ISOLATION OF *MYCOPLASMA BOVIS* AND DETECTION OF ITS ANTIBODIES IN CATTLE IN THREE LOCAL GOVERNMENT AREAS OF ADAMAWA STATE, NIGERIA

BY

MARKUS ISA FRANCIS

DEPARTMENT OF VETERINARY MICROBIOLOGY
AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

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BY

Markus Isa FRANCIS, DVM (UNIMAID., 2007)
M.Sc./VET-MED/12853/2010-2011

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AHMADU BELLO UNIVERSITY, ZARIA
NIGERIA

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DECLARATION

I declare that the work in this thesis entitled, "**Isolation of *Mycoplasma bovis* and detection of its antibodies in cattle in three Local Government Areas of Adamawa State, Nigeria**", has been performed by me in the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, under the supervision of Dr. M. A. Raji, Prof. H. M. Kazeem and Dr. M. M. Suleiman. The information derived from literatures has been duly acknowledged in the text and a list of references provided. No part of this thesis has previously been presented for the award of degree or diploma at any university.

Markus Isa FRANCIS ……. ……..

Signature Date

CERTIFICATION

This thesis entitled, "ISOLATION OF *MYCOPLASMA BOVIS* AND DETECTION OF ITS ANTIBODIES IN CATTLE IN THREE LOCAL GOVERNMENT AREAS OF ADAMAWA STATE, NIGERIA" by **Markus Isa FRANCIS** meets the regulation governing the award of **Master of Science degree** in Veterinary Microbiology of Ahmadu Bello University, Zaria, and is approved for its contribution to scientific knowledge and literary presentation.

Dr. M. A. Raji
Chairman, Supervisory Committee. ……..... ……..
Signature **Date**

Prof. H. M. Kazeem
Member, Supervisory Committee. ……. ……..
Signature **Date**

Dr. M. M. Suleiman
Member, Supervisory Committee. ……. ……..
Signature **Date**

Prof. H. M. Kazeem
Head, Department of Veterinary Microbiology. ……. ……..
Signature **Date**

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

DEDICATION

I dedicate this thesis to my beloved parents **Mr and Mrs Francis Shamaki** and my beloved wife **Mrs Agnes Markus** for their labour, encouragement, prayer, support and provisions both in cash and kind to me particularly in the course of my M.Sc. programme. May the good Lord, the Rewarder of good deeds reward your kind gesture and generosity.

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ABSTRACT

Mycoplasma bovis is an important pathogen causing pneumonia, mastitis, arthritis and variety of other diseases in cattle worldwide. Pneumonic lungs samples were cultured and recovered isolates were biochemically identified for *M. bovis*. At the same time serum samples were collected and tested for the presence of antibodies to *M. bovis* using ELISA Kit (Bio-X Diagnostics, Jemelle-Belgium). A total of four hundred (400) serum samples and one hundred (100) pneumonic lungs were collected from three Local Government Areas in Adamawa State. A prevalence of *M. bovis* of 2.0% (2/100) was obtained from culture of pneumonic lungs. The isolates were observed to reduced tetrazolium chloride and had phosphatase activity; neither do they hydrolyzed glucose nor catabolized arginine. An overall seroprevalence of *M. bovis* detected by ELISA was 19.5% (78/400). The highest seroprevalence was recorded in Ganye LGA 36 (27.7%), followed by Yola LGA 28 (20.0%) and lowest was Mubi LGA 14 (10.8%), the association is statistically significant ($p<0.05$). Age- specific seroprevalence showed that cattle less than one-year old had the highest prevalence (25.8%), followed by four year-old cattle (22.4%). Based on sex distribution, 20.4% of the females and 17.4% of the males were seropositive to *M. bovis*. Breed susceptibility to *M. bovis* infection showed that White Fulani (21.8%) had the highest prevalence, followed by Cross breed (18.5%), Red Bororo (16.4%) and Adamawa Gudali (11.9%). There was no statistical significant association found among *M. bovis* infection and age, sex and breed distribution ($p>0.05$). The isolation as well as seroprevalence of *M. bovis* showed that this organism can have a serious effect on both calves and adult cattle, although the true extent of the disease is often overlooked. In conclusion, this study suggests that *M. bovis* is endemic in the study area and the culture and serology are important in complementing each other in the diagnosis. There is need for regular routine serological

surveillance that will cover more Local Government Areas to ascertain the prevalence of the disease in the study area.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

ABBREVIATIONS

AI - artificial insemination

BAL – bronchoalveolar lavage

BHV1 – bovine herpes virus type-1

BRD – bovine respiratory disease

BRSV – bovine respiratory syncycial virus

CBPP – contagious bovine pleuropneumonia

CCPP – contagious caprine pleuropneumonia

cfu – colony forming unit

CLSI – Clinical Laboratory Standard Institute

CPPS – chronic pneumonia and polyarthritis syndrome

DNA – deoxyribonucleic acid

ELISA – enzyme-linked immunosorbent assay

G+C – guanine + cytosine

IFN-γ – interferon-gamma

IgA – immunoglobulin A

IgG – immunoglobulin G

IHC – immunohistochemistry

IL – interleukin

kbp – kilo base pair

kDa - kilodalton

LRT – lower respiratory tract

Mab – monoclonal antibody

Mb – mega bases

MIC – minimum inhibitory concentration

M. bovis – *Mycoplasma bovis*

*Mmm*SC – *Mycoplasma mycoides* subspecies *mycoides* small colony

OD – optical density

PBS – phosphate buffered saline

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PI3V – parainfluenza type-3 virus

PPLO – pleuropneumonia-like organism

pvpA – phase variant protein type A

RIM – resource inventory and management

RNA – ribonucleic acid

SPSS – statistical package for social science

Th - helper cell derived from thymus

TNF-α – tissue necrotic factor alpha

UK – United Kingdom

URT – upper respiratory tract

USA – United States of America

vsp – variable surface lipoprotein

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Mycoplasma bovis is an important pathogen causing respiratory disease, otitis media, mastitis, arthritis and a variety of other disease in cattle worldwide, and is the second most pathogenic mycoplasma after *Mycoplasma mycoides* subspecies *mycoides* small colony the causative agent of contagious bovine pleuropneumonia (Maunsell *et al*., 2011). It is among the several *Mycoplasma* species colonizing the bovine respiratory mucous membranes (Ter Laak *et al*., 1992a); some of them are considered to be pathogenic whereas others are ubiquitous and part of the normal flora (Ter Laak *et al*., 1992a, b). *Mycoplasma bovis* (formerly *Mycoplasma agalactiae* subsp. *bovis*) among others is a significant but sometimes neglected bacterial pathogen of adult dairy cattle and intensively reared beef and dairy calves (Maunsell *et al.,* 2009; Maunsell *et al*., 2011; Rérat *et al*., 2012).

The organism belongs to the Class *Mollicutes,* Order *Mycoplasmatales,* Family *Mycoplasmataceae,* and the Genus *Mycoplasma* (Razin *et al*., 1998) and are among the smallest free-living micro-organisms capable of auto-replication and are highly fastidious bacteria that are difficult to culture and are slow growing (McAuliffe *et al*., 2004). Initially it was named as *Mycoplasma bovimastitidis,* and it was classified in group 5 with *Mycoplasma agalactiae* (Nicholas and Ayling, 2003). Both species are identical in cell- and colony-forms as well as their metabolic behavior and a high number of common antigens (Nicholas and Ayling, 2003). It is difficult to differentiate them by usual morphological, metabolic and serological methods (Gummelt *et al.,* 1996; Kumar, 2000). Later with advancement of

techniques, it was ranked as a species and named *Mycoplasma bovis*, based on the 16S ribosomal RNA sequence (Askaa and Erno, 1976).

Mycoplasma bovis was first isolated in 1961 in the United States from a cow with severe mastitis (Hale *et al*., 1962) and described as a cause of respiratory disease in 1976 (Nicholas and Ayling, 2003; Caswell and Archambault, 2009). Subsequently, the infection has been reported throughout the world, including most European countries (Nicholas and Ayling, 2003). *M. bovis* has been reported to be present in up to 30% of cases of pneumonia (Le Grand *et al*., 2002) and as the most frequently encountered mycoplasma from herds with mastitis (Bennett and Jasper, 1977).

The diseases caused by mycoplasmas in cattle are of major economic importance. Disregarding *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm*SC), the causative agent of Contagious Bovine Pleuropneumonia (CBPP), other *Mycoplasma* species can cause severe respiratory, venereal and other diseases (Maunsell *et al*., 2011). Among these, *M. bovis* is the most important and most pathogenic mycoplasma affecting the bovine species in Europe and North America (Nicholas and Ayling, 2003). This organism is a significant cause of bovine pneumonia (Pfützner and Sachse, 1996), mastitis (Byrne *et al*., 2000), arthritis (Stipkovits *et al*., 1993), genital disorders and abortion (Byrne *et al*., 1999), and reduction of *in vitro* fertility (Eaglesome and Garcia, 1990). Rarely, it can be isolated from other diseases, such as otitis media (Walz *et al*., 1997), meningeal abscesses (Stipkovits *et al*., 1993), decubital abscesses (Kinde *et al*., 1993) and keratoconjunctivitis of calves (Kirby and Nicholas, 1996). Its natural pathogenic role as a disease causing agent is often ignored and underestimated (Thomas *et al*., 1986; Rodriguez *et al*., 1996; Kumar *et al*., 2012).

Mycoplasma bovis is a common inhabitant of the upper and lower respiratory tract of healthy and pneumonic cattle (Thomas *et al*., 2002b; Arcangioli *et al*., 2008). It increases the severity of respiratory disease in calves and can also act as a primary pathogen (Gagea *et al*., 2006). It is a natural inhabitat of respiratory tract in healthy bovines, without showing any clinical symptoms and is shed through their nasal discharges for months or years (Gagea *et al*., 2006). Also the genital tract of both male and female animals can harbor the organism which can be a source of the infection through coitus during natural service to naive animals (Kreusel *et al*., 1989) or through artificial insemination (AI) with deep frozen bull semen (Jurmanova and Sterbova, 1977). Therefore, mycoplasmas can survive in frozen semen for several years (Jurmanova and Sterbova, 1977). Milk may also be a source of infection for suckling calves (Hirose *et al*., 2001). *Mycoplasma bovis* has also been reported from sheep (Bocklisch *et al*., 1987), goats (Egwu *et al*., 2001), rabbits (Boucher *et al*., 2001) and poultry (Hasan *et al*., 2008), and can be transmitted from these carrier animals and birds to bovine species. *Mycoplasma bovis* is quite resistant to environmental conditions (Nagamoto *et al*., 2001); and therefore, its transmission through fomites is a strong possibility. However, this need to be substantiated (Nagamoto *et al*., 2001).

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). Serological detection of *M. bovis* antibody by ELISA is one important method for detecting previous or recent infections, as antibody level detected by ELISA remains high for many months (Nicholas, 2004; Ball and Nicholas, 2010; Maunsell *et al*., 2011). Information about serological status of *M. bovis* in the farm, herd and a geographical area is desirable for

strategic planning and instituting control measures (Radaelli *et al*., 2008; Tambuwal *et al*., 2011).

1.2 Statement of Research Problem

Mycoplasma bovis infection is chronic in nature and poorly responsive to antimicrobial treatment (Ball and Nicholas, 2010). The infection usually co-exists with other bacteria with severe immunosuppression. Cattle exposed to *M. bovis* naturally or experimentally are immunosuppressive (Ball and Nicholas, 2010). Therefore, disease outbreaks usually have high morbidity rates which cause great economic loss, and can be economically devastating to the affected farms, especially in developing countries (Caswell and Archambault, 2009; Wiggins *et al*., 2011).

