification kit and sequenced on Illumina NextSeq^{TM} 500 platform. Reads were assembled by a *de novo* strategy using the SPAdes, while strains comparison was performed by a gene-by-gene approach. Pangenome analysis was performed with USEARCH and virulence factors were identified by similarity searching against a specialized virulence factor database. Thirty nine (8.13%) *Mycoplasma* species were isolated from the samples, 25 (8.80%) and 14 (7.14%) from Adamawa and Taraba States respectively. Four (0.83%; 4/480) isolates were identified as *M. bovis*. Out of these, 1 (0.91%) was isolated from pleural fluid in Adamawa State, whereas 2 (2.86%) and 1 (3.57%) were respectively isolated from lungs and ear swab samples in Taraba State. Similarly, 33 (6.87%; 3/380) of the isolates were identified as *Mmm*. Out of these, 12 (10.91%) were isolated from both lung tissues and pleural fluid in Adamawa State, whereas, 5 (7.14%) and 4 (5.71%) were isolated respectively from lung tissues and pleural fluid in Taraba State. No *Mmm* was isolated from nasal and ear swab samples in the study area. Histopathological examination of the positive lung showed severe congestion and fibrin exudation into

interalveolar spaces with the collapse of almost all the alveoli. Four *M. bovis* isolates were confirmed positive by PCR with the presence of one band of 734-bp; two isolates from lung tissues and one isolate from both pleural fluid and ear canal. All the 33 *Mmm* isolates were confirmed to be *Mycoplasma mycoides* subspecies based on amplification of CAP-21 genomic region yielding a band size of 574-bp. Following digestion of the amplicon with restriction endonuclease *Vsp*1, the production of two restricted fragments of 180-bp and 380-bp indicated typical fingerprinting pattern of *Mmm*. Whole genome sequencing was undertaken for the 20 field strains of *Mmm* isolates, comprising of thirteen isolates from Adamawa State and seven from Taraba State. The 20 genome assemblies were highly similar and resulted in 19 and 115 contigs with N50 value from 25,646 to 119,472. The total length of the genomes varied by approximately 22,000-bp and were between 1,180,728-bp and 1,202,919-bp per genome. Smallest genome size of 1,180,728-bp was observed in strain AL103 and largest genome size of 1,202,919-bp in strain AP68b. The G+C content of the *Mmm* strains varied between 23.92% and 24.03% and the genomes harboured about 2,053 to 2,127 predicted protein-coding genes per genome. The annotation of the genome revealed CDS between 2,016 and 2,087, and between 29 and 33 tRNA were identified per genome. Six (6) rRNA was identified per genome, 2 each of 5S rRNA, 16S rRNA and 23S rRNA. Genomic comparisons of the 20 field strains of *Mmm* revealed high orthologous average nucleotide identity (OrthoANI) values of between 99.59 and 99.92%. BLAST nucleotide comparison showed 90-100% relatedness of these strains to vaccine strain T1/44. The pan-genome of the strains of *Mmm* contains 3,081 protein-coding genes which comprised of 1,707 core genes (55.4% of the pangenome) and 1,374 accessory genes (44.6% of the pangenome). Functional annotation of the 20 *Mmm* gene products assigned a COG category for just 1,198 ortholog cluster of proteins occurring mainly in the core genome. Phylogenetic tree of

the 20 strains showed 3 distinct phylogroups of 8, 4 and 8 strains MAT-1, MAT-2 and MAT-3 respectively and were confirmed by a Neighbor-Joining tree. The 20 field strains of *Mmm* had a virulence factor range between 9 and 25 and virulence genes varying between 11 and 68 with some strain (AP103, AP68b, AL90, AL107) harbouring high number of virulence factors. In conclusion, this work has established the presence of *M. bovis* and *Mmm* in Adamawa and Taraba States, Nigeria. We thus recommend large scale epidemiological studies and genomic analysis of *Mmm* strains circulating in other States of Nigeria.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENIDICES

Appendix **Title** **Page**

LIST OF ABBREVIATIONS

AA	-	Amino acid
AI	-	Artificial insemination
AL	-	Adamawa lung
AP	-	Adamawa pleural fluid
AU-IBAR	-	African Union-Interafrican Bureau for Animal Resources
BAL	-	Bronchpo alveolar lavage
BHV-1	-	Bovine herpes virus type-1
BLAST	-	Basic local alignment search tool
BRD	-	Bovine respiratory disease
BRIG	-	BLAST ring image generator
BRSV	-	Bovine respiratory syncytial virus
BVDV	-	Bovine viral diarrhea virus
CAPs	-	Cleaved amplified polymorphic sequence
CBPP	-	Contagious bovine pleuropneumonia
CDS	-	Coding sequences
CFT	-	Complement fixation test
c-ELISA	-	Competitive enzyme-linked immunosorbent assay
COG	-	Clusters of orthologous group
CPPS	-	Chronic pneumonia and polyarthritis syndrome
EBL	-	Embryonic bovine lung
DNA	-	Deoxyribonucleic acid
EPS	-	Exopolysaccharide
FAO	-	Food and Agriculture Organisation

G+C	-	Guanine plus cytocine
GlpO	-	Glycerol phosphate oxidase
H&E	-	Haematoxylin and Eosin
H_2O_2	-	Hydrogen per oxide
IBR	-	Infectious bovine rhinotracheitis
IBT	-	Immunoblotting
ICE	-	Integrated and conjugated element
IFN-γ	-	Interferon gamma
IHC	-	Immunohistochemistry
IS	-	Insertion sequence
IZSAM	-	Istuto Zooprofilattico Sperimentale dell' Abruzzo e del Molise
LAT	-	Latex agglutination test
Lpp	-	Lipoprotein
Mab	-	Monoclonal antibody
MAT	-	Markus Adamawa Taraba
M. bovis	-	*Mycoplasma bovis*
MLSA	-	Multilocus sequence analysis
Mmm	-	*Mycoplasma mycoides* subspecies *mycoides*
MoI	-	Multiplicity of infection
NCBI	-	National Centre for Biotechnoogy Information
NGS	-	Next Generation Sequencing
NK	-	Natural killer cells
NVRI	-	National Veterinary Research Institute
OIE	-	Office of International Epizootics
ORF	-	Open reading frame

OrthoANI	-	Orthologous average nucleotide identity
PBMC	-	Peripheral blood mononuclear cell
PCR	-	Polymerase chain reaction
PFGE	-	Pulse field gel electrophoresis
PIV	-	Parainfluenza virus
PPLO	-	Pleuropneumonia-like organism
RFLP	-	Restriction fragment length polymorphism
RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
RNS	-	Reactive nitrogen species
ROS	-	Reactive oxygen species
SNP	-	Single Nucleotide Polymorphism
TCA	-	Tricarboxylic acid
TL	-	Taraba lung
TP	-	Taraba pleural fluid
tRNA	-	Transfer ribonucleic acid
URT	-	Upper respiratory tract
USA	-	United States of America
Vmm	-	Variable surface protein of *Mycoplasma mycoides*
Vsp	-	Variable surface protein
WGS	-	Whole genome sequesncing

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background of the Study

Mycoplasma bovis (*M. bovis*) and *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) are the two most important pathogenic species of mycoplasmas of cattle (Egwu *et al*., 1996; Brown *et al*., 2015). The former has been reported worldwide causing respiratory disease, mastitis, arthritis, otitis media and a variety of other diseases in cattle (Maunsell *et al*., 2011); while the later is the etiological agent of contagious bovine pleuropneumonia (CBPP), which is considered by the World Organisation for Animal Health (OIE) to be one of most severe infectious animal diseases affecting sub-Saharan-African countries, causing major losses of animals with subsequent socioeconomic repercussions, with a particular emphasis in international trades (Nicholas *et al*., 2008; Li *et al.*, 2009; Dupuy *et al*., 2012; OIE, 2014; Fischer *et al*., 2015).

Mycoplasma bovis and *Mmm* are low Gram positive bacteria belonging to the Class *Mollicutes*, Order *Mycoplasmatales*, Family *Mycoplasmataceae*, and the Genus *Mycoplasma* (Razin *et al*., 1998). *Mycoplasma bovis* was first isolated in 1961 in the United States from a cow with severe mastitis (Hale *et al*., 1962), whereas *Mmm* was the first *Mycoplasma* to be described (Nocard and Roux, 1898). They are both extracellular bacteria that live in close association with their host cells (Mtui-Malamsha, 2009). They lack cell wall and have the capacity of self replication (Westberg *et al*., 2004). *Mycoplasma bovis* has the genomic size of 1,080-kbp while that of *Mmm* is 1,211-kbp (Westberg *et al*., 2004; Calcutt *et al*., 2018).

Mycoplasma bovis is a common inhabitant of the upper and lower respiratory tract of healthy and pneumonic cattle (Thomas *et al*., 2002a; Arcangioli *et al*., 2008). It increases the severity of respiratory diseases in calves and can also act as a primary pathogen (Gagea *et al*., 2006). It is a natural inhabitant of respiratory tract of healthy bovines, without showing any clinical symptoms and is shed through their nasal discharges for months or years (Gagea *et al*., 2006). The genital tract of both male and female animals can also harbour the organism which can be a source of the infection through coitus with naive animals (Kreusel *et al*., 1989) or through artificial insemination (AI) with deep frozen bull semen (Jurmanova and Sterbova, 1977). Milk may also be a source of infection for suckling calves (Hirose *et al*., 2001). The main reservoir of *Mmm* is cattle from which the organism has been recovered during both clinical disease and after recovery (Windsor and Masiga, 1997; Kusiluka *et al*., 2000a). *Mycoplasma mycoides* subsp. *mycoides* infects lung tissues and can be transmitted through inhalation of infectious aerosols of the organism by a susceptible animal (Provost *et al*., 1987; OIE, 2015) and is characterized by severe inflammatory, exudative lesions at lung and pleural membranes. In calves however, infection of *Mmm* results mainly in swollen painful limbs (arthritis) and associated lameness, but rarely in pulmonary lesions (OIE, 2014). Other factors such as movement of trade cattle, seasonal migration and transhuman normadism also enhances the spread of the infection (Done *et al*., 1995).

Diagnosis of *M. bovis* infection can be performed by several methods, including isolation of the agent (Stipkovits *et al*., 2001), immunohistochemical staining (Adegboye *et al*., 1995) and the use of specific PCR probe on the lung samples (Hayman and Hirst, 2003) as well as the detection of specific antibodies in the serum (Byrne *et al*., 2001; Le Grand *et al*., 2001). The OIE recommended procedures for CBPP

diagnosis include complement fixation test (CFT) modified by Campbell and Turner, competitive enzyme-linked immunosorbent assay (c-ELISA) and polymerase chain reaction (PCR) (OIE, 2010). Isolation and identification of mycoplasmas is still the gold standard (Nicholas, 2002; Egwu *et al*., 2012a). However, isolation of mycoplasmas requires a well-equipped laboratory with expertise for these very fastidious and slow-growing organisms (Persson *et al*., 1999; McAuliffe *et al*., 2005).

Mycoplasma isolation is generally compromised by the overgrowth of fast growers and less significant *Mycoplasma* and *Acholeplasma* species, other bacterial contaminants and inhibitors used in the growth medium (Nicholas and Baker, 1998; OIE, 2008) thus making its isolation on artificial media tedious. The use of more sophisticated PCR for the rapid and specific identification of *M. bovis* and *Mmm* has its limitations in developing countries like Nigeria, where molecular tools cannot be utilized routinely because they are too expensive, but detection of these species by PCR has been shown to be highly more efficient than culturing techniques (Wade, 2010). Therefore, where such facilities are available, they should be employed in order to obtain better results (Egwu *et al*., 2012a).

1.2 Statement of Research Problem

Mycoplasmas cause some of the most serious and economically important diseases of cattle with global distribution (Parker *et al*., 2018). *Mycoplasma bovis* is currently recognized as one of the most important and frequently isolated *Mycoplasma* species associated with disease in cattle worldwide (Arcangioli *et al*., 2008; Parker *et al*., 2018). In the United Kingdom, it was estimated that up to 1.9 million cattle are affected annually which cost the cattle industry €45 million (Reeve-Johnson, 1999; Arcangioli *et al*., 2008). Furthermore, approximately 157,000 calves die annually as a result of

pneumonia and related infections, which may have a potential market worth of about €99 million (Vanden-Bush and Rosenbusch, 2003). It is most likely that *M. bovis* is accountable for at least a quarter to a third of these losses (Nicholas *et al*., 2000b).

Diseases caused by *M. bovis* have not been given sufficient attention it deserves; although, the organism is widespread in many European countries (Arcangioli *et al*., 2008; Caswell *et al*., 2010) and in some African countries. In Nigeria however, there is no data on economic losses as a result of the disease. Studies have been focused on the culture and seroprevalence of the organism in Adamawa State (Francis, 2014; Francis *et al*., 2014; Francis *et al*., 2015a) and the north-western region (Ajuwape *et al*., 2003; Tambuwal, 2009; Tambuwal *et al*., 2011a).

Contagious bovine pleuropneumonia (CBPP) caused by *Mmm* causes greater losses in cattle than any other disease after rinderpest and is regarded as the most important infectious disease of cattle in the African region (Mariner *et al*., 2006a). It threatens livestock production, limits international trade and is therefore of huge economic concern in affected countries (Fadiga *et al*., 2013). Annual economic losses of up to €30.1 million due to CBPP were estimated in 12 endemic sub-Saharan African countries (Tambi *et al*., 2006). This posed serious implication for food security and livelihood of people in affected countries representing a major constraint to cattle production and trade (Tambi *et al*., 2006; Amanfu, 2009; Dedieu *et al*., 2010; Gourgues *et al*., 2016). In Nigeria, estimated CBPP morbidity and mortality rate of up to 50% and 25% respectively have been documented with annual economic losses of more than ₦2.2 billion (Fadiga *et al*., 2013).

Despite vaccination campaigns in Nigeria, CBPP continues to occur with increased frequency leading to heavy losses (Aliyu *et al*., 2000). Outbreaks of the disease still

occur in the northern region which harbors three-quarter of the country's 19.5 million cattle (Ikhatua, 2011; Tambuwal *et al*., 2011b). This is thought to be as a result of several factors including absence of or improper implementation of control programs, poor vaccination coverage due to limited vaccine supplies and constraints in field mobility and support funds (Aliyu *et al*., 2000; Tambuwal *et al*., 2011b), lack of vaccine efficacy, illiteracy, poor management systems (Fadiga *et al*., 2011) and reduced disease surveillance in the field, abattoir and the laboratories (Amanfu, 2009; FDL, 2010).

In 2011, CBPP was reported to the AU-IBAR by 18 African countries, spreading across the west, central and southern African regions (AU-IBAR, 2013). During the reporting period, 304 epidemiological units were affected by CBPP across Africa involving 16,836 cases and 3,007 deaths, with an estimated case fatality of 17.9% (AU-IBAR, 2011). At that time, Nigeria reported 22 outbreaks of CBPP to AU-IBAR involving 489 cattle with an estimated case fatality of 19.63% (AU-IBAR, 2011). Despite high socioeconomic impact of these pathogens to the cattle industry, there is dearth of information on the molecular characterization of these organisms from Adamawa and Taraba States.

1.3 Justification

Mycoplasma bovis infection is endemic in Nigeria (Egwu *et al*., 1996; Tambuwal *et al*., 2011a; Francis *et al*., 2014; Francis *et al*., 2015a; Francis, 2017). Currently, very few reports are available on the detection of the infection in African countries including Nigeria. The highly contagious nature of these *Mycoplasma* species, their poor responsiveness to treatment and associated culling implications for affected herds make it necessary for rapid and accurate diagnosis for control and prevention of disease outbreak (Parker *et al*., 2018). In recent years, molecular methods have successfully

been employed for the identification and classification of mycoplasmas (Hotzel *et al*., 1996b; Ghadersohi *et al*., 1997).

Contagious bovine pleuropneumonia has been eradicated from most continents; but the disease still persists in Africa in general (OIE, 2008; Thiaucourt *et al*., 2011) and Nigeria in particular. The outbreaks tend to be concentrated in precise areas, for instance, along the upper Benue river, in the north-east part of the country, along the Sokoto and Hadejia rivers in the north-western region and along the river Niger in the north-central region (Nwanta and Umoh, 1992). This is because these regions happens to be the grazing areas and receive large numbers of nomadic cattle as a result of mass trade cattle movement and seasonal migration by the pastoralists in search of grazing land and water during dry season (Aliyu *et al*., 2000; Aliyu *et al*., 2003; Tambuwal *et al*., 2011b). As the control policies for CBPP at the national borders are insufficient, the disease may spread to neighbouring countries or be imported (Provost *et al*., 1987; Masiga *et al*., 1996).

Several epidemiological studies have been conducted in Nigeria to assess the situation of CBPP in south-western region (Babalobi, 2007), North-western region (Tambuwal, 2009; Tambuwal *et al*., 2011b), North-central region (Nwanpka *et al*., 2004; Mailafiya *et al*., 2010; Alhaji and Babalobi, 2015) and North-eastern region (Ameh *et al*., 1998; Halle *et al*., 1998; Aliyu *et al*., 2000; Francis *et al*., 2018a). To the best of our knowledge, recent information about the disease is scanty and no extensive work had been done on genomic characterization of *Mmm* isolates in the study area. Against this background, it is therefore necessary and important to conduct an abattoir based study that will isolate and identify *M. bovis* and *Mmm* as well as carry out genomic characterization of *Mmm* isolates in cattle in the study area. This will be of great

importance in order to clarify the relationship of strains circulating in Adamawa and Taraba States, providing additional support to CBPP investigation and control.

1.4 Aim of the Study

The aim of the present study was to isolate and identify *Mycoplasma bovis* (*M. bovis*) and *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) in order to characterize the genome of *Mmm* strains isolated from slaughtered cattle in Adamawa and Taraba States, North-eastern Nigeria.

1.5 Specific Objectives

The objectives of this study were to:

1. Determine the isolation rate for *Mycoplasma* species from pneumonic lungs, nasal swabs, ear swabs and pleural fluids using culturing method.
2. Identify *Mycoplasma bovis* isolates using conventional polymerase chain reaction (PCR) targeting 16S rRNA.
3. Identify subcluster of *Mycoplasma mycoides* isolates by conventional PCR and perform molecular typing to identify specific *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) using PCR- Restriction fragment length polymorphism (RFLP)**.**
4. Sequence the genomes of field isolates of *Mmm* using next generation sequencing (NGS) technique.
5. Compare *Mmm* sequenced strains isolated from Adamawa and Taraba States for similarity based on orthologous average nucleotide identity (OrthoANI) values.
6. Carry out pangenomic investigation of *Mmm* strains from Adamawa and Taraba States using cluters of orthologous groups (COG).

7. Identify virulence genes among the *Mmm* strains isolated from Adamawa and Taraba States.

1.6 Research Questions

1. What could be the isolation rate for *Mycoplasma* species from suspected pneumonic lungs, nasal swab, ear swab and pleural fluid/swab of slaughtered cattle in Adamawa and Taraba States?
2. What proportion of this *Mycoplasma* isolates could actually be *M. bovis*?
3. What could be the rate of confirming *Mmm* isolates from suspected cattle in Adamawa and Taraba State?
4. What are the sequences the genome of *Mmm* isolates from cattle in Adamawa and Taraba States?
5. What are the differences in *Mmm* strains isolated from Adamawa and Taraba States?
6. To what extent would clusters of orthologous group (COG) be used to investigate the pangenome of *Mmm* strains in Adamawa and Taraba States?
7. What are the virulence genes present in the strains of *Mmm* isolated from Adamawa and Taraba States?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Classification of Mycoplasmas

Mycoplasmas are a group of obligate parasitic bacteria that evolved from Gram positive organisms by reductive evolution; in the course of their evolution have lost many dispensable genes and are thought to maintain only regulatory systems essential for their survival *in vivo* (Caswell and Archambault, 2007; Halbedel and Stulke, 2007). The name "mycoplasma" is conventionally used to designate *Mollicutes*, a class of the smallest prokaryotes that lack a cell wall and have often been portrayed as "minimal self-replicating organisms" because of their small genome size and the paucity of their metabolic pathways (Chazel *et al*., 2010; Citti and Blanchard, 2013). They are responsible for causing many important diseases of ruminants in Africa (Francis *et al*., 2015b).

The genus *Mycoplasma* comprises the simplest life form that includes most important human and animal pathogens that cause diseases whose occurrence has long been undervalued (Citti and Blanchard, 2013). Taxonomically, they belong to the Phylum *Firmicutes*, Class *Mollicutes*, Order *Mycoplasmatales*, Family *Mycoplasmataceae* and Genus *Mycoplasma* (Razin, 1993). They are named *Mollicutes* (from the Latin *mollis*, soft; *cutis*, skin), a group of bacteria so named because they lack cell wall, instead being enveloped by a complex plasma membrane (Basema and Tully, 1997). The class *Mollicutes* consists of nine genera namely: *Mycoplasma*, *Ureaplasma*, *Acholeplasma*, *Anaeroplasma*, *Asteroleplasma*, *Entomoplasma*, *Mesoplasma*, *Spiroplasm*a and *Phytoplasma*; and over 200 species and subspecies, of which 119 are under the genus *Mycoplasma* (Bizarro and Schuck, 2007).

The class *Mollicutes* are separated from all other bacteria by the total lack of a rigid cell wall envelope, reduced cellular (0.1 - 0.3 μm diameter) and genome (0.58 - 2.20-Mbp) sizes, DNA with low G+C content (23 - 40 mol %) and sterol incorporation into their own plasma membrane (Razin *et al*., 1998; Chazel *et al*., 2010; Parker *et al*., 2018). The smallest genome led *Mycoplasma* species to completely economise genetic resources and biosynthetic capacities, and adapt to an obligate parasitic existence and also serves as a blueprint for the design of synthetic live organisms (Razin, 1997; Check, 2002). These bacteria lack the tricarboxylic acid cycle (TCA), and their small genome size limits the range of their metabolic activities so they are largely dependent on external sources of amino acids, nucleic acid precursors and lipids (Khan *et al*., 2005). One product of metabolism is hydrogen peroxide, which may be a pathogenicity factor in mycoplasma infections (Khan *et al*., 2005; Zhao *et al*., 2017). Table 2.1 shows the most important pathogenic *Mollicutes* and the diseases they cause in cattle.

Table 2.1: The most important pathogenic *Mollicutes* in cattle

***Mollicutes* species**	**Disease**
Mycoplasma mycoides subsp. *mycoides*	Contagious bovine pleuropneumonia
Mycoplasma bovis	Pneumonia, polyarthritis, mastitis, abortion, sterility, keratoconjunctivitis
Mycoplasma leachi	Pneumonia and arthritis
Mycoplasma dispar	Pneumonia
Mycoplasma californicum	Mastitis
Mycoplasma canadense	Mastitis
Mycoplasma bovigenitalium	Mastitis and genital disease
Mycoplasma bovocculi	Conjunctivitis
Ureaplasma diversum	Sterility and abortion
Eperythrozoon wenyoni	Anaemia

Source: Frey, 2002

2.2 Bovine Mycoplasmas

Mycoplasmas are wall-less prokaryotes that contain a diverse group of organisms; they are considered the smallest self-replicating organisms. Among the approximately 200 species of *Mollicutes* from animals, only a small number, mainly *Mycoplasma* species, are described as pathogenic (Shahriar and Clark, 2003). *Mycoplasma bovis* (*M. bovis*) was first definitively identified in the USA in 1961, although clinical signs associated with the organism were described beforehand (Caswell and Archambault, 2007). It is now recognised worldwide as the most important *Mycoplasma* pathogen in cattle and as a major cause of infection in dairy calves (Nicholas *et al*., 2002; Maunsell and Donovan, 2009). *Mycoplasma bovis* is considered the second most pathogenic mycoplasma after *Mycoplasma mycoides* subspecies *mycoides* (Shahriar and Clark, 2003). The organism is being increasingly recognised as one of the major emerging pathogens of cattle in industrialized as well as developing countries threatening livestock production (Nicholas, 2011; Francis, 2017; O'Brien *et al*., 2017).

Mycoplasma mycoides subspecies *mycoides* (*Mmm*) is the causative agent of contagious bovine pleuropneumonia (CBPP) and was the first *Mycoplasma* species described and cultured at the end of the 18th century (Taylor-Robinson and Tully, 1998). It is still one of the most important threats to the cattle industry in parts of the world and is a reportable disease in North America (Shahriar and Clark, 2003). The disease is also a major constraint to cattle production in sub-Saharan Africa (Lorenzon *et al.*, 2003). In Nigeria, however, CBPP has been reported as the major militating factor against cattle production (Aliyu *et al*., 2000). Table 2.2 shows the comparative properties of two most important mycoplasmas of cattle.

Table 2.2: Comparative properties of two most important cattle mycoplasmas

Properties	***Mycoplasma bovis***	***Mycoplasma mycoides* subsp. *mycoides***
Disease	Calf pneumonia, mastitis, arthritis, abortion, keratoconjunctivitis	Contagious bovine pleuropneumonia in cattle, and occasional arthritis in calves
Distribution	Worldwide	Sub-saharan Africa, probably in parts of Middle East, Central Asia
Host	Cattle	Cattle, goats (sheep)
Histopathological lesions	Interstitial pneumonia, lympho-histiocytis bronchitis, catarrhal broncho-pneumonia	Fibrinous bronchopneumonia with necrosis
Clinical signs	Respiratory distress, mastitis, arthritis	Few signs, respiratory distress evident after excercise
Diagnosis	Serology, isolation, PCR	Isolation, serology, PCR, abattoir surveillance
Treatment	Chemotherapy	Chemotherapy not recommended because it encourages carrier status
Control	Management e.g., improved ventilation, reduced stocking density	Vaccination, movement control, slaughter

Source: Nicholas and Ayling, 2003

2.3 The Disease

2.3.1 *Mycoplasma bovis* infection

Mycoplasma bovis (*M. bovis*) is widely known as a primary cause of enzootic pneumonia of calves, mastitis of cows, and arthritis of young and adult bovine animals, although, pneumonia in adult cattle has also been reported (Shahriar and Clark, 2003; Radaelli *et al*., 2008). In normal infections, *M. bovis* can be isolated in pure culture from the mammary gland of cows with mastitis (Gonzalez *et al*, 1992) and from the joints, tendon sheaths, or periarticular tissues of cattle with arthritis, tenosynovitis, or chronic pneumonia and polyarthritis syndrome (CPPS) (Adegboye *et al*., 1996; Shahriar *et al*., 2002; Gagea *et al*., 2006). *Mycoplasma bovis* is the principal pathogen isolated from the middle ear of calves with otitis media (Francoz *et al*., 2004; Lamm *et al*., 2004; Maunsell *et al*., 2012). However, the role of *M. bovis* in the multifactorial bovine respiratory disease (BRD) complex is not ascertained (Maunsell *et al*., 2011). *Mycoplasma bovis* is often isolated from the lungs of cattle with pneumonia (Thomas *et al*., 2002), and identified inside lesions using immunohistochemistry (IHC) (Gagea *et al*., 2006).

Mortality related with *M. bovis* infection can range from 5-10% or even higher in severe cases, and morbidity can approach 35% (Nicholas *et al*., 2008). Clinical signs are not pathognomonic, a harsh dry hacking cough accompanied by low grade fever, mild depression, runny eyes and ear droop are characteristic signs that may be observed (Nicholas *et al*., 2008). *Mycoplasma bovis* infection is not considered to be zoonotic, although a few cases have been reported in immunocompromised patients who have had close contact with infected cattle or their products such as infected milk or faeces (Pitcher and Nicholas, 2005).

2.3.2 Contagious bovine pleuropneumonia

Contagious bovine pleuropneumonia (CBPP) is an economically important and highly infectious disease of cattle characterized by localization in the lungs and pleura, caused by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) previously further specified as small colony (SC) type (Manso-Silván *et al.*, 2009). It causes a pulmonary disease that ranges from a persistent, sub-clinical infection to an acute, sometimes fatal disease (Gull *et al.*, 2013). Naive herds can experience losses up to 80%, and many cattle that survive remain chronic carriers which may suffer from recurrent low-grade fever, loss of condition, and respiratory signs upon exercise, and might introduce the causative agent into uninfected herds (Campbell, 2015). The disease was eradicated from the United States and Europe in the early 20th century, though some of them have experienced sporadic outbreaks with affected herds manifesting little distinctive clinical signs and lower mortality than those found in Africa (Nicholas *et al.*, 1996; Nicholas *et al.*, 2008). The main manifestations of CBPP are anorexia, fever and respiratory signs, such as dyspnea, polypnea, cough and nasal discharges (Radostits *et al.*, 2007). The chronic form of CBPP may occur upon initial infection or following either acute or sub-acute disease however, clinical signs are not always evident and could be confused with other respiratory disease symptoms. Most infections are limited to the respiratory tract, although arthritis occurs in calves usually less than 6 months of age (Gull *et al.*, 2013).

2.4 Etiology of *M. bovis* Infection and Contagious Bovine Pleuropneumonia

Mycoplasma bovis infection is caused by *M. bovis* which is a primary or secondary respiratory pathogen in cattle. The organism is usually associated with infections such as pneumonia, arthritis, tensynovitis and other disorders like mastitis, otitis and sinusitis (Nicholas and Ayling, 2003; Maunsell *et al*., 2012). *Mycoplasma bovis* is commonly

isolated from both dairy and beef calves suffering from non-responsive respiratory infections and pneumonia. Most often, it infects an already weakened calf suffering from a viral infection such as IBR (Maunsell *et al*., 2011). *Mycoplasma bovis* is the most frequently occurring mycoplasma pathogen linked to pneumonia, mastitis and arthritis in cattle (Castillo-Alcala *et al*., 2012; Timsit *et al*., 2012). However, there is evidence showing that *M. bovis* can infect otherwise healthy calves and cause clinical signs of disease (Timsit *et al*., 2012). *Mycoplasma bovis* can be found in the respiratory secretions of normal healthy and disease cattle (Maunsell *et al*., 2011). Even dairy calves with no signs of respiratory disease can have the organism present in their respiratory tract. It is believed that *M. bovis* can spread from a few animals carrying the bacteria to other animals within the herd via respiratory secretions, direct contact and aerosol (Gagea *et al*., 2006). An additional source of *M. bovis* infection comes from feeding calves milk infected with *M. bovis* (Maunsell *et al*., 2011).

Contagious bovine pleuropneumonia is caused by *Mmm*, a member of the *Mycoplasma mycoides* cluster in the family *Mycoplasmataceae* (OIE, 2015). This organism was previously specified as the small-colony (SC) type; however, *M. mycoides* subsp. *mycoides* large colony (LC) type are now considered to belong to *M. mycoides* subsp. *capri*. Nevertheless, many sources continue to use the full designation *M. mycoides* subsp. *mycoides* SC. *Mycoplasma mycoides* subsp. *mycoides* can be grouped into at least two major lineages (African and European), or into at least 3 or 4 genetic groups. Strains can also differ in virulence (OIE, 2015).

2.5 Biological Properties of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

Like all mycoplasmas, *Mycoplasma bovis* is small and pleomorphic; it has a genomic size of 1,080-kbp, lacks a cell wall, complex nutritional requirement and has a low G+C

ratio of 23.8-32.9 mol % (Calcutt *et al*., 2018). *Mycoplasma bovis* is also similar to *M. agalactiae* in its biochemical properties, as it neither ferments glucose nor hydrolyses arginine but instead it uses organic acids such as lactate and pyruvate as energy sources for growth (Miles *et al*., 1988; Calcutt *et al*., 2018). The film and spot formation can also be seen on the surface of solid media, indicating the possession of lipolytic activity (Nicholas *et al*., 2003).

Mycoplasmas are usually considered to be highly susceptible to various environmental factors such as high temperature and dryness. Despite this, *M. bovis* can survive outside the host environment especially if protected from sunlight at 4°C for nearly 2 months in sponges and milk; for 20 days on wood and for 17 days in water and at 20°C the survival periods drop to one-two weeks and at 37°C to one week. In deep frozen semen the agent remains infective for years (Pfutzner, 1984). Mycoplasmas lack a cell wall that should make them susceptible to environmental pressure; however, they do survive for long periods indicating survival in bedding sand for 8 months (Justice-Allen *et al*., 2010). Previous finding has partly explained extended survival through the presence of biofilms (McAuliffe *et al*., 2006).

Mycoplasma bovis is usually susceptible to the commonly used disinfectants although the biological materials (milk, discharges) can dramatically reduce their efficacy (Pfutzner *et al*., 1983). Formalin and paracetic acid are proved to be very effective for general disinfecting purposes. Iodophores are also efficient. This enables their use for teat dipping (Pfutzner *et al*., 1983). Unfortunately disinfecting materials based on hypochlorites are unsuitable for this purpose, because of the high concentrations and long exposure periods needed to obtain suitable efficacy (Pfutzner *et al*., 1983). This can

be a problem, because these compounds are widely used in disinfection of milking machines (Pfutzner *et al*., 1983).

Whereas *Mmm* also lacks a cell wall, has a genomic size of 1,280-kbp, low G+C content of 23.2-27.1 mol % (Hermann, 1992; Westberg *et al*., 2004) and is among the simplest self-replicating organisms known (Minion, 2002). *Mycoplasma mycoides* subspecies *mycoides* is sensitive to digitonin (like all members of the order *Mycoplasmatales*), does not produce 'film and spots', ferments glucose, reduces tetrazolium salts (aerobically or anaerobically), does not hydrolyse arginine, has no phosphatase activity, and has no or weak proteolytic properties (OIÈ, 2012). The biochemical properties of the most frequent bovine mycoplasmas compared to the ones of *M. bovis* and *Mmm* are summarised on Table 2.3.

Table 2.3: Comparative biochemical properties of bovine mycoplasmas, including *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*

Mycoplasma	Glucose	Arginine	Urease	Film	Casein	Phosphatase	Tetrazolium	
							Aerobic	Anaerobic
M. alkalescens	–	+	–	–	–	+	–	–
M. bovis	–	–	–	+	–	+	+	+
M. bovirhinis	+	–	–	–	+/–	+/–	+	+
M. bovigenitalium	–	–	–	+	–	+	–	+
M. bovoculi	+	–	–	+	–	+/–	+	+
M. canis	+	–	–	–	+/–	–	–	+
M. californicum	–	–	–	–	nk	+	–	Most strains +
M. canadense	–	+	–	–	nk	weak +	–	+
M. dispar	+	–	–	–	nk	–	+	+
M.mycoides mycoides	+	–	–	–	+	–	+	+
U. diversum	–	–	+	–	–	–	nk	nk

Source: Nicholas *et al*., 2008

\+ = positive
\- = negative
nk = not known

2.6 Other Properties of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

2.6.1 Interactions of *Mycoplasma bovis* with other microorganisms

In naturally infected cattle, *M. bovis* is commonly identified in association with other microorganisms leading to the hypothesis that synergism could be involved in the severe lung lesions observed during necropsy (Shahriar *et al*., 2002; Booker *et al*., 2008). The most common microorganisms that are identified in association with *M. bovis* are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, *Trueperella pyogenes*, other *Mycoplasma* species, and viruses including bovine respiratory syncytial virus (BRSV), bovine herpes virus 1 (BHV-1), bovine viral diarrhea virus (BVDV), parainfluenza virus type 3 (PIV-3), adenovirus and infectious bovine rhinotracheitis (IBR) virus (Booker *et al*., 2008; Taylor *et al*., 2010).

Natural infection with *M. bovis* can result in exudative bronchopneumonia with occasional extensive foci of coagulative necrosis, whereas experimental infection produces subclinical pneumonia and less severe lung lesions (Rodriguez *et al*., 1996). Hence, severe pulmonary disease symptoms caused by *M. bovis* are suspected to be the consequence of synergistic interactions between mycoplasmas and other microorganisms causing significant problems for farm management (Rodriguez *et al*., 1996). In addition, the age as well as the immune status of individual animals are probably also important for the development of disease, as *M. bovis* alone was described to be able to cause pneumonia in very young calves, while synergistic viral or bacterial infections may be necessary to cause the typical extensive caseonecrotic lesions in the lungs of adult animals in feedlots (Prysliak *et al*., 2011; Hermeyer *et al*., 2012).

More specifically, *M. bovis* seems to interplay with the bacterial pathogens *H. somni*, *M. haemolytica* and *P. multocida*; this was shown in experimentally induced bovine respiratory disease where *M. bovis* acted as a predisposing factor for severe symptoms in calves after secondary infection with *P. multocida* (Gourlay and Houghton, 1985). Other studies considered an onset of *M. bovis*-mediated symptoms in tissue pre-damaged through lesions caused by *P. multocida*, *M. haemolytica* or *H. somni*. At such circumstances, *M. bovis* was supposed to withstand the curing of primary infections caused by the Pasteurellaceae by antibiotics or by the host immune system (Gagea *et al*., 2006). In another study, a significant association of *H. somni* and *M. bovis* was observed in feedlot calves, where 80% of *H. somni* cases were positive for *M. bovis*, whereas no significant association was seen between *M. bovis* and BVDV or *M. haemolytica* (Booker *et al*., 2008).