Mycoplasmas cause some of the most serious and economically most costly diseases of cattle. *M. bovis* with its incriminated diseases has not been given sufficient attention it deserves; although, the organism is wide spread in many European countries (Arcangioli *et al*., 2008; Caswell *et al*., 2010) and perhaps in some African Countries particularly in Nigeria where culture and seroprevalence of the organism (*M. bovis*) has been documented only in the north-western region (Ajuwape *et al*., 2003; Tambuwal, 2009). The epidemiological status of the organism and associated disease in other region of the country remains unknown.

The pattern of animal husbandry practice (nomadism) by the cattle owners coupled with transboundary nature of the study area could be responsible for the continued spread of *M. bovis* in the study area.

1.3 Justification

Infection is usually introduced to *Mycoplasma bovis* free herds by clinically healthy calves or young cattle shedding mycoplasma. Infected cattle shed the mycoplasma via the respiratory tract for many months and even years, acting as reservoirs of infection (Nicholas and Ayling, 2003).

The clinical signs and pathological lesions of *M. bovis* infection are not pathognomonic. Therefore, laboratory diagnosis is necessary for identification and confirmation of infection. Identification of mycoplasmas is carried out by cultural and serological methods (Simecka *et al*., 1993). False results due to contamination and cross-reactions are also possible (Sachse *et al*., 1993).

Mycoplasma bovis is endeminc in Nigeria (Egwu *et al*., 1996; Tambuwal *et al*., 2011). Currently, very few reports are available on the detection of *M. bovis* infection in African countries including Nigeria where Contagious Bovine Pleuropneumonia (CBPP) due to *Mycoplasma mycoides* subspecies *mycoides* SC infection is predominantly documented (Egwu *et al*., 1996; Aliyu *et al*., 2000; Egwu *et al*., 2001; Tambuwal, 2009). Nevertheless, intensive cattle production in African countries, particularly Nigeria is gaining prominence with some intensive herds being cross bred with imported cattle breeds (RIM, 1992). The aberrant occurrence of *M. bovis* in caprine mycoplasmal mastitis in Nigeria was reported (Egwu *et al*., 2001) which may have a role in the epidemiology of the disease in bovine species. The prevalence and pattern of distribution of this infection across the country are yet to be documented particularly in the north eastern Nigeria that habour about 2.8 million of the total cattle population of the country. Because the status of *M. bovis* infection in cattle in Adamawa State remains unknown, culture and isolation of the organism as well as detection

of its antibodies in the State is important to provide more meaningful control measures for this emerging economic disease.

1.4 Research Questions

1. Is *Mycoplasma bovis* associated with pneumonia in cattle in Adamawa State?

2. Are there *Mycoplasma bovis* antibodies in cattle in Adamawa State?

1.5 General Aims of the Study

To isolate *Mycoplasma bovis* and serologically detect its antibodies in cattle in Adamawa State.

1.6 Specific Objectives

i. To isolate and identify *M. bovis* from suspected pneumonic lungs of cattle in Adamawa State.

ii. To determine the seroprevalence of *M. bovis* from cattle in Adamawa State using enzyme linked immunosorbent assay (ELISA).

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification

Mycoplasmas are the smallest micro-organisms that are capable of self- replication. They are considered as bacteria even though they lack a typical bacterial cell wall. They are classified as;

Class: *Mollicutes*

Order: *Mycoplasmatales*

Family: *Mycoplasmataceae*

Genus: *Mycoplasma, Hepatoplasma, Ureaplasma*.

Mycoplasmas are fastidious and delicate micro-organism, they require enriched media for growth. The basic medium is good quality beef infusion with supplements such as yeast extract. Additionally, *Mycoplasma* and *Ureaplasma* species required cholesterol and this is usually provided by adding 20- 30% horse, calf, rabbit or pig serum to the culture medium (Quinn *et al*., 1994).

Mycoplasmas belong to the class *Mollicutes* (from the Latin *mollis*, soft; *cutis*, skin), a group of bacteria so named because they lack cellwall, instead being enveloped by a complex plasma membrane (Basema and Tully, 1997). Mollicutes are also characterized by their tiny size, small genomes (0.58 - 2.2 Mb), and low G + C content (24 - 33 mol %) (Razin *et al*., 1998). Perhaps as a direct consequence of the limited biosynthetic capacity of their small genome, mycoplasmas usually form an intimate association with host cells to obtain growth and nutritional factors necessary for survival (Rosengarten *et al*., 2001). Mollicutes are found in a wide range of hosts including mammals, birds, reptiles, fish, arthropods and plants

(Razin, 1992). Their individual relationship with the host varies from primary or opportunistic pathogens to commensals or epiphytes. The most important species of mycoplasmas and the diseases they cause in domestic animals and farmed crocodiles are shown in Table 2.1.

In mammalian hosts, mollicutes typically inhabit mucosal surfaces, including those of the respiratory, urogenital and gastrointestinal tracts, eyes, and the mammary gland (Rosengarten *et al*., 2000). Typical of many mucosal pathogens, pathogenic species of mollicutes may inhabit some mucosal sites without causing disease (Rottem and Naot, 1998; Hickman-Davis, 2002). Disease occurs when host and/or pathogen factors result in dissemination to other sites (e.g from the nasal mucosa to the lower respiratory tract), invasion and destruction of host tissues, or a detrimental inflammatory response. Haematologic dissemination from mucosal surfaces can occur, with the joints being a frequent site of secondary colonization (Simecka *et al*., 1992). Mollicutes are very effective at evading and modulating the host immune response and the immune response contributes significantly to the pathogenesis of many mollicute-associated diseases (Simecka *et al*., 1992; Rosengarten *et al*., 2000).

Table 2.1: Major pathogenic mollicutes of animals (Joachim, 2002).

Animal Hosts/Mollicutes species	**Diseases**

Bovines	
Mycoplasma mycoides subsp. *mycoides* SC	Contagious bovine pleuropneumonia (CBPP)
Mycoplasma sp. bovine group 7	Pneumonia and arthritis
Mycoplasma bovis	Mastitis, pneumonia(calf), polyarthritis(calves), metritis, abortion, sterility
Mycoplasma dispar	Pneumonia (calves)
Mycoplasma californicum	Mastitis
Mycoplasma canadense	Mastitis
Mycoplasma bovigenitalium	Mastitis and genital disease
Mycoplasma bovocculi	Conjunctivitis
Mycoplasma wenyonii	Anaemia
Sheep and goats	
M. capricolum subsp.*capripneumoniae*	Contagious caprine pleuropneumonia (CCPP)
M. capricolum subsp. *capricolum*	Mastitis, arthritis
M. mycoides subsp. *capri*	Pneumonia, mastitis, arthritis, septicaemia (goats)
M. mycoides subsp. *mycoides* LC	Pneumonia, mastitis, arthritis, septicaemia (goats)
M. agalactiae	Infectious agalactia
M. ovipneumoniae	Atypical pneumonia (lambs)
M. conjunctivae	Infectious conjunctivitis (sheep)
Wild caprine (ibex, chamois, gazelles)	
M. conjunctivae	Infectious conjunctivitis (ibex, chamois)
Poultry	
M. gallisepticum	Chronic respiratory disease (chickens), sinusitis, infectious air sacculitis (turkey)
M. synoviae	Air sacculitis, arthritis, tendosynovitis
M. maleagridis	Air sacculitis, arthritis, sinusitis (turkey)
Swines	
M. hyopneuminiae	Enzootic pneumonia
M. hyorhinis	Pneumonia, arthritis
M. hyosynoviae	Arthritis
M. suis	Anaemia
Horses	
M. felis	Pleuritis
M. equirhinis	

Pathogenic mollicutes of animals (continued)

Dogs and cats	
M. cynos	Pneumonia
M. felis	Conjunctivitis, pneumonia (cats)
M. haemocanis	Anaemia (dogs)
M. haemofelis	Anaemia (cats)
Small rodents	
M. arthritidis	Arthritis (rat)
M. pulmonis	Respiratory and genital tract infection (rat and mouse)
Crocodiles	
M. crocodyli	Polyarthritis in crocodiles
M. alligatoris	Polyserositis and arthritis in crocodiles and alligators

2.2 Biological Properties

Like all mollicutes, *Mycoplasma bovis* is small and pleomorphic; it has a genomic size of 1,080kbp, lacks a cell wall and has a low G+C ratio of 27.8-32.9 mol% (Hermann, 1992). *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, lactate and pyruvate as energy sources for growth (Miles *et al*., 1988). The film and spot formation can also be seen on the surface of solid media, indicating the possession of lipolytic activity. The biochemical properties of the most frequent bovine mycoplasmas compared to the ones of *M. bovis* can be seen in Table 2.2.

Table 2.2: Biochemical properties of the most frequent bovine mycoplasmas (Nicholas and Ayling, 2003).

Mycoplasma	Glucose	Arginine	Urease	Film	Casein	Phosphatase	Tetrazolium/ Aerobic	Tetrazolium/ Anaerobic
M. bovis	-	-	-	+	-	+	+	+
M. bovirhinis	+	-	-	-	+/-	+/-	+	+

M.bovigenitalium	-	-	-	+	-	+	-	+
M. bovoculi	+	-	-	+	-	+/-	+	+
M. canis	+	-	-	-	+/-	-	-	+
M. californicum	-	-	-	-	Nk	+	-	+**
M. canadense	-	+	-	-	Nk	+	-	+
M. dispar	+	-	-	-	Nk	-	+	+
M. mycoides SC	+	-	-	-	+	-	+	+
U. diversum	-	-	+	-	-	-	Nk	Nk

NB:
Nk= not known

*= weak

**= most strains

-= negative

+= positive

Mycoplasmas are usually considered to be highly susceptible to various environmental factors such as high temperature and dryness. Despite this, *Mycoplasma bovis* can survive outside the host environment 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).

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).

2.3 Antigenic Variation

There has been a great deal of interest in the antigenic variation observed in *Mycoplasma bovis*. A group of Scientists at The Hebrew University published their initial work on antigenic variation in this species of mycoplasma (Behrens *et al*., 1994; Rosengarten *et al*., 1994). In their initial studies, the family of lipid-modified variable surface lipoprotein (vsp), consisted of at least four classes, vspA-D, though this has now been enlarged to at least 13 different members (Lysnyansky *et al*., 2001). These proteins exhibit both phase switching (ON-OFF states) and size variation. These proteins differ from other mycoplasma size variant proteins in that their immunoblot patterns display irregular periodic spacing, suggesting that the mechanism responsible for the variation may differ from other mycoplasma species (Behrens *et al*., 1994). Each of the members of this group could be distinguished using monoclonal antibodies directed against unique antigenic epitopes (Behrens *et al*., 1994; Rasberry and Rosenbusch, 1995), but they also share a common antigenic epitope found on the phase variant protein type A (PvpA) protein of *Mycoplasma gallisepticum* (Minion, 2002). This epitope was expressed in 246 of 250 different *M. bovis* strains. Hybridization analysis with oligonucleotides sharing sequences among the vsp genes indicates that the vsp

repertoire varies in size and composition among different isolates (Minion, 2002). All of the vsp proteins were organized on the surface of the mycoplasma in evenly distributed B clusters. In addition, vsp homologues have been identified in *Mycoplasma agalactiae* (Flitman-Tene *et al*., 1997). An oligonucleotide representing a conserved region in all vsp genes reacted to multiple bands upon Southern hybridization. Oligonucleotides representing unique distinct vsp regions failed to react indicating that the vsp family in this species was analogous but distinct from the vsp family in *M. bovis* (Minion, 2002).

Mycoplasma bovis demonstrates significant interstrain variability within the 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.

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. 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).

In the presence of antibodies from experimentally-infected or naturally-infected calves, selective pressure of specific vsp phenotypes *in vitro* resulted in the repression or shortening of some vsp proteins while others appeared in the cell population. When the antibody was removed, the cells reverted to the original phenotype. This shows that there is a preferred vsp phenotype during *in vitro* growth and that selective pressure from the host humoral response can alter or modulate that phenotype (Le Grand *et al*., 1996). Whether this modulation is operative *in vivo* where other selective pressures such as adherence receptors exist to enhance maintenance of specific phenotypes is yet to be determine. In addition, it is not known if variation in the vsp phenotype can affect tissue tropism and enhance invasiveness within the host (Minion, 2002).

A second vsp unrelated protein that was identified undergo phase switching and size variation is the membrane protein of 67kDa molecular weight (pMB67). Unlike the vsp proteins, pMB67 was not lipid modified and did not contain the vsp-like repetitive domains. It appeared to be a predominant antigen recognized during *M. bovis* infections or disease and may be suitable for the development of vaccines or diagnostic preparation (Behrens *et al*., 1996).

2.4 Epidemiology of *Mycoplasma bovis*

Mycoplasma bovis is well adapted to colonization of mucosal surfaces, where it can persist without causing clinical disease. The upper respiratory tract (URT) mucosa is the primary 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). Bacteremia during *M. bovis* infection has been well documented (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 shed *M. bovis* for a few months or less (Punyapornwithaya *et al*., 2010), some cattle can shed the organism intermittently for many months or years (Biddle *et al*., 2003). The factors responsible for intermittent shedding have not been determined. 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 associated 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).

2.5 Molecular Epidemiology

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*., 2000; Butler *et al*., 2001; McAuliffe *et al*., 2004; Biddle *et al*., 2005; Miles *et al*., 2005). Considerable genomic heterogeneity among 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*., 2000; 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*., 2000; Butler *et al*., 2001; McAuliffe *et al*., 2005). In contrast, endemically-infected open herds, including dairy calf ranches,

harbor numerous genetically diverse strains of *M. bovis*. This has been attributed to introduction of animals from multiple sources over time (Butler *et al*., 2001).

2.6 Transmission

2.6.1 Risk factors for infection with *Mycoplasma bovis*

The introduction of asymptomatically infected animals is thought to be the primary means by which *Mycoplasma bovis*-free herds become infected (Maunsell *et al*., 2011). 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 1to 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 traditionally been regarded as a contagious mastitis pathogen, with udder-to-udder spread being the major 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, ingestion 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). However, other means of transmission must also be

important, as the disease can occur in calves that are fed milk replacer or pasteurized milk (Maunsell *et al*., 2011). Once established in a multiage facility, *M. bovis* is very difficult to eradicate, suggesting ongoing transmission from older to incoming calves. Calves could also become infected from adults in the calving area. Congenital *M. bovis* infections appear to occur infrequently (Maunsell *et al*., 2011).

Transmission of *Mycoplasma bovis* through respiratory secretions is considered important in the epidemiology of infection, although there is little experimental data to support this contention. However, *M. bovis* might be transmitted in respiratory secretions via 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). It therefore, seems prudent to assume that infection can occur via this route. 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 (Maunsell *et al*., 2011). *Mycoplasma bovis* has been shown to persist 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).

2.6.2 Mycoplasma co-infection with other pathogens

Mycoplasma bovis may act singly or in combination with other microbes and weakens the host immune system, leading to invasion by other pathogenic bacteria or viruses, which may

explain the chronic and polymicrobial nature of *M. bovis* infections (Rosengarten and Citti, 1999; Snowder *et al*., 2006; Soehnlen *et al*., 2011). An association between bovine viral diarrhea virus infection and *M. bovis* has been reported (Shahriar *et al*., 2002; Fulton *et al*., 2009). Bacterial coinfections in *M. bovis*-associated pneumonia (Gagea *et al*., 2006; Fulton *et al*., 2009) and otitis media (Lamm *et al*., 2004) are extremely common. For example, *M. bovis* was isolated from the lungs of 82% of feedlot calves with fibrinosuppurative pneumonia from which *Mannheimia haemolytica* was isolated (Gagea *et al*., 2006). In other surveys of feedlot pneumonia *M. bovis* related pneumonia was commonly identified in combination with *Histophilus somni* (Fulton *et al*., 2009), and coinfection with *Pasteurella multocida* is common in younger calves (Stipkovits *et al*., 2000).

2.7 Pathogenesis of *Mycoplasma bovis* Infection

Mycoplasma bovis infections of cattle are an important disease 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). 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. 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 cellwall, and exposed membrane proteins form the primary interface with the host. These membrane proteins facilitate adherence to mucosal surfaces, although *Mycoplasma bovis* adhesions are not yet well characterized (Buchenau *et al*., 2010). *M. bovis* has a large family of immunodominant variable surface lipoproteins (vsps), 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 phospholipases, hydrogen peroxide, and superoxide radicals which damage host cells. *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 confined to the initial area of colonization, the respiratory tract. Consequently, organisms rapidly gain access to multiple organ systems. This suggests that in order to survive, *M. bovis* may alter its gene expression as it encounters different host environments and different selective pressures. Among those genes thought to be regulated to the advantage of the microbe and to the detriment of the host would be adhesions (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 is a complex process. Early studies showed that the mechanism of adherence to embryonic bovine lung cells may involve interactions with sialic acid-containing receptors or sulfated lipids. 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. 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).

2.8 Clinical Signs Associated with Mycoplasma Infection

2.8.1 Mastitis

The herd presentation of mycoplasma mastitis varies from endemic 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 production. Cows of any age or stage of lactation are affected, including 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 color. 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).

2.8.2 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, hyperpnoea, dyspnoea, 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 be accompanied by cases of otitis media, arthritis, or

both, in the same animal or in other animals in the herd. 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).

2.8.3 Otitis media

Mycoplasma bovis-associated otitis media occurs in dairy and beef calves as enzootic disease or as outbreaks, and also occurs sporadically in feedlot cattle. 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 sequel. 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 have 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).

2.8.4 Arthritis, synovitis and periarticular infection

In contrast to *Mycoplasma bovis* infections of the upper and lower respiratory tracts, *M. bovis*-induced arthritis is presumed to be a consequence of mycoplasmemia (Chima *et al*.,

1981; Thomas *et al*., 1986). Arthritis was preceded by mycoplasmemia in one calf that was inoculated intratracheally with *M. bovis* (Thomas *et al*., 1986). Infections of other body systems that occasionally accompany polyarthritis are also likely to be a consequence of mycoplasmemia (Stipkovits *et al*., 1993). 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 (Gonzalez *et al*., 1993; Stipkovits *et al*., 2005). Although uncommon, outbreaks of disease where arthritis was the predominant clinical presentation have been reported (Stipkovits *et al*., 1993; Butler *et al*., 2000). Clinical signs are typical of septic arthritis with affected joints being painful and swollen, and calves exhibit varying degrees of lameness and may be febrile in the acute phase of disease (Stipkovits *et al*., 1993; Step and Kirkpatrick, 2001). Cattle of any age can be affected by *M. bovis* arthritis. Cases tend to be sporadic and are often concurrent with cases of pneumonia or mastitis, although outbreaks of *M. bovis* arthritis as the predominant clinical manifestation have been reported in calves and dairy cows (Wilson *et al*., 2007). 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. The animal might be febrile and anorectic. 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).

2.8.5 Other diseases

2.8.5.1 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* (Kirby and Nicholas, 1996; Levisohn *et al*., 2004; Alberti *et al*., 2006). An outbreak of severe keratoconjunctivitis, from which *M. bovis* was the only consistently isolated pathogen, was reported in a group of 20 calves. 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). In a report by Alberti *et al*., (2006), an outbreak of *M. bovis*-associated keratoconjunctivitis in beef calves in Italy was followed by cases of pneumonia and arthritis.

2.8.5.2 Meningitis

Meningitis can 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).

2.8.5.3 Decubital abscesses

Kinde and his colleugues (1993) reported that 50 calves developed *Mycoplasma bovis*-infected decubital abscesses over the brisket and joints with some of the calves having concurrent *M. bovis*-associated pneumonia (Kinde *et al*., 1993).

2.8.5.4 Cardiac Disease

Mycoplasma bovis was identified concurrently with *Hemophilus somni* in the hearts of 4 of 92 feeder calves dying from myocarditis (Haines *et al*., 2004). In another report, a heifer with clinical signs of cardiac insufficiency was found to have mural and valvular endocarditis with *M. bovis* isolated from the chronic active fibrinopurulent endocarditis (Helie *et al*., 2007).

2.8.5.5 Genital Disorders

In isolated and predominantly experimental cases, *Mycoplasma 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).

2.9 Pathology Associated with *Mycoplasma bovis* Infection

The macroscopic and microscopic lesions of the respiratory tract in experimental *M. bovis* infection vary considerably among studies, probably 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). Macroscopic lesions have consisted of cranioventral lung consolidation, sometimes accompanied by multiple necrotic foci (Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996). 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 (Lopez *et al*., 1986; Thomas *et al*., 1986; Rodriguez *et al*., 1996).

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. Macroscopically, affected lung lobes are deep red in color 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 is typically accompanied by hyperplasia of the lymphoid tissues in both the upper respiratory tract (URT) and lower respiratory tract (LRT) (Thomas *et al*., 1986; 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 markedly 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. In another report (Thomas *et al*., 1986), intratracheal inoculation of *M. bovis* resulted in arthritis in one calf, and mycoplasmas were isolated from the blood during the first week post-inoculation. 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. Other investigators identified *M. bovis* antigen within foci of mononuclear cell infiltrates in the liver and kidneys of 2 calves with chronic *M. bovis* pneumonia (Adegboye *et al*., 1995).

2.10 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).

2.10.1 Detection of antibodies

Mycoplasma bovis-specific serum antibodies can be detected by indirect ELISA (Uhaa *et al*., 1990), 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).

2.10.2 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. 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. 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). PCR 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; Sachse *et al*., 2010). 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). 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 preincubated in broth. *Mycoplasma bovis* can be detected *in situ* by immunohistochemistry (IHC) on formalin fixed paraffin-embedded tissues (Gagea *et al*., 2006). An indirect fluorescent antibody test for detection of *M. bovis* in fresh, frozen lung tissue has also been described (Knudtson *et al*., 1986).

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*., 2002a), 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. 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).

2.10.3 Sample collection and handling

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*., 2002b).

Samples should be refrigerated, or frozen if time to 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).

2.10.4 Postmortem findings

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). Although some *M. bovis* lesions are characteristic, many are grossly indistinguishable from other pathogens. 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).

2.10.4.1 Pneumonia

The presence of *Mycoplasma 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. 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. 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).

2.10.4.2 Other infections

A diagnosis of mycoplasma mastitis is usually made clinically rather than at necropsy, but lesions are characterized as mild to severe fibrinosuppurative to caseonecrotic mastitis (Maunsell *et al*., 2011). In mycoplasma otitis media, the affected tympanic bullae contain suppurative to caseous exudate and have often undergone extensive osteolysis. During field necropsy the ventral aspect of the bulla can be opened and swabbed or aspirated for culture (Lamm *et al*., 2004; Van Biervliet *et al*., 2004). Joints with *M. bovis* arthritis contain non-

odorous fibrinous to caseous exudate accompanied by fibrosis. Periarticular involvement is common and can involve tendons, synovial sheaths, muscle, and connective tissue. Affected periarticular tissues contain foci of caseous necrosis, linear necrotic lesions, and extensive fibrosis. Immunohistochemistry (IHC) reveals *M. bovis* antigen at the edges of necrotic lesions and within exudates (Adegboye *et al*., 1996; Gagea *et al*., 2006).

2.11 Treatment of *Mycoplasma bovis* Infection

2.11.1 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of large *M. bovis* populations can be useful to make generalizations about resistance. However, the value of antimicrobial susceptibility testing in making evidence-based herd, or individual-level treatment decisions for *M. bovis*-associated disease has not been determined. Susceptibility testing for mycoplasmas in animals is not currently standardized and should be interpreted with caution (Maunsell *et al*., 2011).