Mycoplasma bovis co-infections with BVDV were shown to result in more severe respiratory disease in cattle as a result of immuno-suppressive effects of the virus (Martin *et al*., 1990; Potgieter, 1995). However, contradictory data are available on feedlot cattle regarding the association between *M. bovis* and BVDV (Shahriar *et al*., 2002; Gagea *et al*., 2006; Booker *et al*., 2008; Prysliak *et al*., 2011). Concerning co-infection of *M. bovis* with BRSV, no significant increase in the severity of clinical signs was observed when compared to single infections with *M. bovis* (Thomas *et al*., 1986). Interestingly, experimental co-infection with BHV-1 and *M. bovis* were performed to induce classical *M. bovis* lesions in organs (Prysliak *et al*., 2011). Indeed, co-infection of 6- 8 month-old feedlot calves resulted in caseonecrotic bronchopneumonia typical of *M. bovis* infection, while infection with *M. bovis* alone caused only small consolidations in the lung (Prysliak *et al*., 2011). Moreover the mortality rate was high, indicating the

severe synergistic effect of this co-infection between *M. bovis* and BHV-1 (Prysliak *et al*., 2011).

2.6.2 Antigenic variation in *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

Phase variation is a common mechanism among *Mycoplasma* species and it was considered to be involved in the survival of microorganisms leading to the emergence of varied intra-clonal populations that become accustomed to new environments (Pilo *et al*., 2007). Some of the mycoplasmas posses high number of such phase-variant proteins particularly surface-exposed lipoproteins (Denison *et al*., 2005). Highly variable antigenic profiles of *M. bovis*, which are strain independent, have been observed (Poumarat *et al*., 1994). The finding suggested that high frequency phase (ON–OFF) and size variation of membrane surface lipo-proteins occur as have been demonstrated for several other *Mycoplasma* species (Razin *et al*., 1998). The antigenic heterogeneity of *M. bovis* strains is unrelated to the geographical origin, organ of isolation or type of disease induced by single strains but is variable among different subclones of the same strain (Rosengarten *et al*., 1994). This variability was shown to be based on several prominent amphiphilic, integral, membrane proteins, containing cross-reactive epitopes acting as major immunogens (Rosengarten *et al*., 1994). High frequency antigenic switching was demonstrated to be affected by the presence of cognate *M. bovis* antibodies *in vitro* (Le Grand *et al*., 1996). These points supported earlier assumptions indicating that this system serves as a successful strategy to maintain diversity in strain subpopulations allowing *M. bovis* to evade the host immune system. Thereby, the host's attempts to eliminate this bacterium are massively compromised contributing to the chronic manifestation of diseases caused by *M. bovis* (Buchenau *et al*., 2010).

Mycoplasma bovis demonstrates significant interstrain variability within the variable surface lipoprotein (*vsp*) loci at both the protein and DNA levels (Beier *et al*., 1998). The ON-OFF phase switch for the *vsp* genes involves DNA rearrangements or inversions, possibly involving a recombinatorial mechanism. These inversions sometimes result in the creation of chimeric genes and new *vsp* phenotypes (Lysnyansky *et al*., 2001). In some cases, two site-specific DNA inversions between inverted 35-base pair sequences were needed for phase variation. These inversions resulted in the juxtaposition of a promoter-like sequence to a silent *vsp* gene promoting transcription initiation (Beier *et al*., 1998).

Size variation in the *vsp* proteins is due to repetitive sequences that sometimes make up 80% of the coding sequence. When expressed, these sequences produce periodic polypeptide structures. Eighteen distinct repetitive domains of different lengths and amino acid sequences have been identified (Lysnyansky *et al*., 1999). The sequences are arranged in blocks of similarity and the number of repeats can vary between strains. It is thought that slipped strain mispairing at high frequency during DNA replication causes the unequal copy of the number of repetitive sequence units producing siblings with a different number of repeat units (Minion, 2002). Since there are multiple repetitive domains of different lengths within each *vsp*, replication errors result in the irregular periodic spacing seen in the immunoblot patterns (Minion, 2002).

A second *vsp* unrelated protein that was identified undergoes phase switching and size variation is the membrane protein of 67kDa molecular weight (pMB67) (Burki *et al*., 2015). Unlike the *vsp* proteins, pMB67 was not lipid modified and did not contain the *vsp*-like repetitive domains. It appeared to be a major antigen recognized during *M.*

bovis infections or disease and may be appropriate for the development of vaccines or diagnostic preparation (Behrens *et al*., 1996; Burki *et al*., 2015).

A variable surface protein of the bovine pathogen *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) designated *vmm* has been identified and characterized (Pilo *et al*., 2007). Variable surface protein is a protein of 16kDa and is specific to *Mmm* and is expressed by nearly all strains that were analyzed, where it showed a reversible ON-OFF phase variation at a frequency of 9×10^{-4} to 5×10^{-5} per cell generation (Persson *et al*., 2002; Pilo *et al*., 2007). Genes resembling the variable surface protein of *Mmm* (*vmm*) gene were also found in other species of mycoplasma, but the *vmm*-like proteins in these species could not be detected with a specific monoclonal antibody directed to *vmm* of *Mmm*. Variable surface protein of *Mmm* was specific for this organism and was expressed by 68 of 69 analyzed *M. mycoides* strains (Frey *et al*., 2003). The *vmm* gene was present in all of the 69 tested *Mmm* strains and encodes a lipoprotein precursor of 59 amino acids (AA), where the mature protein was predicted to be 36 amino acids and was anchored to the membrane by only the lipid moiety, as no transmembrane region could be identified. DNA sequencing of the *vmm* gene region from ON and OFF clones showed that the expression of *vmm* was regulated at the transcriptional level by dinucleotide insertions or deletions in a repetitive region of the promoter spacer (Persson *et al*., 2002). Variable surface protein of *Mmm* (*vmm*)-like genes were also found in four closely related mycoplasmas, *Mycoplasma capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae*, *Mycoplasma leachi*, and *Mycoplasma putrefaciens*. However, *vmm* could not be detected in whole-cell lysates of these species, suggesting that the proteins encoded by the *vmm*-like genes lack the binding epitope for the monoclonal antibody used in the research, or alternatively, that the *vmm*-like proteins were not expressed (Persson *et al*., 2002).

The function of *vmm* is currently not known, 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 escape the host immune system (Pilo *et al*., 2007).

2.6.3 Adhesion of mycoplasmas to cell membrane

Adhesion to the cell membrane is one of the first steps during mycoplasma infection (Rottem, 2003). Since mycoplasmas do not seem to secrete toxins that could act over long distance, adhesion in particular is important in mycoplasmal virulence and pathogenesis (Pilo *et al*., 2007). Therefore adhesins expressed on the mycoplasmal membrane are of great importance because of their direct contact with host cells and are responsible for host specificity and tissue tropism (Sachse *et al*., 1996). The intimate contact of mycoplasmas with host cells is crucial for mycoplasmal survival (Sachse *et al*., 1996). Because of their small genomes, mycoplasmas lack a battery of genes involved in essential biosynthetic pathways and have to acquire essential substances such as amino acids, nucleotides and lipids from the host (Razin and Jacobs, 1992). For this purpose, a fusion between the mycoplasmas and the host membranes was proposed to allow the exchange of membrane and intracellular components (Razin and Jacobs, 1992). Although there are several adhesins detected in other *Mycopasma* species, specific adhesins have been postulated to occur but not yet detected in *Mmm* (Pilo *et al*., 2007).

Interestingly, there is no evidence of the presence of a tip organelle in *M. bovis* serving as a structure for a polar accumulation of major adhesins as the case with *M. pneumoniae* (Razin and Jacobs, 1992; Behrens *et al*., 1996). Putative adhesins of *M. bovis* are probably spread over the surface in the form of membrane proteins (Behrens *et al*; 1996; Rottem, 2003). In *in vitro* assays, *M. bovis* strain PG45 adhesion to embryonic

bovine lung cells (EBL) was shown to be temperature dependent with maximal adhesion at 37°C (Behrens *et al*., 1996). The cell receptor binding capacity was limited with saturation reached at a multiplicity of infection (MOI) of 225:1 in EBL cells and 100:1 in bovine bronchial epithelial (BBE) cells (Sachse *et al*., 1996; Thomas *et al*., 2003b). Large variation in cytoadherence rates (3.4-19.1%) were recorded among various *M. bovis* strains independently from the organ of isolation but determined by the type of host cells tested (Sachse *et al*., 1996; Thomas *et al*., 2003a). Significantly lower adherence was observed in less or non-pathogenic strains compared to virulent strains (Thomas *et al*., 2003a; Thomas *et al*., 2003b). Moreover, cytoadherence rates were lower using a fibroblast cell line and primary BBE cells compared to rates in epithelial cell lines (Thomas *et al*., 2003a; Thomas *et al*., 2003b).

Additionally, *M. bovis* strains seem to loose adherence ability after continuous *in vitro* passaging (Thomas *et al*., 2003a; Thomas *et al*., 2003b). *Mycoplasma bovis* adhesion is driven by protein interactions since trypsin treatment of *M. bovis* leads to a partial decrease in adherence (Sachse *et al*., 1996; Thomas *et al*., 2003b). Moreover, sialic acid residues in *M. bovis* proteins were shown to play a role in cytoadhesion (Sachse *et al*., 1993; Sachse *et al*., 1996). At the level of specific proteins, the 32 kDa membrane surface-exposed protein P26 was shown to be a major adhesin of *M. bovis* in the EBL cell model (Sachse *et al*., 1993; Sachse *et al*., 1996). However, the monoclonal antibody 4F6 directed against P26 did not significantly reduce adhesion to BBE cells (Thomas *et al*., 2003b). Another adhesion-related factor of *M. bovis* is the membrane-associated glycolytic enzyme α-enolase, since it induces mycoplasma adherence to EBL cells by binding plasminogen (Song *et al*., 2012). In fact, pretreating EBL cells with plasminogen augmented *M. bovis* adhesion by 11.9% (Song *et al*., 2012). Pre-treatment of *M. bovis* with low concentrations of trypsin increased their proteolytic activity and

adhesion rate to EBL cells, indicating that other proteolytic enzymes activated by partial digestion with minimal amounts of trypsin are also involved in adhesion (Song *et al*., 2012).

Variable surface proteins (*vsp*) were also shown to play a role in *M. bovis* cell adhesion (Sachse *et al*., 1996) since host cells bound *vsp* in Western blots (Sachse *et al*., 1996). Moreover, addition of purified *vsp* decreased *M. bovis* cytoadhesion and *vsp* were retained on host cell layers during cell adhesion experiments (Sachse *et al*., 2000). This was evidenced through the partial cytoadherence inhibition of *M. bovis* PG45 toward EBL cells, using oligopeptides from repetitive domains of *vsp* (Sachse et al., 2000).

The *M. bovis* specific monoclonal antibodies 1E5 and 4D7 (both against common epitopes in *vsp*A, *vsp*B and *vsp*C), 2A8 (against *vsp*C) and 9F1 (against *vsp*F) were used to investigate if mycoplasmal *vsps* contact sites are accessible to antibodies (Thomas *et al*., 2003b). Results showed a partial blocking of adherence of *M. bovis* strains PG45 and 0435 by all specific monoclonal antibodies due to their binding to adherence sites of *vsps* (Thomas *et al*., 2003b). However, the inhibition of adherence was dependent on the cell line used (Sachse *et al*., 1996; Sachse *et al*., 2000; Thomas *et al*., 2003b). Variation of surface antigens through insertion or deletion of the repetitive units in *vsps* generates additional epitopes or removes some epitopes, resulting in an increase or decrease in cytoadhesion also leading to a wider or smaller range of ligands to bind to (Sachse *et al*., 2000; Burki *et al*., 2015).

2.6.4 Cell invasion by *Mycoplasma bovis*

In *Mycoplasma bovis* infected calves, mycoplasmas were found in the cytoplasm of various cell types such as macrophages, neutrophils, hepatocytes, bile duct epithelial cells, renal tubular cells or axons of facial nerves (Rodriguez *et al*., 1996; Maeda *et al*.,

2003). *Mycoplasma bovis* antigen was additionally detected in monocytes, lymph nodes and also occasionally in bronchiolar epithelial cells (Adegboye *et al*., 1995; Rodriguez *et al*., 1996; Maeda *et al*., 2003; Kleinschmidt *et al*., 2013). The survival of *M. bovis* in phagocytes indicates intracellular persistence of the bacteria presumably through an alteration of one of the steps following engulfment during the process of phagocytosis (Kleinschmidt *et al*., 2013). In an *in vitro* assay, *M. bovis* strain *Mb*1 was found to persist in various bovine peripheral blood mononuclear cells (PBMC) subpopulations such as T cells, T helper cells, B cells, monocytes, γδ T cells, cytotoxic T cells, natural killer (NK) cells and dendritic cells, as well as in bovine erythrocytes (van der Merwe *et al*., 2010). Dependent on the cell type used and the time of infection, different intracellular localizations of *M. bovis* were identified. *M. bovis* associated with the cytosolic side of the cell membrane, present in vacuole-like structures as well as a diffuse distribution were observed (Burki *et al*., 2015). Different extent and rate of invasion between cell types and PBMC subpopulations may occur due to distinct receptors required for *M. bovis* adhesion and invasion, or to differential signaling induced by *M. bovis* reliant on the cell type (van der Merwe *et al*., 2010). Interestingly, *M. bovis* was not identified inside alveolar epithelial cells of the udder (Burki *et al*., 2015). However, recent *in vitro* studies using fluorescence and transmission electron microscopy confirmed an intracellular stage of virulent *M. bovis* in primary embryonic calf turbinate cells (Burki *et al*., 2015). Overall, *M. bovis* invasion of epithelial cells, as well as immune cells could contribute to the dissemination of the pathogen to different infection sites in the host and impair control of *M. bovis* infection through antibiotic treatment (Burki *et al*., 2015). However, further investigations are necessary to dissect the molecular mechanisms involved in persistence of *M. bovis* in different bovine cell types (Burki *et al*., 2015).

2.6.5 Modulation of the host's immune system by *Mycoplasma bovis*

Mycoplasmal membrane proteins are important because of their direct interaction with the host immune system. *M. bovis* host cell interactions seem to be dependent on the cell type, or the subset of PBMCs. van der Merwe *et al*., (2010) observed induction of IFN-γ in T cells, T helper cells, cytotoxic T cells, NK cells and γδ T cells, whereas no IFN-γ induction was measured in monocytes, dendritic cells or B cells (Burki *et al*., 2015). Similarly, there are studies concerning *M. bovis* induced apoptosis of PBMCs (Vanden Bush and Rosenbusch, 2002; Mulongo *et al*., 2014). Under *in vitro* conditions, the induction of apoptosis in lymphocytes by *M. bovis* was reported (Vanden Bush and Rosenbusch, 2002), whereas a delay of the apoptosis process in bovine monocytes infected with *M. bovis* was observed in another study (Mulongo *et al*., 2014). Moreover, there are reports suggesting suppression as well as stimulation of the host immune system by *M. bovis* (Kauf *et al*., 2007; van der Merwe *et al*., 2010). Immune stimulation seems to occur through macrophages, T cells, or complement activation as well as by the expression of cytokines up-regulating the immune response (Jungi *et al*., 1996; Razin *et al*., 1998; Rosati *et al*., 1999; Vanden Bush and Rosenbusch, 2003; Robino *et al*., 2005; Kauf *et al*., 2007; van der Merwe *et al*., 2010). Immune suppression is suspected to occur by the expression of anti-inflammatory cytokines or chemokines like IL-10 and by the suppression of expression of pro-inflammatory cytokines such as IFN-γ and TNF-α (Mulongo *et al*., 2014). Interleukin-10 then shifts the adaptive immune response to express T helper cells type 2 (Th2), resulting in strong expression of IgG1, leading to poor opsonization and immunity. Alternatively, suppression of the host immune system could be accomplished by the down-regulation of lymphocyte proliferation, by a putative mycoplasmal lympho-inhibitory protein, or through interference with the lympho-proliferative response to phytoagglutinin (Thomas *et al*.,

1990; Vanden Bush and Rosenbusch, 2003; van der Merwe *et al*., 2010). Thereby the proliferation of lymphocytes is down- regulated, whereas their cytokine expression is not altered (Burki *et al*., 2015). *Mycoplasma bovis* seems to suppress the lymphocyte-mediated immune response via a decrease of their population (van der Merwe *et al*., 2010). Another strategy of *M. bovis* to restrain the host immune response is to bind neutrophils and thereby inhibit their oxidative burst (Thomas *et al*., 1991). The modulation of the host immune response is compatible with a prolonged survival and systemic dissemination of *M. bovis* in the host as observed in infected cattle (Mulongo *et al*., 2014).

2.6.6 Biofilm formation and secondary metabolites of *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*

Biofilm formation where bacterial cells adhere to a surface and surround themselves in a polysaccharide matrix that are mostly resistant to bacteriocidal activity of the host and to antibiotics is thought to be an important factor in disease initiation and persistence for many bacterial species (McAuliffe *et al*., 2007; Pilo *et al*., 2007). Biofilm production contributes to bacterial persistence in the environment and inside the host leading to the chronicity of a disease (McAuliffe *et al*., 2006). Additionally, biofilms can also increase damage in host tissues since phagocytes are attracted, releasing lysosomal enzymes, reactive oxygen, and nitrogen species (ROS and RNS), whereas phagocytosis is rather inefficient under these conditions (McAuliffe *et al*., 2006; Hermeyer *et al*., 2011). The role of bacterially derived biofilms in causing human disease has been known for some time (Wilson, 2001), and an increasing appreciation of biofilms in bovine mastitis was reported to be emerging (Melchior *et al*., 2006; Gomes *et al*., 2016). It is therefore plausible that biofilms elaborated by *M. bovis* may influence some aspect of the disease course or pathogenicity in cattle (Calcutt *et al*., 2018).

Conversely, *Mmm* does not form biofilms as shown in a published report, where 25 strains from different origins and with variable degrees of virulence were tested for biofilm production (McAuliffe *et al*., 2006). Contrary to this McAuliffe *et al*. (2007) examined biofilm formation by *Mmm* using a simple model without an air/liquid interface and have found that adherent *Mmm* was more resistant to many stresses, including heat, drying, osmotic shock and oxidative stress. Biofilms of *Mmm* also exhibited remarkable persistence and were able to survive for up to 20 weeks in stationary phase (McAuliffe *et al*., 2007). Several *Mycoplasma* species including *M. bovis* produce biofilms apparently independently of their pathogenicity (McAuliffe *et al*., 2006). The capacity of several *Mycoplasma* species to adhere to host cells appears to be essential since adherence to coverslips was observed as the initial step of biofilm formation (McAuliffe *et al*., 2006). The extent of biofilm formation under *in vitro* growth is very diverse among *M. bovis* strains and correlates with the different molecular types or *vsp* profiles (McAuliffe *et al*., 2006). Since *vsps* are supposed to be involved in adhesion (Sachse *et al*., 1996, Sachse *et al*., 2000), certain *vsp* patterns may display different adhesion capabilities, resulting in a variable ability to form biofilms (McAuliffe *et al*., 2006). Biofilm production increases bacterial resistance toward environmental stressors and host defenses (Mah and O'Toole, 2001). Indeed, biofilm forming *M. bovis* strains were more resistant to heat and desiccation enabling their survival in the environment, whereas no change in minimal inhibitory concentrations (MIC) of fluoroquinolones and tetracyclines was detected (McAuliffe *et al*., 2006).

Oxygen uptake and H_2O_2 production were identified as particular characteristics in fermentative *Mycoplasma* species and were expected to influence the virulence of pathogenic mycoplasmas (Pilo *et al*., 2007). Secondary metabolites were shown to be involved in the pathogenesis of *Mycoplasma* species (Pilo *et al*., 2005; Hames *et al*.,

2009). Hydrogen peroxide (H_2O_2) production is a major virulence factor of several *Mycoplasma* species, leading to cell death, inhibition of ciliary action or peroxidation of lipids (Tryon and Baseman, 1992; Pilo *et al*., 2005; Hames *et al*., 2009). In necropsy material, H_2O_2 production was identified in all *M. bovis* isolates (Schott *et al*., 2013). Additionally, ROS and RNS were detected following the recruitment and stimulation of macrophages and neutrophils (Hermeyer *et al*., 2011). The combination of mycoplasmal H_2O_2 and ROS/RNS from white blood cells may result in the severe typical caseonecrotic lung lesions (Hermeyer *et al*., 2011; Schott *et al*., 2013). Hydrogen peroxide formation by *M. bovis* type and field strains was also measured *in vitro* showing strain variability concerning the amount of H_2O_2 produced (Khan *et al*., 2005). Amounts of H_2O_2 generated from NADH oxidation varied from 0 to 1.1 mol H_2O_2/mol O_2 depending on the strains (Khan *et al*., 2005). This is proposed to be due to NADH-oxidase mainly producing either water or H_2O_2 (Khan *et al*., 2005). *In vitro* passage of strains led to a reduction of H_2O_2 levels, whereas oxidation-rates of other substrates did not change (Khan *et al*., 2005). This was demonstrated by the loss of a 32 kDa protein after extensive passaging of a *M. bovis* strain in axenic medium that correlated with a 50% reduction of H_2O_2 production (Khan *et al*., 2005). However, L-α-glycerophosphate (GlP) was never oxidized by any strain tested, indicating the absence of glycerophosphate oxidase (GlpO) in all non- fermentative, non-arginine-hydrolyzing *M. bovis* strains (Khan *et al*., 2005).

Mycoplasma mycoides subsp. *mycoides* strains isolated from the re-emerging European outbreaks of CBPP in 1980-1999 were reported to 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 suggested that a glycerophosphate oxidase could represent a significant virulence factor of *Mmm* (Rice *et al*., 2001; Pilo *et al*., 2005). The role of

glycerol metabolism in the virulence of *Mmm* was conducted, based on the observation that African strains contain an efficient active glycerol import system, GtsABC, specified by an ABC transporter protein, while the European strains, which are considered less virulent and cause a disease that appears to be largely chronic showing few clinical signs and low mortality are devoid of this transporter protein (Vilei and Frey, 2001). Glycerol is metabolized after uptake and phosphorylation to dihydroxyacetone phosphate (DHAP) by an oxidative process leading to the release of the highly toxic compound H_2O_2. Blocking the glycerol uptake proteins GtsABC by specific antibodies resulted in a significant reduction of H_2O_2 production (Vilei and Frey, 2001). Furthermore, it was shown that European strains that lack the GtsABC transporter produce significantly lower amounts of H_2O_2 (Vilei and Frey, 2001; Pilo *et al*., 2007).

A proposed model for triggering cellular damage to eukaryotic cells is that glycerol present in the interstitial fluid is incorporated actively via the highly active ABC glycerol transporter GtsABC and is subsequently phosphorylated into G3P. This, in turn, is oxidized by GlpO into DHAP, which enters in the glycolysis cycle of the mycoplasma, and H_2O_2 is released (Pilo *et al*., 2007). Facilitated by the intimate contact of the mycoplasma with the host cell membrane, H_2O_2 or ROS enters the host cell. Inside the host cells, these toxic compounds act as powerful mediators of cell injury and inducers of inflammatory processes (Figure 2.1). They are expected to damage the host either by directly impairing tissue cells or by inducing host gene expression, e.g. of pro-inflammatory genes via activation of the nuclear factor kappa B (NF-κB) (Baeuerle and Henkel, 1994; Pilo *et al*., 2007), or via the Fenton reaction (Crichton *et al*., 2002).

Mycoplasmas have been reported to induce a respiratory burst in phagocyte cells, suggesting that host-generated ROS might further contribute to tissue damage (Vilei and Frey, 2001). Furthermore, it was shown that *Mmm* strains are also able to induce the tumor necrosis factor alpha (TNF-α) in bovine alveolar macrophages (Jungi *et al*., 1996). TNF-α also acts as an inflammatory mediator and, in association with NF-κB, can act as primary signals inducing apoptosis in host cells (Pilo *et al*., 2007). Schematic representation of the various virulence pathways of *Mmm* is shown in Figure 2.1.

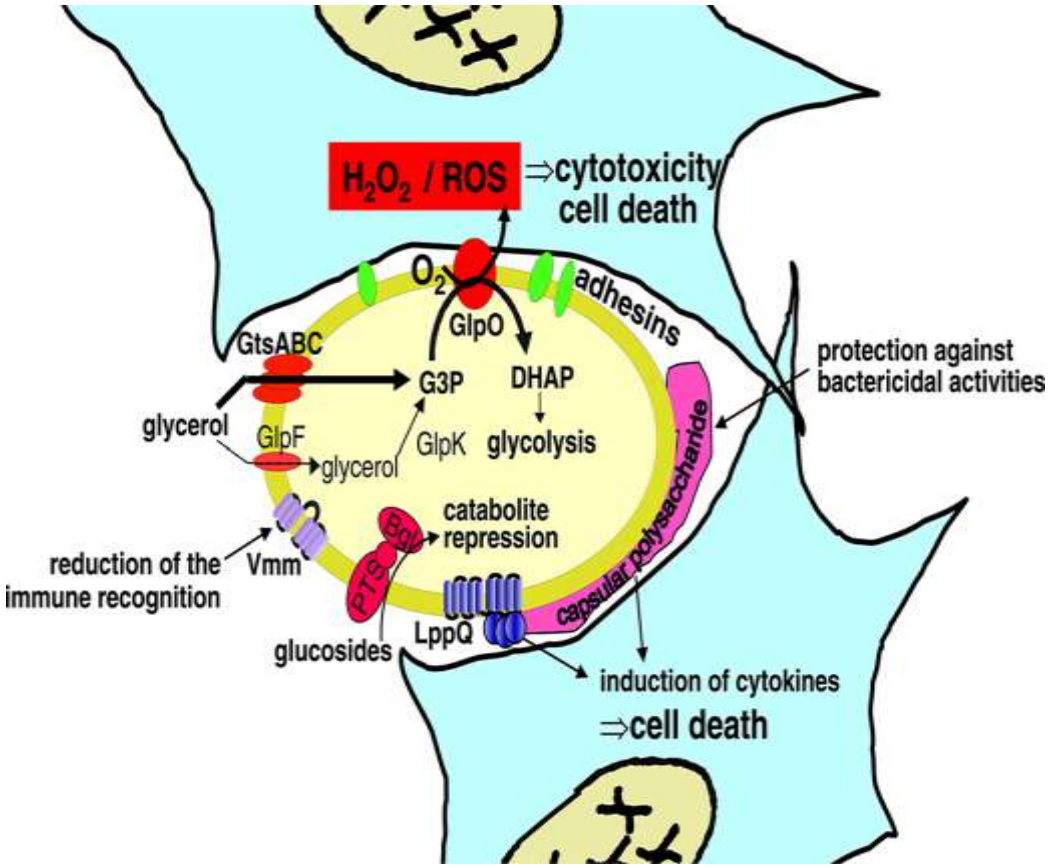


Figure 2.1: Schematic representation of the various virulence pathways of *Mycoplasma mycoides* subsp. *mycoides* (Pilo *et al*., 2007)

2.6.7 Mechanism for pathogenicity of *Mycoplasma mycoides* subsp. *mycoides*

To cause disease, *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) possesses particular mechanisms to adhere to the host tissue, evade the host's immune defence, enable persistence and dissemination in the infected animal, and cause inflammation and disease signs through cytotoxicity (Pilo *et al*., 2007). The loss of any of these mechanisms can lead to loss of virulence (Pilo *et al*., 2007). In contrast to other pathogenic bacteria, where virulence is determined mainly by cytolysins and invasins, no such typical primary virulence genes have been found on the genomes of the ten *Mycoplasma* species that have been sequenced completely (Vasconcelos *et al*., 2005; Pilo *et al*., 2007). Mycoplasmas seem rather to use intrinsic metabolic and catabolic functions to cause disease in the affected host and to ensure the microbe's survival (Pilo *et al*., 2005; Pilo *et al*., 2007). *Mycoplasma mycoides* subsp. *mycoides* is the most pathogenic mycoplasma among all the *Mycoplasma* species of cattle. Its virulence is probably the result of a coordinated action of various components of an antigenically and functionally dynamic surface architecture (Pilo *et al*., 2007). The different virulence attributes allow the pathogen to evade the host's immune defence, adhere tightly to the host cell surface, persist and disseminate in the host causing mycoplasmaemia, efficiently import energetically valuable nutrients present in the environment, release and simultaneously translocate toxic metabolic pathway products to the host cell where they cause cytotoxic effects that are known to induce inflammatory processes and disease (Pilo *et al*., 2007). This strategy enables the mycoplasma to exploit the minimal genetic information in its small genome, not only to fulfil the basic functions for its replication but also to damage host cells in intimate proximity thereby acquiring the necessary bio-molecules, such as amino acids and nucleic acid precursors, for its own biosynthesis and survival (Pilo *et al*., 2007).

2.6.8 Lipoproteins of *Mycoplasma mycoides* subspecies *mycoides*

Lipoproteins play an important role in triggering mechanisms of pathogenicity as a result of its interaction with adhesions on their surfaces of most mycoplasmas. Because of their antigenic properties, they are considered as valuable targets for specific and sensitive diagnosis (Pilo *et al*., 2007). Few lipoproteins of *Mycoplasma mycoides* subsp. *mycoides* have been detailly characterized. Majority of the lipoproteins plays a role as strong antigens and are easily detected in serum of infected cattle on immunoblots (Pilo *et al*., 2007). Lipoprotein A of *Mmm* is strongly conserved among mycoplasmas of the *M. mycoides* clusters, hence it cannot be used as a specific target for serodetection (Monnerat *et al.,* 1999). Its role in Th1 and Th2 immunity is currently under investigation. Lipoprotein B is found only in *Mmm* strains belonging to the African/Australian cluster, but it is not found in strains isolated from the re-emerging European outbreaks in 1980-2000. It is, however, also present in other mycoplasmas of the *M. mycoides* cluster (Vilei *et al.,* 2000). The role of two further lipoproteins *Lpp*C and *Lpp*D is under investigation. Lipoprotein Q (*Lpp*Q) seems to be a particular lipoprotein of *Mmm* as it is specific to this organism. It has a particularly strong antigenic N-terminal part which is located on the outer surface of the membrane, while its C-terminal part is involved in membrane anchoring (Abdo *et al.,* 2000). The high specificity and strong antigenicity of *Lpp*Q have been exploited for the development of a robust indirect ELISA test for serological diagnosis and for epidemiological investigations of CBPP (Bruderer *et al.,* 2002). Structural analysis of *Lpp*Q showed strong analogies to proteins with super-antigenic character (Abdo *et al.,* 2000). A study has shown that cattle immunized with purified recombinant *Lpp*Q, using different adjuvant methods, were significantly more susceptible to challenge with *Mmm* than cattle that were not vaccinated with *Lpp*Q (Frey *et al*., 2003). Hence, *Lpp*Q is assumed

to play an adverse reaction in vaccination similar to the peptidoglycan-associated lipoprotein *Pal*A of *Actinobacillus pleuropneumoniae*, which was shown to inhibit completely beneficial effects of efficient subunit vaccines when animals were vaccinated simultaneously with *Pal*A (van den Bosch and Frey, 2003). In this respect, it must be noted that the currently used life vaccines express *Lpp*Q, a matter to be considered in the development of new vaccine strains (Frey *et al*., 2003). Lipoprotein Q (*Lpp*Q) may therefore contribute to the immunopathologies induced by *Mmm* (Pilo *et al*., 2007). The known antigens of *Mycoplasma mycoides* subsp. *mycoides* are shown in Table 2.4.

Table 2.4: Known antigens of *Mycoplasma mycoides* subspecies *mycoides*

Antigen	Function	Location	Impact in virulence	Effect in vaccine
*Lpp*A	Lipoprotein	Membrane	Strong antigen	Under investigation
*Lpp*B	Lipoprotein	Membrane	Unknown	Probably no
*Lpp*C	Lipoprotein	Membrane	Unknown	Under investigation
*Lpp*Q	Lipoprotein	Membrane	Strong (super) antigen	Adversary
*Lpp*D	Lipoprotein	Membrane	Unclear	Under investigation
*Gts*ABC	Glycerol uptake + phosphorylation	Membrane	Yes	Possible candidate
*Glp*O	Glycerol-3-phosphate oxidase	Membrane	Yes (anti-*Glp*O block cytotoxicity)	Good candidate
Bgl	6-phospho-beta glucosidase	Cytosol	Bacterial survival	Under investigation
Vsp	Variable surface protein	Membrane	Escape of hots's immune defence	Probably no

Source: Frey *et al*., 2003

Lpp = lipoprotein
*Gts*ABC = ATP-binding cassette transporter system
Vsp = variable surface proteins
*Glp*O = glycerophosphate oxidase

2.6.9 Exopolysaccharides secretion by *Mycoplasma mycoides* subspecies *mycoides*

Molecules located on the outer surface or in the plasma membrane of *Mycoplasma* species, such as lipoproteins, capsular polysaccharides and biofilms composed mainly of fibrillar polycarbohydrates surrounding cells, are generally assumed to protect the pathogen from the bactericidal activity of complement and other host defence functions, and to trigger the inflammatory process in the infected host (Pilo *et al*., 2007). The capsular polysaccharide galactan, composed of 6-O-β-D-galactofuranosyl-D-galactose and was shown to increase the virulence of the strongly attenuated *Mmm* vaccine strain KH3J and produced transient apnea, increased pulmonary arterial pressure and pulmonary edema when injected intravenously in calves (Pilo *et al*., 2007). The impact 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 (Pilo *et al*., 2007).

In the absence of classical virulence determinants, the pathogenicity of *Mmm* is thought to rely on intrinsic metabolic functions and specific components of the outer cell surface (Bertin *et al*., 2013). *Mycoplasma mycoides* subsp. *mycoides* has been shown to produce two types of polysaccharides: a capsular one, namely galactan, and an extracellular one, named exopolysaccharide (EPS) (Branda *et al*., 2005). The purification of free EPS has been jeopardized by polysaccharide contaminants in the complex culture medium used to grow mycoplasmas. Exopolysaccharide is immunologically related to the capsular galactan and has been found in large amounts as a free *Mmm* product unassociated with cells, both in culture supernatants and in the blood of infected cattle (Bertin *et al*., 2013). *Mycoplasma mycodes* subsp. *mycoides* strains able to produce large amounts of capsular polysaccharide proved less sensitive to growth inhibition by bovine antisera

(March *et al*., 1999) and displayed longer bacteremia than strains with little capsular polysaccharide in a mouse infection model (March and Brodlie, 2000). This clearly pointed towards a role of capsular polysaccharides in *Mmm* persistence and dissemination. The capsular polysaccharide galactan has been notably demonstrated to play a role in *Mmm* persistence and dissemination (Bertin *et al*., 2013). The free exopolysaccharides (EPS) also produced by *Mmm* and shown to circulate in the blood stream of infected cattle, have received little attention so far. Indeed, their characterization has been hindered by the presence of polysaccharide contaminants in the complex *Mycoplasma* culture medium (Bertin *et al*., 2013). A method was developed where large quantities of EPS were produce by transfer of *Mycoplasma* cells from their complex broth to a chemically defined medium and subsequent purification. The intraclonal *Mmm* variants that produce opaque/translucent colonies on agar were analyzed. First, colony opacity that was related to the production of a capsule was demonstrated, as observed by electron microscopy (Bertin *et al*., 2013). Then the EPS extracts were compared and showed that the non-capsulated, translucent colony variants produced higher amounts of free EPS than the capsulated, opaque colony variants. This phenotypic variation was associated with an antigenic variation of a specific glucose phosphotransferase permease (Bertin *et al*., 2013). Finally, *in silico* analyses of candidate polysaccharide biosynthetic pathways were conducted in order to decode the potential link between glucose phosphotransferase permease activity and attachment/release of galactan. The co-existence of variants producing alternative forms of galactan (capsular versus free extracellular galactan) and associated with an antigenic switch constitutes a finely tuned mechanism that may be involved in virulence (Bertin *et al*., 2013).

2.7 Epidemiology of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

Mycoplasma bovis is well adapted to colonization of mucosal surfaces, where it can persist without causing clinical disease (Francis, 2017). The upper respiratory tract (URT) mucosa is the most important site of *M. bovis* colonization in cattle after URT exposure (Nicholas *et al*., 2002). After intramammary exposure, the mammary gland appears to be the major site of colonization (Fox *et al*., 2008). Regardless of the route of exposure, *M. bovis* can be isolated from multiple body sites during early infection, particularly the URT, mammary gland, conjunctiva and urogenital tract (Punyapornwithaya *et al*., 2010). Mycoplasmemia during *M. bovis* infection has been well reported (Nicholas *et al*., 2002; Fox *et al*., 2008). The URT mucosa and the mammary gland appear to be the most important sites of persistence and shedding of the organism (Punyapornwithaya *et al*., 2010). Although many cattle can shed *M. bovis* for few months or less (Punyapornwithaya *et al*., 2010), some cattle shed the organism intermittently for months or years (Biddle *et al*., 2003). The factors liable for intermittent shedding of the organism have not been established. However, cattle with clinical disease usually shed large numbers of *M. bovis* (Maunsell *et al*., 2011). Stressful conditions such as transportation, comingling, entry into a feedlot, and cold stress are connected with increased rates of nasal shedding of *M. bovis* (Fox *et al*., 2008). Chronic asymptomatic infection with intermittent shedding of *M. bovis* appears critical to the epidemiology of infection, especially the maintenance of the agent within a herd and exposure of naive populations (Maunsell *et al*., 2011; Francis, 2017).