2.11.2 Antimicrobial resistance

The β-lactam antimicrobials are not effective against these pathogens, because mycoplasmas lack a cellwall. Similarly, mycoplasmas do not synthesize folic acid and are therefore intrinsically resistant to sulfonamides. Mycoplasmas, as a class, are generally susceptible to drugs (tetracyclines, macrolides, linosamides, and florfenicol) that interfere with protein or DNA (fluoroquinolones) synthesis. However, *M. bovis* is resistant to erythromycin (Francoz *et al*., 2005; Rosenbusch *et al*., 2005).

2.11.3 Isolates for antimicrobial susceptibility testing

Isolates obtained from the site of infection from representative early, untreated cases should be used. Samples collected at necropsy are ideal. If live cattle with respiratory disease are sampled, broncho-alveolar lavage (BAL) samples should be used; antimicrobial susceptibility data of paired *M. bovis* isolates obtained from nasal swabs and BALs were found to differ considerably (Thomas *et al*., 2002b).

2.11.4 Appropriate method for antimicrobial testing

Microbroth dilution, agar dilution, and the E-test (AB BIODISK, Solna, Sweden) can be used to determine minimum inhibitory concentrations (MICs) for *M. bovis*. There is currently no MIC testing control standards for veterinary mycoplasmas, although the Clinical Laboratory Standards Institute (CLSI) is in the process of developing these. Breakpoints have not yet been determined and so MIC results cannot be defined as susceptible, intermediate, or resistant (Maunsell *et al*., 2011).

2.11.5 Available data on antimicrobial susceptibility of *Mycoplasma bovis* isolates

Selected data on the antimicrobial susceptibildty profiles of *Mycoplasma bovis* isolates are presented in Table 2.3. Most isolates originated from the respiratory tract of diseased cattle (Ayling *et al*., 2000; Francoz *et al*., 2005; Rosenbusch *et al*., 2005; Gerchman *et al*., 2009). Minimum inhibitory concentrations (MICs) of tilmicosin and spectinomycin tend to have a bimodal distribution, and many isolates have high MICs for tetracyclines, findings that are suggestive of acquired resistance. Resistance to the fluoroquinolones and florfenicol appears uncommon, although enrofloxacin resistance has been identified in a subpopulation of Israeli *M. bovis* isolates (Gerchman *et al*., 2009). MICs of tulathromycin have been reported for 63 European *M. bovis* isolates with the MIC_{50} being 4 µg/mL, MIC_{90} □ 64 µg/mL, and MIC range 0.125 to □ 64 µg/mL (Godinho, 2008). However, tulathromycin was efficacious in the

treatment of calves infected with a strain of *M. bovis* that had a MIC of □ 64 µL/mL, so the clinical relevance of tulathromycin MIC values is unknown (Godinho *et al*., 2005a).

Table 2.3: Selected minimum inhibitory concerntration (MIC) values for *Mycoplasma bovis*, all values are reported as µg/mL (Maunsell *et al*., 2011)

	Rosenbusch[a]	**Gerchman[b]**	**Ayling[c]**	**Francoz[d]**
Enrofloxacin				
MIC_{50}	0.25	0.16	-	0.19
MIC_{90}	0.5	0.36	-	0.25
MIC range	0.03- 4	0.08- 2.5	-	0.047- 0.5
Florfenicol	1	-	4	-
MIC_{50}	4	-	16	-
MIC_{90}	0.06- 8	-	1-64	-

MIC range				
Oxytetracycline/Tetracycline	2	4	32	4
MIC_{50}	16	8	64	8
MIC_{90}	0.125 to □ 32	0.5- 16	1-128	0.094 to □ 256
MIC range				
Spectinomycin	2	2	4	2
MIC_{50}	4	□1,024	□128	□1,021
MIC_{90}	1 to □ 16	0.5 to □ 1,024	1->128	0.38 to □ 1,021
MIC range				
Tilmicosin	64	128	□128	-
MIC_{50}	□128	□128	□128	-
MIC_{90}	0.5 to □ 128	0.5 to □ 128	4 - □ 128	-
MIC range				

NB:

[a]Rosenbusch *et al*., 2005. 223 US isolates, microbroth dilution method.

[b]Gerchman *et al*., 2009. 17 Israeli isolates, microbroth dilution method except for spectinomycin, where the E- test was used.

[c]Ayling *et al*., 2000. 62 UK isolates, microbroth dilution method.

[d]Francoz et al., 2005. 55 Canadian isolates, E- test.

[e]Data from the Francoz study are for tetracycline, other data are for oxytetracycline.

2.11.6 Other information on treatment of *Mycoplasma bovis* infection

There is little information on how pharmacokinetic and pharmacodynamic data, where available, should be applied in the treatment of *Mycoplasma bovis* infections (Maunsell *et al*., 2011). Two antimicrobials are currently 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. Enrofloxacin is only approved for treatment of BRD associated with *Manheimia haemolytica, Pasteurella multocida,* and *Histoophilus 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).

Some controlled trials have evaluated the efficacy of antimicrobials for the treatment of experimentally induced *M. bovis*-associated disease. In an industry sponsored study, calves that developed respiratory disease after experimental *M. bovis* infection were treated with tulathromycin (Godinho *et al*., 2005a). Treated calves had lower temperatures, lower rate of removal from the trial for welfare reasons, and lower lung lesion scores than control calves. In another study, tilmicosin given at the onset of clinical disease was associated with reduced numbers of *M. bovis* in the lungs of calves experimentally infected with *M. haemolytica* plus *M. bovis* (Gourlay *et al*., 1989). Calves treated for 10 days with oral valnemulin or oral enrofloxacin beginning 10 days after experimental infection with *M. bovis* had improved clinical scores and fewer *M. bovis* recovered from their lungs compared with untreated calves (Stipkovits *et al*., 2005).

There is little information on the treatment of naturally occurring *M. bovis*-associated disease in cattle, despite a huge volume of literature on the treatment of undifferentiated BRD. Oxytetracycline and tilmicosin resulted in clinical improvement in calves with pneumonia that included a mycoplasma component (Maunsell *et al*., 2011). In an industry-sponsored

study, tulathromycin and florfenicol proved effective treatments for BRD that included an *M. bovis* component (Godinho *et al*., 2005b). For the treatment of *M. bovis*-associated diseases other than BRD, there are few data available. Cattle with *M. bovis*-associated arthritis have an especially poor response to treatment. Aggressive early treatment before the development of extensive tissue necrosis seems most likely to be successful. Fluoroquinolones, tetracyclines, and macrolides tend to have good distribution into joints (Adegboye *et al*., 1996). Myringotomy with irrigation of the middle ear has been recommended for the treatment of otitis media in calves. There is a report of successful surgical treatment of a calf with *M. bovis*-associated otitis media-interna in which a bilateral tympanic bulla osteotomy was performed (Van Biervliet *et al*., 2004).

Early recognition and treatment of cases are likely to be very important in successful therapy, because improved efficacy is observed when treatment is initiated early in the course of experimental disease. There seems to be no published studies that have critically evaluated the duration of therapy for *M. bovis*-associated disease. However, given that *M. bovis* disease often becomes chronic, continuing antimicrobial treatment until clinical resolution could be important and would involve extending treatment beyond most label recommendations. Research is needed to evaluate the effect of treatment duration on cost and outcome (Maunsell *et al*., 2011).

2.12 Prophylactic Treatment of *Mycoplasma bovis*-Associated Disease

In experimental *Mycoplasma bovis* infections, response to treatment when antimicrobials are given early in the course of disease is often better than response rates reported for natural disease (Godinho *et al*., 2005a). Prophylaxis might therefore be more successful than treatment after clinical *M. bovis*-associated disease develops. There is little doubt that

strategic treatment of cattle at high risk of developing undifferentiated BRD is beneficial in reducing the incidence and severity of disease, and some data support treatment of calves at high risk of *M. bovis*-associated disease. For example, in a blinded, randomized study, veal calves in a facility in which *M. bovis* was the predominant respiratory pathogen were treated with florfenicol or oral tilmicosin during a BRD outbreak (Catry *et al*., 2008). Prophylactic florfenicol resulted in higher weight gain, better clinical status, and reduced rates of BRD compared with tilmicosin or untreated controls. Tulathromycin is the only drug currently approved for prophylactic use in the control of BRD associated with *M. bovis* in the United States (Godinho *et al*., 2005a). In an industry-sponsored, blinded, randomized field trial in high-risk cattle, significantly fewer cattle developed BRD after tulathromycin prophylaxis than after no treatment or treatment with tilmicosin. *Mycoplasma bovis* was isolated from affected cattle along with other BRD pathogens (Godinho *et al*., 2005b). Given the limited data available, prophylactic use of antimicrobials is probably justified when high levels of morbidity and mortality because of *M. bovis*-associated disease are being sustained or can be expected in high-risk cattle, although *M. bovis*-specific efficacy data and economic analyses are needed (Maunsell *et al*., 2011).

2.13 Immunity

2.13.1 Passive immunity

There is a strong association between failure of transfer of passive immunity and increased risk and severity of bovine respiratory disease (BRD) in calves. However, the role of maternal immunity in protection against *Mycoplasma bovis*-associated disease is unknown (Maunsell *et al*., 2011).

2.13.2 Host immune responses

Innate immune responses are critical in the early phase of mycoplasma infections. Alveolar macrophages in particular are important in the early clearance of mycoplasmas from the lungs. However, inappropriate activation of alveolar macrophages by mycoplasmas can promote an excessive inflammatory response (Kauf *et al*., 2007). Detrimental inflammatory responses in *M. bovis* infections have been partly attributed to excessive TNF-α production by alveolar macrophages (Maunsell *et al*., 2011). Activation of macrophages results in the recruitment of neutrophils to sites of inflammation, and neutrophils are a prominent cell type in the lungs, middle ear, and joints of *M. bovis* infected calves (Lamm *et al*., 2004; Gagea *et al*., 2006). Excessive neutrophil recruitment with the subsequent release of large amounts of inflammatory mediators can occur, and the extent of neutrophil recruitment is directly correlated with the severity of mycoplasma disease. Although bovine neutrophils are able to kill opsonized *M. bovis*, unopsonized *M. bovis* can adhere to neutrophils and inhibit respiratory burst activity (Maunsell *et al*., 2011).

Despite a substantial body of work examining adaptive immune responses to mycoplasma infections, the optimal responses for protection and the types of responses contributing to disease remain poorly defined. Adaptive responses that are in place at the time of exposure can help control new infections (Kauf *et al*., 2007; Maunsell *et al*., 2011). For example, prior *M. bovis* mastitis seems to protect cows from developing the severe mastitis that is typically observed on primary infection; most reinfections result in subclinical or mild disease. However, adaptive responses are often ineffective at eliminating established mycoplasma infections and ongoing ineffective responses result in chronic inflammation (Maunsell *et al*., 2011). Exactly how mycoplasmas manage to avoid clearance by the host is not well understood. However, mycoplasmas can induce a broad range of immunomodulatory events

that might induce ineffective immune responses, and variation of surface antigens could help mycoplasmas to avoid clearance mediated by adaptive responses (Maunsell *et al*., 2011).

Experimental respiratory infection of calves with *M. bovis* usually elicits a strong humoral response characterized by high levels of serum IgG1 and very little IgG2 (Vanden Bush and Rosenbusch, 2003), and local mucosal IgG and IgA responses (Maunsell *et al*., 2011). Similarly, *M. bovis* inoculation of the mammary gland results in serum IgG and local mucosal IgG and IgA responses. Humoral responses of naturally infected cattle are more variable. Together with innate responses, humoral immune responses appear to be important in protection from *M. bovis*. Systemic antibody is particularly important in preventing disseminated infections, and serum *M. bovis* IgG titers are correlated with protection from arthritis (Nicholas *et al*., 2002). On mucosal surfaces, however, local antibody is likely to be more important. For example, anti-*M. bovis* antibody concentrations in milk, but not in serum, are correlated with resistance to reinfection in cows following *M. bovis* mastitis. Immunoglobulin G (IgG) concentrations in bronchoalveolar lavage (BAL) fluid have been correlated with resistance to *M. bovis*-associated respiratory disease (Maunsell *et al*., 2011).

It is widely accepted that mycoplasma respiratory infections have substantial immunopathological components, characterized by large accumulations of lymphocytes in affected tissues, the production of proinflammatory cytokines, and lung inflammation. Mycoplasmas, including *M. bovis*, can also modulate some inflammatory responses. However, little is known about the cytokine environment in the lungs of calves with *M. bovis* infections (Maunsell *et al*., 2011). In one study (Vanden Bush and Rosenbusch, 2003), peripheral blood mononuclear cells from *M. bovis*-infected calves secreted IFN-γ and IL4 in response to *M. bovis* antigen, and there was a strong systemic IgG1 response with little IgG2 produced. These findings indicate that *M. bovis* induces a mixed Th1-Th2 cytokine response,

although the lack of IgG2 production was more consistent with a Th2-biased response (Maunsell *et al*., 2011).

2.14 Vaccination against *Mycoplasma bovis*-Associated Infection

In general, attempts to vaccinate cattle against *Mycoplasma bovis*-associated disease have been unrewarding. However, several *M. bovis* bacterins are licensed for marketing in the United States for the control of *M. bovis*-associated respiratory disease or, in one case, mastitis. In addition, a number of US companies produce autogenous *M. bovis* bacterins. However, there is virtually no data demonstrating field efficacy of the available *M. bovis* vaccines. To date, no *M. bovis* vaccines are commercially available in Europe (Maunsell *et al*., 2011).

As discussed earlier, adaptive immune responses that are in place at the time of mycoplasma exposure can help control infection. So it is not surprising that vaccination can, in some instances, protect cattle from experimentally induced *M. bovis*-associated disease (Nicholas *et al*., 2002). In a number of instances *M. bovis* vaccines have appeared promising in challenge studies but have been ineffective or resulted in increased severity of disease when applied in field trials. For example, a *M. bovis* bacterin prevented respiratory disease in calves that were challenged 3 weeks after vaccination (Nicholas *et al*., 2002). However, when the same vaccine was used in a field trial, an increased rate and severity of respiratory disease was observed in the vaccinated group (Nicholas *et al*., 2006). In another blinded, controlled field trial, a commercial *M. bovis* bacterin was no different to a placebo in preventing *M.bovis*-associated disease in high-risk dairy calves (Maunsell *et al*., 2009). In both these studies a substantial proportion of calves were identified as infected with *M. bovis* before vaccination. Increased disease severity has also been observed in vaccinated, experimentally infected

calves. For example, vaccination with *M. bovis* membrane proteins was associated with enhanced severity of respiratory disease following aerosol challenge, compared with control calves (Bryson *et al*., 1999). Increased severity of clinical mastitis was reported in cows vaccinated with an *M. bovis* bacterin compared with controls after intramammary inoculation of *M. bovis* (Boothby *et al*., 1986).

It is therefore apparent that vaccination against *M. bovis*-associated disease is sometimes possible in a controlled setting, but the vaccines critically evaluated to date are not protective in the field. The early age at which calves often become infected also presents a challenge to the development of a successful *M. bovis* vaccine (Maunsell *et al*., 2011).

2.15 Control and Prevention of *Mycoplasma bovis*

2.15.1 Biosecurity for *Mycoplasma bovis*

The best way to prevent *Mycoplasma bovis* infections is probably to maintain a closed herd or, if that is not possible, to screen and quarantine purchased animals. Mycoplasma biosecurity practices targeted to the individual operation should be developed. 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. When purchasing lactating cows, milk samples should be submitted for mycoplasma detection (culture, PCR, or sELISA), 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).

2.15.2 Management of mycoplasmal mastitis

Monitoring programmes to detect *Mycoplasma bovis* should be in place in herds that are attempting to remain mycoplasma- free, as well as in herds managing a mycoplasma problem. Herd level detection of *M. bovis* mastitis is usually achieved by testing of bulk tank milk by culture, PCR, or sELISA (Gonzalez and Wilson, 2002; Gonzalez and Wilson, 2003). Bulk tank testing should be performed at least monthly, with more frequent sampling indicated for large herds, herds undergoing expansion, or when managing a mycoplasma problem. Sampling of clinical mastitis cases, high somatic cell count cows, and cows and heifers (especially new purchases) at calving is also important. Whole herd sampling is sometimes used when attempting to eliminate *M. bovis*, but low-test sensitivity means that repeated sampling is required. Mastitis records, including response to treatment, should be monitored (Maunsell *et al*., 2011).

The approach to management of *M. bovis* mastitis needs to be tailored to each operation and can range from culling of all *M. bovis*-infected cows to only culling cows with chronic clinical mastitis. *Mycoplasma bovis* mastitis can be eliminated from dairy herds through aggressive surveillance and culling of infected cows (Gonzalez and Wilson, 2003), and where a closed herd can be maintained and a small proportion of cows are infected this could be feasible. Conversely, for expansion of herds or dairies where a large proportion of the lactating herd is infected, eradication of *M. bovis* might not be appropriate or economical. However, it should be emphasized that the worst outbreaks of clinical mycoplasma disease

observed by some of the authors have occurred after mycoplasma mastitis was detected and not eliminated. Attempted elimination of all adult cows with intramammary mycoplasma infections remains the strongly recommended course of action. Economic analyses of the various approaches for managing mycoplasma mastitis in today's large dairy herds are critically needed to help guide veterinarians in recommending the most appropriate strategy (Byrne *et al*., 1998; Maunsell *et al*., 2011).

In herds where the ultimate goal is eradication of *M. bovis* but not all infected cows can be immediately culled, strict segregation of infected cows has been used effectively to limit new infections (Gonzalez and Wilson, 2003). As transmission might occur via routes other than the udder, cows should be segregated at all times, not just in the milking parlor. Strict milking parlour hygiene is recommended to reduce udder-to-udder transmission of *M. bovis* (Gonzalez and Wilson, 2003). Cows with clinical mycoplasma mastitis should be culled. As discussed earlier, *M. bovis* can survive well in some bedding substrates. Ideally, bedding found to be mycoplasma positive, usually recycled bedding processed on the farm, should not be used to bed dairy animals of any age (Maunsell *et al*., 2011).

2.15.3 Management of *Mycoplasma bovis* in calves

Surveillance for *M. bovis* in calf facilities should include monitoring of health records and the submission of appropriate samples from suspected cases for diagnostic testing. For the control of *M. bovis* infections in calves, general infectious disease control principles based on reducing exposure and maximizing host defenses can be used. *Mycoplasma bovis* exposure via infected milk can be eliminated by pasteurization or by feeding milk replacer. Batch pasteurization of milk (Butler *et al*., 2000) at 65^{o}C for 10 minute or 70^{o}C for 3 minutes or high-temperature (72^{o}C) short-time (flash) pasteurization will inactivate *M. bovis* (Stabel *et*

al., 2004). Other potential routes of exposure of calves to *M. bovis* include colostrums and respiratory secretions of infected animals. Exposure to infected colostrum could be reduced by pasteurization, by not pooling colostrum, and by not feeding colostrum from cows known to be infected with *M. bovis*. Exposure to airborne *M. bovis* could be reduced by good ventilation and low-stocking density. Calves with clinical mycoplasma disease shed very large numbers of organisms (Pfutzner and Sachse, 1996), and moving sick calves to a separate hospital area might reduce transmission in calf facilities. All-in all-out practices or segregation of age groups might also limit transmission of *M. bovis* in multiage facilities. Removing fence-line contact with other cattle and limiting the time the calf spends in the maternity area will also reduce the potential for exposure. Proper sanitization of buckets, housing, and other equipment between uses, wearing gloves, and handling sick calves last could reduce formite-mediated transmission. Although *M. bovis* survives surprisingly well in the environment, it is highly susceptible to heat and to most commonly used chlorine-, chlorhexidine-, acid-, or iodine-based disinfectants. Addressing nonspecific factors related to respiratory health such as air quality, colostrum management, and nutrition could also help limit the impact of *M. bovis*-related disease. Appropriate vaccination and control programmes should be in place for respiratory viruses, as controlling other pathogens could decrease the risk of *M. bovis* coinfections (Maunsell *et al*., 2011).

2.15.4 Management of *Mycoplasma bovis* in stocker and feeder cattle

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. Strategic antibiotic treatment of high-risk animals on arrival or during an outbreak of BRD might be useful in reducing the incidence of mycoplasma disease. Segregating affected cattle and keeping the hospital pen separate from new arrivals

could reduce exposure of high-risk animals to *M. bovis*. Using appropriate hygiene measures for handling sick cattle (use separate equipment or personnel or clean equipment among animals, feed last) could reduce the chances of formite-mediated *M. bovis* transmission (Maunsell *et al*., 2011).