Contagious bovine pleuropneumonia (CBPP) caused by *Mmm* is a notifiable disease to the World Organization for Animal Health (OIE) because of its major impact on livestock production and a potential for rapid spread across national borders (Egwu *et*

al., 1996). The disease has been reported causing enormous problems in Africa with severe socio-economic consequences (Windsor, 2000; Jores *et al*., 2013). Because of the method of spread, outbreaks tend to be more extensive in housed animals and in those in transit by train or on foot and the incubation period can last from a few days up to several months (Mariner *et al*., 2006b). In groups of susceptible cattle the morbidity approaches 90%, the morbidity and mortality may be as high as 50% and 25% respectively and the infected ones remain as recovered carriers with or without clinical signs (Cassel *et al*., 1985; ter Laak, 1992). The epidemiology of CBPP is characterized by direct contact, long incubation period, and possibility of early excretion of *Mmm* (up to 3 weeks before appearance of clinical signs) during the course of the disease and after recovery in "lungers" (up to two years) (Gedlu, 2004). These epidemiological features on the one hand, and the lack of reliable screening tests to pick up early carriers and lungers on the other hand, make it essential to control cattle movements in order to minimize the spread of the disease (Gedlu, 2004).

The epidemiology of CBPP in Africa is subject to different factors; the species affected are cattle; there is absence of reservoir host in wild animals, although the organism has been reported in small ruminants (Egwu *et al*., 2012), transmission is through the direct contact of clinical cases or chronic carriers with susceptible animal and, cattle movements play a very significant role in the maintenance and extension of the disease (Bessin and Connor, 2000).

In Nigeria, control of CBPP was achieved in the early sixties, even though it was for short period as the disease re-surfaced a few years later, most probable from chronic carriers from any of the close by countries of Niger, Chad and Cameroun (Egwu *et al*., 1996). High number of outbreak of CBPP was reported in the north during winter period

when apparently healthy and carrier animals concentrate near the rivers, streams and ponds for watering and grazing leading to transmission of infection (Nwanta and Umoh, 1992). Contrary to that, a survey conducted in north eastern Nigeria reported more cases of the disease during rainy season between the months of June and October than in dry season (Egwu *et al*., 1996). It is however a fact that the disease is endemic in northern Nigeria and north east in particular because outbreak do occur throughout the year irrespective of the season (Francis *et al*., 2018a).

2.8 Molecular Epidemiology of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

Mycoplasma bovis is well equipped to generate genetically diverse populations, and has been observed to undergo DNA recombination and rearrangement events at high frequency (Lysnyansky *et al*., 1996; Poumarat *et al*., 1999; Nussbaum *et al*., 2002). The *M. bovis* genome contains a large number of insertion sequences which are also likely to lead to heterogeneous populations (Miles *et al*., 2005; Thomas *et al*., 2005). There have been several molecular epidemiological studies of *M. bovis* utilizing a variety of DNA fingerprinting techniques including randomly-amplified polymorphic DNA analysis, amplified fragment length polymorphism analysis, restriction fragment length polymorphism analysis, pulsed-field gel electrophoresis (PFGE) analysis, and insertion-sequence profile analysis (Poumarat *et al*., 1994; Kusiluka *et al*., 2000b; Butler *et al*., 2001; McAuliffe *et al*., 2004; Biddle *et al*., 2005; Miles *et al*., 2005). Considerable genomic heterogeneity between field isolates of *M. bovis* has been reported, especially when isolates were collected from diverse geographical regions and over a period of several years (Poumarat *et al*., 1994; McAuliffe *et al*., 2004; Miles *et al*., 2005). Correlations between particular DNA fingerprint types and geographic location, year of isolation, and type or severity of pathology have not been identified (Poumarat *et al*.,

1994; Kusiluka *et al*., 2000b; McAuliffe *et al*., 2004; Miles *et al*., 2005). This may reflect the frequent movement of cattle among herds in modern management systems, as well as the ability of *M. bovis* to create genetically diverse populations (Maunsell *et al*., 2011).

Comparison of pulse field gel electrophoresis (PFGE) patterns for isolates of *Mycoplasma bovis* or *Mycoplasma californicum* obtained at necropsy from multiple body sites in seven cows with mycoplasmal mastitis was reported (Biddle *et al*., 2005). Within each cow, the same PFGE pattern was found in 100% of isolates from sites in the mammary system (milk, mammary parenchyma and supra-mammary lymph nodes). Forty-one percent of isolates obtained from the respiratory system and 90% of isolates obtained from other body systems had PFGE patterns identical to that of the mammary isolates (McAuliffe *et al*., 2005). These findings indicated that the same strain of *M. bovis* often colonizes multiple body sites, but also that multiple strains may be present within an animal. Isolates of *M. bovis* from multiple sites of pathology within the same animal or from multiple animals in the same disease outbreak typically are closely related or identical by DNA typing methods, especially when the herd is closed (Gonzalez *et al*., 1993; Kusiluka *et al*., 2000b; Butler *et al*., 2001; McAuliffe *et al*., 2005). In contrast, endemically-infected open herds, including dairy calf ranches, harbour numerous genetically diverse strains of *M. bovis*. This has been ascribed to introduction of animals from several sources over time (Butler *et al*., 2001).

Strains of *Mmm* causing CBPP were considered very homogeneous until 1995, when various techniques such as enzymatic restriction of whole DNA or Southern blotting showed that this was not the case (Lorenzon *et al*., 2003). These techniques are unfortunately difficult to standardize and require the extraction of DNA from an *Mmm*

culture. A study was conducted to investigate the possibility of constructing a molecular epidemiology tool based on multilocus sequence analysis (MLSA) with PCR amplification of various loci followed by sequencing (Lorenzon *et al*., 2003). Six loci were found suitable for this purpose and an additional PCR was designed to detect the presence of an 8.8 kb deletion earlier described in some strains. Fifteen different MLSA profiles were evidenced in the study. They allowed a clear distinction between European, south-western African and sub-Saharan strains (Lorenzon *et al*., 2003).

However, polymerase chain reactions (PCR) can detect *Mmm* strains in blood, tissues and body fluids of individual cattle, even in chronic cases (Schnee *et al.,* 2011), as well as differentiating these strains according to their geographical lineages (Poumarat & Solsona, 1995; Miles *et al.,* 2006). Molecular typing using restriction fragment length polymorphism (PCR-RFLP) was used to identify *Mycoplasma mycoides* sub /clusters and *Mmm* with a molecular size of 575-bp as well as 180-bp and 380-bp respectively (Bashiruddin *et al*., 1994; Muuka *et al*., 2013)). A study aimed at epidemiologically detecting and identifying *Mmm* strains under field conditions in asymptomatic pastoral cattle of Niger State, north-central Nigeria was conducted (Alhaji and Babalobi, 2015). The authors established control strategies of the infection since virulence consequences of *Mmm* differ widely depending on the clusters, and have modified two PCR assays of conventional PCR and fingerprinting that target *Mmm* strains genes (Alhaji and Babalobi, 2015).

2.9 Genome Sequencing of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

Whole genome sequencing (WGS) is a widely used and accepted tool for investigating microbial genome sequences as high throughput sequencing becomes faster, cheaper and more readily available (Parker *et al*., 2018). It involves sequencing the entire genome of selected isolates which can then be used for clinical diagnostics, disease outbreak investigation, controlling microbial resistance (Edwards and Holt, 2013) and has also been used to compare the genetic diversity between a large number of isolates (Parker *et al*., 2016).

The complete and fully assembled genome sequence of *M. bovis* type strain PG45 was performed for this species and offers a framework for comparison with additional pathogenic isolates (Wise *et al*., 2011). The single circular chromosome of 1,003,404-bp reveals multiple gene sets and mechanisms involved in variable expression of surface antigens and the incursion of numerous and assorted mobile elements, despite its reduced size (Wise *et al*., 2011). The genome has a 29.3% G+C content and contains 826 open reading frames (ORFs; including 61 pseudogenes) with 89% coding density, and has limited sets of 6 rRNA and 34 tRNA genes, characteristic of *Mollicutes* (Wise *et al*., 2011). A large set of 54 IS elements (comprising seven distinct categories) (Lysynyansky *et al*., 2009) is scattered throughout the chromosome. Two integrative and conjugative elements (ICE) also known as conjugative transposons occur. ICEB-1 (23,271-bp, 18 ORFs) is a counterpart to ICEA, an element similarly positioned in the highly syntenous *M. agalactiae* PG2 genome (Nouvel *et al*., 2010), thereby, suggesting that integration occurred in a common ancestor prior to speciation. ICEB-2 (37,408-bp, 33 ORFs) is inserted into an IS element that is implicated in the inversion of a 483-kb region of the *M. bovis* PG45 chromosome, relative to *M. agalactiae* PG2. The potential

for genome plasticity among strains of *M. bovis* is underscored by these features (Wise *et al*., 2011).

The genome of *M. bovis* strain Hubei-1 isolated in 2008 from a case of calf pneumonia in a Chinese farm was sequenced (Li *et al*., 2011). The genome contains a single circular chromosome of 953,114-bp with a 29.37% G+C content. Also identified were 803 open reading frames (ORFs) that occupies 89.5% of the genome (Li *et al*., 2011). The authors reported 45 lipoproteins, 12 extracellular proteins and 18 transmembrane proteins which are phase variable proteins and these helps the organism escape host immune response (Li *et al*., 2011). The genomic analysis validated lateral gene transfer between *M. bovis* and the *Mmm* (Li *et al*., 2011).

Reference strain of *Mmm* PG1 was completely sequenced more than a decade ago (Westberg *et al*., 2004). It was reported that the genome comprises of a single circular chromosome of 1,211,703-bp with the lowest G+C content (24 mol %) and a highest density of insertion sequences (13% of the genome size) of all sequenced bacterial genome (Westberg *et al*., 2004). The genome of *Mmm* was also reported to contain 985 putative genes with an average length of 982-bp, of which 72 are part of insertion sequences and encode transposases (Westberg *et al*., 2004). About 27% of the putative genes are unique to *Mmm* and 14% of which have an unknown function (Westberg *et al*., 2004).

The Italian strain 57/13 of *Mmm* which was isolated in 1992 during CBPP outbreak in Italy was also sequenced (Orsini *et al*., 2015). The genome of which was reported to contain 1,192,498-bp nucleotide chromosome with 2 rRNA operons, 30 tRNAs, and 1,077 protein coding genes, of which 332 encode hypothetical protein conserved among other *Mmm* isolates (Orsini *et al*., 2015). As compared with other European strains, the

isolates were reported to lack the *Gts*C and *Lpp*B genes (Vilei *et al*., 2000). Compared to the reference *Mmm* PG1 type strain, the *Mmm* strain 57/13 has been reported to show overall identity of 99% and one major insertion (Orsini *et al*., 2015).

The genome of *Mmm* strain T1/44, a vaccine strain commonly used in Africa was sequenced (Gourgues *et al*., 2016). It consists of a 1,188,848-bp chromosome with a G+C content of 23.92% and encoded 1,112 coding sequences, 2 rRNA sets (5S rRNA, 16S rRNA, 23S rRNA), and 30 tRNAs. Its genome organization was reported to be similar to the previously sequenced reference strain PG1 except for an inversion of 173-kb between two IS-rich loci. This remarkable inversion (positions 1,173,545 to 1,188,848 and 1 to 157,822) includes the predicted chromosomal replication origin (Gourgues *et al*., 2016).

2.10 Economic Impact of *Mycoplasma bovis* Infection and Contagious Bovine Pleuropneumonia

There are limited data available relating to the economic impact that *M. bovis*-associated disease had upon production and care of cattle. Additionally there are concerns regarding the accuracy of the economic reports due to the difficulty of attributing acute pneumonia cases to one particular pathogen (Caswell and Archambault, 2007). Despite this, the economic burden caused by BRD is one of the most profound in food animal production (Snowder *et al*., 2006). Economic costs associated with *M. bovis* has not been determined; a detailed analysis of all cost factors such as mortality, veterinary costs, treatment, milk loss, added housing/feed costs through lack of weight gain would yield a more complete appreciation of the full economic burden (Calcutt *et al*., 2018). *Mycoplasma bovis* has been shown to be able to infect non-bovine hosts, such as broiler chickens, bison, and white-tailed deer, but the economic impact of these infections are unknown (Dyer *et al*., 2004; Dyer *et al*., 2008; Ongor *et al*., 2008; Janardhan *et al*.,

2010). Although the economic loss estimates are likely largely underestimated, the importance of *M. bovis*-associated infections is clear. Most cost estimates are related to the direct and indirect *M. bovis*-disease associated costs, however, there are also concerns related to the impact upon international trade. International trade is directly linked to animal welfare, which may be influenced by *M. bovis* infections (Soehnlen, 2011).

Contagious bovine pleuropneumonia is considered to be a disease of economic importance in Africa. The disease is associated with high mortality rate, production loss, increase in production cost due to disease control, loss of weight and working ability. Delay in marketing, reduced fertility, loss due to quarantine, loss of cattle trade, reduced investment in livestock production can all be attributed to the disease (Tambi *et al*., 2006; Radostits *et al*., 2007). The direct losses result from mortality, reduced milk yield, vaccination campaign costs, disease surveillance and research programmes. The indirect costs are mainly due to chronic nature of the disease and include loss of weight and working ability, delayed marketing, reduced fertility and losses due to quarantine (Egwu *et al*., 1996). In addition to these, it leads to imposition of rigorous limitations to international trades on CBPP-affected countries in accordance with world organization of Animal Health (OIE) regulations (Muuka *et al*., 2011; Sacchini *et al*., 2012). The economic effects of CBPP can be enormous, resulting in heavy losses in cattle populations. The financial and economic loss caused by disease in Africa is significant. Otte *et al.* (2004) reported that the continent has lost approximately 2 billion US dollar per year due to death of livestock from the disease. Contagious bovine pleuropneumonia has been reported causing significant economic loss on the agriculture sectors and the national economy (Admassu *et al*., 2015). Egwu *et al*. (1996) reported losses in excess of 1.5 million US dollar per year of cattle from CBPP in Nigeria.

2.11 Transmission

2.11.1 Transmission of *Mycoplasma bovis* infection

Mycoplasma bovis is highly contagious, transmission is generally thought to be by aerosol and introduction of asymptomatically infected animals as the primary means by which *M. bovis*-free herds become infected (Calcutt *et al*., 2018). Transmission is delayed until, and if, shedding occurs. This delay can make it difficult to identify the source of infection and mycoplasma disease outbreaks can occur in seemingly closed herds (Wilson *et al*., 2007; Maunsell *et al*., 2011). Little is known about the time of onset and duration of nasal shedding of *M. bovis* in young calves. It has been suggested that *M. bovis* prevalence is highest in calves between 1 and 4 months of age, but it is not known at which point in those months is highest regardless of differences between geographic locations (Soehnlen *et al*., 2011).

Once present in a herd, *Mycoplasma bovis* can be readily transmitted from infected to uninfected cattle. In dairy cattle, *M. bovis* has usually been considered as a contagious mastitis pathogen, with udder-to-udder spread being the most important means of transmission (Gonzalez and Wilson, 2003). Whether URT transmission with internal dissemination to the mammary gland is important in the epidemiology of *M. bovis* mastitis has not been determined, but *M. bovis* can be isolated from nasal secretions of cows with mastitis (Punyapornwithaya *et al*., 2010). For young calves, drinking of infected milk is an important means of *M. bovis* transmission. Calves fed on contaminated milk with *M. bovis* have much higher rates of nasal colonization than those fed uncontaminated milk, and feeding of contaminated milk or nursing of cows with *M. bovis* mastitis has been associated with disease in calves (Maunsell *et al*., 2011). Although, other means of transmission must also be important, as the disease can occur in calves that are fed milk replacer or pasteurized milk (Parker *et al*., 2018). It has been

documented that the teat canal and genital tract are also routes of infection and that *M. bovis* can be passed from an infected cow to its foetus (Wrathall *et al*., 2007). Once established in a multiage facility, *M. bovis* is very difficult to eliminate, suggesting ongoing transmission from older to incoming calves. Calves could also become infected from adults in the calving area (Maunsell *et al*., 2011; Parker *et al*., 2018).

Transmission of *Mycoplasma bovis* through respiratory secretions is considered vital in the epidemiology of infection, although there is little experimental data to support this contention. However, *M. bovis* might be transmitted in respiratory secretions by means of aerosols, nose-to-nose contact, or indirectly via feed, water, housing, or other formites (Nicholas *et al*., 2002). The importance of aerosols in calf-to-calf transmission of *M. bovis* is unknown, but the organism has been isolated from air in barns containing diseased calves, and calves can be experimentally infected by inhalation of *M. bovis* (Nicholas *et al*., 2002). Formite-mediated transmission of *M. bovis* in respiratory secretions is likely given that fomites can be important vehicle in the transmission of mycoplasma mastitis (Gonzalez and Wilson, 2003). Mycoplasmas are susceptible to desiccation and sunlight, but can survive for long periods in protected environments with greatest survival in cool and humid conditions (Parker *et al*., 2018). *Mycoplasma bovis* has been shown to persevere for months in recycled sand bedding (Justice-Allen *et al*., 2010), and has been found in cooling ponds and dirt lots on dairies (Maunsell *et al*., 2011; Parker *et al*., 2018).

2.11.2 Transmission of contagious bovine pleuropneumonia

Mycoplasma mycoides subsp. *mycoides* is mainly transmitted from animal to animal in respiratory aerosols (OIE, 2015). This organism also occurs in saliva, urine, fetal membranes and uterine discharges (Campbell, 2015). Close, repeated contact is

generally thought to be necessary for transmission; however, *Mmm* might be spread over longer distances (up to 200 meters) if the climatic conditions are favourable (Tardy *et al*., 2011; Campbell, 2015). Carrier animals, including subclinically infected cattle, can retain viable organisms in encapsulated lung lesions (sequestra) for several months to two years (Tardy *et al*., 2011). These animals are thought to be capable of shedding organisms, particularly when stressed. Transplacental transmission is also possible (Campbell, 2015).

Although there are a few sketchy reports of transmission on fomites, mycoplasmas do not survive for more than a few days in the environment and indirect transmission is thought to be unimportant in the epidemiology of this disease (Campbell, 2015; OIE, 2015).

2.12 Pathogenesis

2.12.1 Pathogenesis of *Mycoplasma bovis* infection

Mycoplasma bovis infection is an important disease of cattle worldwide. *Mycoplasma bovis* causes pneumonia, arthritis and mastitis in cattle and can be isolated from a variety of tissues including all areas of the reproductive tract and the eye (Minion, 2002; Francis, 2017). During natural infections, *M. bovis* can be easily observed lining the epithelial surfaces of the respiratory tract (Adegboye *et al*., 1995). *Mycoplasma bovis* has characteristics that enable it to colonize and persist on mucosal surfaces, to invade tissues, and to persist at sites of disease despite an aggressive immune response (Minion, 2002). Molecules involved in adherence, antigenic variation, invasion, immunomodulation, biofilm formation, and production of toxic metabolites are likely to

be important in pathogenesis, but exactly how *M. bovis* interacts with the host is poorly understood (Maunsell *et al*., 2011).

Mycoplasmas lack a cell wall, and exposed membrane proteins form the primary interface with the host. These membrane proteins facilitate adherence to mucosal surfaces, although *M. bovis* adhesions are not yet well characterized (Buchenau *et al*., 2010). *Mycoplasma bovis* has a large family of immunodominant variable surface lipoproteins (*vsp*), which undergo high frequency phase and size variation *in vitro* and *in vivo*, and exhibit extensive strain variation in their coding sequences (Buchenau *et al*., 2010). Particular *vsp* variants can be selected by exposure to antibodies. These characteristics impart a vast capacity for antigenic variation in *M. bovis* populations that likely contribute to immune evasion and persistence and provide a challenge for vaccine development (Maunsell *et al*., 2011).

Mycoplasma bovis antigen is often seen only at the periphery of necrotic areas suggesting that the organism is not actively involved in the necrosis itself. During infections, macrophages and neutrophils are actively recruited to infected areas (Khan *et al*., 2005). *Mycoplasma bovis* antigen can be detected within epithelial cells, inflammatory cells in the airways and in alveolar walls. The organism is not limited to cattle. Experimental infections in goats have also been reported (Rodriguez *et al*., 2000; Khan *et al*., 2005).

Mycoplasma bovis has several other properties that enhance pathogenesis. After adherence, *M. bovis* generates products such as hydrogen peroxide, superoxide radicals, phospholipases and biofilms which damage host cells (Gagea *et al*., 2006). *Mycoplasma bovis* can also form biofilms *in vitro* that impart increased resistance to desiccation and heat stress (McAuliffe *et al*., 2006). The ability to undergo antigenic variation by

phenotypic alteration of immunodominant surface lipoproteins and the resulting modulation of the host immune response can facilitate the persistence of *M. bovis* and the development of chronic infection in the face of an immune response and prolonged antibiotic therapy (Gagea *et al*., 2006).

Mycoplasma bovis is highly invasive and is not restricted to the initial area of colonization, the respiratory tract. As a result, organisms rapidly gain access to multiple organ systems (Minion, 2002). A possible mechanism for this is due to the reported ability of the organism to attach to peripheral blood mononuclear cells (PBMC) and erythrocytes (van der Merwe *et al*., 2010). This suggests that in order to survive, *M. bovis* may alter its gene expression as it encounters different host environments and different selective pressures (Minion, 2002). In this way, specific adhesins could be expressed only when needed, preventing an early hosts immune response that could block colonization of specific tissues (Maunsell *et al*., 2011). Adherence of *M. bovis* to epithelial cells as well as embryonic bovine lung cells may involve interactions with sialic acid-containing receptors or sulfated lipids (Minion, 2002). One potential adhesin was identified as a 32kDa antigen, P26, based upon inhibition of adherence by monoclonal antibody (Mab) 4F6. The adherence was sensitive to trypsin, neuraminidase and temperature (Minion, 2002). Purified P26 was shown to block mycoplasma adherence in competition assays. A second Mab directed against a common epitope of the *vsp* family, Mab 1E5, also showed adherence-blocking activity suggesting that one of the *vsp* antigens may also be involved in adherence to embryonic bovine lung cells (Minion, 2002). To further examine this possibility, peptides representing different repeating units of four *vsp* proteins were used to map reactivity of convalescent sera and to study adherence of *M. bovis* to embryonic bovine lung cells (Sachse *et al*., 2000). The

effectiveness of several of the peptides in blocking adherence suggests that *vsp* proteins are involved in cytadherence (Minion, 2002).

Lung invasion by *M. bovis* results in injury to the host cells and inflammation. It is possible for *M. bovis* to adhere to the bovine tracheobronchial epithelial cells leading to colonization in the lung, however, unlike other animal mycoplasmas this adherence does not lead to ciliostasis (Howard *et al*., 1987; Caswell *et al*., 2010). Following infection, *M. bovis* may continuously evade the host immune defenses causing chronic colonization in the lungs and the eventual dissemination to other organs, especially the joints and middle ear (Maunsell and Donovan, 2009; Caswell *et al*., 2010). It has been suggested that ascending infection of the Eustachian tube is the likely route of spread to the inner ear from the respiratory tract (Maunsell and Donovan, 2009).

2.12.2 Pathogenesis of contagious bovine pleuropneumonia

Contagious bovine pleuropneumonia is typical example of multi-factorial diseases, where factors such as intercurrent infections, crowding, inclement climatic conditions, age, genetic constitution and stress from transportation, handling and experimentation are important determinants of the final outcome of infection (Admassu *et al*., 2015). An essential part of the pathogenesis of the disease is thrombosis in the pulmonary vessels, probably prior to the development of pneumonic lesions. The mechanism of development of the thrombosis is not well understood, but is considered, at least in part, mediated through induction of cytokines (Rosendal, 1993; Admassu *et al*., 2015). Contagious bovine pleuropneumonia is lobar variety of pneumonia in which the inter-lobular septa are dilated and prominent due to great out pouring of plasma and fibrin in to them and this dilated septa that give the “marbled” effect to the lung in these areas (Radostits *et al*., 1994; Admassu *et al*., 2015).

Bronchitis, bronchiolitis and alveolitis with predominantly neutrophils and mononuclear cellular response constitute the very early inflammation in *Mycoplasma* pneumonia. Contagious bovine pleuropneumonia is characterized by substantial unilateral pulmonary necrosis, sometimes sequestration and marked serosanguinous fluid accumulation in interstitial and pleura (FAO, 1997; Pilo *et al*., 2007). Vasculitis appears to be an important component of the pathological changes in this disease, explaining the marked exudation and pleurisy. Thrombosis can explain ischemic necrosis and infarcts of the lung. Death results from anoxia and presumably from toxemia (Walker, 1999; Admassu *et al*., 2015). There are various substances produced by the *Mollicutes*, which are potentially important in disease pathogenesis. Peroxide and super-oxide production may be important in disruption of host cell depending on the résistance level of the animal and integrity (Quinn *et al*., 2002; Mtui-Malamsha, 2009).

Mycoplasma phospholipases are potentially important in pneumonia for they may reduce surface tension of the alveolar surfactants, thus resulting in atelectasis. A galactan polymer in *Mmm* has been shown to modulate the immune response and promote dissemination (FAO, 1997; Admassu *et al*., 2015).

2.13 Clinical Signs

2.13.1 Clinical signs associated with *Mycoplasma bovis* infection

2.13.1.1 Pneumonia

Mycoplasma bovis-associated pneumonia occurs in any age in cattle, including dairy and beef calves, beef cattle after arrival at a feedlot, and adults (Tenk *et al*., 2004; Caswell and Archambault, 2007). Clinical signs are nonspecific and include fever, hyperpnea, dyspnea, and decreased appetite, with or without nasal discharge and

coughing. Poor weight gain is observed in chronically affected animals (Caswell and Archambault, 2007). The severity of calf pneumonia is further compounded by animal husbandry, the environment, low efficacy of many antimicrobials, and unknown efficacy of vaccines (Nicholas *et al*., 2009; Soehnlen *et al*., 2011). *Mycoplasma* pneumonia can go along with cases of otitis media, arthritis, or both, in the same animal or in other animals in the flock. Chronic pneumonia and polyarthritis syndrome (CPPS), where animals develop polyarthritis in association with chronic pneumonia, occurs in beef cattle several weeks after feedlot entry (Maunsell *et al*., 2011; Francis, 2017).

2.13.1.2 Mastitis

The herd presentation of mycoplasma mastitis varies from general subclinical disease to severe clinical mastitis outbreaks (Gonzalez and Wilson, 2003). Many infections are subclinical, and subsets of subclinically infected cows do not have a marked increase in somatic cell count or reduced milk yield. Cows of any age or stage of lactation are affected as well as prepubertal heifers and dry cows (Fox *et al*., 2008). When the disease is clinical, signs are nonspecific and classically more than one quarter is affected. There is a drastic decrease in milk production and signs of systemic illness are relatively mild (Gonzalez and Wilson, 2003). The mammary gland might be swollen but is not usually painful. Secretions vary from mildly abnormal to gritty or purulent, and are sometimes brownish in colour. A history of mastitis that is resistant to treatment with antimicrobials is common, and clinical disease can persist for several weeks (Gonzalez and Wilson, 2003; Maunsell *et al*., 2011). Return to production is possible but slow. Arthritis, synovitis, joint effusion or combinations, or respiratory disease in mastitic or nonmastitic cows can accompany *M. bovis* mastitis (Gonzalez and Wilson, 2003; Wilson *et al*., 2007; Maunsell *et al*., 2011).

2.13.1.3 Otitis Media

Mycoplasma bovis-associated otitis media occurs in dairy and beef calves as enzootic disease or as outbreaks, and also occurs intermittently in feedlot cattle (Francis, 2017). In early or mild cases calves remain alert with a good appetite, but as disease progresses they become febrile and anoraexic. Clinical signs are because of ear pain and cranial nerve VII deficits, especially ear droop and ptosis (Lamm *et al*., 2004; Francoz *et al*., 2004). Ear pain is evidenced by head shaking and scratching or rubbing ears. Epiphora and exposure keratitis can develop secondary to eyelid paresis. Clinical signs can be unilateral or bilateral, and purulent aural discharge can be present if the tympanic membrane has ruptured (Maunsell *et al*., 2011). Concurrent cases of pneumonia, arthritis, or both are common. Otitis interna and vestibulocochlear nerve deficits can occur as consequence. Head tilt is the most common clinical sign, but severely affected animals can exhibit nystagmus, circling, falling, or drifting toward the side of the lesion and vestibular ataxia (Van Biervliet *et al*., 2004). In advanced otitis media-interna, meningitis can develop. Spontaneous regurgitation, loss of pharyngeal tone, and dysphagia has also been reported and are indicative of glossopharyngeal nerve dysfunction with or without vagal nerve dysfunction (Van Biervliet *et al*., 2004; Maunsell *et al*., 2011).

2.13.1.4 Arthritis, Synovitis and Periarticular Infection

In contrast to *M. bovis* infections of the upper and lower respiratory tracts, *M. bovis*-induced arthritis is alleged to be a consequence of mycoplasmemia (Maunsell *et al*., 2011). Arthritis was preceded by mycoplasmemia in one calf that was inoculated intratracheally with *M. bovis* (Fox *et al*., 2008). Infections of other body systems that occasionally accompany polyarthritis are also likely to be a consequence of

mycoplasmemia (Stipkovits *et al*., 2005). Clinical cases of *M. bovis*-induced arthritis in dairy calves tend to be sporadic and are typically accompanied by respiratory disease within the herd and often within the same animal (Stipkovits *et al*., 2005). Although uncommon, outbreaks of disease where arthritis was the main clinical presentation have been reported (Butler *et al*., 2000). Clinical signs are typical of septic arthritis with affected joints being painful and swollen, and calves show signs of varying degrees of lameness and may be febrile in the acute phase of disease (Step and Kirkpatrick, 2001). Cattle of any age can be affected by *M. bovis* arthritis. Chronic pneumonia and polyarthritis syndrome (CPPS) have been described in feedlot cattle (Gagea *et al*., 2006). Clinical signs are typical of septic arthritis, including acute nonweight bearing lameness with joint swelling, pain, and heat on palpation (Wilson *et al*., 2007). Involvement of tendon sheaths and periarticular soft tissues is common. Large rotator joints (hip, stifle, hock, shoulder, elbow, and carpal) are commonly affected, although other joints such as the fetlock or even the atlantooccipital joint can be involved. Poor response to treatment is a common feature (Wilson *et al*., 2007).

2.13.1.5 Other diseases

2.13.1.5.1 Keratoconjunctivitis

Mycoplasma bovis can be isolated from the conjunctiva of healthy and diseased cattle (Alberti *et al*., 2006; Fox *et al*., 2008), although *M. bovis*-associated ocular disease is considered uncommon (Brown *et al*., 1998). However, there are several reports of outbreaks of keratoconjunctivitis involving *M. bovis* alone, or in mixed infections with *Mycoplasma bovoculi* (Levisohn *et al*., 2004; Alberti *et al*., 2006). Clinical signs included mucopurulent ocular discharge, severe eyelid and conjunctival swelling, and

corneal oedema and ulceration. Most clinical signs resolved within 2 weeks but some animals had residual corneal scarring (Kirby and Nicholas, 1996).

2.13.1.5.2 Meningitis

Mycoplasma can cause brain infections (meningitis) in calves; these can sometimes be difficult to recognize, as calves may just appear to have fevers and be depressed. Signs of apparent neck pain and abnormal eye movements may also be evident (Wustenberg *et al*., 2003). Meningitis can also occur as a complication of mycoplasma otitis media-interna. *Mycoplasma bovis* has also been isolated from the cerebral ventricles of young calves with clinical signs of meningitis in conjunction with severe arthritis, suggesting disseminated septic disease (Maunsell *et al*., 2011).

2.13.1.5.3 Genital Disorders

In isolated and primarily experimental cases, *M. bovis* has been associated with genital infections and abortion in cows and seminal vesiculitis in bulls. However, there is little evidence to support an important role for *M. bovis* in naturally occurring bovine reproductive disease (Maunsell *et al*., 2011).

2.13.2 Clinical signs associated with contagious bovine pleuropneumonia

Typically, when first introduced into a herd, CBPP is severe and mortality relatively high. A small proportion of cattle may die rapidly without showing any signs other than fever. It may be possible to link the onset of disease to previous contact with other cattle three to six weeks earlier, but this is not always the case as the incubation period can be as long as six months. Clinical signs may become apparent only several months after the contact (Radostits *et al*., 2007; Campbell, 2015). The disease can therefore become

established in a herd before it is noticed, and tracing back to the origin can be difficult. This is particularly so where routine vaccination has been practised with long intervals between campaigns, and where antibiotics have been used to treat clinical cases. Both case reduce the incidence of clinical disease, making its recognition more difficult (OIE, 2015). After some time, the disease in the herd becomes chronic, clinical signs become less severe and the mortality rate falls. However, losses continue to occur. Not all the animals are affected in the same way and often the disease has a chronic course from its onset (Campbell, 2015).

The hyper acute form, involving up to 10% of infected animals, may be observed at the onset of an outbreak. The clinical signs observed in the hyper acute form are much accelerated. Affected animals may die within a week exhibiting classical respiratory signs and clinical diagnosis is difficult (Hirst *et al*., 2004; Campbell, 2015).

The acute form is observed in approximately 20 percent of diseased animals. The course is usually from 5-7 days. The earliest signs are a sudden onset of fever up to 107°F (41.5°C), and a drop in milk yield in milking cows. Sick cattle tend to isolate themselves from the herd and may stop eating (Radostits *et al*., 2007). A typical respiratory disease develops; breathing is laboured and obviously painful. Abdominal breathing with a respiratory rate of 50-55 breaths/minute may be seen and cattle may "grunt" when breathing out. Some animals develop a shallow, dry and painful cough, particularly noticeable on exercise (Radostits *et al*., 2007; OIE, 2015). Application of pressure between the ribs is painful and resented by affected cattle, which sometimes react violently. On percussion, the ventral part of the chest sounds dull owing to the presence of fluid in the chest cavity. Acutely affected cattle stand with head and neck extended and forelegs spread apart, with dilated nostrils and mouth open panting for air (Radostits *et al*., 2007). There may be nasal discharge, sometimes streaked with blood, and frothy

saliva accumulates around the mouth. Some animals develop swellings of the throat and dewlap (Radostits *et al*., 2007). Pregnant cows and heifers may abort, and diarrhea has been recorded. The animal becomes recumbent and dies after 1-3 weeks. While classical respiratory signs may be evident in calves, articular localization of the causative agent with attendant arthritis usually predominates (Radostits *et al*., 2007; Campbell, 2015).

The subacute form occurs most frequently in about 40 to 50 percent of the animals affected. The symptoms resemble those of the acute form, but are less severe and fever is intermittent. This form usually becomes chronic (Radostits *et al*., 2007; Campbell, 2015).

The chronic form is a natural evolution of both acute and subacute forms but in some animals it may develop directly. The clinical signs regress but cattle can still have intermittent fever, together with loss of both appetite and weight. Calves in the first six months of life more often show lameness from swollen, hot, painful limb joints (Radostits *et al*., 2007). The mortality rate is variable, rarely exceeding 50 percent, and depends on a range of factors, such as age, breed, nutrition, presence of other infections or infestations, and the type of management (Radostits *et al*., 2007). Many affected cattle appear to recover fully, yet the lesions in the lungs take a long time to heal completely. The causative agent can survive for as long as two years within the lesions. Up to 25 percent of affected cattle can become chronic carriers of infection. They are often referred to as “lungers” and are believed to play a role in initiating new outbreaks when they are introduced into susceptible herds (Radostits *et al*., 2007; OIE, 2015). Chronically affected cattle usually exhibit signs of varying intensity for 3-4 weeks, after which the lesions gradually resolve and the animals appear to recover. Subclinical cases

occur and may be important as carriers. Infected calves may present primarily with polyarthritis that is seen as swelling of joints and lameness (Campbell, 2015).

2.14 Pathology

2.14.1 Pathology associated with *Mycoplasma bovis* infection

The gross and histological lesions of the respiratory tract in experimental *M. bovis* infection vary significantly among studies, most likely reflecting differences in the route of inoculation, the dose and strain of *M. bovis*, the age and health status of the host and the duration of infection (Rodriguez *et al*., 1996). Gross lesions have consisted of cranioventral lung consolidation, sometimes accompanied by multiple necrotic foci (Rodriguez *et al*., 1996; Clark, 2002; Shahriar *et al*., 2002). Histologically, experimental lung infections with *M. bovis* are characterized by peribronchiolar lymphoid hyperplasia or cuffing, often accompanied by acute or subacute suppurative bronchiolitis, thickening of alveolar septa due to cellular infiltration, atelectasis, and, in some cases, foci of coagulative necrosis (Rodriguez *et al*., 1996; Shahriar *et al*., 2002).