CHAPTER THREE

MATERIALS AND METHODS

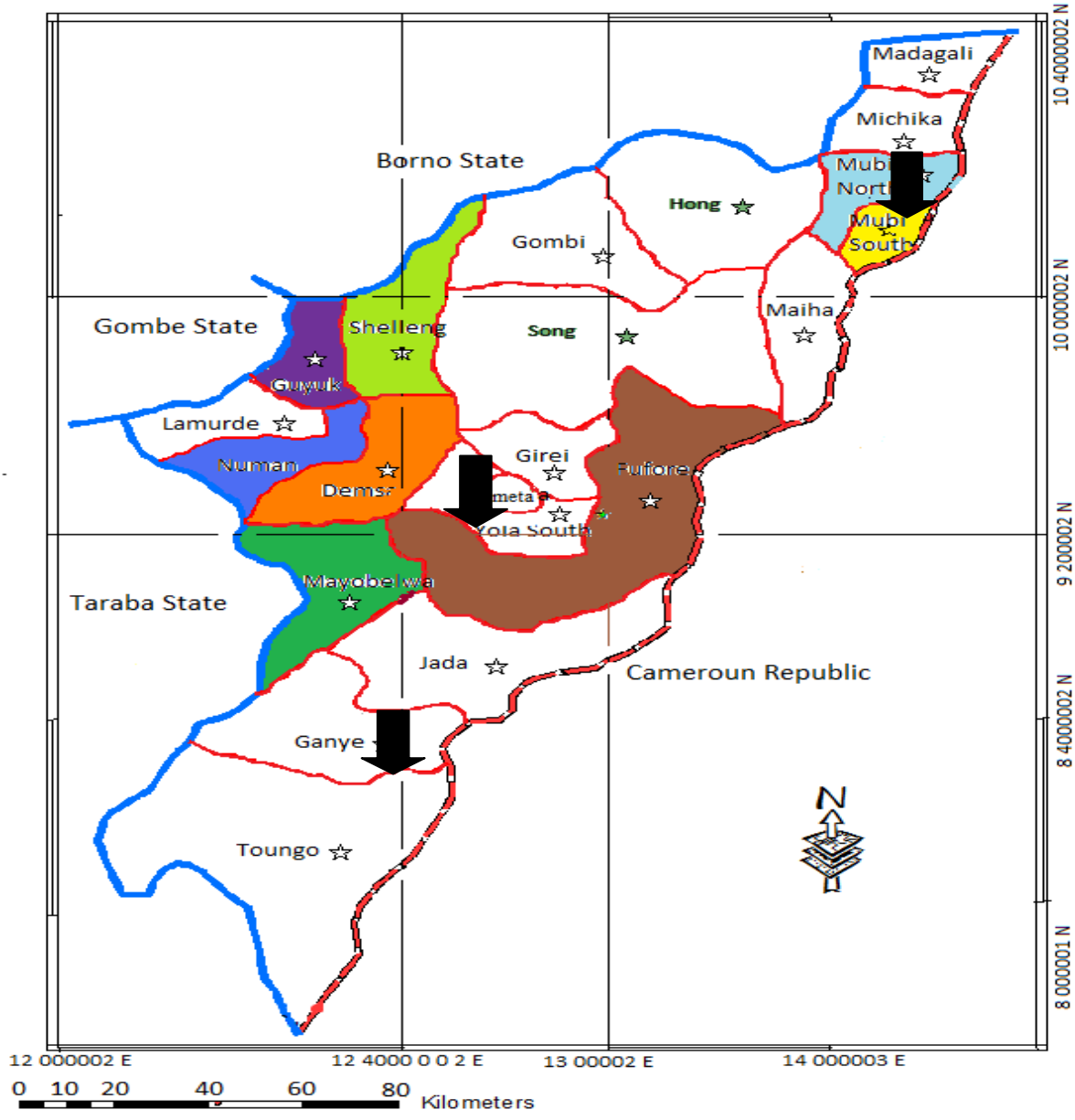
3.1 Study Area

Adamawa State is located at the North Eastern part of Nigeria. It lies between latitude 7° and 110°N and between Longitude 11° and 140°E. It shares boundary with Taraba State in the South and West, Gombe State in its North-West and Borno State to the North. The State has an international boundary with the Cameroon Republic along its eastern side. It has a land area of about 38,741 km^2 (Adebayo, 1999). The State is divided into 21 Local Government Areas (Figure 3.1). Adamawa State has a 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 Northwest to 1600 mm in the extreme southern part of the State (Adebayo, 1999). The State has 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 State has an estimated cattle population of 2.8 million (Anonymous, 1994) made up of White Fulani (Bunaji), Red Bororo (Rahaji), Adamawa Gudali (Bokoloji) breeds and their crosses, which constitutes 88% of cattle breeds in Nigeria (Ngere, 1983). The cattle used in this study were under extensive management system.

3.2 Study Design

One Local Government Area from each of the three Senatorial zones of the State (Yola, Mubi and Ganye) was used for the study (Figure 3.1). Abattoirs in each of the study area were used

for sampling of suspected pneumonic lung specimens. Fulani herds were selected based on their willingness to cooperate for the blood sample collection.



NB: = where the samples are collected in Adamawa State

Figure 3.1: Map of Adamawa State showing international, interstate boundaries and sample sites (Adamawa State Ministry of Land and Survey, 2010)

3.3 Sample Size

Sample size was calculated using the formula of Araoye (2004)

$$n = \frac{Z^2 pq}{d^2}$$

Where

n = sample size for the study

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

p = expected prevalence of 66% (Tambuwal *et al*., 2011)

q = 1- p

d = desired absolute precision (5% or 0.05)

Therefore,

$$n = \frac{(1.96)^2 \times 0.66 \times (1-0.66)}{(0.05)^2} = 345$$

However, for effective investigation, the figure was jack up to four hundred (400) to increase precision.

For the lung samples, one hundred (100) suspected pneumonic lungs were collected (50 samples was collected from Yola abattoir, 30 from Mubi and 20 from Ganye, sample distribution was based on the number of cattle slaughtered in each of these abatoirs daily.

3.4 Sampling Procedure

The abattoir was visited twice weekly, on every other day, in each of the senatorial zones and lung samples were collected for a period of four (4) months (January- April 2013). The samples were collected from animals with suspected pneumonia. The samples were excised from lungs with areas that have normal and consolidation using sterile scalpels; and then minced using scissors after homogenization with phosphate buffered saline (PBS).

The lung samples were collected into sample bottles containing modified Hayflick's broth (Medium-B broth) with phenol red and glucose (Erno and Stipkovits, 1973). The samples were kept on ice packs containers and transported to the Mycoplasma laboratory of National Veterinary Research Institute Vom and stored at -20°C until used.

Cattle in the pastoral herds in each LGA were selected with the help of Local Government Staffs in the Veterinary Unit using systematic random sampling method. Each animal was properly restrained and its age (using dentition), sex and breed were recorded before sample was collected. Six (6) ml of blood was aseptically collected from the jugular vein of the animal, using a sterile 10 ml syringe and 18G hypodermic needle. The blood was dispensed into sample bottles free from anticoagulant and kept in a slanting position and allowed to

coagulate and for serum to be harvested. The serum samples obtained was decanted into serum vials and stored at -20^{o}C until use.

3.5 Laboratory Examination

3.5.1 Microbiological culture and identification

The samples were processed according to the method described by Thiaucourt *et al*., (1992). The samples are kept on the bench for 10 minutes and allowed to thaw. Then a four serial ten-fold dilution from 10^{-1} to 10^{-4} in modified Hayflick's broth containing pleuropneumonia-like organism (PPLO) broth (Difco) 2.1 g, 20% decomplemented horse serum, 10% fresh yeast extract, 0.2% glucose, 0.4% sodium pyruvate and 0.04% ampicillin, 1% phenol red 0.2ml and deionized water 70ml was carried out. One hundred and eighty microliters (180µL) of broth were dispensed into wells of microtitre plate (see details on the preparation of agar and broth as attached Appendix I). Twenty (20) microlitre of broth containing the homogenized lung tissue was taken and added into a well containing 180µL broth medium and mixed thoroughly. Again, 20 µL of broth was taken from each well and mixed into the next broth medium well (180 µL) for each sample. The last dilution was inoculated on to solid Hayflick's medium made by solidifying the broth media by adding 1% agar noble (Difco). The broth cultures were incubated at 37°C in aerobic conditions and examined within 3- 5 days for turbidity and pH change and those with growth, indicated by turbidity, were subcultured onto agar plates from the lowest-dilution tube that showed turbidity.

The plates were incubated at 37°C in 5% carbon dioxide and examined for *Mycoplasma*- like colonies ("fried egg" colony) every 3- 5 days using Stereomicroscope [h33 hund WETZLAR 195-040439 (X35)]. Visible single colonies were triple cloned to obtain a pure growth.

Both broth and agar media were considered negative if no *Mycoplasma*- like colony was seen after 14- 20 days before discarding any culture. The isolated colonies were inoculated into stock suspension (50% broth/50% horse serum) and stored frozen at –20°C.

Series of Biochemical tests that included glucose fermentation, arginine hydrolysis, phosphatase test, reduction of 2, 3, 5, triphenyltetrazolium hydrochloride and spot and film test were performed on the isolates for identification of *Mycoplasma bovis* as described by Poveda (1998) (attached Appendix II).

3.5.2 Enzyme-linked immunosorbent assay (ELISA) test

Serum samples were examined with BIO-X *Mycoplasma bovis* antibody ELISA Kit (Bio-X Diagnostics, Jemelle- Belgium).

3.5.2.1 Principle of the test

The test uses 96-well microtitration plates sensitised by a recombinant protein from *Mycoplasma bovis* expressed by *E. coli.* A gene from *M. bovis* is expressed by this recombinant *E. coli* culture. The entire surface of each microplate has been sensitised with recombinant from *M. bovis.*

The test blood sera were diluted in the dilution buffer. The platewas incubated and washed, then the conjugate, protein G peroxidase-labelled, is added to the wells. The plate was incubated a second time at 21^{o}C +/- 3. After the second incubation, the plate was washed again and the chromagen (tetramethylbenzidine) added. The chromagen has the advantages of being more sensitive than the other peroxidase chromagens and not being carcinogenic. If specific *Mycoplasma bovis* immunoglobulins are present in the test sera, the conjugate remains bound to the microwell that contains the bacterial recombinant antigen and the

enzyme catalyses the transformation of the colorless chromagen into a pigmented compound. The intensity of the resulting blue color is proportionate to the titre of specific antibody in the sample.

3.5.2.2 Procedure of the test

The test was carried out according to the Manufacturer's instruction as follows;

- ✓ All the reagents before use were brought to a room temperature.
- ✓ 1 mL aliquots of the dilution buffer were prepared in 5 or 10 mL hemolysis tubes. 10µL serum samples were added in each tube (dilution 1/100) and shaken briefly on mechanical agitator.
- ✓ Positive and negative sera were diluted 1/100 in a dilution buffer.
- ✓ Sera samples and the positive and negative sera were distributed (100µL/well). One well per sample. The plate was incubated at room temperature for one hour.
- ✓ Then the plates were rinsed with the washing solution, emptying the contents by flipping it over sharply above a sink. The washing step was repeated two more times.
- ✓ The conjugate was diluted 1:50 in the dilution buffer. 100µL of the conjugate solution was added to each well and incubated for one hour at room temperature.
- ✓ The plates were washed as described before.
- ✓ After washing, 100µL of the chromagen solution was added in each well on the plate.
- ✓ The plates were incubated for 10 minutes at room temperature.

- ✓ Then 50µL of stop solution was added to each microwell.
- ✓ The optical densities (OD) at 450nm in the microwell were read with the aid of APPAR Plate Reader (UniEquip GmbH®, Martinsried, Munich, Germany).

The test can be validated only if the difference between the optical density readings of the positive control serum and negative control serum (OD positive serum- OD negative serum) at ten minutes is greater than 0.7000 and the negative serum yields an optical density that is lower than 0.400.

The optical density (OD) was calculated from the measured OD values of the samples and the negative and positive serum samples using the following formula;

$$Sample's\ Coeff. = \frac{\text{Sample OD} - \text{Negative serum OD}}{\text{Positive serum OD} - \text{Negative serum OD}} \times 100$$

A sample is considered negative if its coefficient is less than 37% and positive if greater than or equal to 37%.

3.6 Statistical Analysis

The results obtained were presented as tables and chart. Chi-square was used to test the level of significance with an aid of the statistical products for savings and solutions (SPSS) version 17.0. (SPSS Inc., Chicago, IL, USA) and values of $p < 0.05$ were considered significant.

CHAPTER FOUR

RESULTS

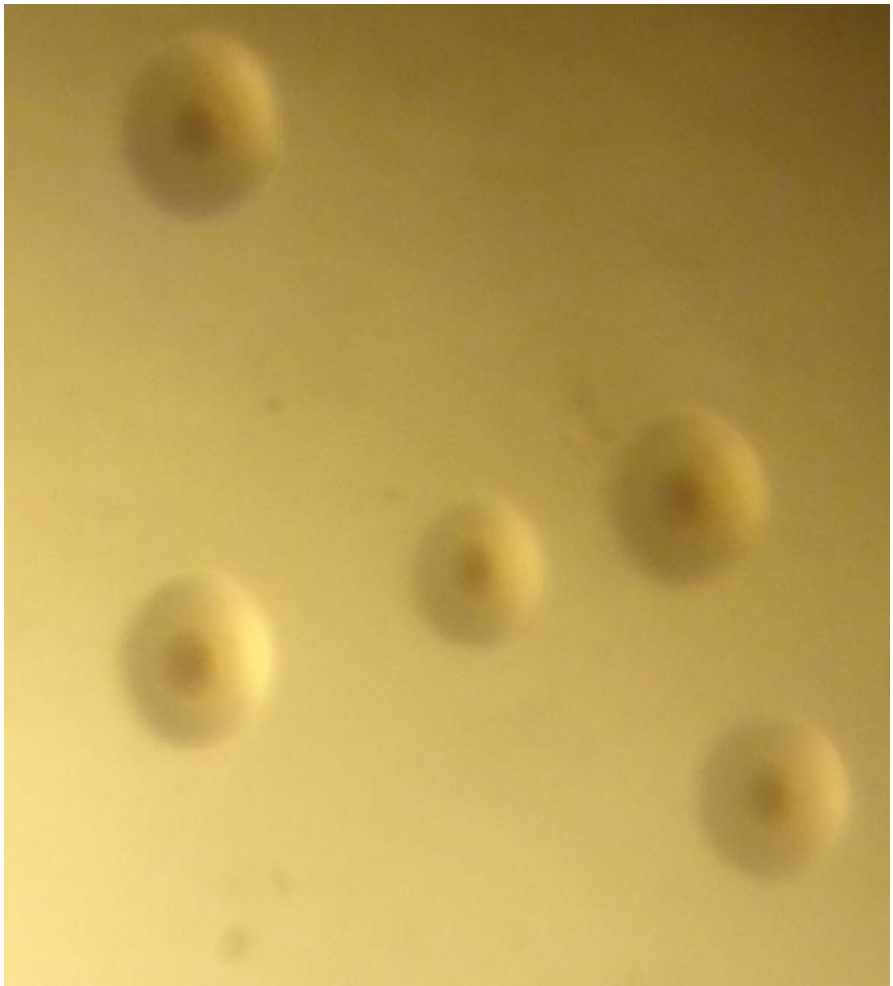
4.1 Results of Mycoplasma Isolation

Three (3.0%) out of the 100 suspected pneumonic lung samples collected yielded growth on mycoplasma agar were from Ganye 1 (5.0%) and Yola 2 (4.0%) with no growth observed in Mubi (Table 4.1). The growths observed were small in size, circular, convex with raised central part darker than the periphery, giving a 'fried-egg' appearance (Plate I and II).

Biochemical identification of the *Mycoplasma* isolates indicated that two (2.0%) of the isolates were observed to reduce tetrazolium chloride and had phosphatase activity. They did not hydrolyze glucose nor catabolise arginine and produce spot and film on the surface of the agar medium and were conclusively identified as *Mycoplasma bovis* (Plate II). The other isolate was observed to hydrolyze glucose and also reduce tetrazolium chloride and did not have phosphatase activity nor catabolise arginine and was suspected to be a *Mycoplasma* species other than *M. bovis* (Table 4.2). The results of the biochemical test are shown on Plate III.

Table 4.1: Isolation rate of *Mycoplasma* species from bovine lung samples from three LGAs of Adamawa State

LGA	Examined samples	No. of isolates	Percentage (%)
Ganye	20	1	5.00
Mubi	30	0	0.00
Yola	50	2	4.00
TOTAL	**100**	**3**	**3.00**



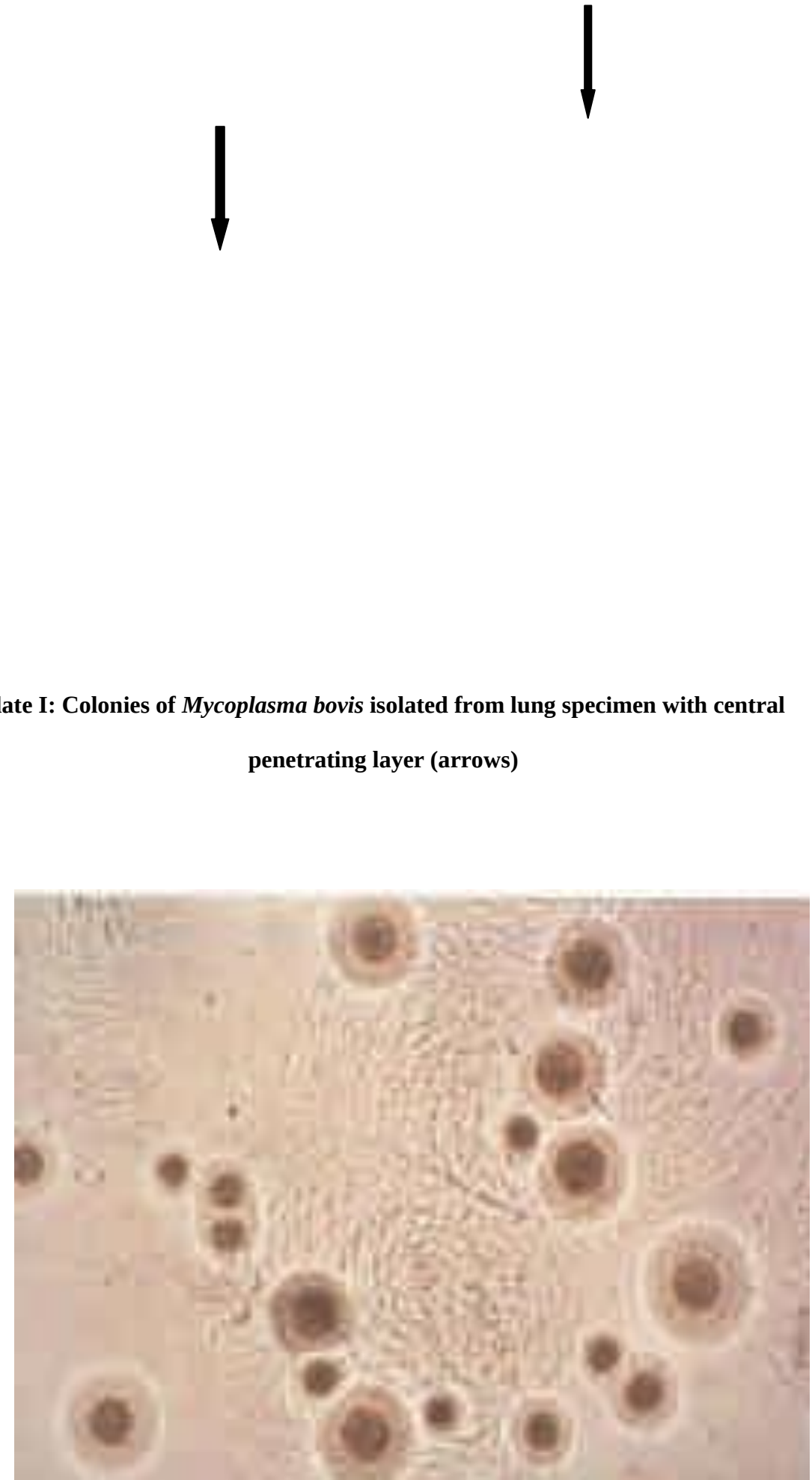


Plate I: Colonies of *Mycoplasma bovis* isolated from lung specimen with central penetrating layer (arrows)

Plate II: Typical *Mycoplasma bovis* colonies isolated from specimens with typical film and spots (arrows) on the medium

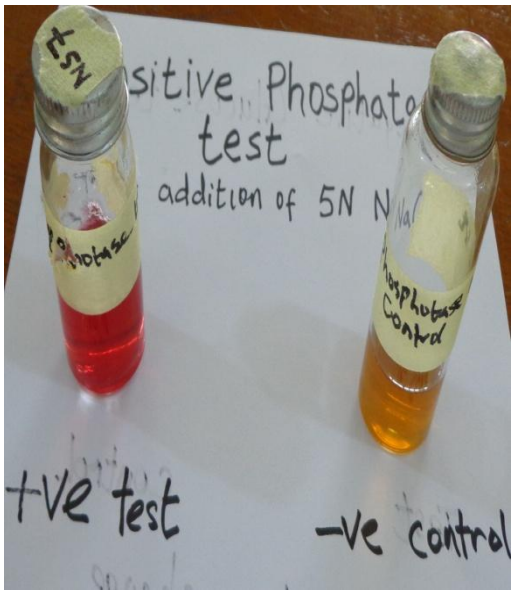
Table 4.2: Biochemical characteristics of bovine mycoplasmas isolated from cattle in Adamawa State

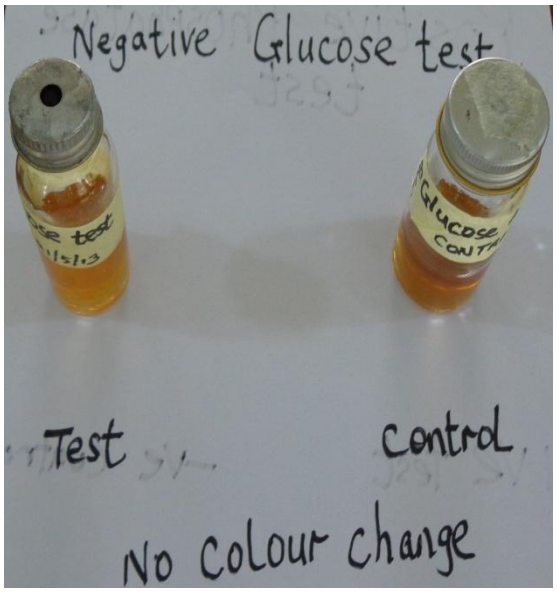
Sample	Number examined	Number positive recovered	GF	PT	AU	TR	Remarks
Lungs	100	2 (2%)	-	+	-	+	*Mycoplasma bovis* (2%)
		1 (1%)	+	-	-	+	*Mycoplasma* spp (1%)

Key:

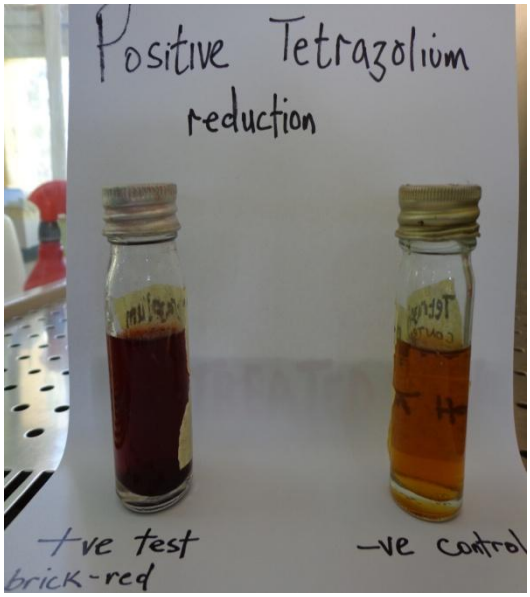
GF: glucose fermentation **PT**: phosphatase test

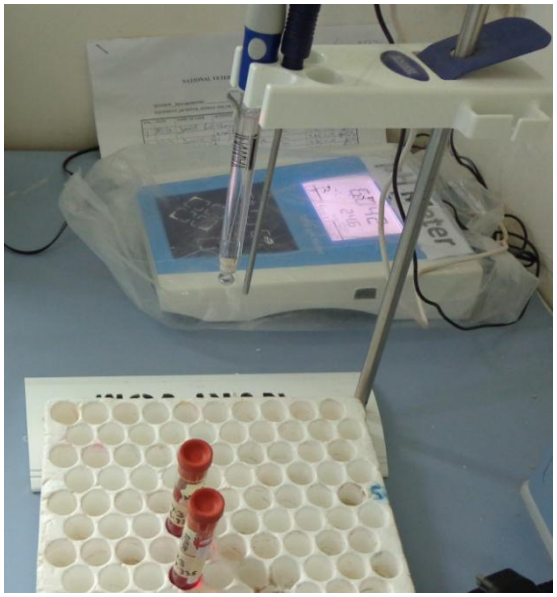
AU: arginine utilisation **TR**: tetrazolium reduction





a **b**





c **d**

Plates III: Biochemical tests results after 7 days of incubation

a: Phosphatase (+ve) b: Glucose (–ve) c: Teterazolium aerobic (+ve)

d: Arginine; initial adjusted pH 7.3; final pH 7.335 (-ve)

4.2 Detection of Antibodies

A total of four hundred (400) serum samples from cattle in the three Local Government Areas in Adamawa State were examined using BIO K260 ELISA kits [(BIO-X Diagnostics, Belgium) for *Mycoplasma bovis* antibodies. The results obtained showed that the positive and negative controls had an optical density (OD) of 1.473 and 0.072 (averaged) respectively (Plate IV).