Lesions described for the lungs of calves with natural *M. bovis* infections are similar to those described for experimental disease, although often of much greater severity. Grossly, affected lung lobes are deep red in colour and have varying degrees of consolidation, often accompanied in subacute to chronic cases by multifocal necrotizing lesions (Clark, 2002; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006). Lesions usually have a cranioventral distribution, but can involve whole lung lobes and the cranial portions of the caudal lobes. Necrotic lesions can vary from 1-2 mm to several centimetres in diameter and contain yellow caseous material. They are distinct from typical lung abscesses in that they are not usually surrounded by a well-

defined fibrous capsule (Clark, 2002; Khodakaram-Tafti and Lopez, 2004). Diffuse fibrinous or chronic fibrosing pleuritis are sometimes observed, and interlobular septae may contain oedema fluid or linear yellow necrotic lesions (Bashiruddin *et al*., 2001; Step and Kirkpatrick, 2001; Clark, 2002; Gagea *et al*., 2006). Histologically, lung lesions in naturally-occurring *M. bovis* infections are characterized by a subacute to chronic suppurative bronchopneumonia that is usually necrotizing (Adegboye *et al*., 1995; Rodriguez *et al*., 1996; Clark, 2002; Shahriar *et al*., 2002; Khodakaram-Tafti and Lopez, 2004; Gagea *et al*., 2006).

Experimental and natural infections with *M. bovis*-associated respiratory disease are typically accompanied by hyperplasia of the lymphoid tissues in both upper and lower respiratory tract (Gagea *et al*., 2006). Foci of caseous necrosis in bronchial and mediastinal lymph nodes of affected calves have been observed (Gagea *et al*., 2006).

In calves with *M. bovis*-associated otitis media, affected tympanic bullae are filled with fibrinosuppurative to caseous exudate (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). Histologically, extensive fibrinosuppurative exudates fill the tympanic bullae and the normal architecture may be obliterated (Walz *et al*., 1997; Maeda *et al*., 2003; Lamm *et al*., 2004). The tympanic mucosa may have areas of ulceration and/or squamous metaplasia and is distinctly thickened due to infiltrates of macrophages, neutrophils, and plasma cells, and proliferation of fibrous tissue (Walz *et al*., 1997; Lamm *et al*., 2004; Van Biervliet *et al*., 2004).

Mycoplasma bovis-associated lesions have occasionally been identified in other body systems in both experimentally- and naturally-infected calves (Maeda *et al*., 2003; Ayling *et al*., 2005). Ayling *et al*., (2005) described a 10-month-old calf with a history of respiratory disease that had lesions of endocarditis and encephalitis from which *M.*

bovis was the only pathogen isolated. At necropsy, investigators observed perivascular mononuclear cell infiltration in portal areas of the liver, and immunohistochemical staining revealed *M. bovis* in association with these lesions (Ayling *et al*., 2005).

2.14.2 Pathology associated with contagious bovine pleuropneumonia

Except for young calves, pathological lesions are generally confined to the thoracic cavity where usually one lung and pleura are affected and a large volume of pleural exudate containing clots of fibrin is common (Egwu *et al.,* 1996; Nicholas *et al*., 2008). Large fibrin deposits make the lung adhere to the chest wall and at these sites, the pleura is thickened and opaque (Nicholas *et al*., 2008). The interlobular septa of affected lung regions are also thickened due to adsorption of the exudates and "frame" the lung lobules, which vary in colors of red, grey and yellow due to different stages of inflammatory lesions giving the characteristic "marbled" appearance. Pleural exudates are rarely seen in chronic cases of CBPP, but adhesions between lung lobes and to the chest wall are more common (Nicholas *et al*., 2008).

Necrotic lesions in the lung are surrounded by capsules of fibrous connective tissue forming structures called sequestra. Sizes of sequestra can vary from 1-30 cm in diameter, the capsule can be up to 1 cm thick and sequestra of different sizes can be found simultaneously in one lung (Nicholas *et al*., 2008). The inner parts of the sequestra often retain the (lung) structure of the acute lesion but the necrotic material may liquefy or become caseous over time. Fibrous scars can replace small sequestra over time, but large sequestra may persist for years. A sequestrum is believed to be a source of infection if it is ruptured or drained by a bronchus, but no evidence of this has been published (Scudamore, 1995; Egwu *et al.,* 1996; Masiga *et al.,* 1996).

Although the disease is characterized by respiratory signs, animals develop mycoplasmemia and demonstrate systemic effects. Septic arthritis with joint distention, synovitis and cartilaginous erosions may be seen in calves (Gull *et al.*, 2013).

Early on in the course of CBPP, lesions include a bronchiolar necrosis and edema, progressing rapidly to an exudative serofibrinous bronchiolitis with extension to the alveoli and uptake of alveolar fluid into tissue spaces, lymph vessels and ultimately septal lymphatic ducts (Done *et al*., 1995). These rapidly reach saturation and the process is extended centrifugally to the tracheobronchial lymph nodes and centripetally to the pleural lymphatic ducts (Nicholas *et al*., 2008). The mediastinal, sternal, aortic and intercostal lymph nodes may then become enlarged, oedematous or even haemorrhagic. With stasis, lymph vessels become thrombosed and ultimately fibrosed (Gull *et al.*, 2013). The pulmonary lobules become consolidated with alveolar oedema, fibrin and inflammatory cells with coagulative necrosis is commonly observed. *Mycoplasma mycoides* subsp. *mycoides* can be isolated from or demonstrated in these lobules by immunohistochemistry (AU-IBAR, 2013).

Perivascular organisation foci or 'organising centers', found in the interlobular septa, are considered pathognomonic for CBPP. They consist of a center occupied by a blood vessel with proliferation of connective and inflammatory cells surrounded by a peripheral zone of necrotic cells (Nicholas *et al*., 2008; AU-IBAR, 2013).

2.15 Diagnosis

2.15.1 Diagnosis of *Mycoplasma bovis* infection

Rapid and accurate diagnosis of *M. bovis* infections is compromised by the low sensitivity and, in some cases, specificity of the available tests, and subclinical infections and intermittent shedding complicate diagnosis (Maunsell *et al*., 2011).

2.15.1.1 Detection of antibodies

Mycoplasma bovis-specific serum antibodies can be detected by indirect ELISA, usually by 6-10 days after experimental infection. However, in natural infections, individual animal titres are poorly correlated with infection or disease as not all diseased animals develop high titres, that can remain increased for months (Le Grand *et al*., 2002), and maternal antibody results in high titres in calves. On a group level, however, seroconversion or high titres are predictive of active *M. bovis* infection. Serology is therefore, best applied in surveillance or as part of a biosecurity programme (Le Grand *et al*., 2002). Antibody titres in milk have been used to identify *M. bovis*-infected mammary glands (Byrne *et al*., 2000).

2.15.1.2 Detection in clinical materials

Mycoplasma culture requires complex media, specialized equipment, and technical skill. Growth is often apparent by 48 hours, but 7-10 days incubation is recommended before samples are called negative (Nicholas *et al*., 2008; Francis *et al*., 2015b). The sensitivity of culture for the detection of *M. bovis* in clinical material is quite low. Intermittent and low-level shedding, uneven distribution of *M. bovis* throughout diseased tissue, suboptimal sample handling or culture conditions, and the presence of mycoplasma

inhibitors in samples likely contribute to the low sensitivity (Nicholas *et al*., 2008). Sensitivity of milk culture for diagnosis of mycoplasma intramammary infection has been reported as approximately 50% for bulk tank samples and 30% in individual cows without clinical mastitis (Biddle *et al*., 2003; Gonzalez and Wilson, 2003), although it is higher in cows with clinical mastitis. The sensitivity of *M. bovis* culture for other clinical material has not been reported. Sensitivity can be enhanced by repeated sampling, optimal sample handling, and the use of various laboratory techniques (Biddle *et al*., 2003; Punyapornwithaya *et al*., 2009). Mycoplasmas isolated in culture should be speciated by antibody-based tests (immunofluorescence or immunoperoxidase tests) or, preferably, polymerase chain reaction (PCR) (Maunsell *et al*., 2011).

Mycoplasma bovis can be detected directly in clinical specimens by PCR (Cremonesi *et al*., 2007). Polymerase chain reaction can be especially useful for stored samples; PCR had a similar sensitivity to culture for detection of *M. bovis* in fresh milk but was much more sensitive than culture in milk frozen for 2 years (Pinnow *et al*., 2001). Realtime PCR systems with high sensitivity and specificity have been described for the detection of *M. bovis* in clinical samples (Cai *et al*., 2005; Rossetti *et al*., 2010; Sachse *et al*., 2010). Multiplex PCR that allows simultaneous detection of *M. bovis* with other *Mollicutes* has been reported (Cornelissen *et al*., 2017; Parker *et al*., 2017). Other techniques, including denaturing gradient gel electrophoresis PCR and melting-curve analysis of PCR products, appear promising for the simultaneous detection and differentiation of multiple mycoplasma species (Cai *et al*., 2005; McAuliffe *et al*., 2005). The use of 16S rRNA gene in PCR (Gonzalez *et al*., 1995) also produced cross reactivity with *M. agalactiae*. Thereafter, Ghadersohi *et al*. (1997) designed PCR primers from sequences obtained from *M. bovis* specific dot blot hybridization probe which was further modified by Hayman (2003) into a seminested setup. The semi-

nested system has been developed by Hayman and Hirst (2003) whereas, Pinnow *et al*. (2001) developed a specific nested PCR test, with which the preservative-treated milk samples can also be examined. Alberti *et al*. (2006) used 16S rDNA sequence to establish the relatedness of strains. A restriction fragment length polymorphism (RFLP) strategy directed to the identification of phylogenetic clusters was designed to restrict the diagnostic investigation to a few bovine mycoplasma species (Alberti *et al*., 2006). Reverse-transcription PCR and primer extension analysis indicated that both p68 and p48 are transcribed in *M. bovis* under *in vitro* growth conditions. The PCR system designed for *uvr*C gene sequences provide high specificity and clear distinction of *M. bovis* thus *uvr*C gene could distinguish between *M. bovis* and *M. agalactiae* (Subramaniam *et al*., 1998). Rossetti *et al*. (2010) described a new specific real-time PCR assay targeting the *uvr*C gene that was developed to directly detect *M. bovis* from milk and tissue samples without laborious DNA purification. Taqman real-time PCR assay for the detection of *M. bovis* was designed using unique primers targeting the highly conserved house-keeping gene (*uvrC*) and the authors recommended the *uvrC* gene as a good and reliable diagnostic marker for accurate and rapid detection of *M. bovis* with assay much faster than conventional PCR and culture based identification of *M. bovis* (Naikare *et al*., 2015). The Taqman assay results can be obtained within 2½ hours with greater sensitivity and specificity in identifying *M. bovis* DNA from clinical specimens (Naikare *et al*., 2015).

A monoclonal antibody-based sandwich ELISA (sELISA) kit for the detection of *M. bovis* in clinical material is available in Europe (Bio-X Diagnostics, Jemelle, Belgium); sensitivity and assay time are better than conventional culture when samples are pre-incubated in broth and have been shown to be useful as a herd test and also for demonstrating lack of infection (Nielsen *et al*., 2015; Petersen *et al*., 2016).

Mycoplasma bovis can be detected *in situ* by immunohistochemistry (IHC) on formalin fixed paraffin-embedded tissues (Gagea *et al*., 2006).

For the diagnosis of *M. bovis* pneumonia in the live animal, transtracheal wash or bronch-alvealar lavage (BAL) is preferable to upper respiratory tract (URT) samples (Thomas *et al*., 2002), although isolation of *M. bovis* is not well correlated with respiratory disease in the individual animal. Aspirates of affected joints or tendon sheaths can be submitted for *M. bovis* detection (Francoz *et al*., 2004). In live calves with otitis media, the sensitivity or specificity of URT *M. bovis* culture has not been reported, and samples are not typically collected from the middle ear of live calves. Imaging (radiography, computed tomography) has been used as an aid in the diagnosis of otitis media/interna in calves (Francoz *et al*., 2004; Van Biervliet *et al*., 2004).

2.15.2 Diagnosis of contagious bovine pleuropneumonia

Confirmatory diagnosis of CBPP is achieved by the demonstration of typical pathology and/or the presence of *Mmm* after postmortem examination and isolation of the etiologic agent from pneumonic lesions characteristic of CBPP when combined with other serological tests (Egwu *et al*., 2012b).

Conventional methods for the detection, isolation and identification of mycoplasmas systematically involve enrichment steps in selective broth followed by morphological, biochemical and immunological tests (Nicholas *et al*., 2008). These techniques are well established but have some important shortcomings. The morphological and biochemical characteristics are in general not discriminative, while immunological cross-reactions have been frequently reported as well. Moreover, these classical techniques are often

labour intensive and hardly ever useful to differentiate strains belonging to the same species (Stakenborg, 2005).

Clinical diagnosis of CBPP is difficult as early signs may or may not exist and are impossible to differentiate from any other severe pneumonia (OIE, 2014). Antibiotic treatment may also alleviate clinical signs (Nicholas *et al*., 2000a). In practice, clinical diagnosis is unreliable and when respiratory disease occurs within a livestock population thus CBPP should be confirmed by serological, pathological, culture and molecular based methods (Hamsten, 2009; OIE, 2014).

2.15.2.1 Isolation method of diagnosis

Mycoplasma mycoides subspecies *mycoides* can be isolated from live animal samples such as nasal and ear swabs, bronco-alveolar lavage and pleural fluid; or at post-mortem examination from infected lung tissues, pleural fluid, lymph nodes of the broncho-pulmonary tract or synovial fluid from animals with arthritis (OIE, 2014). Tissue samples should be collected at the interface between diseased and normal parts. For swab samples, a transport medium should be used to protect *Mmm* and prevent proliferation of cell-walled bacteria (OIE, 2014). After collection all samples must be packed in ice or kept refrigerated at 4°C and sent to the laboratory within 24 hours. For longer periods they should be kept frozen at or below -20°C (OIE, 2014).

The presence of *Mmm* varies with the stage of disease and it may be difficult to isolate from chronic lesions, especially if antibiotic treatment is involved (Bashiruddin, 1998; Aliyu *et al.*,. 2000; OIE, 2014) hence, a negative isolation result is not conclusive (OIE, 2014). Unlike many other mycoplasmas, *Mmm* is relatively easy to grow given the right media. There are several media compositions used in different

reference laboratories but, essentially, they should contain a basic medium such as heart-infusion or pleuropneumonia-like organisms (PPLO) broth or agar base; yeast extract (preferably fresh) and inactivated horse serum (15-20%) (Nicholas *et al*., 2008). Besides, several other components can be added, such as glucose, glycerol, DNA, sodium pyruvate and fatty acids, but the effects vary with the strains. To avoid growth of other bacteria and fungi, inhibitors, such as penicillin and amphotericin B respectively, are necessary (Nicholas *et al*., 2008; OIE, 2014).

The media should be used both as broth and solid and all culture media prepared should be subjected to quality control and must support growth of a reference strain (T1/44 vaccine strain), which should be cultured in parallel with the suspicious samples to ensure that the tests are performed correctly (OIE, 2014). To ensure the best conditions for mycoplasma growth, inoculated media are incubated at 37°C in a 5% CO_2 atmosphere and should be inspected daily for about 7-10 days (Nicholas *et al*., 2008). After this time if there is no growth, the sample is considered negative. Positive samples in liquid medium show a homogeneous cloudiness, usually within 2-4 days, frequently with a silky, fragile filament called a 'comet'. During the following days a uniform opacity develops which forms swirls when shaken (Nicholas *et al*., 2008). On agar media, the colonies are small (approximately 1 mm in diameter) and have the classical appearance of 'fried eggs' with a dense centre (Nicholas *et al*., 2008; OIE, 2014). At this stage, biochemical tests and subsequently, immunological tests such as growth inhibition tests, monoclonal antibodies (mAbs) and/or immunofluorescence based methods can be performed to identify the colonies (Rice *et al.,*. 2000; Rurangirwa *et al.,* 2000; Brooks *et al.,*. 2009). However, closely related *Mycoplasma* species may cross-react in these tests (OIE, 2014).

2.15.2.2 Serological methods of diagnosis

Contagious bovine pleuropneumonia diagnostic systems based on serology are generally regarded as not sensitive enough (Bellini *et al*., 1998) and cross-reactions with closely related *Mycoplasma* species are frequent (Stark *et al*., 1995). Diagnosing chronic carriers of CBPP is also challenging and as a consequence, serological diagnosis of CBPP is valid on a herd level only since the individual animal can either be in an early stage of disease prior to generation of specific antibodies or in a late chronic stage when few animals remain seropositive (OIE, 2008). Despite these shortcomings, serological diagnosis is still appropriate for large scale disease monitoring, especially in Africa where PCR-based methods are not always feasible (Nicholas *et al*., 2000b; Francis *et al*., 2017). The OIE approved serological tests for CBPP are; complement fixation test (CFT) and competitive enzyme-linked immunosorbent assay (c-ELISA) (OIE, 2008).

2.15.2.2.1 Complement fixation test (CFT)

The CFT has been used for CBPP eradication campaigns in many countries and has been verified to be specific, but lacks in sensitivity (Le Goff and Thiaucourt, 1998). In a thorough evaluation of CFT in which over 33,000 sera from healthy herds were tested in the 1990s 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%. During the Italian outbreaks, abattoir surveillance detected nearly as many outbreaks as serological monitoring, while clinical examination was much less useful (Regalla *et al*., 1996). It followed that by using CFT as a screening test, some CBPP affected cattle, in the early or later stages of infection were missed, accounting for the then persistence of the disease in Portugal (Hamsten, 2009). Even though the CFT is

thought to only detect a small proportion of animals in early stages of the disease or chronic carriers, it is still considered to detect almost 100% of infected herds (OIE, 2008).

2.15.2.2.2 Competitive enzyme-linked immunosorbent assay (cELISA)

The c-ELISA, using a monoclonal antibody (mAb) targeting the variably expressed *PtsG* surface protein, was developed in an effort to improve serological diagnosis of CBPP (Le Goff and Thiaucourt, 1998; Gaurivaud *et al*., 2004). Competitive ELISA offers practical advantages of ease of standardization and has equal sensitivity and better specificity as compared to the CFT (OIE, 2014).

The antibody classes; these 2 methods (CFT and c-ELISA) primarily detection can in part explain the differences in CBPP detection over time. In the early stages of infection, immunoglobulin M (IgM) responses dominate and are characterized by lesser affinity (in general, compared to following immunoglobulin G (IgG) responses) but higher complement fixating ability (Goldsby *et al*., 2003). This is in favour of the CFT, relying on complement activation, over the c-ELISA relying on affinity to reduce mAb binding. But as infection progress, IgM responses change to IgG responses that have comparably higher affinities but less complement fixating abilities. This favours the c-ELISA and responses have indeed been detected longer than CFT titres (Niang *et al*., 2006).

It is not possible to use CFT or c-ELISA to assess vaccination efficiency as vaccination with strains such as T1/44 or T1-SR does not always induce detectable antibody responses. However, as post-vaccinal antibodies do not persist after 3 months, CFT or c-ELISA can be used for the detection of natural infections,

especially in areas where vaccination is practiced (Le Goff and Thiaucourt, 1998).

2.15.2.2.3 Other serological diagnostic methods

A rapid latex agglutination test (LAT), giving results in two minutes using serum or whole blood, for screening in the field has been developed (Ayling *et al*., 1999). Slide agglutination tests had earlier been described, but were not recommended due to false-positive reactions (March *et al*., 2003). The LAT uses a specific polysaccharide antigen extracted from the *Mmm* capsule which is bound to latex beads (Nicholas *et al*., 2000b). The test has been evaluated on European and African sera and the sensitivity was comparable to the CFT while the LAT was simpler and more rapid to perform (Nicholas *et al*., 2000b). In general, LATs allow simple, rapid and inexpensive disease monitoring at the herd level in the field, which is very suitable for the CBPP situation in Africa (March *et al*., 2003). Although none of the LATs for CBPP diagnosis have been approved by the OIE, but the product is in the market and has been used to carry out on-field diagnosis of CBPP in Nigeria (Okaiyeto *et al*., 2011).

An immunoenzymatic test based on western blotting known as immunoblotting test (IBT), has also been developed and is of diagnostic value (OIE, 2014). A field evaluation of the IBT found the sensitivity to be 92.6% (compared to 77.5% for the CFT) and the specificity was 100% (compared to 99.9% for c-ELISA) with no false positive result (Regalla *et al*., 1996). The IBT is highly specific and the most sensitive serological test so far described for CBPP (Anonymous, 2001), but it is not suitable for mass screening and should primarily be used to confirm uncertain CFT or c-ELISA results (OIE, 2014). World Organisation of Animal Health (OIE) recommended that diagnostic tests have limited sensitivity and are primarily useful at herd but not at individual level (Marobela-Raborokgwe *et al*., 2003).

2.15.2.3 Molecular identification

Numerous important nucleic acid based diagnostic methods are available (Hamsten, 2009). These are mainly used for the detection of *Mmm* directly from clinical sample (Miles *et al*., 2006), and final identification when the organism has been grown and isolated (Hamsten, 2009). Based on the polymerase chain reaction (PCR), robust diagnostic systems have been developed for the rapid detection, identification and differentiation of members of the *Mycoplasma mycoides* cluster and the specific identification of *Mmm* (AU-IBAR, 2013).

Polymerase chain reaction is a sensitive, specific, rapid and relatively easy technique to perform (Hamsten, 2009). The first PCR test for *Mmm* identification was reported by Bashiruddin *et al*. (1994) that required digestion of one of the amplicons with restriction enzyme followed by separation of the corresponding fragments for specific identification of *Mmm*. This method was later refined to use colorimetric detection of the amplicons, but still required separate analysis of the digested amplicon (Bashiruddin *et al*., 1999). A PCR was also reported which used restriction of the amplicon or dot blot hybridization of the amplicon to a labelled probe to confirm and/or enhance the specificity for *Mmm* (Dedieu *et al*., 1994). A nested PCR using primers amplifying all members of the *Mycoplasma mycoides* cluster in a first reaction, followed by a species-specific amplification was then developed by Hotzel *et al*. (1996a). Another nested PCR, targeting the p72 (*Lpp*A) gene and having a 10^3-10^5 fold higher sensitivity compared to single step PCR using the same primers or primers from previously mentioned methods was later presented (Miserez *et al*., 1997). Two methods based on PCR amplification of the two 16S rRNA genes from mycoplasmas in the *M. mycoides* cluster were later presented, which differed in the

specific detection of *Mmm* (Persson *et al*., 1999). A PCR, able to specifically detect T1/44 vaccine strain based on the IS1296 element in those strains, was reported by Lorenzon *et al*. (2000) to help distinguish CBPP outbreaks among vaccinated cattle. Miles *et al.* (2006) developed PCRs based on the insertion sequence IS1296 which have enabled not only the specific identification of *Mmm* but also in the differentiation of African/Australian strains from the European strains.

Even though a quick, sensitive and specific method, PCR has problems which include the high risk of cross-contamination and carryover-contamination, especially with nested PCR (Lorenzon *et al*., 2000). Thus the strict separation of laboratory space used for PCR preparation and space possibly contaminated with PCR products such as electrophoresis facilities is required. Nonetheless, PCR has become the primary tool for identification of *Mmm* (OIE, 2008). Reports suggesting that single PCR tests were inadequate for diagnosis of CBPP (Le Grand *et al*., 2004, Bashiruddin *et al*., 2005) led to the development of real-time PCR assays, several of which have been described to reduce the risk of contamination and have increased sensitivity compared to a conventional PCR-based detection of *Mmm* (Lorenzon *et al*., 2008). Vilei and Frey (2010) described a TaqMan real-time PCR they designed specifically to target the *Lpp*Q gene, which may be useful if an *Lpp*Q-devoid vaccine is successfully developed. Schnee *et al*. (2011) also described a novel multiplex real-time PCR they demonstrated to be specific and sensitive when assessed using experimentally infected cattle.

2.15.3 Sample collection and handling for mycoplasma

A key to isolation success lies in collecting good quality samples. *Mycoplasma bovis* and *Mmm* can be isolated from samples taken either in live animals or at necropsy

(Nicholas *et al*., 2008). Samples to be collected for mycoplasma isolation both in live animals and at necropsy are shown on Table 2.5.

Optimal sample handling is vital to ensure mycoplasma survival. Because mycoplasmas are cell-surface associated, it is important to swab vigorously when sampling. Wooden-shaft cotton swabs should be avoided as they can inhibit mycoplasma growth. Swabs should be placed immediately into aerobic bacterial or mycoplasma transport media (Biddle *et al*., 2004). Tissue samples should be formalin fixed for histopathology and immunohistochemistry (IHC) or placed in plastic bags on ice for culture. When tissue cannot be processed rapidly after necropsy, postmortem broncho-alveolar lavage (BAL) samples or swabs of lesions might be preferable; mycoplasmas remain viable in BAL fluid for a few days at 4^{o}C, whereas isolation from lung tissue decreases markedly over a few hours because of the release of mycoplasma inhibitors from disrupted tissue (Thomas *et al*., 2002a).

Samples should be refrigerated, or frozen, if time for processing will exceed 2 days. Significant reductions in mycoplasma recovery rates occur with increased time to processing, regardless of whether samples are refrigerated or frozen, and best recovery rates are achieved when samples are processed fresh within a few hours of collection (Biddle *et al*., 2004).

Table 2.5: Samples for *Mycoplasma* species isolation in Cattle

Species	Growth *in vivo*	Host	Samples from live animals	Samples from dead animals
M. bovis	Good	Cattle	Nasal swabs, joint fluids, ocular swabs, milk, bronchoalveolar washings, pleural fluid, semen	Lung lesions, joints fluid, udder, brain
M. mycoides subsp. *Mycoides*	Good	Cattle	Nasal swabs, nasal discharges, bronchoalveolar washings, pleural fluid	Lung lesions, pleural fluid, bronchoalveolar lymph nodes
M. dispar	Fastidious	Cattle	Nasal swabs, bronchoalveolar washings, pleural fluid	Lung lesions
M. bovigenitalium	Good	Cattle, sheep	Vulvo-vaginal swabs, discharges from reproductive systems, milk, semen	Endometrium and other reproductive sites
M. putrifaciens	Good	Cattle	Milk	Udder and associated lymph nodes
M. canis	Good	Cattle	Nasal swabs, nasal discharges, bronchoalveolar washings, pleural fluid	Lung lesions, pleural fluid, bronchopulmonary lymph nodes
Ureaplasma spp.	Requires specialize medium containing urea	Cattle, sheep	Semen, vulvo-vaginal swabs/ washes/ discharges, nasal swabs	Lung lesions, pleural fluid, bronchopulmonary lymph nodes

Source: Nicholas *et al*., 2008; Francis *et al*., 2015b

2.15.4 Postmortem lesions

2.15.4.1 Postmortem lesionss for M. bovis infection

With the exception of mastitis, *Mycoplasma bovis*-associated disease is best diagnosed by necropsy; a definitive diagnosis is based on demonstration of *M. bovis* in affected tissues by immunohistochemistry (IHC) or by culture, polymerase chain reaction (PCR), or sand-witch enzyme-linked immunosorbent assay (sELISA) (Adegboye *et al*., 1995; Maunsell *et al*., 2011). Although some *M. bovis* lesions are characteristic, many are grossly impossible to differentiate from other pathogens (Maunsell *et al*., 2011). Additionally, *M. bovis* pneumonia can resemble contagious bovine pleuropneumonia (CBPP). Therefore, tissues should be submitted to a diagnostic laboratory for verification of field necropsy findings (Maunsell *et al*., 2011).

The presence of *M. bovis* in pneumonic lungs must be interpreted together with histopathology and other findings, given that *M. bovis* can be isolated from lungs of cattle without lesions (Gagea *et al*., 2006). Macroscopically, affected lung often contains multiple necrotic foci filled with dry yellow to white caseous material (Caswell and Archambault, 2007). These raised nodular lesions can be a few millimetres to several centimetres in diameter. Interlobular septae can contain linear necrotic lesions (Gagea *et al*., 2006). Extensive fibrosis is common, and necrotic sequestra can be present. Acute fibrinous to chronic fibrosing pleuritis occurs in some cases. Histologically, naturally occurring *M. bovis* pneumonia is characterized as subacute to chronic bronchopneumonia that can be suppurative and is usually necrotizing (Gagea *et al*., 2006; Caswell and Archambault, 2007). Immunohistochemical (IHC) staining reveals large amounts of *M. bovis* antigen, especially at the periphery of lesions (Gagea *et al*., 2006). Mixed infections often complicate the characterization of lesions, and IHC

can be useful in determining *M. bovis* involvement in these cases (Maunsell *et al*., 2011).

2.15.4.2 Postmortem lesions for contagious bovine pleuropneumonia

The thoracic cavity may contain up to 10 litres of clear yellow or turbid fluid mixed with fibrin flakes, and the organs in the thorax are often covered by thick deposits of fibrin. The disease is largely unilateral, with more than 80-90% of cases affecting only one lung and the affected portion is enlarged and solid (OIE, 2015). On section of the lung, the typical marbled appearance of pleuropneumonia is evident because of the widened interlobular septa and subpleural tissue that encloses gray, yellow, or red consolidated lung lobules (Campbell, 2015). Microscopically, there was severe, acute, fibrinous pneumonia with fibrinous pleurisy, thrombosis of pulmonary blood vessels, and areas of necrosis of lung tissue; the interstitial tissue is markedly thickened by edema fluid containing much fibrin. In chronic cases, the lesion has a necrotic center sequestered in a thick, fibrous capsule, and there may be fibrous pleural adhesions. Organisms may survive only within the inner capsule of these sequestra, and these animals may become carriers (Campbell, 2015).

2.16 Treatment

2.16.1 Treatment of *Mycoplasma bovis* infection

The good news about *M. bovis* infection is that unlike other mycoplasma diseases, antimicrobials are recommended for its treatment even though there is scanty information about pharmacokinetic and pharmacodynamic data on the treatment of *M. bovis* infections (Maunsell *et al*., 2011; Francis, 2017). There is no effective therapy for mycopasmal mastitis and only little success in treatment has been reported for

respiratory and joint infections caused by *M. bovis* infection (Stipkovits *et al*., 1993; Caswell and Archambault, 2007). Few antimicrobials have been approved in the United States for treatment of bovine respiratory disease (BRD) associated with *M. bovis*. These are tulathromycin (Draxxin, Pfizer Animal Health, New York, NY) and florfenicol (Nuflor Gold, Intervet/Schering-Plough Animal Health, Summit, NJ). Another macrolide, gamithromycin (Zactran Injectable Solution, Merial Canada, Baie d'Urfe, Quebec, Canada), is approved for treatment of *M. bovis*-associated BRD in Canada (Maunsell *et al*., 2011). Oxytetracycline, tilmicosin, and tylosin have a theoretical basis for efficacy against *M. bovis* and are approved in the United States for treatment of BRD. Spectinomycin is no longer available for treatment of BRD in the United States (Maunsell *et al*., 2011). Enrofloxacin is only approved for treatment of BRD associated with *Manheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*, and extralabel use is prohibited in the United States. However, in countries where fluoroquinolones and spectinomycin do carry appropriate labels, these drugs could be considered for treatment of *M. bovis* infections (Maunsell *et al*., 2011; Sulyok *et al*., 2014). Antibiotic treatment must be done early in the course of the disease, and pain relief should be provided for sick cows and calves (Jensen, 2015).

2.16.2 Treatment of contagious bovine pleuropneumonia

Treatment of CBPP is recommended only in endemic areas because the organisms may not be eliminated, and carriers may develop (Nicholas *et al*., 2012). Antibiotics are reported to be ineffective in chronic cases. A study was carried out an *in vitro* trial of the effects of five commonly used antibiotics on a number of strains of *Mmm*, and concluded that tilmicosin and danofloxacin were effective both in terms of mycoplasmastatic and mycoplasmacidal activity; florfenicol and a tetracycline provide

intermediate effectiveness while spectinomycin was unsuccessful against some strains (Ayling *et al*., 2000). Tetracyclines, macrolides and fluoroquinolones are reported to be useful in treatment, but individual drugs may differ in their effects (Mitchell *et al*., 2012; Nicholas *et al*., 2012). Tylosin (10 mg/kg, IM, bid, for six injections) and danofloxacin 2.5% (2.5 mg/kg/day for 3 consecutive days) have been reported to be effective (Campbell, 2015). Certainly treatment of single animals will not control the spread of disease among infected herds because complete elimination of mycoplasmas is reported to be rare (Nicholas *et al*., 2012). The degree of risk from treated animals is still uncertain; however, treatment is controversial, and some countries do not permit antibiotics to be used (Nicholas *et al*., 2012, AU-IBAR, 2013).

2.17 Prevention, Control and Vaccination

2.17.1 Prevention and control of *Mycoplasma bovis* infection

The best way to prevent *M. bovis* infections is probably to maintain a closed herd or, if that is not possible, to screen and quarantine purchased animals. The use of sick boxes for sick cows and good sectioning of different age groups of calves and young animals are very important to prevent outbreaks (Jensen, 2015). For dairy herds, it is recommended that the bulk tank culture history of the herd of origin be examined when purchasing heifers or adults. If this history is unavailable, the bulk tank can be sampled at least 3 times spaced 3-4 days apart (Gonzalez and Wilson, 2002). Where possible, calf health records should be examined to determine if *M. bovis*-associated diseases such as otitis media have been observed as well as avoiding the mixing of calves from different age groups (Nicholas *et al*., 2016). When purchasing lactating cows, milk samples should be submitted for mycoplasma detection (culture, PCR, or ELISA), keeping in mind the low sensitivity of a single sample for detection of subclinical *M.*

bovis mastitis. Testing for *M. bovis* antibodies in milk might be useful to identify infected cows (Gonzalez and Wilson, 2002). Testing purchased dry cows, purchased heifers, and heifers raised offsite at calving and isolating them until results are obtained has been recommended. Serology has been used to help identify uninfected groups of cattle before purchase (O'Farrell *et al*., 2001; Maunsell *et al*., 2011).

Recommendations for the control and prevention of *M. bovis*-associated disease in stocker and feeder cattle focus on maximizing respiratory system health and immune function rather than *M. bovis*-specific measures (Pfutzner and Sachse, 1996; Maunsell *et al*., 2011). Strategic antibiotic therapy of high-risk animals on arrival or during an outbreak of BRD might be useful in reducing the incidence of mycoplasma disease (Butler *et al*., 2000; Stabel *et al*., 2004). Using proper hygiene measures for managing sick cattle (use separate equipment or personnel or clean equipment among animals, feed last) could reduce the likelihood of formite-mediated *M. bovis* transmission (Maunsell *et al*., 2011).

2.17.2 Prevention and control of contagious bovine pleuropneumonia

The control of CBPP in Africa has been achieved by the live T1/44 vaccine, which has been used for over 60 years (Olorunshola *et al*., 2017). Vaccination has been shown to be successful when coverage of the cattle population of a country or region is high and maintained over five successive years (Molungo *et al*., 2015; Olorunshola *et al*., 2017). Furthermore, mathematical modelling has shown that mass vaccination campaign over a 5-year period is not likely to achieve eradication unless other strategies are used (Mariner and Catley, 2003; Molungo *et al*., 2015). A policy of strict movement control and test and slaughter is at this time not possible to implement in most regions because of public resistance, fragmented veterinary services and lack of funds for compensation

(Mariner *et al*., 2006a). Publicly funded mass vaccination programs have not been sustainable leading to infrequent or sporadic control (Jores *et al*., 2013).

The slaughter of diseased cattle and strict control of animal movements plus crude vaccination were factors in the successful eradication efforts of the early 20th century in the USA, Japan and parts of Western Europe (Masiga and Domenech, 1995; Egwu *et al*., 1996). Quarantines and serological testing of imported animals are helpful. Outbreaks are eradicated with quarantines, movement controls, slaughter of infected and in-contact animals, and cleaning and disinfection (Xin *et al*., 2012). These methods were enhanced and used in the eradication of CBPP in Australia in the early 1970s (Kusiluka and Sudi, 2003). The resurgence of CBPP in southern Europe during the 1980s and 1990s was controlled by efficient disease monitoring and slaughter of infected herds (Egwu *et al*., 1996; Nicholas *et al*., 2000b). This can in part be attributed to successful European collaborative efforts on CBPP diagnosis and control initiated during the 1990s (Hamsten, 2009).

Slaughtering of infected herds is not realistic in Africa, although it is still the most efficient method to eradicate CBPP (Windsor and Wood, 1998). Epidemiological factors such as transhumance, nomadism and trekking of trade cattle present unique challenges that are aggravated by problems deploying control measures due to economic factors, political instability and lack of movement controls (Masiga *et al.*, 1996). Ultimately, African countries with endemic CBPP cannot afford eradication by "stamping-out" of all infected herds (Kusiluka and Sudi, 2003; Jores *et al*., 2008). This was demonstrated by a stamping-out eradication of CBPP in Botswana in 1996, which led to negative effects on short-term economics and increased malnutrition in children (Windsor and Wood, 1998; Boonstra *et al*., 2001). Mass vaccination and

chemotherapy are the remaining options for CBPP control in Africa and of these, vaccination is still preferred (Windsor, 2000; March, 2004).

2.17.3 Vaccination

Although vaccines has been reported to be available in developed nations against *M. bovis* infection, but has not been proved protective (Nicholas *et al*., 2009; Ayling *et al*., 2015). A quadrivalent inactivated vaccine containing respiratory syncycial virus, parainfluenza type 3, *M. dispar* and *M. bovis* showed some protection against respiratory disease in the field (Nicholas *et al*., 2009). A vaccine prepared from formalin inactivated strains of *M. bovis* and *Manhaemia haemolytica* taken from target herd reduced losses from pneumonia (Nicholas *et al*., 2002). A saponised inactivated vaccine was shown to be safe, highly immunogenic and protective against strong experimental challenge of virulent *M. bovis*, vaccinated calves showed few respiratory signs while all unvaccinated calves developed signs of pneumonia (Nicholas *et al*., 2002). The vaccine also reduced the spread of *M. bovis* to internal organs including the joints (Nicholas *et al*., 2002, Nicholas *et al*., 2009).

The first use of crude CBPP vaccine was published by Louis Willems in 1852 and consisted of pleural exudates from diseased animals (Egwu *et al*., 1996). Crude vaccination using pleural exudate or lung tissue, placed under the skin at the nose or the tip of the tail, had been used long before that in Africa and is still in use (Blancou, 1996). This can confer some protection but is discouraged as it frequently gives harsh adverse reactions known as the Willems' reaction at the point of incorporation (Provost *et al*., 1987).

Since then, diverse live attenuated *Mmm* strain vaccines have been used and the vaccine presently recommended by the OIE is the live strain vaccine T1/44 isolated in Tanzania in 1950s, semi-attenuated by 44 passages in embryonated eggs and has been in use in most African countries to date (Egwu *et al*., 1996; Gourgues *et al*., 2016). Contagious bovine pleuropneumonia vaccine T1/44 currently in used in Nigeria has been produced in National Veterinary Research Institute (NVRI) Vom which produced immunity for about 12 months (Tambuwal *et al*., 2011b). However, short duration of action, partial protection to the vaccinated animals, cold chain requirement and adverse tissue reaction has been reported as the major setbacks of the vaccine (Mbulu *et al*., 2004; Tambuwal *et al*., 2011b; Schieck *et al*., 2014). Adverse reactions leading to occasional deaths also occur which are seen as accepted side effects of vaccination or even essential for immunity (Thiaucourt *et al*., 2000; Nicholas *et al*., 2009). Reservations about the efficacy of T1/44 vaccine and its variants were raised after failed campaigns in the 1990s including loss of immunogenicity, increased virulence of outbreaks and insufficient vaccine titres (Tulasne *et al*., 1996; Nicholas *et al*., 2000a; AU-IBAR, 2013). March (2004) recommended enhanced buffering of T1/44 vaccine to improve its viable dose while Dedieu and Balcer-Rodrigues, (2006) stated that eradication of CBPP will only be achieved by the development of an efficient long-term protective vaccine.

A better understanding of the immunology associated with CBPP and the protective response is also vital in vaccine development. Different views about the protective role of CD4+ T cells in the course of a primary infection have been reported; Totté *et al.* (2008) indicating CD4+ T cells have a major contribution in animals that have recovered from infection whereas Sacchini *et al*. (2011) reported that CD4+ T lymphocytes have a minor role in the control of a primary infection of CBPP in cattle. A better vaccine that protects animals for more than two years, requires only a single

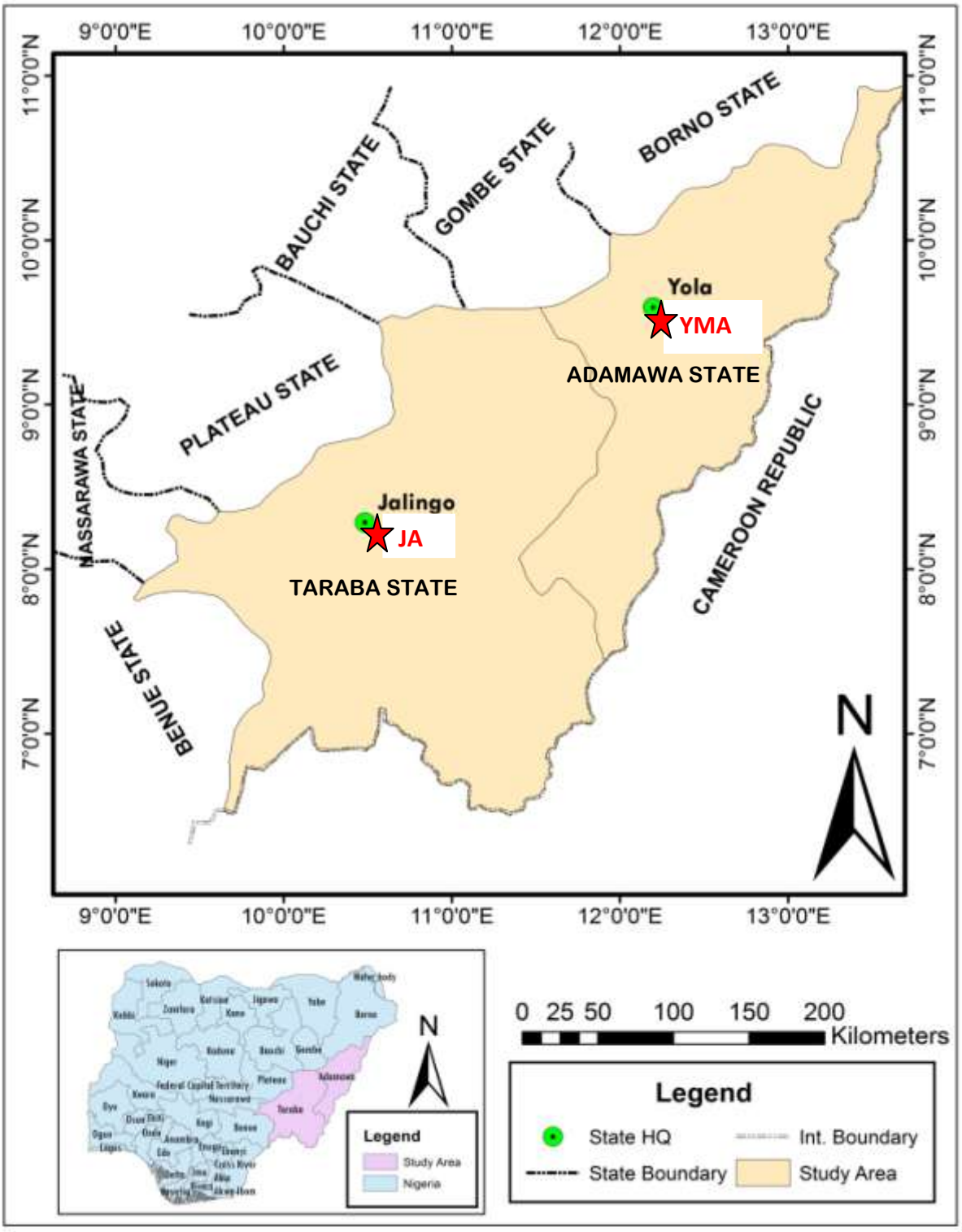
injection, does not need a cold chain and is not associated with adverse reactions is vital for the progressive control of CBPP within all regions. With these recommendations, it is apparent that novel vaccines that require less cold storage are needed (Ayling, 2013; Olorunshola *et al*., 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

Adamawa and Taraba States are located in the Northeastern part of Nigeria (Figure 3.1). They lie between latitude 7°0′0″ and 11°0′0″N and between Longitude 9°0′0″ and 13°0′0″E. The Yola Modern Abattoir based on 3D Global Positioning System (GPS) is located on the Latitude 9°13’33.3”N and Longitude 12°27’10.1”E. An average of 70 - 80 cattle is slaughtered daily in the abattoir and higher during festives period. Jalingo Abattoir based on 3D Global Positioning System (GPS) is located on the Lattitude 8°54’12.1”N and Longitude 11°21’14.3”E with an average of 40 - 50 cattle slaughtered daily. Adamawa State shares boundaries with Taraba, Borno, Gombe States while Taraba State is bounded by Gombe, Bauchi, Plateau, Nassarawa and Benue States. They both share an international boundary with the Cameroon Republic along the south-eastern border. Adamawa and Taraba States have a land area of about 91,390 km^2 with tropical wet and dry climate. Dry season lasts for a minimum of five months (November - March) while the wet season spans April to October. Mean annual rainfall in the State ranges from 700 mm in the north to 1800 mm in the extreme southern part with low humidity and high temperature. The climate in the area is also characterized by high evapo-transpiration especially during the dry season (Adebayo, 1999). The two states are among the lead producers of livestock in Nigeria with an estimated cattle population of 3.5 Million (Francis *et al*., 2018b).



★ = Location of the abattoir; YMA:- Yola Modern Abattoir, JA:- Jalingo Abattoir

Figure 3.1: Map of Adamawa and Taraba States, North-eastern Nigeria
Source: Adamawa State Ministry of Land and Survey, 2010.

3.2 Study Design

The study focused on one abattoir each from the two states (Taraba, Jalingo Abattoir and Adamawa, Yola Modern Abattoir).

3.3 Sample Collection

Purposive sampling technique (Portney and Watkins, 2007) was used and a total of four hundred and eighty (480) samples of lung tissues suspected of pneumonic, nasal swab, ear swab and pleural fluid were collected from 190 heads of cattle at slaughter (120 from Adamawa State while 70 from Taraba State). Two hundred and eighty four (284) and one hundred and ninety six (196) samples (tissues and swabs/pleural fluids) from cattle were collected from the abattoirs in Adamawa and Taraba States respectively. Four different types of samples in the following order: lung tissues (n=180), pleural swabs/fluid (n=180), nasal swabs (n=60) and ear swabs (n=60) were collected from suspected cattle with pneumonia.

Lung tissues (n=17) of slaughtered cattle suspected with pneumonia were also collected for histopathological examination. A tissue sample of about 5 gram was cut at the junction between normal and affected area, placed directly into a sample bottle containing 70% neutral buffered formalin and labelled appropriately for histopathology processing. The cattle sampled were local breeds normally presented for slaughter in the study area and comprised of White-Fulani, Red Bororo, Adamawa Gudali and their crosses. The animals were aged 3-7 years using standard procedure for aging described by Lasisi *et al*., (2002).

3.4 Sampling Procedure

The abattoirs were visited thrice weekly alternatively in each State and lung samples were collected for a period of thirteen (13) months between February 2016 and February 2017. The samples were collected from animals with cases of suspected pneumonia indicative of *M. bovis* infections and CBPP.

Lungs of slaughtered cattle were visually examined for gross lesions after which samples were obtained by incision at the junction of the diseased and normal tissues using scissors or meat-inspector's knife. The samples were thereafter placed in sterile polythene bags, labelled properly and placed in a cool box containing ice packs. Nasal and ear swabs were collected pre-slaughter from the ear and nasal passages using swab sticks wetted with PPLO broth and rubbed gently in the ear and nasal passages, labelled properly and placed in a cool box containing ice packs. Five millilitres of pleural fluid was collected from slaughtered cattle by using 10 ml syringes fitted with 21G needles. The syringes containing the pleural fluids were labelled properly and then placed in a cool box containing ice packs.

All the collected samples from abattoirs in Yola and Jalingo were transported to the National Veterinary Research Institute Zonal Offices in Yola and Jalingo respectively. In the designated Laboratories, individual samples of lungs were seared with hot spatula; pieces were cut with a scissors and placed in 2 ml sterile Nalgene® cryo vials containing 1.5 ml of pleuropneumonia-like organism (PPLO) broth. Swab sample tips containing specimens (nasal and ear swabs) were cut directly into the sterile Nalgene® cryo vials containing 1.5 ml of broth, while the pleural fluids were dispensed from the syringes into sterile Nalgene® cryo vials containing 1.5 ml of broth and labelled appropriately.

The samples were kept at -20°C at the Federal Medical Centre Yola and Federal Medical Centre Jalingo and later transported in refrigerated Coleman box to the Mycoplasma Laboratory, National Veterinary Research Institute (NVRI) Vom, Plateau State and then stored at -20°C and processed immediately.

3.5 Laboratory Examination

3.5.1 Microbiological culture of mycoplasmas

The samples were processed according to the method described by Thiaucourt *et al*. (1992). The transport media (PPLO broth) containing various samples were filtered by passing through 0.45µm millipore antibacterial filters which is permeable to organisms smaller than 0.45 µm in size such as *Mycoplasma* and viruses. The filtrate was then incubated at 37^{o}C in screw-capped bottles for three (3) days under CO_2 and later cultured onto PPLO agar. These were incubated at 37^{o}C in a 5% CO_2 atmosphere with maximum humidity and examined daily for evidence of growth for a period of 1-2 weeks using Stereomicroscope. Isolated colonies having the classical 'fried eggs' appearance with dense raised centres (nipples) were triple-cloned by aseptically transferring a small agar block containing an isolated *Mycoplasma* colony into 10 ml PPLO broth and incubated for one week. Both broth and agar media were considered negative if no comet or *Mycoplasma*-like colony was observed after 14 days of incubation. Cloned isolates were stored in triplets at -4°C, -20ºC and -80°C. These isolates were later transported under cold chain by FedEx® Red Star Express Courier to OIE Reference Laboratory for Contagious Bovine Pleuropneumonia (CBPP) in Istituto Zooprofilattico Sperimentale dell' Abruzzo e del Molise (IZSAM) "G. Caporale", Teramo, Italy. On receiving the isolates, they were stored at -20°C, the isolates were

then removed, verified and then registered under exotic disease unit before being processed.

3.5.2 Histopathological examination of positive lung tissues

Seventeen (17) positive and one (1) normal lung tissues were processed at the Histopathology laboratory, Department of Veterinary Pathology, Ahmadu Bello University Zaria for histopathological lesions according to the method described by Baker *et al*. (2000). Tissue samples of lungs of slaughtered cattle suspected with pneumonia were collected and fixed in 10% neutral buffered formalin. The samples were processed for histopathological assessment by dehydration in graded concentrations of alcohol (70%, 80%, 95% and 100%), cleared using Xylene, impregnated in plastic embedding rings and sectioned at 5µm using microtone. The tissues were then depolarized with Xylene, rehydrated in graded concentrations of alcohol (100%, 95%, 80% and 70%), stained with Haematoxylin and Eosin (H&E) stain and viewed under light microscope.

3.6 Identification of *Mycoplasma bovis* based on 16S rRNA Genes

3.6.1 DNA extraction

Mycoplasma bovis DNA from the presumptive culture was extracted from 500 µl of late exponential phase broth cultures using commercially available automated nucleic acid extraction system the Maxwell® 16 Tissue/cell DNA Purification kits (Promega, USA) with Maxwell® 16 instrument (Promega, USA) following manufacturer's instructions. The extracted DNA was resuspended in 500 µl TE buffer (10 mM Tris- HCl, 1 mM EDTA, pH 7.6) and was stored at -20°C until analyzed.

3.6.2 Polymerase chain reaction (PCR) identification of *Mycoplasma bovis*

For each sample, the following mix was used: 25 µl of master mix (Taq Polymerase, dNTPs, $MgCl_2$, Taq Buffer), 18 µl nuclease free water, 1 µl forward primer, 1 µl reverse primer and 5 µl of DNA sample as described by Bashiruddin *et al*. (2005a).

Primers designed for 16S rRNA were used to amplify 734-bp of 16S rRNA gene for *Mycoplasma bovis* (Table 3.1). The forward primer was MBOF2: 5'-GAA GCG AAA GTA GCA TAG GAA ATG AT-3' and the reverse primer was MBOR2: 5'-CGT CGT CCC CAC CTT CCT CCC G-3' (Johansson *et al*., 1996). The samples were then subjected to amplification reactions consisting of one cycle of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds, extension at 72°C for 30 seconds, a final extension step at 72°C for 5 minutes and standby tempearature at 4°C. The amplified products were detected by electrophoresis in 1% agarose gel electrophoresis. The resultant bands were stained with ethidium bromide and visualised under ultraviolet light. *Mycoplasma bovis* reference strain PG45 (Wise *et al*., 2011) was used as positive control, while negative control contained only the master mix.

Table 3.1: Primer Sequences used for PCR Identification of *Mycoplasma* species

Primer pairs	Oligonuceaotide sequences (5'-3')	Size of specific amplicon (bp)	Specificity	References
MBOF2	5'-GAAAGCGAAAGTAGCATAGGAAATGAT-3'	734	*M. bovis*	Johansson *et al*., 1996
MBOR2	5'-CGTCGTCCCCACCTTCCTCCCG-3'			
MM450	5'-GTATTTTCCTTTCTAATTTG-3'	574	*Mm* subspecies	Bashiruddin *et al*., 1994
MM451	5'-AAATCAAATTAATAAGTTTG-3'			

3.7 Identification and Confirmation of *Mycoplasma mycoides* subspecies *mycoides* Isolates

3.7.1 DNA extraction

Deoxyribonucleic acid (DNA) was extracted from 500 µl of PPLO broth presenting comet using Maxwell® 16 Tissue/cell DNA Purification kits (Promega, USA) with Maxwell® 16 instrument (Promega, USA) according to the manufacturer's instructions. Briefly, the cultures in the Eppendorf tubes were centrifuged at 1600g for 10 minutes. The supernatant was discarded and the pellet re-suspended with 100 µl of lysine buffer in Eppendorf tubes and then pipetted into the first chamber of the DNA purification cartridge. The DNA purification kit was inserted into the Maxwell® 16 instrument and then run for 36 minutes and the extracted DNA was kept at -20℃ until analyzed.

3.7.2 Molecular identification of *Mycoplasma mycoides* subspecies using PCR

This protocol is a PCR-based test for the specific identification of *Mycoplasma mycoides* subspecies according to Bashiruddin *et al*. (1994). For each sample, the following mix was prepared: 25 µl of Qiagen master mix (Taq Polymerase, dNTPs, $MgCl_2$, Taq Buffer), 18 µl of nuclease free water, 1 µl of forward primer MM450 5'-GTATTTTCCTTTCTAATTTG-3' 50 pmol/µl, 1 µl of reverse primer MM451 5'-AAATCAAATTAAGTTTG-3' 50 pmol/µl both designed from CAP-21 genomic region and 5 µl DNA of the sample as shown in Table 3.1 (Bashiruddin *et al*., 1994).

The samples were then subjected to amplification reactions consisting of initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, a final extension at 72°C for 5 minutes and standby at 4°C in GeneAmp® PCR System 9700 (Applied Biosystems). The CAP-21 genomic region of *Mycoplasma mycoides*

subcluster was used as positive controls and a band size of 574-bp was expected for *Mycoplasma mycoides* subclusters.

The PCR products were electrophoresed on 1% agarose gel and the samples run for 30 minutes. The resultant bands were stained with ethidium bromide and visualised under ultraviolet light (Bashiruddin *et al*., 1994).

3.7.3 Differentiation of *Mycoplasma mycoides* subspecies *mycoides* using PCR-RFLP

The typing method was adapted from the protocol used in OIE Reference Laboratory for CBPP and that of Bashiruddin *et al*. (2005), but varied with respect to *Vsp*1 (8-12 U/µl concentration) which replaced *Asn*1 endonuclease in this study.

The master mix comprised of: 0.4 µl of restriction endonuclease (*Vsp*1), 3.6 µl of nuclease free water, 1.0 µl of specific buffer and 5.0 µl of the PCR product. The mixture was centrifuged at 16,875g for 45 seconds and then incubated at 37°C for 2 hours. Polymerase chain reaction products were digested with restriction enzyme *Vsp*1. The products were run on a 3% agarose gel electrophoresis at 100 volts for a period of 40 minutes. At the end of the run, the resulting DNA fragments were analyzed by visualization under ultraviolet light to determine the expected base pairs of the fragments.

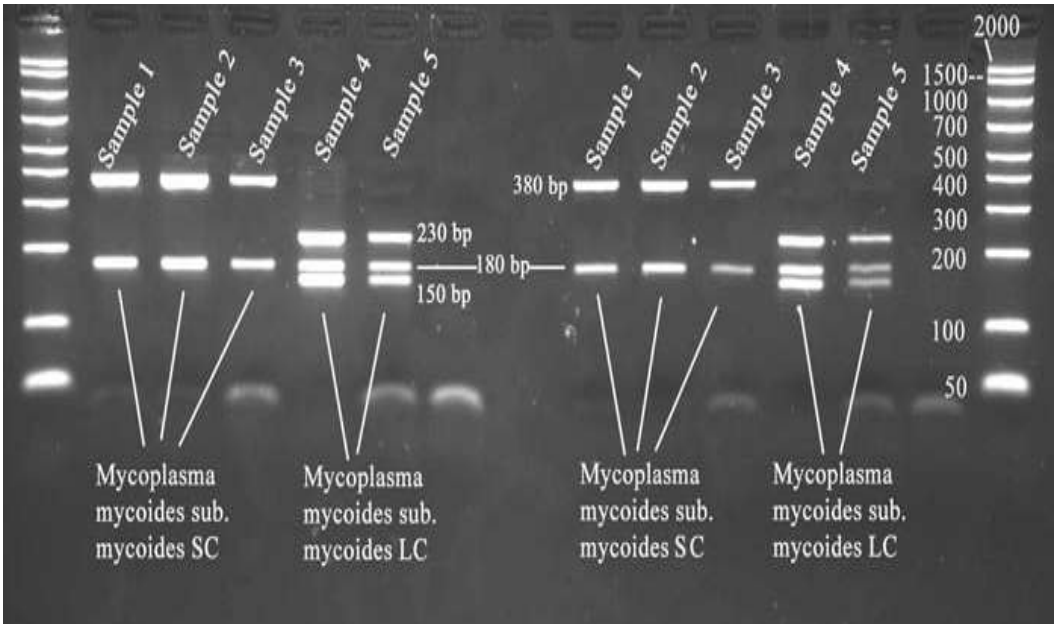


Figure 3.2: Resrtriction Fragment Length Polymorphism (RFLP) profiles of *Mycoplasma mycoides* subsp. *mycoides* with two restricted bands at 180-bp and 380-bp and *Mycoplasma mycoides* subsp. *capri* with three restricted bands at 150-bp, 180-bp and 230-bp (Bashiruddin *et al.*, 2005b).

3.8 Next Generation Sequencing of *Mycoplasma mycoides* subspecies *mycoides*

Next Generation Sequencing technique was carried out using the method described by Dupuy *et al*. (2012) to sequence *Mmm* field strains isolated from Adamawa and Taraba States, for phylogeny studies. Genomic DNA was extracted from culture using the Maxwell 16 Tissue/cell DNA purification kits (Promega) according to manufacturer's instruction. The extracted DNA was submitted for Next Generation Sequencing (NGS). Library preparation was carried out by Nextera XT DNA library 300-bp paired end preparation kit (Illumina Inc.). The libraries were loaded onto NextSeq 500/550 Mid Output Reagent Cartridge (Version 2.0) 300 cycles kit (Illumina Inc.) and then sequenced on Illumina NextSeq^{TM} 500 platform (Illumina USA) producing 150-bp paired-end reads. Following library preparation, thirteen (13) isolates failed quality control analysis and were therefore excluded from sequencing. This resulted in twenty (20) isolates being successfully sequenced.

3.8.1 Genome analysis

Fast QC:Read QC (Version 10.1) was used for quality control of sequence reads, and adaptor sequences and trailing bases were removed by using Trimmomatic (Version 1.0.0) (Lohse *et al*., 2012). A minimum read length of 150 bases was chosen. Assembly of the sequencing reads was performed by using the SPAdes genome assembler tool (Version 3.5) with k-mer sizes of 21, 33, 55, and 77 (Bankevich *et al*., 2012). For annotation, the genomes were submitted to a local installation of ProKKa (Version 2.0) (Overbeek *et al*., 2014). Genome-Genome Orthologous Average Nucleotide Identity (OrthoANI) was measured by using the OrthoANI tool (Lee *et al*., 2016).

Basic Local Alignment Search Tool (BLASTn) comparisons were performed with BLAST Ring Image Generator (BRIG) application (Alikhan *et al*., 2011). *Mycoplasma*

mycoides subsp. *mycoides* strain T1/44 (vaccine strain) was used as the central reference sequence, and 50% and 90% identities were chosen as the lower and upper identity thresholds respectively.

3.8.2 Pangenome analysis and phylogenetic inference

Core/Pan-genome analysis was performed with USEARCH (Version 8.1)) using a minimum identity of 0.6 (Meier-Kolthoff *et al*., 2013). The core genome of the 20 isolates was determined as the set of genes shared by the 99-100% of strains and soft-core (genes shared by 95% to 99% of the isolates), while the accessory genome was the set of genes present in a subset of strains or in a single strain.

The accessory genome was split into shell (genes common to at least 15% and up to 95% strains), and cloud (unique genes and genes shared by less than the 15% of isolates).

The core genome was further used to derive the phylogeny of the strains. Tree building was performed by using FastTree (Version 2.1.7) (Price *et al*., 2009; Price *et al*., 2010) with the general time reversible (GTR) gamma model of nucleotide evolution and 1,000 bootstrapping replicates.

Phylogenetic tree based on SNP matrix was performed by kSNP3 (Version 3.0) software (Guindon and Gascuel, 2003). The whole SNPs matrix was imported into the MEGA 6.0 package to derive a Neighbour-Joining tree with Transition/Transversion model and 1,000 bootstrapping replicates.

3.8.3 Virulence factors identification

Virulence factors/genes were identified by similarity searching against a specialized virulence factor database (*http://www.mgc.ac.cn/VFs/*) accessed in January 2018, using

the BlastP algorythm with default parameters, using the results of annotation as query. Proteins were assigned to a particular class of virulence factors if both coverage and similarity were higher than 80%.

3.9 Data Analysis

Data collected were analysed using simple percentages, tables and figures. Next Generation Sequence data analysis include processing the data to remove adapter sequences and low-quality reads, mapping of the data to a sequence genome, and analysis of the compiled sequenced data using bioinformatics software packages. Evolutionary relationship between *Mmm* field strains, vaccine strain T1/44, Italian strain 57/13 and Indian strain BEN32 in the gene bank were determined. Phylogenetic tree were constructed with PHYLogeny Inference Package (PHYLIP) using neighbour-joining to support the conformation of the relationship between the strains.

CHAPTER FOUR

4.0 RESULTS

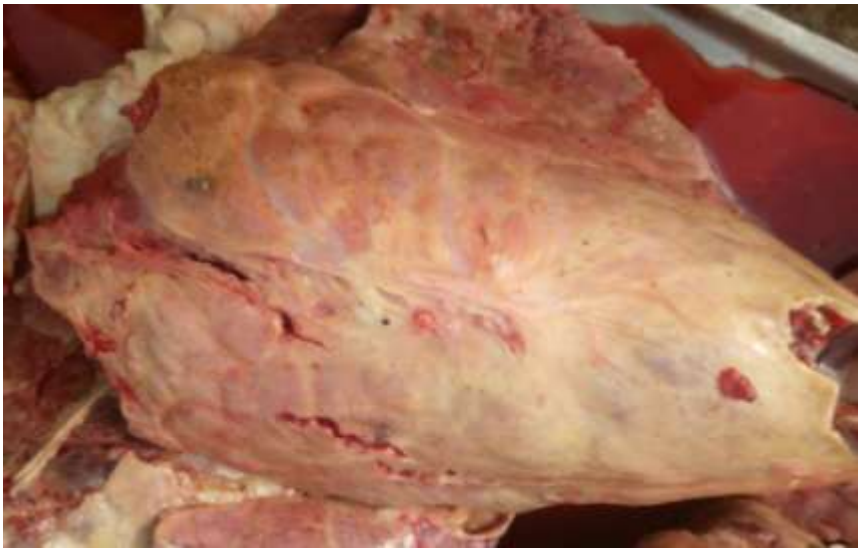
4.1 Isolation and Recovery Rates of *Mycoplasma* species Identified by Culture and Polymerese Chain Reaction

Four hundred and eighty (480) samples of lung tissues (Plate I), pleural fluid, nasal swabs and ear swabs were collected and processed at Mycoplasma Laboratory, National Veterinary Reseach Institute Vom, Plateau State Nigeria for *Mycoplasma* identification. Thirty nine of the 480 samples tested, 39 (8.13%) were positive for mycoplasma (Plate IIa and IIb). In Adamawa State, 12 (10.91%) lung tissues and 13 (11.82%) pleural fluid gave a positive growth on PPLO agar media with 25 (8.80%) isolation rates, while samples of nasal and ear swabs were negative for mycoplasma. In Taraba State, 8 (11.43%) lung tissues, 5 (7.14%) pleural fluid and 1 (3.57%) ear swabs yielded characteristic 'fried egg' colonies typical of *Mycoplasma* (Plate II and III) with an isolation rate of 14 (7.14%) while nasal swab samples yielded no growth on PPLO media.

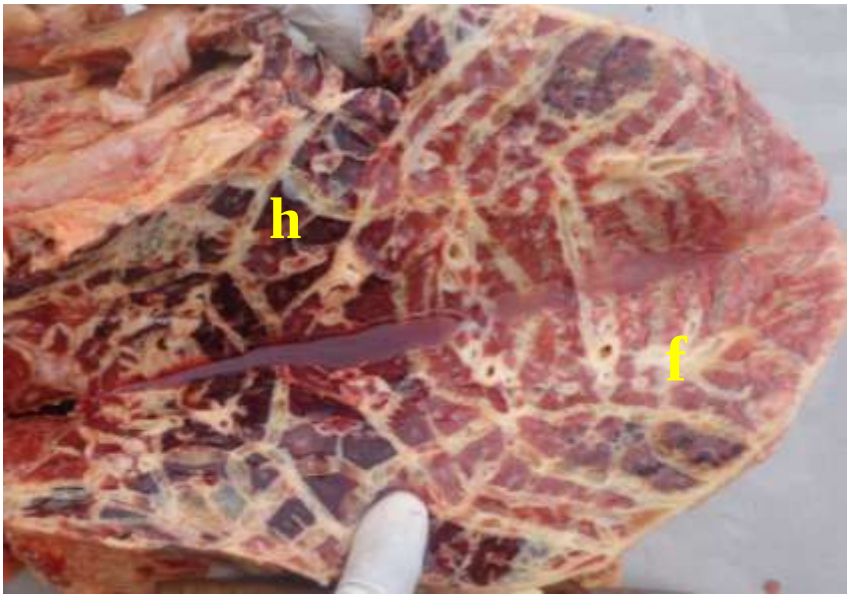
The thirty nine (39) pure isolates of *Mycoplasma* species were shipped to OIE Reference Laboratory for CBPP in Teramo, Italy (Appendix II), 37 isolates were confirmed on both PPLO media and specific PCR. Thus, 19 (10.56%) lung tissues, 17 (9.44%) pleural fluid and 1 (1.67%) ear swab yielded positive result giving an overall *Mycoplasma* species recovery rate was 7.70% (37/480), out of which Adamawa State had 25 (8.80%) and Taraba States had 12 (6.12%). The resuts did show that more isolates came from Adamawa State compared to Taraba State. The isolation and recovery rates of *Mycoplasma* species is shown on Table 4.1

Table 4.1: Rate of isolation and PCR confirmation of *Mycoplasma* species from cattle in Adamawa and Taraba States northeast Nigeria

Source of sample	Sample type	No. of samples collected	No. of *Mycopasma* cultured (%)	No. of isolates identified by PCR (%)
Adamawa State	Lungs	110	12 (10.91)	12 (10.91)
	Pleural fluid	110	13 (11.82)	13 (11.82)
	Nasal swab	32	0 (0.00)	0 (0.00)
	Ear swab	32	0 (0.00)	0 (0.00)
Sub-total		***284***	***25 (8.80)***	***25 (8.80)***
Taraba State	Lungs	70	8 (11.43)	7 (10.00)
	Pleural fluid	70	5 (7.14)	4 (5.71)
	Nasal swab	28	0 (0.00)	0 (0.00)
	Ear swab	28	1 (3.57)	1 (3.57)
Sub-total		***196***	***14 (7.14)***	***12 (6.12)***
TOTAL		**480**	**39 (8.13)**	**37 (7.71)**

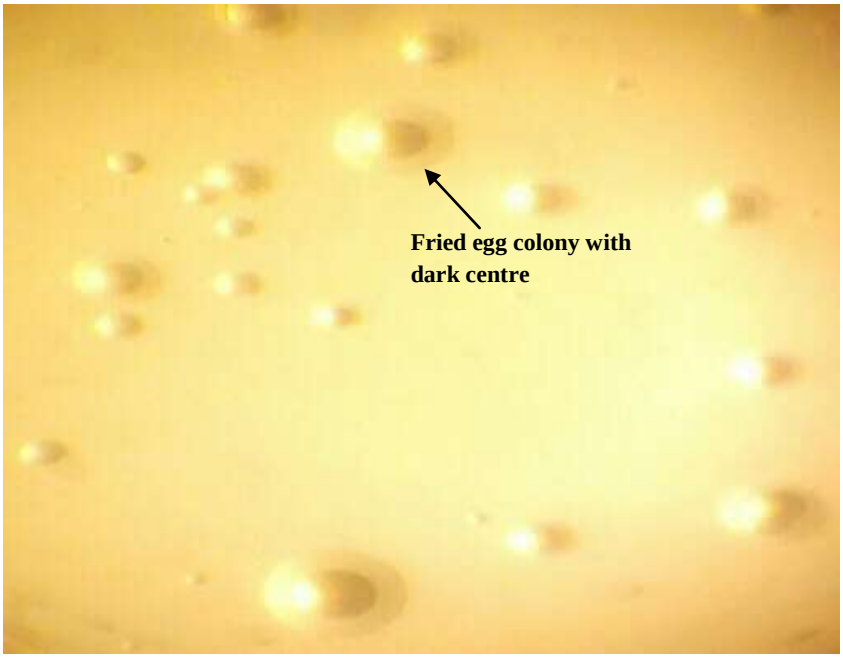


(a)

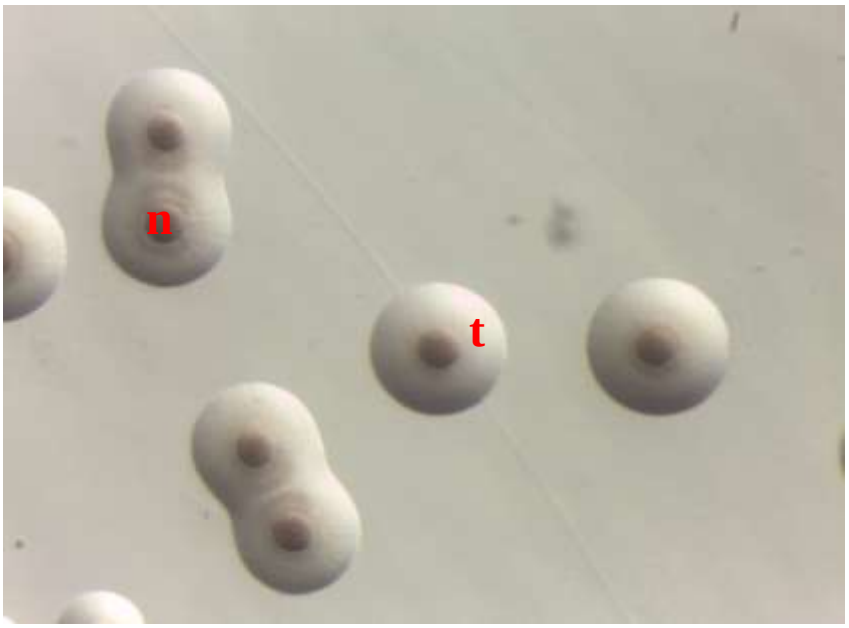


(b)

PLATE I: Typical gross appearance of suspected pneumonic lungs at Yola Modern Abattoir, Adamawa State. A condemned CBPP lung (a) and an opened lung (b) showing gross lesions of extensive fibrosis (f), hepatisation (h) with marble appearance pathognomonic of CBPP



(a)



(b)

PLATE II: Positive culture of *Mycoplasma* species isolates from lung sample from Yola Modern Abattoir (AL90) on PPLO agar showing characteristic 'fried egg shape' colonies, dense raised central area 'nipple' (n) and transparent periphery (t) growth on PPLO agar isolated at NVRI Vom after 72 hours (a) and recovered from isolates shipped to IZSAM Teramo, Italy after5 days (b) of incubation viewed under Stereomicrospe (X35)

4.2 Isolation rates of *Mycoplasma* species from Cattle in Adamawa and Taraba States northeastern Nigeria

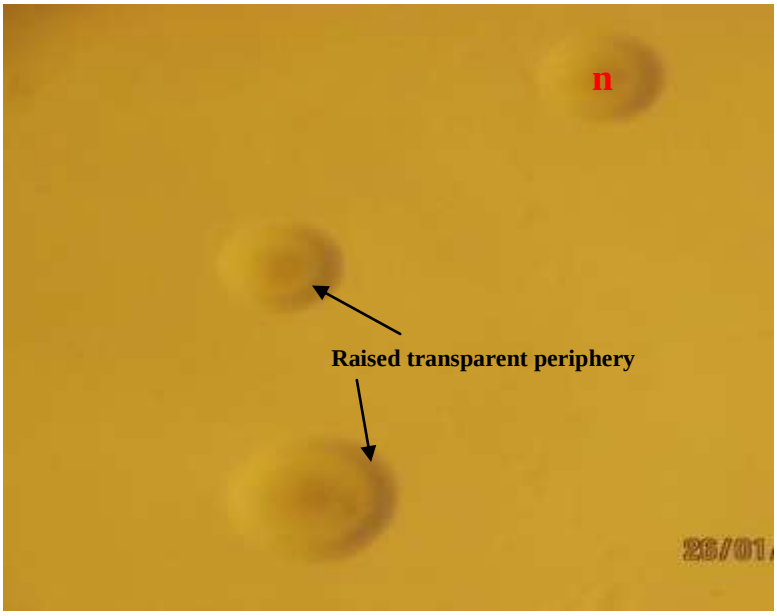
The results of the two States showed 4 (0.83%) isolation rate of *M. bovis* (Plate IIIa). In Adamawa State, 1 (0.91%) *M. bovis* was isolated from pleural fluid, and all the samples from lung tissues, nasal swab and ear swab were negative. While in Taraba State, 2 (2.86%) and 1 (3.57%) *M. bovis* came from lung tissues and ear swab samples respectively with no isolates from pleural fluid and nasal swab samples (Table 4.2).

On the basis of frequency of isolation, *Mycoplasma mycoides* subsp. *mycoides* (Plate IIIb) was the dominant species 33 (6.87%). In Adamawa State, 12 (10.91%) isolates of *Mmm* came from both lung tissues and pleural fluid. The study revealed that, 5 (7.14%) and 4 (5.71%) isolates of *Mmm* respectively came from lung tissues and pleural fluid in Taraba State. The samples from nasal and ear swab from both Adamawa and Taraba States were negative for *Mmm* (Table 4.2).

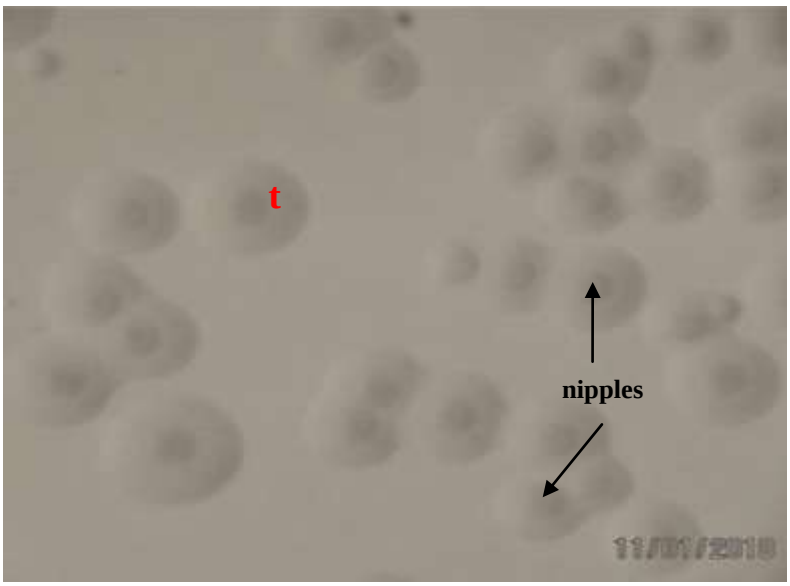
Mycoplasma mycoides subsp. *mycoides* and *M. bovis* were isolated and identified from lung tissue (AL106) and pleural fluid (AP106) respectively of the same animal in the study area (Appendix II)

Table 4.2: Isolation rates of *Mycoplasma* species from cattle in Adamawa and Taraba States, northeast Nigeria

State	Source of examined isolates	No. of samples	No. of positive samples (%)	*M. bovis* (%)	*Mmm* (%)
Adamawa	Lungs	110	12 (10.91)	0 (0.00)	12 (10.91)
	Pleural fluid	110	13 (11.82)	1 (0.91)	12 (10.91)
	Nasal swabs	32	0 (0.00)	0 (0.00)	0 (0.00)
	Ear swabs	32	0 (0.00)	0 (0.00)	0 (0.00)
Taraba	Lungs	70	7 (10.00)	2 (2.86)	5 (7.14)
	Pleural fluid	70	4 (5.71)	0 (0.00)	4 (5.71)
	Nasal swab	28	0 (0.00)	0 (0.00)	0 (0.00)
	Ear swab	28	1 (3.57)	**1 (3.57)**	0 (0.00)
	TOTAL	**480**	**37 (7.70)**	**4 (0.83)**	**33 (6.87)**



(a)

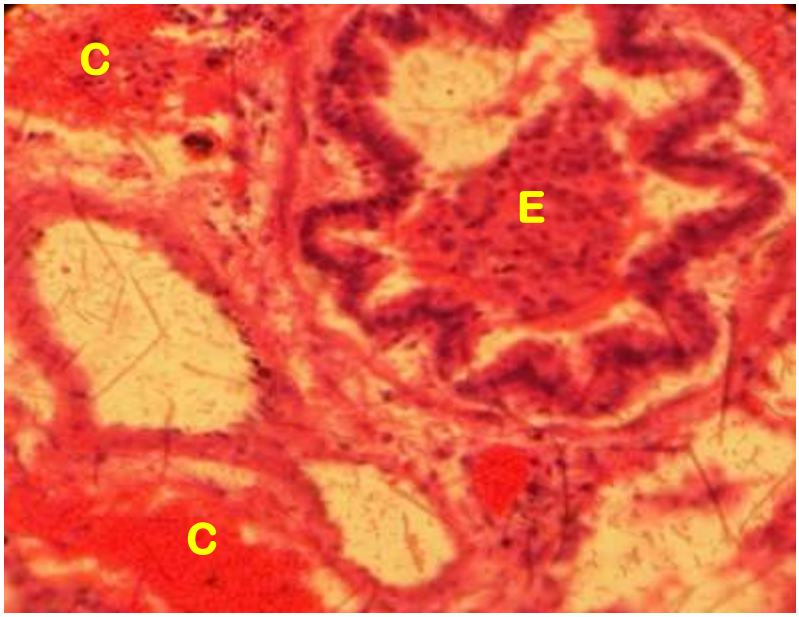


(b)

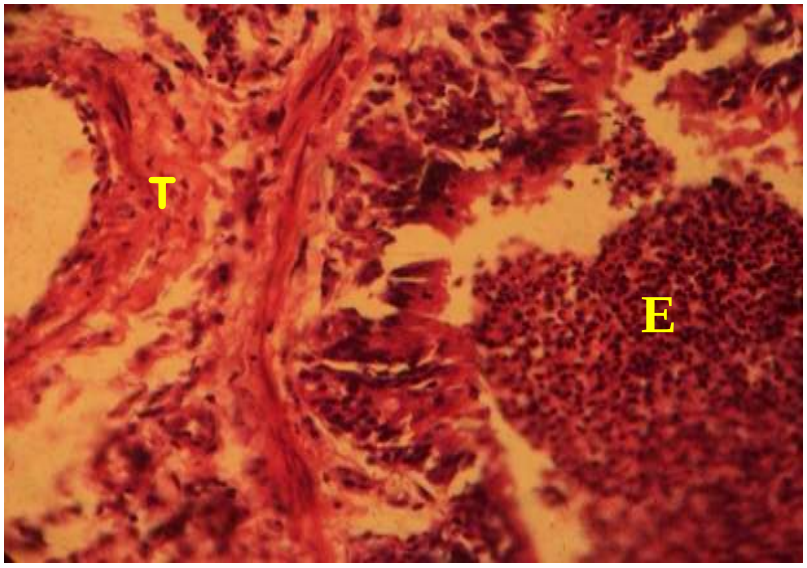
PLATE III: Colonies of *Mycoplasma bovis* (a) and *Mycoplasma mycoides* subspecies *mycoides* (b) from lung sample on PPLO agar showing 'fried egg' colonies with nipples 'n' and raised transparent periphery 't' observed under Stereomicroscope (X40)

4.3 Histopathology of Positive Lungs of Slaughtered Cattle

Histopathological examination was carried out on pneumonic lung tissues collected from 7 year old white Fulani cow (Plate I). Histopathology showed severe congestion of pulmonary blood vessel (Plate IVa) and fibrin exudation into interalveolar spaces with almost all the alveoli collapsed. There was interstitial pneumonia accompanied by mononuclear cellular infiltration with numerous macrophages, few lymphocytes and neutrophils many of which have engulfed erythrocytes. The bronchiolar epithelium was thickened, hyperplastic and folded, with a considerable quantity of edematous fluid and inflammatory cells found in the lumen (Plate IVb). The photomicrogragh of lung positive for *Mmm* is shown on Plate IV.



(a)



(b)

PLATE IV: Photomicrogragh of lung of 7 year White Fulani cow positive for *Mmm* in Adamawa State showing congestion (C), exudation (E) of fluid into the bronchiole, thickened pulmonary blood vessel (T) with alveolar spaces filled with pinkish fluid. H&E X200 (a), X400 (b)

4.4 Molecular Identification of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *mycoides*

4.4.1 Identification of *Mycoplasma bovis* by polymerase chain reaction (PCR)

The PCR analysis showed that of the 37 mycopasma isolates tested, 4 were positive for *M. bovis* by the presence of 734-bp band of 16S rRNA gene (Plate V). Of these four isolates of *M. bovis*, two (2) came from lung samples while one each came from pleural fluid and ear swabs.

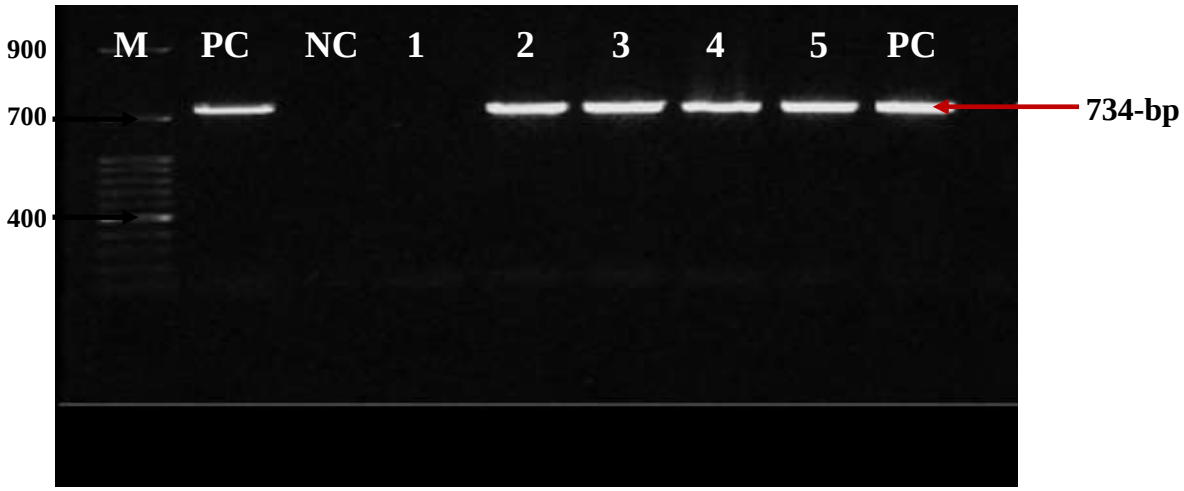


PLATE V: PCR amplification of *Mycoplasma bovis* 16S rRNA gene on 1% agarose gel with primers MBOF2 and MBOR2. M: molecular marker of 50-bp; PC: positive control reference strain PG45 (734-bp); NC: negative control; Lanes 1-5: represents samples TL41, T43, TL46, TES57 and AP106.

4.4.2 Identification of *Mycoplasma mycoides* subspecies *mycoides* by PCR-RFLP

Thirty-three (33) out of the thirty-seven (37) *Mycoplasma* isolates that were culture positive were confirmed to be *Mycoplasma mycoides* subspecies by specific Polymerase Chain Reaction assay based on amplification of CAP-21 genomic region. The production of a band equivalent to 574-bp and at the same distance with positive control indicated *Mycoplasma mycoides* sub-cluster member (Plate VI).

Following digestion of the amplicons with restriction endonuclease *Vsp*1, the production of two bands of approximately 180-bp and 380-bp indicated typical fingerprinting pattern of *Mmm* control and 33 of the isolates of which 16 and 17 were from lung and pleural fluid respectively. None of the PCR products was observed with three bands of 150-bp, 180-bp, and 230-bp specific for *Mycoplasma mycoides* subsp. *capri* in RFLP (Plates VII and VIII).

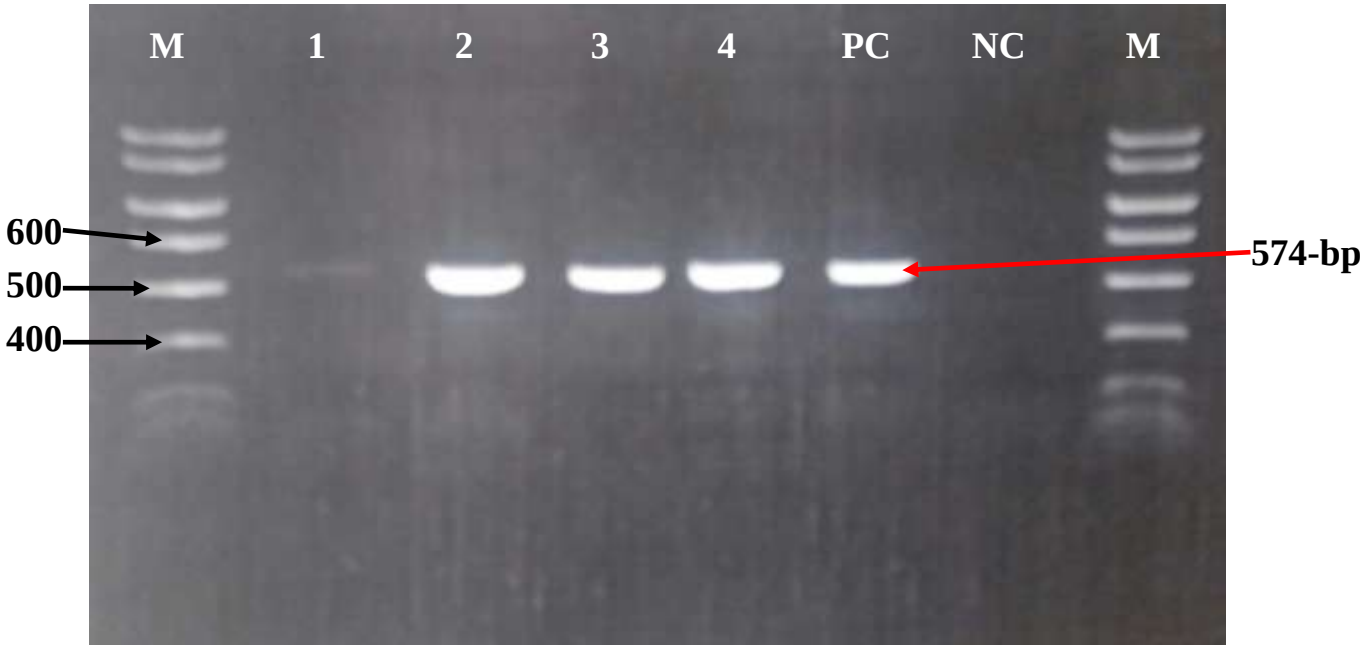


PLATE VI: PCR amplification of *Mycoplasma mycoides* subspecies CAP-21 genomic region (574-bp) on 1% agarose gel for identification using primers MM450 and MM451. Lane M: molecular size marker 100-bp; Lanes 1-4: samples TL42, TP57, AL90, AP108 showing *Mm* subspecies; PC: positive control; NC: negative control.

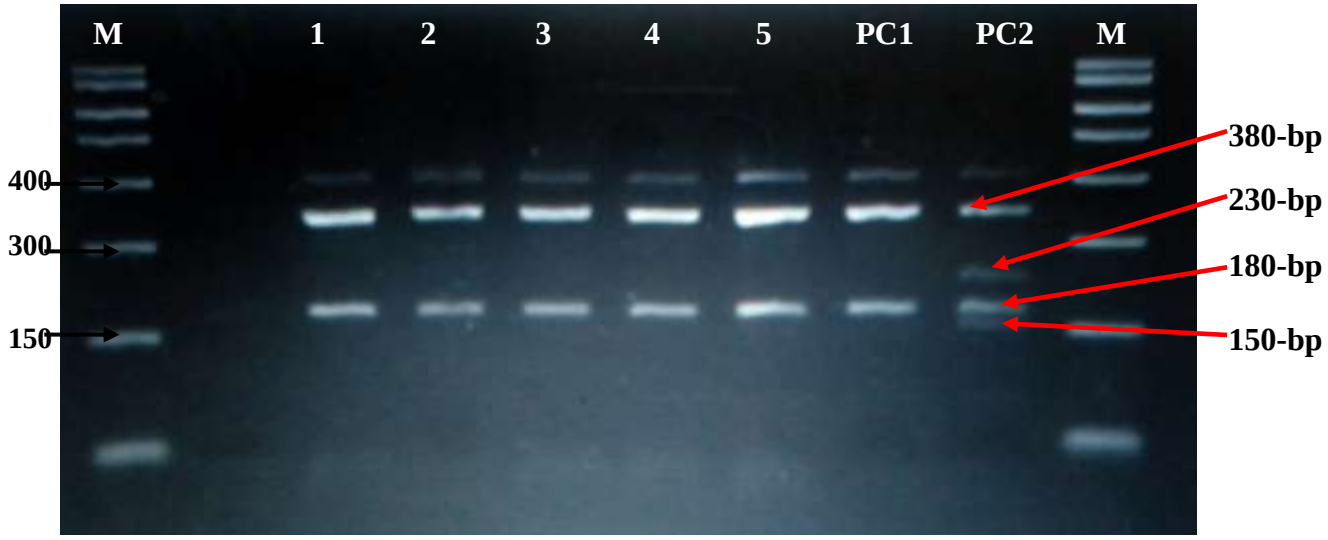


PLATE VII: PCR-RFLP amplicons on 3% agarose gel for the identification of *Mycoplasma mycoides* subspecies *mycoides* following digestion of PCR product with restriction endonuclease *Vsp*1 on the CAP-21 genomic region. Lane M: molecular size marker 100-bp; lane NC: negative control; lane 1-5: positive samples TL41, TP47, TP51, TP57, AL90 showing *Mmm* profile; lane PC1: *Mmm* positive control; lane PC2: *Mm* subsp. *capri* positive control.

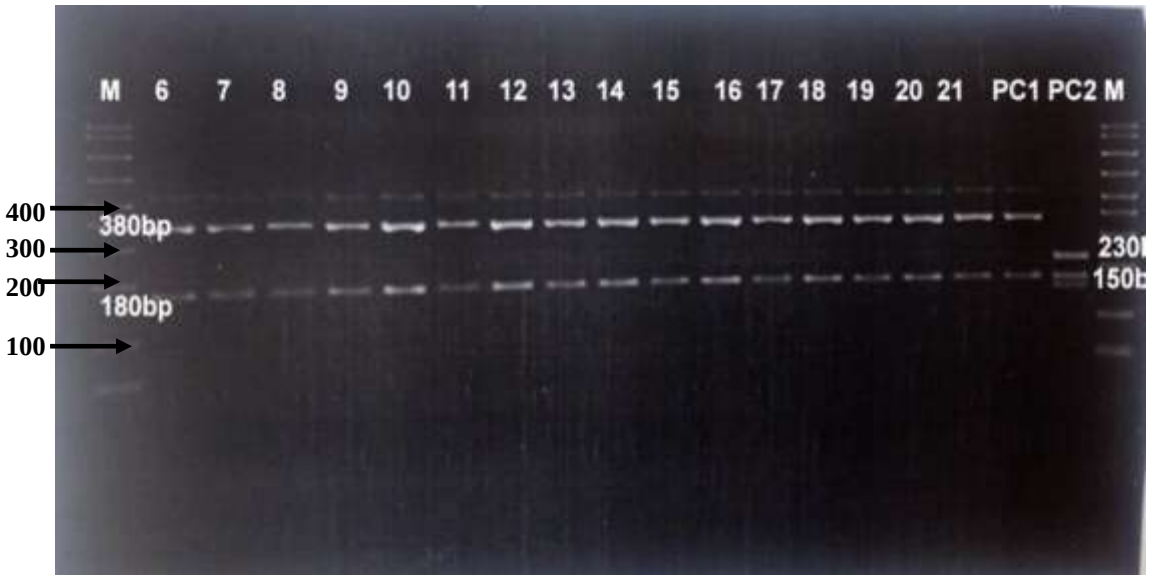


PLATE VIII: PCR-RFLP profiles on 3% agarose gel for the identification of *Mycoplasma mycoides* subspecies *mycoides* following digestion of PCR product with restriction endonuclease *Vsp*1 on the CAP-21 genomic region. Lane M: molecular size marker 100-bp; lane 6-21: samples AL103, AL104, AP104, AL106, AL107, AP108, AL109, AP109, AL111, AP111, AL112, AP112, AL113, AP113, AL114, AP115 showing *Mmm* profile; lane PC1: *Mmm* positive control; lane PC2: *Mm* subsp. *capri* positive control.

4.5 Next Generation Sequence (NGS) Data for *Mycoplasma mycoides* subspecies *mycoides* Strains

Of the thirty three (33) *Mmm* isolates that were subjected to NGS, thirteen (13) isolates were eliminated from further analysis due to their low average sequence depth. The *Mmm* isolates were assigned a sequence identity prior to analysis. The sequence identity and sample identity numbers were used vice versa throughout the analysis of the sequenced results. The sample and sequence identification numbers of *Mycoplasma mycoides* subspecies *mycoides* strains are shown in Appendix V.

4.5.1 Genomic features of field strains of *Mycoplasma mycoides* subspecies *mycoides*

Whole genome sequencing was undertaken for the 20 field isolates of *Mmm,* comprising of thirteen (13) isolates from Adamawa State and seven (7) from Taraba State. The complete fully annotated Reference type strain PG1 was used as a reference genome for the reads of the field strain for the 20 strains of *Mmm*. The 20 draft assemblies were highly similar and resulted in 19 and 115 contigs with a N50 value from 25,646 to 119,472. The total length of the genomes varied by approximately 22,000-bp, and were between 1,180,728-bp and 1,202,919-bp. The G+C content of the *Mmm* strains varied between 23.92% and 24.03% and the genomes harboured about 2,053 to 2,127predicted protein-coding sequences per genome (Table 4.3).

The annotation of the 20 genomes revealed coding sequences (CDS) from 2,016 to 2,087 and between 29 and 33 tRNA were identified per genome. The summary of the assembly and annotation statistics of these draft genomes is shown on Table 4.3. The lowest and highest value for each measured value is shown with yellow colour and red colour respectively and the Reference strain PG1 is shown on blue colour.

The genome sequence of *Mmm* strain **AP108** has been deposited in GenBank with accession number: VCPH1000000. The Illumina raw reads was also been deposited in the National Centre for Biotechnology Information (NCBI) Sequenced Read Archive (SRA) under the accession number SAMN11775871 (*www.ncbi.nlm.nih.gov/genome*).

Table 4.3: Summary of the Assembly and Annotation Statistics of *Mycoplasma mycoides* subspecies *mycoides* strains

Sequence ID	Isolate ID	Assembled	G+C content (%)	No. of Contigs	Average coverage	N50	No of protein coding genes	CDS	rRNA	tRNA
PG1 Ref. strain	**PG1**	**1211703**	**24.00**	**52**	**22929**	**62856**	**2098**	**2067**	**6**	**31**
DS8338267-79	AP68b	**1202919**	23.93	21	57282	91010	**2127**	**2087**	6	**33**
DS8338249-61	TP41	1201122	23.95	38	31609	78460	2106	2067	6	32
DS8338257-69	AP103	1198710	**23.92**	23	52118	89949	2126	2088	6	30
DS8338258-70	AL104	1197542	23.95	25	47902	99603	2114	2077	6	30
DS8338260-72	AL106	1197135	23.93	30	39905	85857	2121	2084	6	30
DS8338266-78	AL114	1195777	23.93	**19**	**62936**	**119472**	2077	2040	6	30
DS8338252-64	TP47	1195248	23.94	25	47810	92320	2104	2067	6	30
DS8338250-62	TL42	1195019	23.96	40	29876	78566	2110	2074	5	30
DS8338251-63	TL47	1194598	23.94	26	45946	86296	2104	2067	6	30
DS8338265-77	AL113	1194250	23.94	56	21326	53781	2081	2044	6	30
DS8338262-74	AP108	1191600	23.95	33	36109	89677	2111	2075	6	**29**
DS8338261-73	AL107	1191162	23.96	38	31346	68244	2105	2068	6	30
DS8338259-71	AP104	1190994	23.94	34	35029	85861	2099	2062	6	30
DS8338264-76	AL112	1190686	23.95	44	27061	63612	2110	2073	6	30
DS8338255-67	AL90	1190636	23.95	29	41056	88246	2081	2044	6	30
DS8338247-59	TP57	1190102	24.02	71	16762	41869	2105	2067	7	30
DS8338253-65	TP51	1189429	23.93	66	18022	38400	2084	2048	6	30
DS8338248-60	TL41	1187679	23.96	54	21994	47698	2101	2064	6	30
DS8338263-75	AL111	1187116	23.95	59	20121	62910	2090	2051	6	32
DS8338256-68	AL103	**1180728**	**24.03**	**115**	**10267**	**25648**	**2053**	**2016**	6	30

Blue Colour: Reference Strain PG1 Red Colour: Highest Value Yellow Colour: Lowest Value

4.5.2 Similarities between *Mycoplasma mycoides* subspecies *mycoides* strains

A high degree of genomic sequence similarity among the *Mycoplasma mycoides* strains was demonstrated by the calculated average genomic identity. Genomic index is an equation to predict the accuracy of genomic values by combining data from multiple traits. Genomic comparisons of the 20 field strains of *Mycoplasma mycoides* subspecies *mycoides* revealed high Orthologous Average Nucleotide Identity (OrthoANI) values between 99.59 and 99.92%, while the recommended cut-off range for species delimitation was 95 to 96%. The highest OrthoANI values indicated by red colour was observed between strain DS8338251-63-TL47 and DS8338266-78-AL114 while the lowest OrthoANI value indicated by yellow colour was observed between strains DS8338247-59-TP57 and DS8338257-69-AL103 (Table 4.4).

Table 4.4: Orthologous Average Nucleotide Identity of *Mycoplasma mycoides* subspecies *mycoides* field strains from Adamawa and Taraba States

	AP90	AP68b	AP108	AP103	AL107	TP57	TL42	TP47	AL112	AL114	TP57	AP104	AL104	TL47	AL111	TL41	AL106	TP41	AL103	AL113
AP90	100	99.87	99.80	99.83	99.78	99.84	99.81	99.86	99.80	99.84	99.76	99.78	99.87	99.87	99.77	99.76	99.83	99.76	99.63	99.78
AP68b	99.87	100	99.85	99.88	99.82	99.84	99.85	99.87	99.80	99.84	99.83	99.87	99.92	99.88	99.82	99.84	99.86	99.85	99.73	99.80
AP108	99.80	99.85	100	99.83	99.86	99.76	99.86	99.87	99.79	99.81	99.80	99.85	99.86	99.85	99.70	99.82	99.83	99.86	99.60	99.79
AP103	99.83	99.88	99.83	100	99.77	99.78	99.84	99.88	99.80	99.85	99.77	99.80	99.88	99.92	99.71	99.81	99.84	99.83	99.62	99.81
AL107	99.78	99.82	99.86	99.77	100	99.77	99.81	99.86	99.77	99.81	99.74	99.83	99.83	99.88	99.77	99.76	99.83	99.84	99.65	99.80
TP51	99.84	99.84	99.76	99.78	99.77	100	99.84	99.82	99.76	99.82	99.71	99.78	99.86	99.85	99.83	99.73	99.85	99.82	99.66	99.77
TL42	99.81	99.85	99.86	99.84	99.81	99.84	100	99.88	99.79	99.85	99.73	99.81	99.88	99.91	99.82	99.77	99.81	99.85	99.65	99.86
TP47	99.86	99.87	99.87	99.88	99.86	99.82	99.88	100	99.83	99.89	99.78	99.89	99.88	99.91	99.83	99.77	99.89	99.86	99.67	99.84
AL112	99.80	99.80	99.79	99.80	99.77	99.76	99.79	99.83	100	99.79	99.81	99.72	99.78	99.80	99.85	99.73	99.77	99.81	99.70	99.79
AL114	99.84	99.84	99.82	99.85	99.81	99.82	99.85	99.89	99.79	100	99.79	99.80	99.87	**99.92**	99.79	99.79	99.86	99.82	99.68	99.83
TP57	99.76	99.83	99.80	99.77	99.74	99.71	99.73	99.79	99.81	99.79	100	99.73	99.78	99.81	99.75	99.74	99.77	99.80	**99.59**	99.77
AP104	99.78	99.87	99.85	99.80	99.83	99.78	99.81	99.89	99.72	99.80	99.73	100	99.88	99.86	99.77	99.74	99.83	99.84	99.66	99.79
AL104	99.87	99.91	99.86	99.88	99.83	99.86	99.88	99.88	99.78	99.87	99.78	99.88	100	99.88	99.74	99.81	99.90	99.83	99.72	99.81
TL47	99.87	99.88	99.85	99.92	99.88	99.85	99.91	99.91	99.80	**99.92**	99.81	99.86	99.89	100	99.82	99.82	99.88	99.88	99.71	99.85
AL111	99.78	99.82	99.70	99.71	99.77	99.83	99.82	99.83	99.85	99.79	99.75	99.77	99.74	99.82	100	99.71	99.80	99.80	99.68	99.78
TL41	99.76	99.84	99.82	99.81	99.76	99.73	99.77	99.77	99.73	99.79	99.74	99.74	99.81	99.82	99.71	100	99.77	99.78	99.61	99.72
AL106	99.83	99.84	99.83	99.84	99.83	99.85	99.81	99.89	99.77	99.86	99.77	99.83	99.90	99.88	99.79	99.77	100	99.87	99.68	99.79
TP41	99.76	99.85	99.85	99.83	99.84	99.82	99.85	99.86	99.81	99.82	99.80	99.84	99.83	99.88	99.80	99.78	99.87	100	99.64	99.86
AL103	99.63	99.73	99.60	99.62	99.65	99.66	99.64	99.67	99.70	99.68	**99.59**	99.66	99.72	99.71	99.68	99.60	99.68	99.64	100	99.71
AL113	99.78	99.80	99.79	99.81	99.80	99.77	99.86	99.84	99.79	99.83	99.77	99.79	99.81	99.85	99.78	99.72	99.79	99.86	99.71	100

Red Colour: Highest Value Yellow Colour: Lowest Value

The genome sequence relatedness of the *Mmm* isolates was illustrated in a BLASTn ring diagram (Figure 4.1), where the 20 *Mmm* field strains under study were compared to the central genome of vaccine strain T1/44. The 20 *Mycoplasma mycoides* subspecies *mycoides* field strains from Adamawa and Taraba States share a high degree of genomic relatedness indicating that the vaccine T1/44 is related to the field strains of *Mmm* in the study area. There was no detectable absent region in the genomes of the 20 *Mmm* field strains and the vaccine strain T1/44 (Figure 4.1).

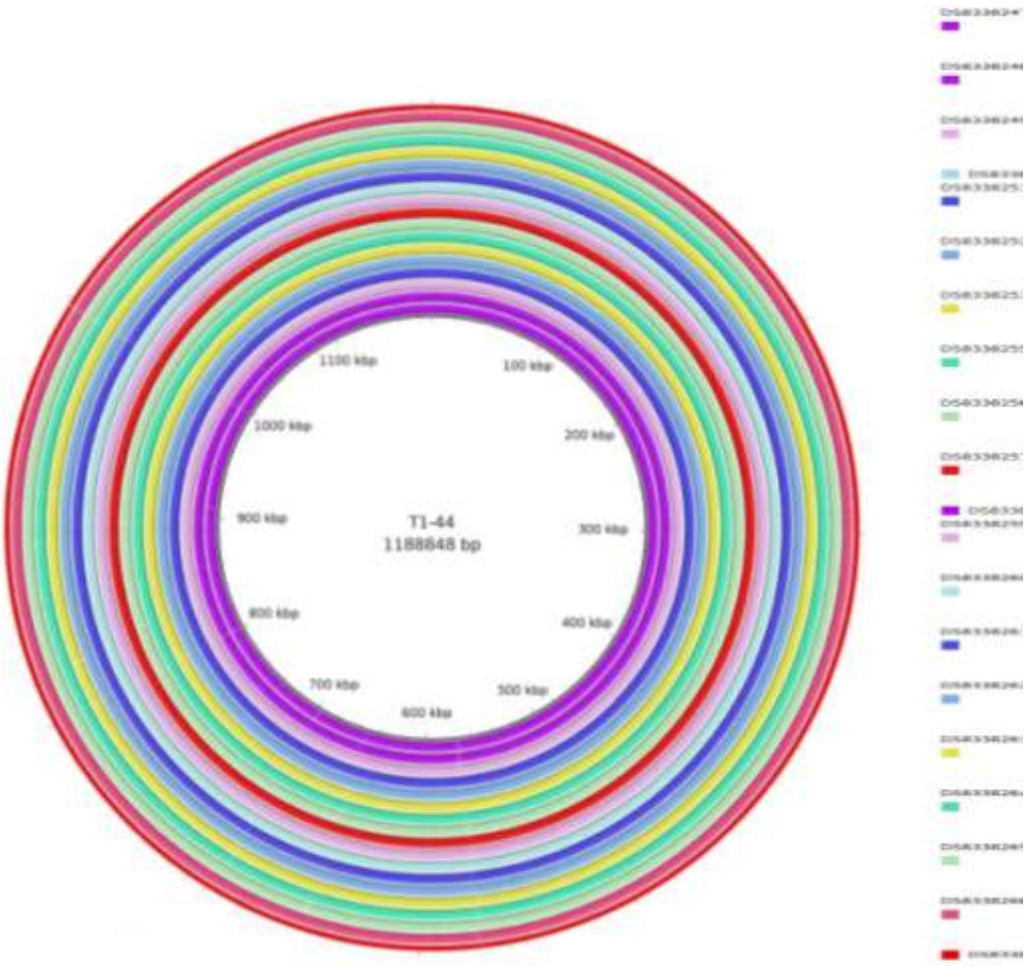


Figure 4.1: Schematic of BLASTn comparison of nucleotide sequences of the draft genomes of *Mmm* strains (shown as concentric rings) to T1/44 strain (central ring). *Myccoplasma mycoides* subsp. *mycoides* strains share a high degree of genomic relatedness. The BLAST matches are coloured on a sliding scale from 50-100% (although all difference were in the range 90-100%), demonstrating the degree of sequence relatedness between the query strains (shown in the key) and the T1/44 strain. No space was detected between the rings indicating the presence of all regions in both the vaccine strain and the 20 *Mmm* field strains. The inner concentric circle showed genome positions in bases where position one is the first base of *dna*A gene.

4.5.3 Pangenome investigation of field strains of *Mycoplasma mycoides* subspecies *mycoides*

Pan-genome represents the cumulative number of clusters of orthologous groups of protein (COG) present in all *Mycoplasma* genomes, while core-genome represents the conserved number of COG. Strain diversity was investigated with a pan-genome analysis. The pan-genome of the 20 field strains of *Mmm* comprised 3,081 genes, with 1,707 core genes (55.4% of the pangenome) and 1,374 accessory genes (44.6% of the pangenome) (Figure 4.2). With between 2,053 and 2,127 predicted protein-coding genes identified in the draft genomes (Table 4.3), the 1707 core genes constituted between 80.3 and 83.1% of the predicted proteome of each isolate and comprised of 1,470 genes in the core and 237 genes in the soft core (Figure 4.2). The accessory genome consists of high frequency (dispensable) and low frequency (unique) genes. The number of high frequency genes family that was conserved in at least two species was 691 genes, and the number of low frequency genes that was present in only one strain was 683 genes (Figure 4.3).

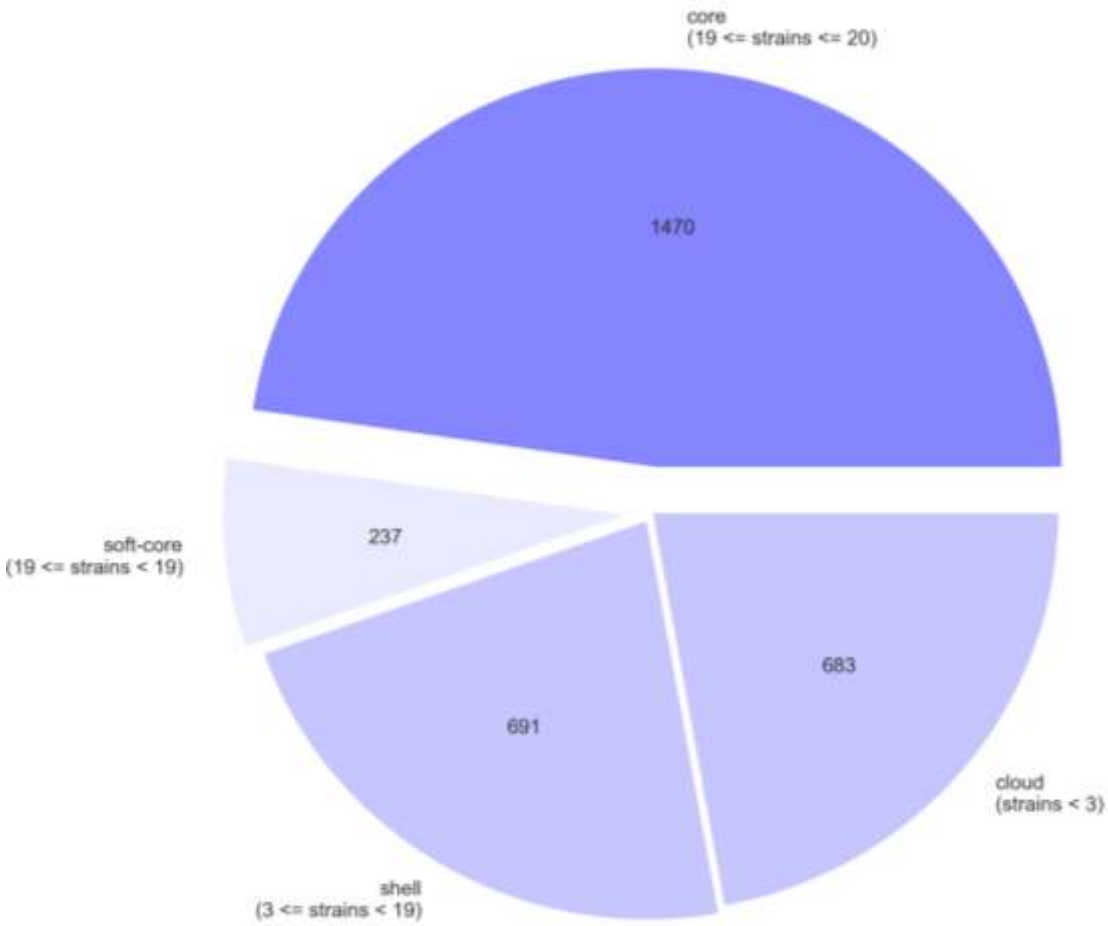


Figure 4.2: Pangenome of 20 field strains of *Mycoplasma mycoides* subsp. *mycoides* comprises 3,081 genes. The core genome constitutes 55.4% of the genes, the high-frequency accessory group constituting 22.4% and the low-frequency accessory group representing 22.2%.

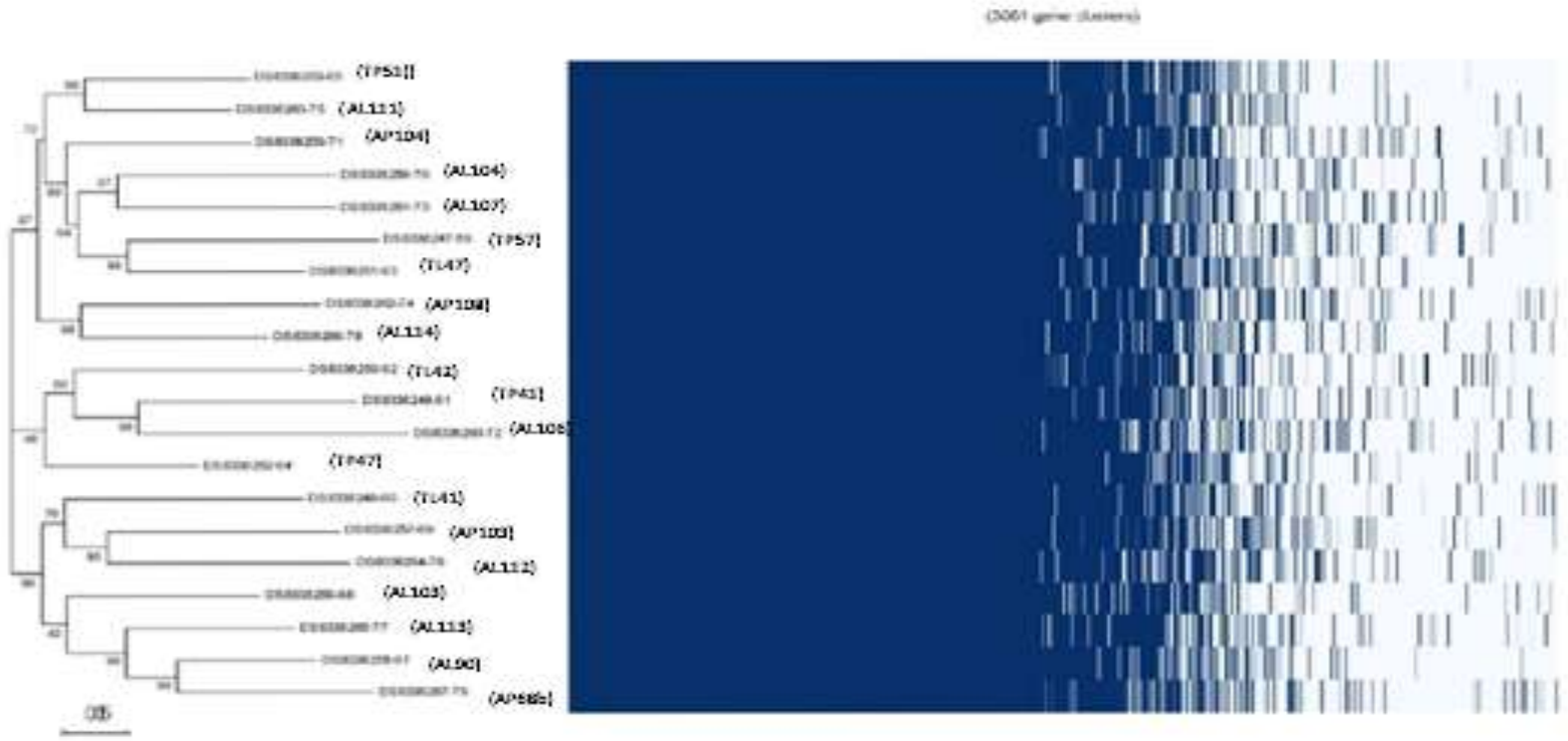


Figure 4.3: Phylogenetic tree showing the relationships of the 20 field strains of *Mycoplasma mycoides* subspecies *mycoides* and accessory genes present or absent in these strains. The tree is based on alignments of orthologous regions of the genomes and was generated by using an approximated maximum likelihood method. All interior node values are 100 or otherwise as marked and represent local support values from 1,000 bootstrap replicates. The scale bar denotes the number of substitutions per site. Accessory genes present or absent in strains are shown to the right of the tree, and blue boxes represent genes that are present, while white boxes represent the absence of genes from the genome.

The clusters of orthologous groups (COG) database provide a framework for analyzing the orthologous relationship among completely sequenced genomes. The 20 *Mmm* genomes were found to exhibit high degree of similarities in COG functional category distributions. Among the 3,081 total genes in the genome of the 20 *Mmm* field strains, 1,198 genes (38.9%) could be assigned to clusters of orthologous groups (COG) comprising of 21 functional categories (Figure 4.4). In the core genome which contains a subset of genes present in all genomes under investigation, the most occurring categories were mostly the genes described to be backbone of the genome which are genes involved in cellular processes and signaling, and information storage and processing, comprising the translation, ribosomal structure and biogenesis (J) and carbohydrate transport and metabolism (G) followed by replication, recombination and repair (L), and ensuring by the housekeeping function of the metabolism, while the least occurring category was secondary metabolites biosynthesis, transport and catabolism (Q). In the accessory genome was instead observed with higher frequency genes related to the mobilome (X), translation, ribosomal structure and biogenesis (J), carbohydrate transport and metabolism (G), followed by the cell wall/membrane/envelope biogenesis (M) with no occurring category in signal transduction mechanism (T) (Figure 4.4). In the core-genome, genes involved in the regulation of gene expression were strongly represented, with this group having the highest relative number of coding sequences involved in transcription processes (K), but also in signal transduction (T) and carbohydrate transport and metabolism (G) conversely to the lipid metabolism (I) category which has been more frequently represented in the accessory genome. Genes involved in RNA processing and modification (A), chromatin structure and dynamics (B), extracellular structure (W), nuclear structure (Y) and cytoskeleton (Z) were completely absent in all the 20 strains of *Mmm* genome sequenced in this study.

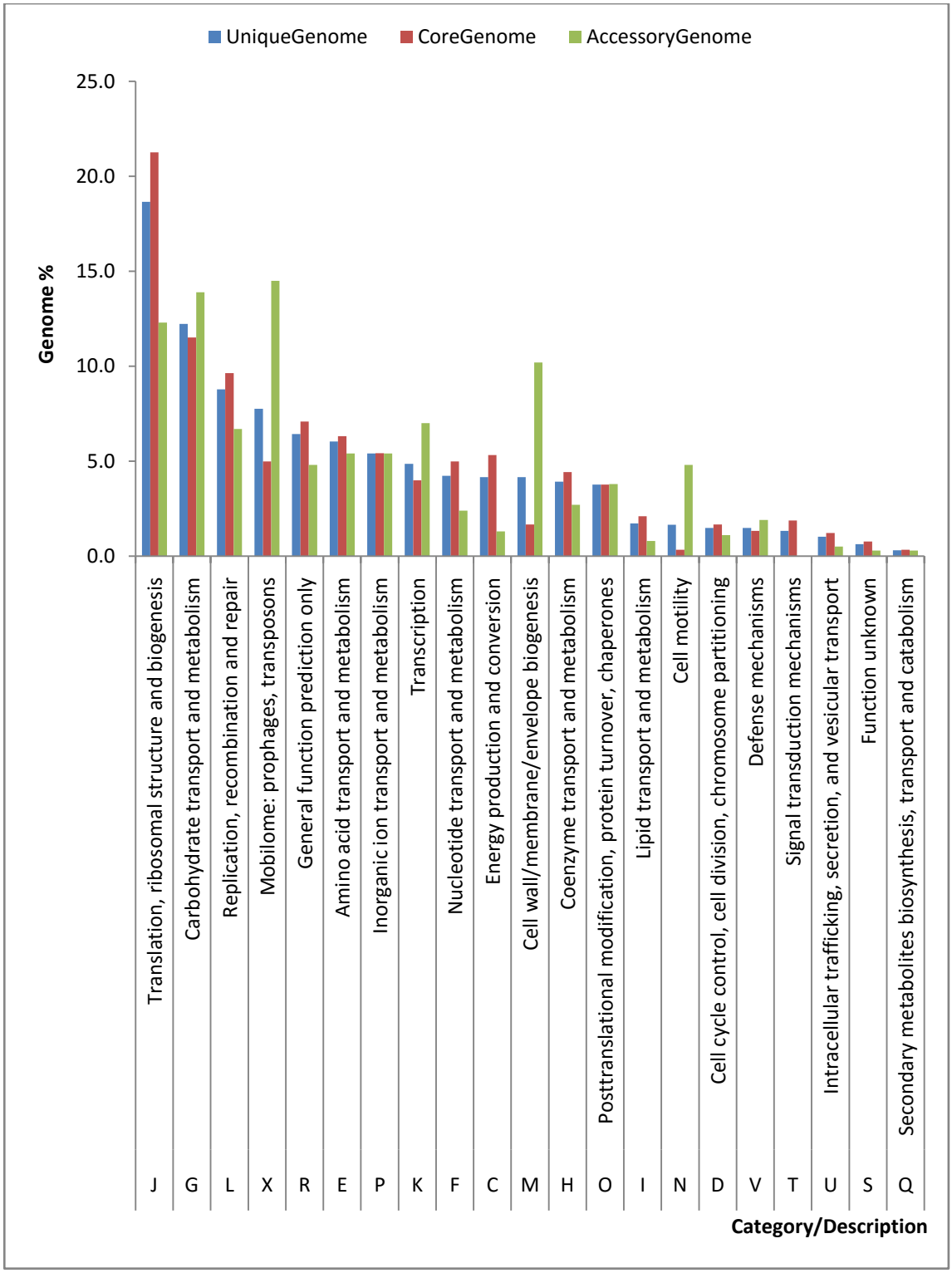


Figure 4.4: Clusters of orthologous groups (COG) functional distribution of genes in the genome of 20 *Mmm* field strains isolated from Adamawa and Taraba States, Nigeria

4.5.4 Phylogeny of *Mycoplasma mycoides* subspecies *mycoides* field strains

To better understand the relationship among these geographically and a temporally diverse strain, a phylogenetic tree of the 20 strains was inferred by using alignments of orthologous genomic regions of the strains and an approximated maximum likelihood approach. The resulting tree shows 3 distinct phylogroups (Strains MAT-1 to 3) of the strains (Figure 4.5). Genes that are specific to certain phylogroups, which are identified during pangenome analysis (Figure 4.4) need further investigation.

Strain MAT-1 isolates formed a further sister sub-clade comprised of eight isolates (DS8338252-64-TP47, DS8338267-79-AP68b, DS8338253-65-TP51, DS8338248-60-TL41, DS8338260-72-AL106, DS8338257-69-AP103, DS8338256-68-AL103 and DS8338255-67-AL90) separated from the other *Mmm* strains. The strain MAT-2 contained the lowest number of strains comprising of four isolates (DS8338264-76-AL12, DS8338263-75-AL111, DS8338266-78-AL114 and DS8338261-73-AL107) all isolated from lung tissues and seperated more distant from each other and from the other strains. Finally, the strain MAT-3 consisting of eight strains (DS8338265-77-AL113, DS8338251-63-TL47, DS8338250-62-TL42, DS8338258-70-AL104, DS8338249-61-TP41, DS8338259-71-AP104, DS8338262-74-AP108 and DS8338247-59-TP57) resulted in a single subclade of generally related strains.

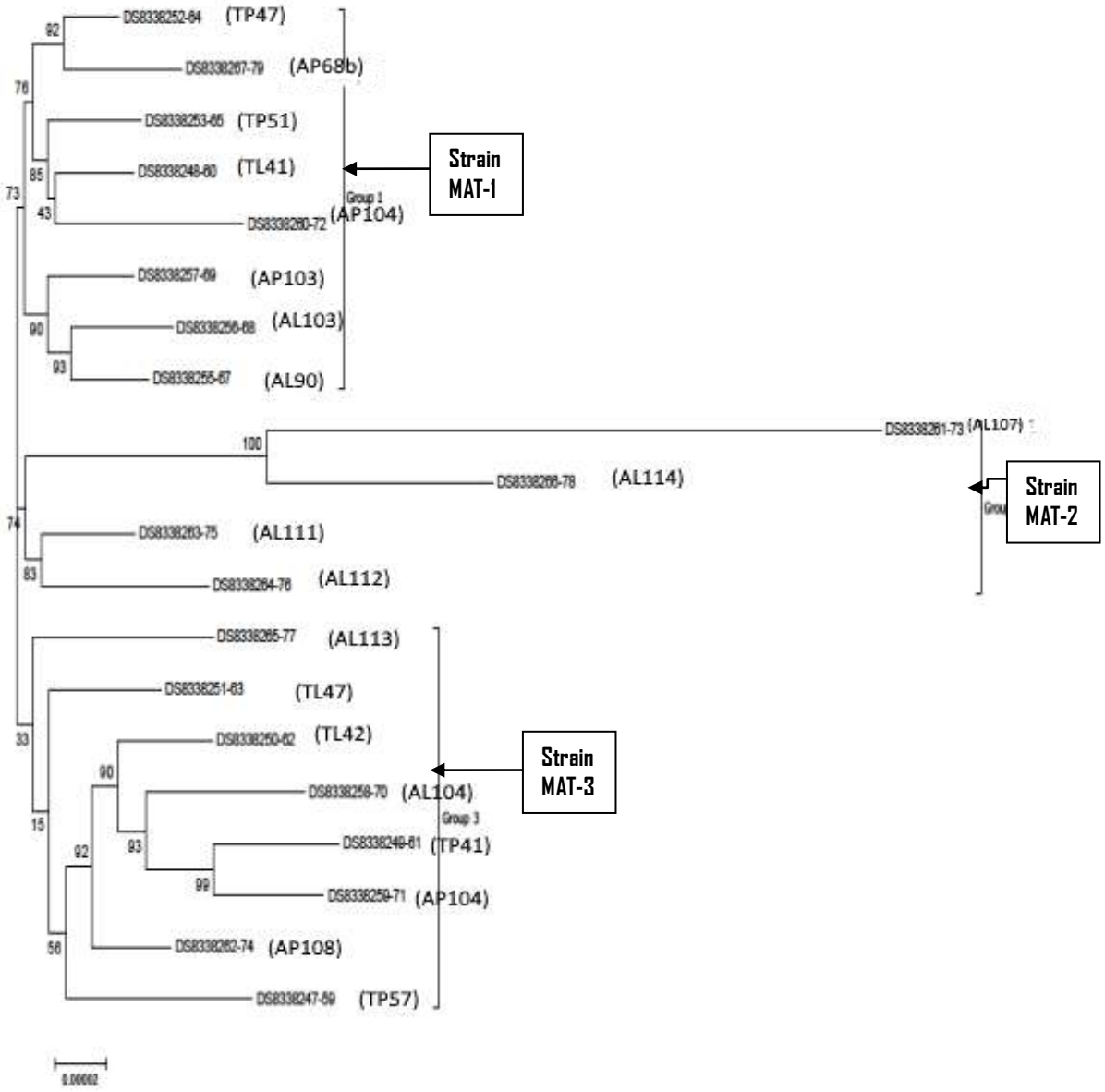


Figure 4.5: Phylogenetic analysis by Maximum Likelihood method of the core genome. The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The tree with the highest log likelihood (-78086.6573) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.0500)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

4.5.5 Evolutionary relationship of the 20 *Mmm* strains to others strains in GeneBank

The Neighbour-Joining tree showing relationships among the 20 *Mmm* field strains to that of *Mmm* strains T1/44 (red colour), 57/13 (blue colour) and BEN326 (black colour) is presented on Figure 4.6. The tree is drawn to scale 50, with branch lengths in the same unit as those of the evolutionary distances used to infer the phylogenetic tree. The strains in groups MAT-1 and MAT-3 cluster together while MAT-2 strains outgroup their corresponding strains in MAT-1 and MAT-2. *Mycoplasma mycoides* subspecies *mycoides* strains T1/44 isolated from Tanzania (NZ-CP014346), 57/13 isolated from Italy (NZ-CP010267) and BEN326 isolated from China (NZ-CP011263) were linked to the outgroup. Most of the strains of *Mmm* isolated from Adamawa and Taraba States were observed to be far from the outgroup strains as shown in Figure 4.6.

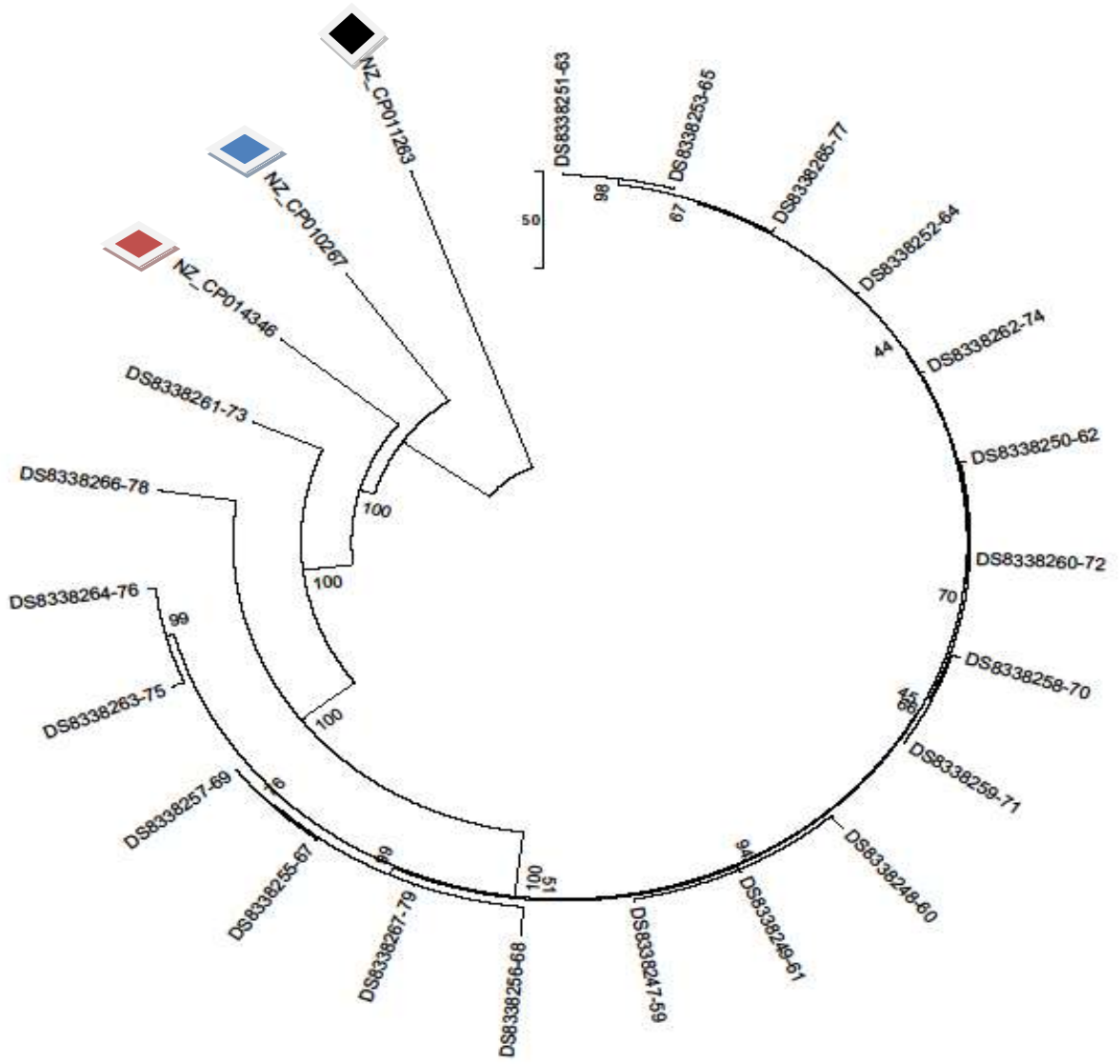


Figure 4.6: Evolutionary relationships of the 20 *Mmm* field strains to that of Vaccine strain T1/44-NZ-CP014346 (Red), Italian strain 57/13-NZ-CP010267 (Blue) and Indian strain BEN326-NZ-CP011263 (Black) all from the GenBank. The evolutionary history was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch length = 605.50000000 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches. The tree is drawn to scale 5.0, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method and are in the units of the number of base differences per sequence. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 604 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

The Phylogeny of the 20 *Mmm* field strains was also confirmed by a Neighbour-Joining Tree inferred by a core SNPs matrix obtained by an alignment free algorithm. Figure 4.7 shows the core-SNPs based tree highlighting that the entire dataset is comprised in a 20-80 SNPs distance range. The tree is drawn with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of different method and are in the units of the number of base differences per sequence. The analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1280 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. The scale 2.0 was the representation of the genetic distance in substitution per nucleotide. All the 20 field strains from the two States seemed to have evolved from a common source.

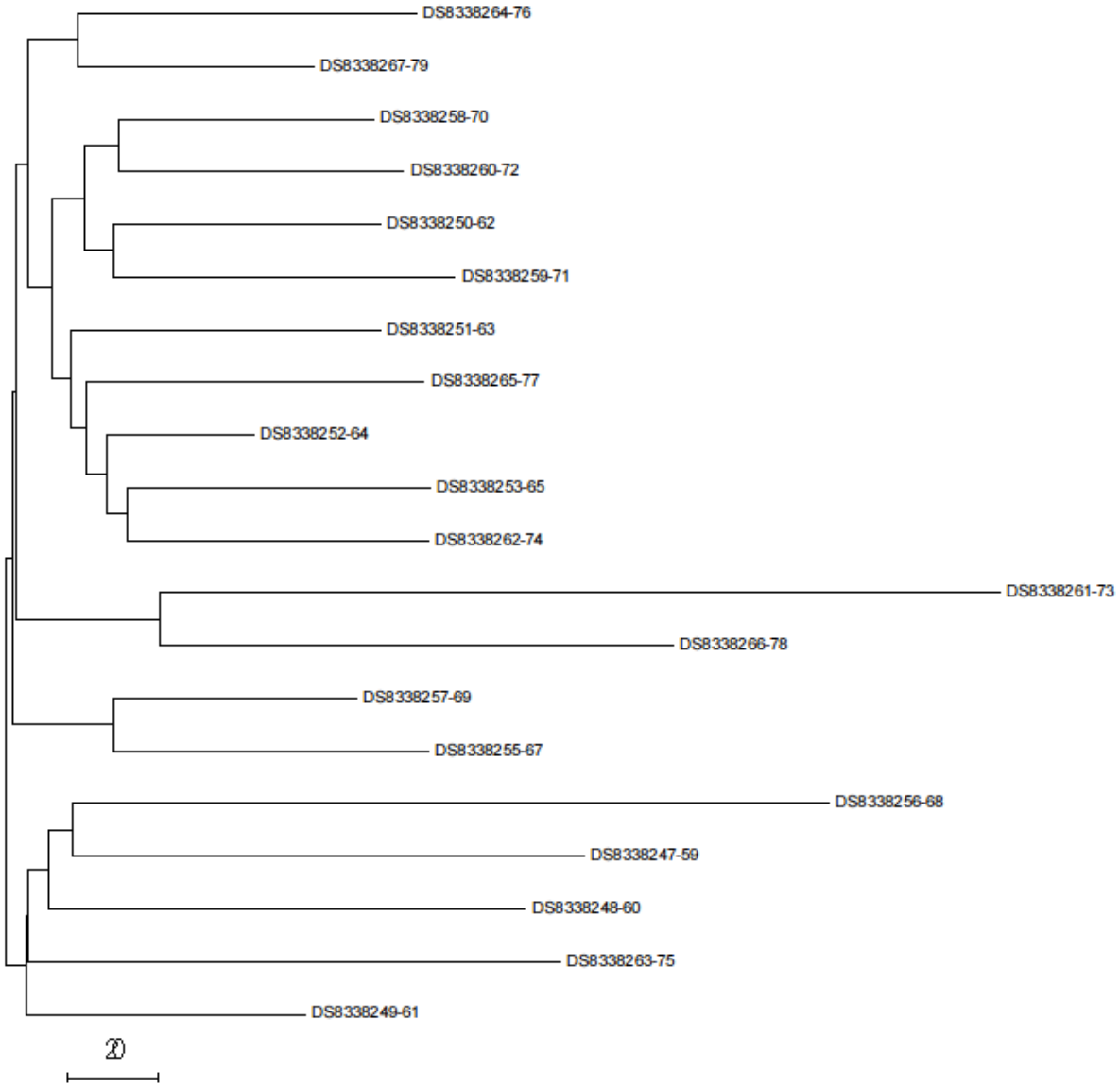


Figure 4.7: Neighbour-Joining SNPs analysis confirming evolutionary history of *Mmm* strains. The optimal tree with the sum of branch length = 1154.68652344 is shown. The tree is drawn to scale 2.0, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of different method and are in the units of the number of base differences per sequence. The analysis involved 20 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1280 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

4.5.6 Virulence factors and genes of 20 *Mycoplasma mycoides* subspecies *mycoides* field strains

The genomes of the 20 field strains of *Mmm* were searched for orthologs of 25 well-characterized *Mmm* virulence factors with the aim of identifying gene candidates that may play roles in its persistence in the host tissue. Several multidrug resistant genes were annotated in the draft genomes. The 20 field strains of *Mmm* had virulence factors ranging between 9 and 25; and virulence genes ranging between 11 and 68. The lowest number of virulence factors of 9 was present in two strains DS8338252-64-TP47 and DS8338264-76-AL112 isolated from pleural fluid and lung tissue respectively while all the 25 virulence factors of *Mmm* were observed in strain DS8338261-73-AL107 isolated from lung tissue in Adamawa State. Similarity searches against the virulence factor database (VFDB) returned a variable number of proteins assignable to well-known virulence factors in the dataset (Table 4.6) which could be re-conducted to 25 established *Mycoplasma mycoides* virulence factors (Table 4.5) that will be the subject of further analyses.

Table 4.5: Established *Mycoplasma mycoides* subsp. *mycoides* virulence factors in the GeneBank

S/N	Virulence Factor Accession ID	Related Gene and its Description
1	VFG016490(gi:42560712)	(*tuf*) translation elongation factor Tu [EF-Tu (CVF587)]
2	VFG016503(gi:42560814)	(*pdh*B) Pyruvate dehydrogenase (lipoamide), beta chain [PDH-B (CVF588)]
3	VFG016513(gi:42560658)	(*hly*A) hemolysin A [Hemolysin (CVF589)]
4	VFG016525(gi:42560667)	(*eps*G) Glycosyltransferase [Capsule (CVF591)]
5	VFG016526(gi:42560668)	(*cps*) Glycosyltransferase [Capsule (CVF591)]
6	VFG016527(gi:42560669)	(*gal*U) UTP-GLUCOSE-1-PHOSPHATE URIDYLYLTRANSFERASE [Capsule (CVF591)]
7	VFG016528(gi:42561487)	(*gal*E) UDP-glucose 4-epimerase [Capsule (CVF591)]
8	VFG016530(gi:42561489)	*(eps*G) Glycosyltransferase [Capsule (CVF591)]
9	VFG016531(gi:42561490)	(*cps*) Glycosyltransferase [Capsule (CVF591)]
10	VFG016532(gi:42561491)	(*opp*F) oligopeptide ABC transporter, permease component [Capsule (CVF591)]
11	VFG016533(gi:42561492)	(*tnp*) IS1634BN transposase [Capsule (CVF591)]
12	VFG016534(gi:42561493)	(*glf*) UDP-galactopuranose mutase [Capsule (CVF591)]
13	VFG016536(gi:42561495)	(*MSC*-0979) hypothetical protein [Capsule (CVF591)]
14	VFG016537(gi:42561496)	(*eps*G) Glycosyltransferase [Capsule (CVF591)]
15	VFG016538(gi:42561497)	(*tnp*) IS1634AV transposase [Capsule (CVF591)]
16	VFG016539(gi:42561498)	(*cps*) Glycosyltransferase [Capsule (CVF591)]
17	VFG016540(gi:42561499)	(*opp*F) Oligopeptide ABC transporter, ATP-binding component Capsule (CVF591)]
18	VFG016541(gi:42561500)	(*glf*) UDP-galactopyranose mutase [Capsule (CVF591)]
19	VFG016542(gi:42561501)	(*gal*E) UDPglucose 4-epimerase [Capsule (CVF591)]
20	VFG016545(gi:42561504)	(*cps*) Glycosyltransferase [Capsule (CVF591)]
21	VFG016546(gi:42561505)	(*tnp*) IS1634CB transposase [Capsule (CVF591)]
22	VFG016548(gi:42561507)	(*MSC*-0991) hypothetical protein [Capsule (CVF591)]
23	VFG016549(gi:42561508)	(*MSC*-0992) hypothetical protein [Capsule (CVF591)]
24	VFG016550(gi:42561509)	(*eps*G) Glycosyltransferase [Capsule (CVF591)]
25	VFG016557(gi:42560930)	(*vmm*) Variable surface protein *vmm* (prolipoprotein) [*vmm* CVF594)]

Table 4.6 Number of virulence factors and genes identified in *Mmm* field strains from Adamawa and Taraba States

Sequence ID	Sample ID	Genes	Virulence Factors
DS8338255-67	AL90	55	23
DS8338267-79	AP68b	61	22
DS8338262-74	AP108	59	20
DS8338257-69	AP103	60	22
DS8338261-73	**AL107**	**68**	**25**
DS8338253-65	TP51	27	17
DS8338250-62	TL42	25	16
DS8338252-64	TP47	16	9
DS8338264-76	AL112	11	9
DS8338266-78	AL114	27	19
DS8338247-59	TP57	64	20
DS8338259-71	AP104	39	19
DS8338258-70	AL104	55	19
DS8338251-63	TL47	42	18
DS8338263-75	AL111	56	19
DS8338248-60	TL41	40	20
DS8338260-72	AL106	52	19
DS8338249-61	TP41	43	20
DS8338256-68	AL103	46	18
DS8338265-77	AL113	42	18

CHAPTER FIVE

5.0 DISCUSSION

Four (4) and thirty three (33) out of the 39 isolates from this study were confirmed to be *Mycoplasma bovis* (*M. bovis*) and *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) respectively using PCR with specific primers for each of the *Mycoplasma* species identified. Two isolates were lost on culture prior to confirmation. The isolation and identification rate of 0.83% (4/480) of *M. bovis* reported in this study was lower than 2.0% isolation by Francis *et al*. (2014) from pneumonic lungs of cattle from Adamawa State and 8.9% isolation by Tambuwal and Egwu (2017) from pneumonic lungs of cattle from two northwestern States of Nigeria. The difference in the isolation rates may be attributed to delayed analysis of the samples after collection. The isolation of *M. bovis* from pneumonic lungs of slaughtered cattle shows that *M. bovis* and its interrelated infection may be occurring in the study area. This finding is of clinical significance because these few infected cattle could likely be a threat and basis of infection to several vulnerable and in-contact herds and farms in the study area. Similar observations were made by Egwu *et al*. (1996); Ajuwape *et al*. (2003); Tambuwal (2009); Francis *et al*. (2014) and Tambuwal and Egwu (2017).

Mycoplasma bovis was isolated and identified from the ear canal of cattle in Taraba State with 3.57% isolation rate. This is of scientific significance as the organism has been previously reported to cause otitis media in cattle (Alberti *et al*., 2006). To the best of our knowledge, this is the first report of isolation of *M. bovis* from bovine ear canal in Nigeria. Considering the ability of other ruminant mycoplasmas to colonise the ear canal, ear involvement by *M. bovis* may not be doubtful. The assumption on the occurrence of *M. bovis* in the ear canal include the anatomical connection between

pharynx and middle ear via Eustachian tube for the pathogenesis of otitis media due to *M. bovis* infection in cattle (Maeda *et al*., 2003; Lamm *et al*., 2004; Arcangiolo *et al*., 2012). Also the mycoplasmaemia developed for the period of acute infection may involve dissemination of mycoplasmas from primary site of infection (the respiratory system) to other organs and systems including the auditory organs where the organism were isolated (Gull *et al*., 2013). The report was consistent with the report of Santos *et al*. (2009) who also for the first time reported *M. bovis* and a crowd of other mycoplasmas from ear canal of cattle in Brazil.

The isolation rate of 6.87% of *Mmm* found in this study was slightly higher than the previously reported by Tambuwal and Egwu (2017) who reported 6.20% isolation of *Mmm* from slaughtered cattle in two northwestern states of Nigeria. However, the findings in this study was higher than the 1.81% reported in Plateau State (Ankeli *et al*., 2016); 1.56% PCR identification reported in three states of northeasten Nigeria (Musa *et al*., 2016); 5.10% reported in five states of northern Nigeria (Nwankpa, 2008) and the 3.40% PCR identification reported in northern Cameroun (Wade *et al*., 2015). The isolation rate of *Mmm* in this study was found to be over 300% higher than 1.56% reported in Borno State (Musa *et al*., 2016). The increased rate observed in the same agro-ecological zone may be connected to security situation in the Northeastern Nigeria, where nomadic herdsmen migrated with their animals from one area to another for safety with fragmented veterinary services (Done *et al*., 1995; Aliyu *et al*., 2000; Francis *et al*., 2018a). To the best of our knowledge, this is the highest reported isolation rate of *Mmm* from field samples in Nigeria. The high isolation rate of *Mmm* obtained in this study may indicate that the disease is very active and endemic in the study area. Contagious bovine pleuropneumonia has been previously reported to be endemic in northern Nigeria (Egwu *et al*., 1996; Aliyu *et al* 2000). The endemic nature of the

disease was compounded by increased and unrestricted cattle movement and porous nature of our borders with neighbouring countries among other factors all of which may contribute to the high rate observed in this study (Aliyu *et al*., 2000). This calls for quick intervention and aggressive response from concerned authorities in order to tackle the menace of this economically important disease of cattle (Tambuwal *et al*., 2011b; Sada *et al*., 2015).

Mycoplasma mycoides subspecies *mycoides* was not isolated from the nasal and ear swab samples collected even though OIE recommended these sites for sampling in live animals (Karahan *et al*., 2010; OIE, 2014). This may be due to contamination of such sites with debris, ear mites, other bacterial and *Mycoplasma* flora, thus suppressing *Mmm* from thriving in such anatomical sites (Santos *et al*., 2009). Broncho-alveolar lavage (BAL) method of sampling was the most preferred method for isolation of mycoplasmas in live animals than nasal and ear swabs because of contamination, although the method was much more difficult under field conditions and animal owners were usually reluctant to allow this procedure (Karahan *et al*., 2010; Akan *et al*., 2014). The results obtained here contrasted the previous report of Ankeli *et al*. (2016) who reported isolation of *Mmm* from ear canal of apparently healthy cattle in Plateau State. We attributed our failure to isolate *Mmm* from nasal and ear swab to limited number of such samples collected, presence of *Mycoplasma* inhibitors in the ear swab samples and time interval between sample collection and laboratory analysis. *Mycoplasma* isolation had been reported to decrease with increased time before laboratory processing regardless of whether samples have been refrigerated or frozen, and that the optimal time for isolation is when fresh samples are inoculated on to media and incubated within a few hours of collection (Biddle *et al*., 2004; Thiaucourt *et al*., 2004; Parker *et al*., 2018).

Mycoplasma bovis (*M. bovis*) and *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) were both isolated and identified from paired sites (lungs and pleural fluid) of cattle in Adamawa State. This could be explained by the fact that, the predilection site for most *Mycoplasma* species is the respiratory system where these organisms multiply and cause pathological lesions accompanied by the production of pleural exudates within the thoracic cavity (Thiaucourt *et al*., 2004; Nicholas *et al*., 2008). The isolation of *M. bovis* and *Mmm* from the same animal host in this study suggests that the aforementioned organisms apart from having similarity in their pathogenesis and pathology, may also be a major contributing factor to the pathology observed in affected animals (Bashiruddin *et al*., 2001). This is corroborates with the report of Tambuwal and Egwu (2017) who reported *M. bovis*, *Mmm* and multitude of other bacterial flora from both normal and pneumonic lungs of slaughtered cattle. The presence of these mycoplasmas in a single host may result in synergistic consequence in precipitating CBPP and *M. bovis*-associated infection and more debilitating pathology on the affected organs (Rodriguez *et al*., 1996; Alberti *et al*., 2006; Tambuwal and Egwu, 2017).

Histopathological examination revealed lesions of interstitial pneumonia accompanied by perivascular and peribronchiolar lymphoid cell infiltration of mostly macrophages with few lymphocytes and neutrophils. Accumulation of cells and serous exudates in bronchioles and interalveolar spaces with catarrhal pneumonia, seen in this study were comparable to the earlier reports (Rodriguez *et al*., 1996; Ajuwape *et al*., 2003; Gagea *et al*., 2006; Kumar *et al*., 2012).

Conventional polymerase chain reaction (PCR) was able to identify all the 37 *Mycoplasmas* isolates. Molecular identification of isolates from broth culture, identified 16S rRNA gene of *M. bovis* isolates TL43, TL46, TES57 and AP106, and was

consistent with previous reports (Johansson *et al*., 1996; Bashiruddin *et al*., 2001; Bashiruddin *et al*., 2005a).

Molecular typing of isolates of *Mycoplasma mycoides* subspecies using the CAP-21 genomic region identified the isolates TP57, TL41, TP41, TL42, TL47, TP47, TP51, TL41b, TL42b, AP68, AL90, AL103, AP103, AL104, AP104, AL106, AL107, AP108, AL109, AP109, AL111, AP111, AL112, AP112, AL113, AP113, AL114, AP114, AP115, AP68b, AP68c, AL90b, and AL90c from lungs and pleural fluid. The confirmation of thirty three isolates out of the original thirty seven was quite significant and showed that CBPP caused by *Mmm* was active, endemic and widespread in the study area. This finding is in agreement with previous reports (Bashiruddin *et al*., 2005b; Muuka *et al*., 2013; Wade *et al*., 2015; Musa *et al*., 2016). In order to confirm the presence of *Mmm,* molecular analysis with PCR-RFLP following digestion with restriction endonuclease (*Vsp*1) identified all the 33 *Mycoplasma* isolates as *Mmm* with amplicons of 180-bp and 380-bp. The digested restriction endonuclease 380-bp fragments delineated *Mmm* from *Mycoplasma mycoides* subspecies *capri*. This finding corroborates the earlier reports (Bashiruddin *et al*., 1994; Bashiruddin *et al*., 1999; Musa *et al*., 2016) and it further revealed strains of *Mmm* circulating in Adamawa and Taraba States, Nigeria.

The utilisation of genome sequences based on Next Generation Sequencing (NGS) technique has enabled analysis of the phylogenome of 20 *Mmm* field strains from Adamawa and Taraba States. The genomic sizes of the 20 *Mmm* field strains varied between 1,180,728 and 1,202,919-bp which was similar to previously sequenced *Mmm* genomes: strain Gladysdale isolated from Australia (Wise *et al*., 2012), strain Afade isolated from northern Cameroun (Fischer *et al*., 2015), strain 57/13 isolated from Italy (Orsini *et al*., 2015), and vaccine strain T1/44 isolated from Tanzania (Gourgues *et al*.,

2016) all deposited in GenBank. The similarities in the genomes of these wide geographical distant strains suggests the common origin of the pathogens which was estimated to be about 3000 years ago as well as its narrow host specificity (Dupuy *et al*., 2012; Fischer *et al*., 2015). The genomic sizes were similar to most African/Australian strains of *Mmm*, because those strains were considered to be highly virulent than the European strains as a result of their glycerol metabolism, even though they have been reported being exported from Europe to Africa during 19th Century (Pilo *et al*., 2007; Thompson *et al*., 2011; Dupuy *et al*., 2012). The genomic sizes of *Mmm* strains reported in this study were higher than the previous sequenced strain BEN326 isolated from China (Li *et al*., 2016). However, the genome sizes were slightly lower than that of PG1 Reference strain of unknown origin (Westberg *et al*., 2004). The genome in addition had a G+C content of 23.92-24.03 which falls within the normal values of G+C content of most mollicutes (Calcutt *et al*., 2018). The assemblies produced in this study are considered draft genomes because they did not surpass minimal standards for genome announcement publication and as such are subject to improvements.

Orthologous average nucleotide identity (OrthoANI) has been widely used to compare two prokaryotic genome sequences based on similarity by calculating average nucleotide identity for taxonomic purposes (Li *et al*., 2015; Lee *et al*., 2016) and has replaced DNA-DNA hybridization method that was considered the gold standard within the last decades (Stropko *et al*., 2014). The technique has been documented to be better suited for large scale comparisons of closely related microorganisms and not for genomes belonging to different species (Kim *et al*., 2014; Lee *et al*., 2016). Genomic comparison of all the 20 field *Mmm* strains revealed OrthoANI value between 99.59 and 99.92% higher than the recommended cut-off range of 95-96% for species boundary (Chun and Rainey, 2014; Lee *et al*., 2016). The isolates DS8338251-63-TL47 and

DS8338266-78-AL114 both from lung tissue had a genomic index value of 99.92% which was the highest value compared to other isolates. This implies that there was high degree of similarities among the strains of *Mmm* circulating in the study area. The technique was successfully applied to determine inter- and intra-species diversity and phenotypic similarity among draft genome assemblies of isolates of *Escherichia coli*, *Bacillus cereus* and *Bacillus anthracis* (Yoon *et al*., 2017; Jain *et al*., 2018). The BLASTn comparison of nucleotide sequences demonstrated 90-100% degree of sequence similarities between *Mmm* strains and vaccine strain T1/44 and this was consistent with earlier report (Li *et al*., 2016).

Pan-genome is the entire set of genes possessed by all members of a particular species, and includes the core genome (containing genes present in every strain sequenced) and accessory genome (containing dispensable genes present in a subset of the strains and strain specific genes). The degree of similarity within the core genome is considered to be one of the best phylogenomic measures for comparing microbial genomes (Segerman, 2012). The pan-genome of the 20 field strains of *Mmm* was found to be composed of approximately 3,081 genes which were twice larger than 1,474 pan-genome genes previously reported for eight *Haemoplasma* species (Guimaraes *et al*., 2014). The core genomes of these strains contained 1,707 genes which was slightly smaller in size than the predicted core genome of other bacteria species (den Bakker *et al*., 2010). The accessory genes were estimated to constitute 44.6% of the pan-genome of the strains and this illustrates a relatively limited amount of strain diversity. The accessory genome of the 20 *Mmm* strains was categorized into high and low frequency genes enabling a linkage of genes in these groups to different evolutionary processes as opined by Cordero and Polz (2014). High frequency genes consists of genes of category X, M, G and T and have been reported to be maintained primarily by vertical

inheritance and homologous recombination encoding core metabolic functions enabling the organism adaptation to new environment and uptake and utilization of different carbon sources (Sakharhar and Chow, 2005; Cordera and Polz, 2014). The low frequency group have been reported to consist of genes conferring resistance to the commonly used antibiotics located on the membrane of mycoplasmas (Liu *et al*., 2012; Kandavelmani and Piramanayagam, 2019).

The backbone genome refers to the core genome which contains a subset of genes present in all genomes of *Mmm* strains (Liu *et al*., 2012). Genes involved in cellular process and signalling (D, M, N, O, T, U, V) and information storage and processing (J, K, L) form the major constituent of the backbone genome. Metabolic genes (C, E, F, G, H, I, P, Q) have a minor contribution toward the backbone. This was because mycoplasmas have been reported to have evolved from Gram positive bacteria by drastic reduction in their genome sizes (Razin *et al*., 1998; Chen *et al*., 2012), thereby losing several genes implicated in diverse biosynthetic/metabolic pathways, depending primarily on their host for supply of nutrients (Chen *et al*., 2012; Kandavelmani and Piramanayagam, 2019). Genes of the sub-category: RNA processing and modification (A), chromatin structure and dynamics (B), extracellular structure (W), nuclear structure (Y) and cytoskeleton (Z) were completely lost in the sequenced genomes of the 20 *Mmm* strains in this study due to degenerative evolution of the organism and this observation was consistent with previous report (Kandavelmani and Piramanayagam, 2019).

Phylogeny of the field strains of *Mmm* based on maximum likelihood approach has resulted in separating these strains into three phylo-groups of 8, 4 and 8 isolates. The strains in phylogroup MAT-1 and MAT-3 had 8 isolates each with equal proportion of sites of isolation (lungs and pleural fluid) and they cluster together, signifying similarity

among the group members as well as having the same lineage in the course of their evolution (Stanborough *et al*., 2017). The strain DS8338260-72-AL106 in MAT-1which was isolated from lung tissue was more distant, and might have probably evolved later than other members of the same lineage. The MAT-2 members consisted of 4 strains that were isolated from lung tissues (AL112, AL111, AL114 and AL107). The group members formed a clade that was more distant from each other and from other strains, suggesting their different times of evolution although having the same lineage. Considering the phylogenetic tree where most of the strains cluster in phylogroups were identified, strains from Adamawa State (AP68b, AL106, AP103, AL103, AL90, AL113, AL104, AP104 and AP108) did not vary from those that emanated from Taraba State (TP47, TP51, TL41, TL47, TL42, TP41 and TP57) which clustered in MAT-1 and 3 resulting in low diversity among the *Mmm* genome as opined by Dupuy *et al*. (2012). The equal distributions of the *Mmm* strains in the two phylogroups do not correlate with the States of isolation. The different phylogroups observed in this study revealed that three different strains (MAT-1, MAT-2 and MAT-3) of *Mmm* were identified and circulating in the study area which is in agreement with previous reports (Dupuy *et al*., 2012; Stanborough *et al*., 2017). The phylogenetic evaluation of the strains based on Neighbour-Joining tree suggests strong relationship amongst and between the isolates in the two States studied. This may also be connected to strong link in the movement of cattle and trade routes between the two States in search of pasture as well as favourable nearby markets. The exact origin of these animals was not ascertained due to lack of animal identification system in the study area and the entire nation in general.

The Neighbor-Joining of the SNPs and the phylogeny for the comparison of *Mmm* strains confirmed similarities in the isolated strains and might have probably evolved from a common origin. The findings showed that these strains are constantly evolving

with time irrespective of the location. This buttressed the earlier findings of Musa (2016). Contrarily, strains DS8338261-73-AL107 and DS8338266-78-AL114 both isolated from lung tissues within the MAT-2 phylogroup were observed to clustered together and separated from other members forming a distant clade from other strains. Similarly, strains DS8338257-69-AP103 from pleural fluid and DS8338255-67-AL90 from lung tissue belonging to MAT-1 formed a separate clade even though it was not necessarily separated from other strains. The separation of these strains may be attributed to single nucleotide variation that might have occurred due to genetic mutation or horizontal gene transfer in the accessory genome resulting in the alteration of nucleotide arrangement as buttressed by Thiaucourt *et al*. (2011) and Dupuy *et al*. (2012).

The present study was able to identify all 25 well known virulence factors in strain DS8338261-73-AL107 isolated from lung tissue with highest number of genes (68), followed by 23 virulence factors in strain DS8338255-67-AL90 from lung tissue and 22 virulence factors in DS8338267-79-AP68b and DS8338257-69-AP103 both from pleural fluid. The strain AL107 was observed to harbour all the virulence factors and highest number of genes indicating the pathogenicity of the strain. This may explain the probable reason why it appeared to be distant from other strains of the same phylogroup and the other strains in the phylogenetic tree. The successful identification of high number of virulence factors and genes in some *Mmm* strains (AP103, AP68b, AL90, AL107) showed that these pathogens might be a good potential candidates for CBPP vaccine development buttressing a previous report of reverse vaccinology (Rappuoli, 2000).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study has established;

i. Isolation rates of 0.83% for *Mycoplasma bovis* and 6.87% for *Mycoplasma mycoides* subsp. *mycoides* were reported in this study.

ii. Isolation of both *M. bovis* and *Mmm* in a paired sample of lung (AL106) and pleural fluid (AP106) in this study.

iii. High number of *Mmm* field strains 33 (6.87%) was recovered in this study which is probably the first of its kind in Nigeria.

iv. This study demonstrated the first isolation of *M. bovis* from ear canal of a 6 year old cow at slaughter in Taraba State, Nigeria.

v. The genomic similarity and relatedness among the field strains of *Mmm* was evident in the study area.

vi. The estimated genomic size of the filed strains varied by 22-Kb and were between 1.18 and 1.20-Mb with the strain AP68b having the highest genomic size, protein coding genes, CDS and tRNA.

vii. High orthologous average nucleotide identity (OrthoANI) value of 99.59-99.92% among the strains of *Mmm* with strains AL103/AL113 exhibiting the highest OrthoANI value of 99.92%.

viii. The pan-genome of the 20 field strains of *Mmm* comprised of 3,080 protein coding genes with 1,707 genes in the core-genome (55.4% of pan-genome) and 1,374 genes in the accessory genome (about 44.6% of the pan-genome).

ix. This study established three strains of *Mmm* (MAT-1, MAT-2 and MAT-3) circulating in the study area.

x. This study documented high number of virulence factors and genes in some strains of *Mmm* (AP103, AP68b, AL90, AL107) that might likely be a potential candidate for CBPP vaccine development.

6.2 Recommendations

The following are recommended;

1. Provision of adequate facilities in our institutional laboratories to enable the use of cutting-edge technology.
2. Molecular typing of *M. bovis* and *Mmm* field strains from the rest of northern Nigeria that harbour two-third of the 19.5 million cattle population.
3. Large scale epidemiological studies that will reveal the prevalence and distribution of *M. bovis* and *Mmm* in cattle population in Nigeria.
4. National and international borders should be strengthened and strict movement control be revived in order to minimise the spread of this dreaded and internationally recognised disease of cattle.
5. The need for sequencing of *M. bovis* isolates to better understand the strain circulating in the study area.
6. Integration of genetic data with information on pattern of animal movement as an additional support for CBPP outbreak investigation.
7. Genomic analysis of *Mmm* strains circulating in other states of Nigeria which may represent an important support for more targeted disease control programs.
8. Further studies should be carried out in different regions of Nigeria to better understand *Mmm* diversity and correlation within the country.

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APPENDICES

Appendix I: Preperation of Mycoplasma Agar and Broth

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VOM. PLATEAU STATE. NIGERIA

Preparation of broth and agar medium base for mycoplasma cultures

I. Aim and field of application

This protocol aims to describe the procedure for preparation of medium base, which is a component of the growth medium used for the isolation of mycoplasmas. This protocol was adapted from UPRS 15 protocol for CBPP diagnosis, CIRAD, EMVT, France.

II. Principle

The procedure consists in the preparation and sterilisation of a growth medium base, whether liquid (broth) or solid (agar), which will provide a complete culture medium after addition of the appropriate supplement.

This protocol is adapted from:

Provost, A. et al. (1987). *Contagious bovine pleuropneumonia*. Rev. Sci. Tech. Off int. Epiz. 6, (3), 625-679.

Freundt, E. A. (1983). *Culture media for classic mycoplasmas*. In *Methods in Mycoplasmology* (I) 405-410. Edited by S. Razin and J. G. Tully: Academic Press.

III. Related documents

- List of reagents available to the UPR15
- Operating manuals for all materials used (Autoclave)
- Operating procedure "Quality control of mycoplasma growth media"
- Registration sheet

IV. Materials and methods

a) Materials

- Weighing scales
- Glassware (a 3 L beaker, a 2 L bottle, twenty 100 ml bottles, graduated cylinders)
- Magnetic stirrer with heater
- Autoclave

b) Reagents

- PPLO broth Difco (without crystal violet)
- Milli-Q water
- Agar noble Difco
- Autoclaving control tape

e) Specific safety measures

- Risk of burning whilst manipulating hot products

d) Operating procedure

PPLO agar base (1.4 litres for 20 bottles)

1- Measure 1.4 litres of milli-Q water and transfer to a beaker with a capacity of 3 litres

2- Add a magnet and agitate on a magnetic stirrer, heating until it starts boiling

3- Weigh 20 g of agar noble Difco and add to the boiling water

4- Continue heating and agitation until the agar is completely molten (around 10-20 minutes)

5- Add 42 g of PPLO and let dissolve

6- When the mix acquires a translucent appearance, distribute 70 ml of the preparation (still liquid) into 100 ml bottles. Measure the volumes using a 100 ml cylinder and wear protective gloves to prevent skin burns.

7- Close the bottles, stick an autoclaving control tape on the lid and write on it the lot number with a permanent marker.

8- Fill in the registration sheet for production of growth media, indicating the reagents used for future reference.

9- Autoclave at 121°C for 20 minutes.

PPLO broth base (1.4 litres for 20 bottles)

1- Measure 1.4 litres of milli-Q water and transfer to a beaker with a capacity of 3 litres

2- Add 42 g of PPLO and let dissolve

3- Distribute 70 ml of the solution into 100 ml bottles

4- Close the bottles, stick an autoclaving control tape on the lid and write on it the lot number with a permanent marker

5- Fill in the registration sheet for production of growth media, indicating the reagents used for future reference

6- Autoclave at 121°C for 20 minutes

7- The quality of a lot of medium base must be tested at least once whenever a new lot of any of its ingredients is used

e) Storage

Media base may be conserved for up to 1 year at +4°C.

f) Comments - remarks

This protocol permits to obtain twenty bottles of base medium, which will provide, after supplementation, a final volume of 2 litres of ready-to-use culture medium.

Note that 30 ml of supplement are added to 70 ml of base, providing a final volume of 100 ml supplemented medium. For preparation of agar plates, melted base is cooled down to 60°C, supplemented as above and 25 ml doses are immediately added to 9 cm diameter Petri dishes.

3

Document from CIRAD, EMVT, UPR15
Author: Lucia Manso-Silván
Date: 16/11/05

Preparation of supplement for mycoplasma culture medium

I. Aim and field of application

This protocol aims to describe the procedure for preparation of the supplement, which is a component of the growth medium used for the isolation of mycoplasmas. It is destined to the persons attending the practical course on CBPP diagnosis at CIRAD, EMVT department, UPR15.

II. Principle

The procedure consists in the preparation of a mixture of all the reagents that need to be added to the "medium base" in order to produce a complete culture medium.

This protocol is adapted from:

Provost, A. et al. (1987). *Contagious bovine pleuropneumonia*. Rev. Sci. Tech. Off int. Epiz. 6, (3), 625-679.

Freundt, E. A. (1983). *Culture media for classic mycoplasmas*. In *Methods in Mycoplasmology* (I) 405-410. Edited by S. Razin and J. G. Tully: Academic Press.

III. Related documents

- List of reagents available to the UPR15
- Operating manuals for all materials used
- Operating procedure "Preparation of fresh yeast extract"
- Operating procedure "Quality control of mycoplasma growth media"
- Registration sheet

IV. Materials and methods

a) Materials

- Water bath
- Weighing scales
- Glassware (a 2 L bottle, graduated cylinders)
- 0.22 µm filter units
- Horizontal flux hood
- Sterile 50 ml "Falcon" tubes

b) Reagents

- Horse serum tested for mycoplasma culture
- Fresh yeast extract tested for mycoplasma culture
- Milli-Q water

- D-glucose
- Sodium pyruvate
- Ampicillin

c) Specific safety measures

- No identified risks

d) Operating procedure

1- The previous day, defrost two bottles of 500 ml horse serum and one bottle of 250 ml fresh yeast extract

2- De-complement the serum by incubating for 1 hour at 56°

3- Prepare a solution of 250 ml milli-Q water, 10 g glucose, 20 g pyruvate and 2 g ampicillin.

4- Under a horizontal flux hood: Filter this solution through a 0.22 µm filter unit positioned over a 2 L bottle

5- Then filter the fresh yeast extract (250 ml) and finally the horse serum (1 L)

6- Mix well and distribute 30 ml doses into 50 ml 'Falcon' tubes, annotating the lot number

7- Fill in the registration sheet for production of growth media, indicating the reagents used for future reference

9- Take one tube to test the sterility (compulsory) and the efficiency (whenever required)

10- The quality of a lot of supplement must be tested at least once whenever a new lot of any of its ingredients is used

e) Storage

The supplement may be conserved for up to 1 year at -20°C.

f) Comments – remarks

This protocol permits to obtain fifty tubes of supplement which will provide, after supplementation, a final volume of 5 litres of ready-to-use culture medium.

Note that each 30ml of supplement is added to 70ml medium base, producing a final volume of 100ml supplemented medium

However, for isolation of fastidious species such as *M. capricolum* subsp. *capripneumoniae*, additional horse serum may be added (i.e.: 10 ml horse serum are added to the 100 ml supplemented medium).

Appendix II: *Mycoplasma* species from Adamawa and Taraba States isolated at VNRI Vom and shipped to IZSAM Teramo, Italy

S/N	Isolate Identification Code	Tentative Diagnosis	Location
1	TP57	*Mycoplasma* spp	Jalingo Abattoir, Taraba State
2	TL41	,,	Jalingo Abattoir, Taraba State
3	TP41	,,	Jalingo Abattoir, Taraba State
4	TL42	,,	Jalingo Abattoir, Taraba State
5	TL43	,,	Jalingo Abattoir, Taraba State
6	TP44	,,	Jalingo Abattoir, Taraba State
7	TL46	,,	Jalingo Abattoir, Taraba State
8	TL47	,,	Jalingo Abattoir, Taraba State
9	TP47	,,	Jalingo Abattoir, Taraba State
10	TL48	,,	Jalingo Abattoir, Taraba State
11	TES57	,,	Jalingo Abattoir, Taraba State
12	TP51	,,	Jalingo Abattoir, Taraba State
13	AP68	,,	Yola Modern Abattoir, Adamawa State
14	AL90	,,	Yola Modern Abattoir, Adamawa State
15	AL103	,,	Yola Modern Abattoir, Adamawa State
16	AP103	,,	Yola Modern Abattoir, Adamawa State
17	AL104	,,	Yola Modern Abattoir, Adamawa State
18	AP104	,,	Yola Modern Abattoir, Adamawa State
19	AL106	,,	Yola Modern Abattoir, Adamawa State
20	AP106	,,	Yola Modern Abattoir, Adamawa State
21	AL107	,,	Yola Modern Abattoir, Adamawa State
22	AP108	,,	Yola Modern Abattoir, Adamawa State
23	AL109	,,	Yola Modern Abattoir, Adamawa State
24	AP109	,,	Yola Modern Abattoir, Adamawa State
25	AL111	,,	Yola Modern Abattoir, Adamawa State
26	AP111	,,	Yola Modern Abattoir, Adamawa State
27	AL112	,,	Yola Modern Abattoir, Adamawa State
28	AP112	,,	Yola Modern Abattoir, Adamawa State
29	AL113	,,	Yola Modern Abattoir, Adamawa State
30	AP113	,,	Yola Modern Abattoir, Adamawa State
31	AL114	,,	Yola Modern Abattoir, Adamawa State
32	AP114	,,	Yola Modern Abattoir, Adamawa State
33	AP115	,,	Yola Modern Abattoir, Adamawa State
34	TES41	,,	Jalingo Abattoir, Taraba State
35	TL42b	,,	Jalingo Abattoir, Taraba State
36	AP68b	,,	Yola Modern Abattoir, Adamawa State
37	AP68c	,,	Yola Modern Abattoir, Adamawa State
38	AL90b	,,	Yola Modern Abattoir, Adamawa State
39	AL90c	,,	Yola Modern Abattoir, Adamawa State

Key: AL- Adamawa Lung; AP- Adamawa pleural fluid ; ANS- Adamawa nasal swab; AES- Adamawa ear swab; TL- Taraba lung; TP- Taraba pleural fluid; TNS- Taraba nasal swab; TES- Taraba ear swab; *Mycoplasma* spp- *Mycoplasma* species

Appendix III: A White Fulani cow showing suspected signs of CBPP in a herd in Adamawa State. Neck extended towards the direction of wind and dull posture.

Appendix IV: Colonial morphology of isolated *Mycoplasma* species and confirmation by specific PCR

S/N	Isolate ID code	Age (yrs)	Specimen screened	Colony appearance	PCR identification
1	TL41	4	Lung	Round, medium, dark centre	*Mmm**
2	TP41	4	Pleural fluid	Small, rough with tiny centre	,,
3	TL42	3	Lung	Round, clear with dark centre	,,
4	TL47	5	Lung	Medium, small centre	,,
5	TP47	5	Pleural fluid	Large, round, dark centre	,,
6	TP51	6	Pleural fluid	Medium with dark centre	,,
7	TP57	6	Pleural fluid	Large, clear with no centre	,,
8	TL41b	4	Lung	Medium, round, dark centre	,,
9	TL42b	3	Lung	Medium, clear,dark centre	,,
10	AP68	5	Pleural fluid	Round irregular, small centre	,,
11	AL90	7	Lung	Large, dark central area	,,
12	AL103	4	Lung	Large clear, no centre	,,
13	AP103	4	Pleural fluid	Small, rough with tiny centre	
14	AL104	6	Lung	Round, medium with dark centre	,,
15	AP104	6	Pleural fluid	Medium with small centre	,,
16	AL106	3	Lung	Medium, irregular shape, small centre	,,
17	AL107	6	Lung	Large, rough with tiny centre	,,
18	AP108	4	Pleural fluid	Round with dark centre	,,
19	AL109	5	Lung	Medium, small centre	,,
20	AP109	5	Pleural fluid	Medium, dark centre	,,
21	AL111	3	Lung	Small with dark centre	,,
22	AP111	3	Pleural fluid	Round,clearwith dark centre	,,
23	AL112	4	Lung	Medium with dark centre	,,
24	AP112	4	Pleural fluid	Large, darker centre	,,
25	AL113	3	Lung	Large, irregular shape, small centre	,,
26	AP113	3	Pleural fluid	Round with small centre	,,
27	AL114	7	Lung	Medium, dark marked centre	,,
28	AP114	7	Pleural fluid	Large, clear, o centre	,,
29	AP115	4	Pleural fluid	Large, clear, dark centre	,,
30	AP68b	5	Pleural fluid	Round, small dark centre	,,
31	AP68c	5	Pleural fluid	Small, rough with dark centre	,,
32	AL90b	7	Lung	Raised central area, dark	,,
33	AL90c	7	Lung	Round, medium with centre	,,
34	AP106	6	Pleural fluid	Medium, dark centre, presence of films	*M. bovis***
35	TL43	4	Lung	Medium, raised centre	,,
36	TL46	5	Lung	Large, dark raised centre	,,
37	TE57	3	Ear swab	Small, rough with presence of films	,,

NB *: *Mycoplasma mycoides* subsp. *mycoides* **: *Mycoplasma bovis*

Appendix V: Sample and Sequence Identification Numbers of *Mmm* strains

S/N	Sample ID	Sample registration	Sequence ID	Location
1	TP57	2017.TE.26697.1.1	DS8338247-59	Taraba State
2	TL41	2017.TE.26697.1.2	DS8338248-60	‘’
3	TP41	2017.TE.26697.1.3	DS8338249-61	‘’
4	TL42	2017.TE.26697.1.4	DS8338250-62	‘’
5	TL47	2017.TE.26697.1.8	DS8338251-63	‘’
6	TP47	2017.TE.26697.1.9	DS8338252-64	‘’
7	TP51	2017.TE.26697.1.12	DS8338253-65	‘’
8	AL90	2017.TE.26697.1.14	DS8338255-67	Adamawa State
9	AL103	2017.TE.26697.1.15	DS8338256-68	‘’
10	AP103	2017.TE.26697.1.16	DS8338257-69	‘’
11	AL104	2017.TE.26697.1.17	DS8338258-70	‘’
12	AP104	2017.TE.26697.1.18	DS8338259-71	‘’
13	AL106	2017.TE.26697.1.19	DS8338260-72	‘’
14	AL107	2017.TE.26697.1.21	DS8338261-73	‘’
15	AP108	2017.TE.26697.1.22	DS8338262-74	‘’
16	AL111	2017.TE.26697.1.25	DS8338263-75	‘’
17	AL112	2017.TE.26697.1.27	DS8338264-76	‘’
18	AL113	2017.TE.26697.1.29	DS8338265-77	‘’
19	AL114	2017.TE.26697.1.31	DS8338266-78	‘’
20	AP68b	2017.TE.26697.1.36	DS8338267-79	‘’

Appendix VI: Limitations and Contributions to Knowledge

A. Limitations of the work

The present study faced the following limitations;

1. Insufficient finance: Mycoplasma research is highly demanding, time consuming and expensive that requires huge finance at almost all levels.
2. Delay in securing bench space for the isolation and further molecular studies.
3. Failure in recovering of the first batch of isolates shipped to the OIE Reference laboratory of CBPP in Teramo, Italy.
4. Delay in sequencing and analysis of sequenced data for about 2 years.

B. Contributions to Knowledge

To the best of our knowledge, the present was able to;

1. Report for the first time isolation and identification of *Mycoplasma bovis* from ear canal of cattle in Nigeria.
2. Co-habitation of both *M. bovis* and *Mmm* in a cattle in the study area.
3. The study had established three similar and closely related strains circulating in the study area.
4. The study revealed the presence of high number of virulence factors and genes in some strains of *Mmm* that might be a potential candidate for CBPP vaccine development.

Appendix V: Conference Publications

Book of Abstracts

56th Annual National Congress KOGI 2019

PMPM 01 PATHOLOGY, MICROBIOLOGY, PARASITOLOGY AND MEDICINE

Molecular identification of *Mycoplasma bovis* and *Mycoplasma mycoides* subspecies *Mycoides* from slaughtered cattle in Adamawa and Taraba states, North-Eastern Nigeria

Francis, M.I[1]., Kwanashie, C.N[1]., Adamu, J[1]., Allam, L[1]., Raji, M.A[2]., Sacchini, F[3]., Scacchia, M[3]. and Egwu, G.O[4]

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ABSTRACT

This study was carried out to isolate, identify *Mycoplasma bovis* (*M. bovis*) and *Mycoplasma mycoides*subspecies *mycoides*(*Mmm*) by culture andpolymerase chain reaction (PCR) from slaughtered cattle in Adamawa and Taraba States, Northeastern Nigeria. A total of 480 samples of suspected pneumonic lungs (180), pleural fluid (180), nasal swab (60) and ear swab (60) were collected from 190 heads of cattle at slaughter in Yola Modern Abattoir and JalingoAbbatoir. Samples were processed based on standard laboratory protocols, identification and confirmation was achieved with specific PCR and PCR-RFLP. Thirty nine (8.13%) *Mycoplasma* species were isolated from the samples. Four (4) isolates of *M. bovis*and33 isolates of *Mmm*showed positive growth on PPLO agar media. *Mycoplasma bovis*were recovered in 4 isolates giving an overall isolation rate of 0.83% (4/480). In Adamawa State, 1 (0.91%) *M. bovis* was isolated from pleural fluid. While in Taraba State, 2 (2.86%) and 1 (3.57%) *M. bovis* were isolated from lung tissues and ear swab samples respectively. For *Mmm*, 33 isolateswere isolated giving an isolation rate of 6.87% (33/480). In Adamawa State, 12 (10.91%) *Mmm* were isolated from both lung tissues and pleural fluid, whereas, 5 (7.14%) and 4 (5.71%) *Mmm* were isolated respectively from lung tissues and pleural fluid in Taraba State. Four*M. bovis*isolates were confirmed positive by PCR with the presence of one band of 734-bp. Whereas, all the 33 *Mmm* isolates were confirmed positive by the production of two bands of approximyately 180-bp and 380-bp following digestion with *Vsp*1 restriction endonuclease. In conclusion, this work had established the presence of *M. bovis* and *Mmm*in cattle in Adamawa and Taraba States. We thus recommend large scale epidemiology studies that will reveal the prevalence and distribution of *M. bovis* and *Mmm* in cattle population in Nigeria.

Keywords: Abattoir, Isolation, *Mycoplasma mycoides* subspecies*mycoides*, *Mycoplasma bovis*, PCR

PMPM 02 PATHOLOGY, MICROBIOLOGY, PARASITOLOGY AND MEDICINE

Phylogenomic analysis of *Mycoplasma mycoides* subspecies *Mycoides* strains isolated from cattle in Adamawa and Taraba states, North-Eastern Nigeria

[1]Francis, M.I., Kwanashie, C.N., Adamu, J., Allam, L., Raji, M.A., Di Federico M., Orsini M., Ancora M., Krasteva I., Zilli K., Maracci, M., Cammà C., Sacchini, F., Scacchia, M. and[2]Egwu, G.O.

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ABSTRACT

This study was aimed to perform genomic comparison of *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*) strains isolated from cattle in Adamawa and Taraba States by whole genome sequencing.Thirty three (33) isolates of *Mmm* were processed and the genomic DNA was extracted using Maxwell 16 cell/DNA purification kit. Library preparation was carried out by Nextera XT Library preparation, loaded onto NextSeq 500/550 Mid Output Reagent Cartridge and then sequenced onIlluminaNextSeq™ 500 platform. Whole genome sequencing was undertaken for the 20 field strains of *Mmm*. The 20 draft assemblies resulted in 19 and 115 contigs with a N50 value between 25,646 and 119,472. The total length of the genome varying by approximately 22,000-bp and were between 1,180,728-bp and 1,202,919-bp. Smallest genome size of 1,180,728-bp was observed in strain AL103 and largest genome size of 1,202,919-bp in strain AP68b both isolated from Adamawa State. Their G+C content was between 23.92% and 24.03% and the genomes harboured about 2,053 to 2,127 predicted protein-coding genes. The annotation revealed CDS from 2,016 to 2,087 and between 29 and 33 tRNAs were identified per genome.Genomic comparisons of the 20 field strains of *Mmm* revealed high OrthoANI values of between 99.59 and 99.92%. The pan-genome of the strains of *Mmm* contains 3,081 protein-coding genes which comprised of 1,707 core genes (55.4% of the pangenome) and 1,374 accessory genes (44.6% of the pangenome). Functional annotation of the 20 *Mmm* gene products assigned a COG category for just 1198 out of 3081 products occurringmainly in the core genome group. Phylogenetic tree of the 20 strains shows 3 distinct phylogroups of 8, 4 and 8 strains in MAT-1, 2 and 3 respectively. The 20 field strains of *Mmm* had a virulence factors range between 9 and 25 and virulence genes between 11 and 68. In conclusion, this work has established genomic similarity and relatedness among the strains of *Mmm*circulating in Adamawa and Taraba State northeastern Nigeria. We thus recommend integration of genetic data with information on pattern of animal movement as an additional support for CBPP outbreak investigation.

Keywords: Genes, Genome, *Mycoplasma mycoides* subspecies*mycoides*, Strains, Nigeria