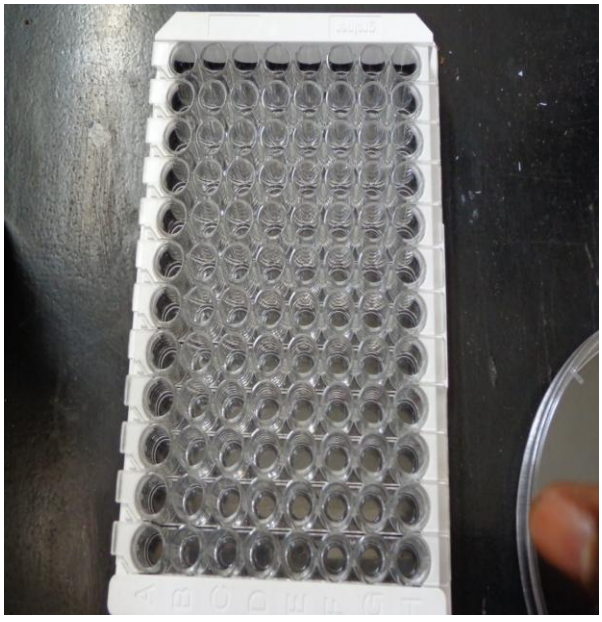
Seventy-eight (19.5%) out of the 400 serum samples examined were seropositive for *M. bovis* (Table 4.3 and Figure 4.1). Out of these 36 (27.7%), 28 (20.0%) and 14 (10.8%) were from Ganye LGA, Yola LGA and Mubi LGA respectively. From the results the highest

seroprevalence was found in Ganye (27.7%), followed by Yola (20.0%) and least in Mubi (10.8%) LGAs which shows a statistical significant difference ($p=0.003$; $p<0.05$).

The ages of the cattle examined ranged between less than 1 and more than 5 years. Seroprevalence in different age groups varied from 2.9 to 25.8% (Table 4.4 and Figure 4.2). Cattle less than one-year-old had the highest seroprevalence of 25.8% (8/31), followed by 4-year old cattle with 22.4% (22/98) and lowest in 1-year old cattle with 2.9% (1/35), although there are no statistically significant differences among the age groups ($p=0.690$; $p>0.05$).

Sex-specific prevalence shows that females had the highest seroprevalence of 20.4% (58/285) than the males 17.4% (20/115) (Table 4.5 and Figure 4.3), although the difference was not statistically significant ($p=0.499$; $p>0.05$).

Seroprevalence of *Mycoplasma bovis* varied in different breeds of cattle examined, ranging from 11.9 to 21.8% (Table 4.6 and Figure 4.4). The highest seroprevalence was found in White Fulani with 21.8% with(33/243), followed by Cross breeds with 18.5% (10/54) and lowest in Adamawa Gudali with 11.9% (5/42). No relationship was observed between the seroprevalence and breed ($p=0.427$; $p>0.05$).



a. ELISA plate before reading b. ELISA plate after reading

Plate IV: ELISA plates before and after reading

Table 4.3: Seroprevalence of *Mycoplasma bovis* in Cattle in three Local Government Areas of Adamawa State using ELISA.

L. G. A	Number examined	Number positive (Prevalence%)
Yola	140	28 (20.0)
Mubi	130	14 (10.8)
Ganye	130	36 (27.7)
Total	**400**	**78 (19.5)**

X^2=11.893 df=2 p=0.003 p<0.05

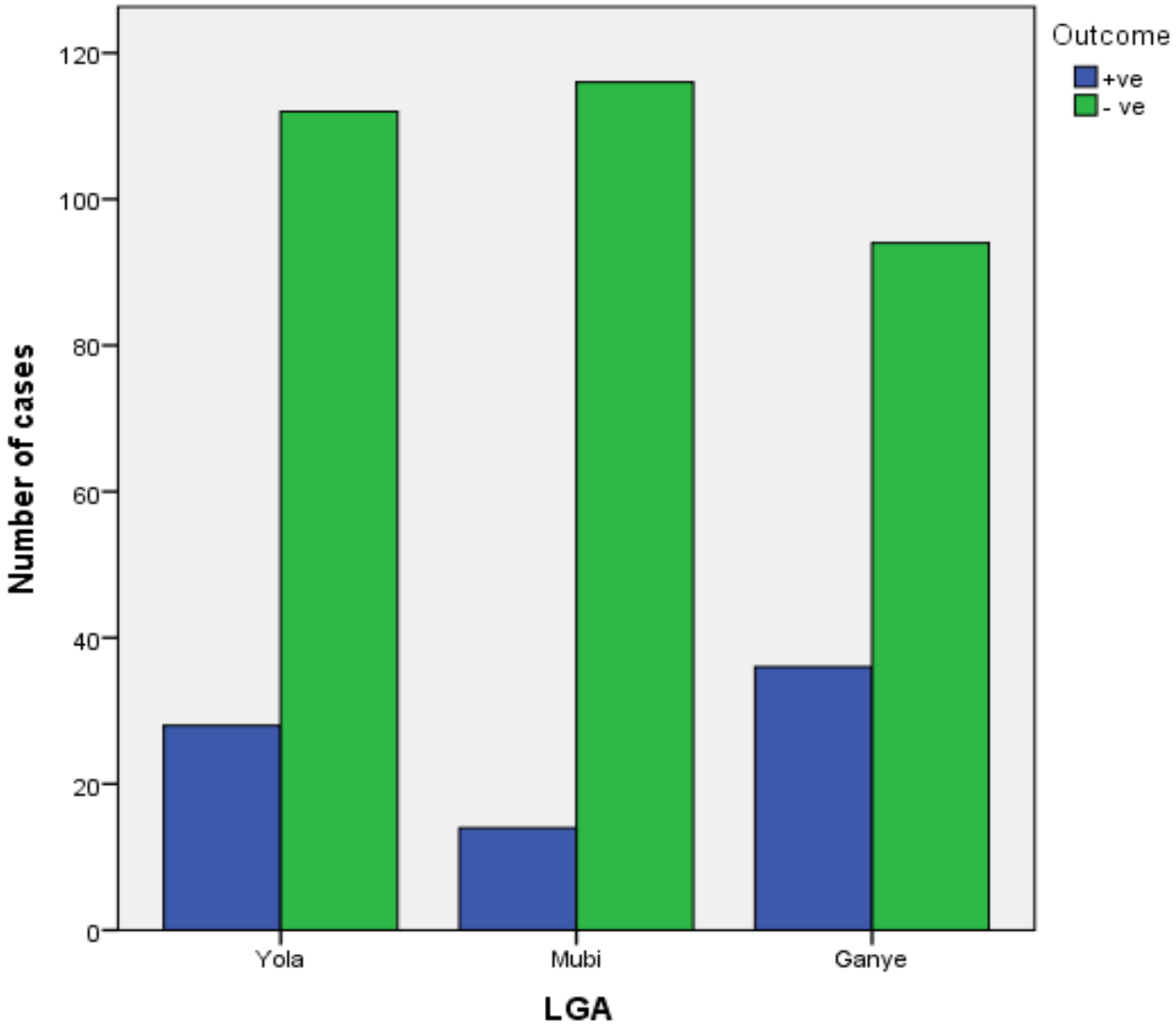


Figure: 4.1 Seroprevalence of *Mycoplasma bovis* in cattle in three Local Government Area of Adamawa State

Table 4.4: Age specific seroprevalence of *Mycoplasma bovis* in Cattle in three Local Government Areas of Adamawa State.

Age in years	Number examined	Number positive (Prevalence%)
<1	31	8 (25.8)
1 - <2	35	1 (2.9)
2 - <3	100	21 (21.0)
3 - <4	93	19 (20.4)
4 - <5	98	22 (22.4)
≥5	43	7 (16.3)

X^2=0.159 df=1 p=0.690 p>0.05

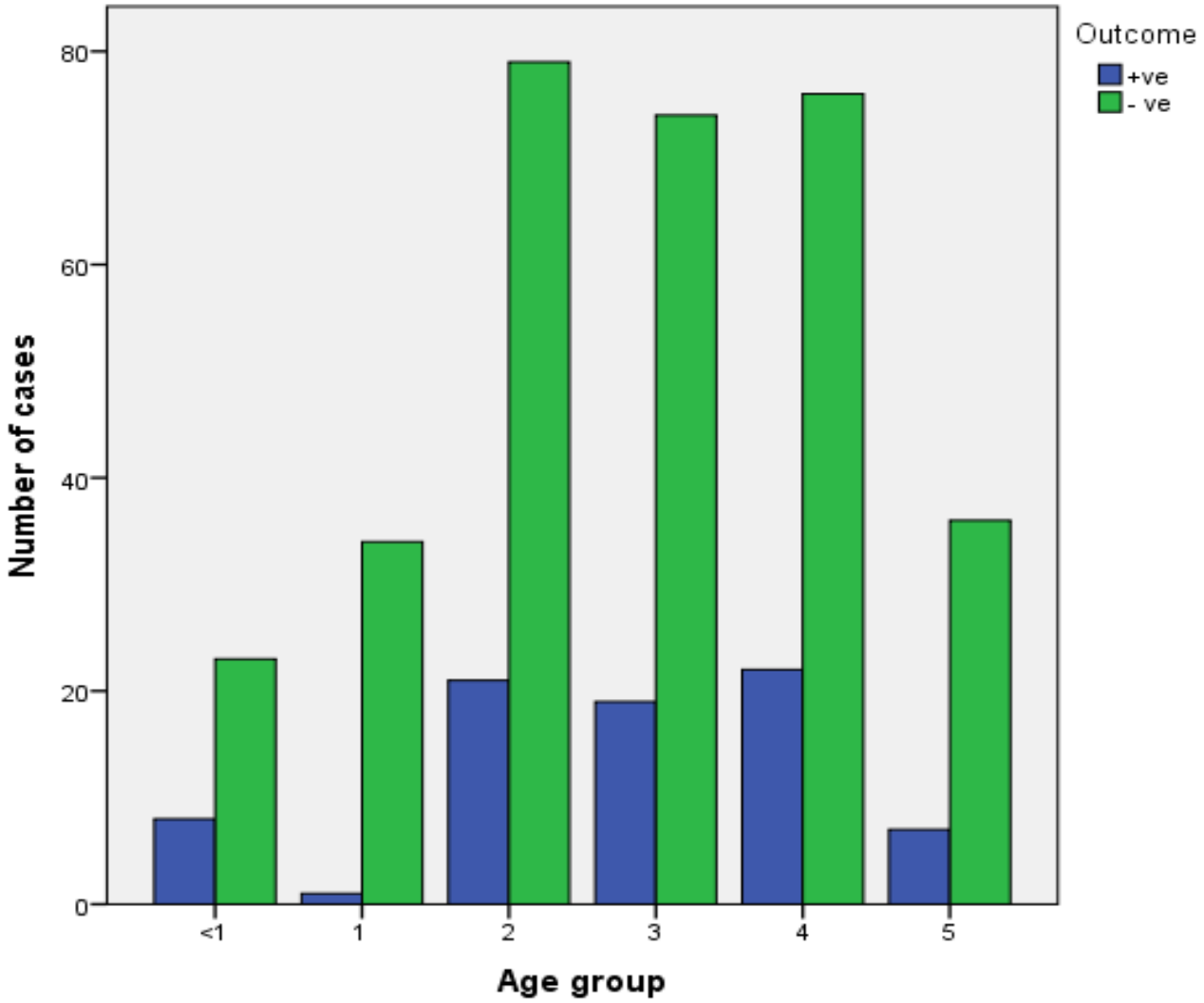
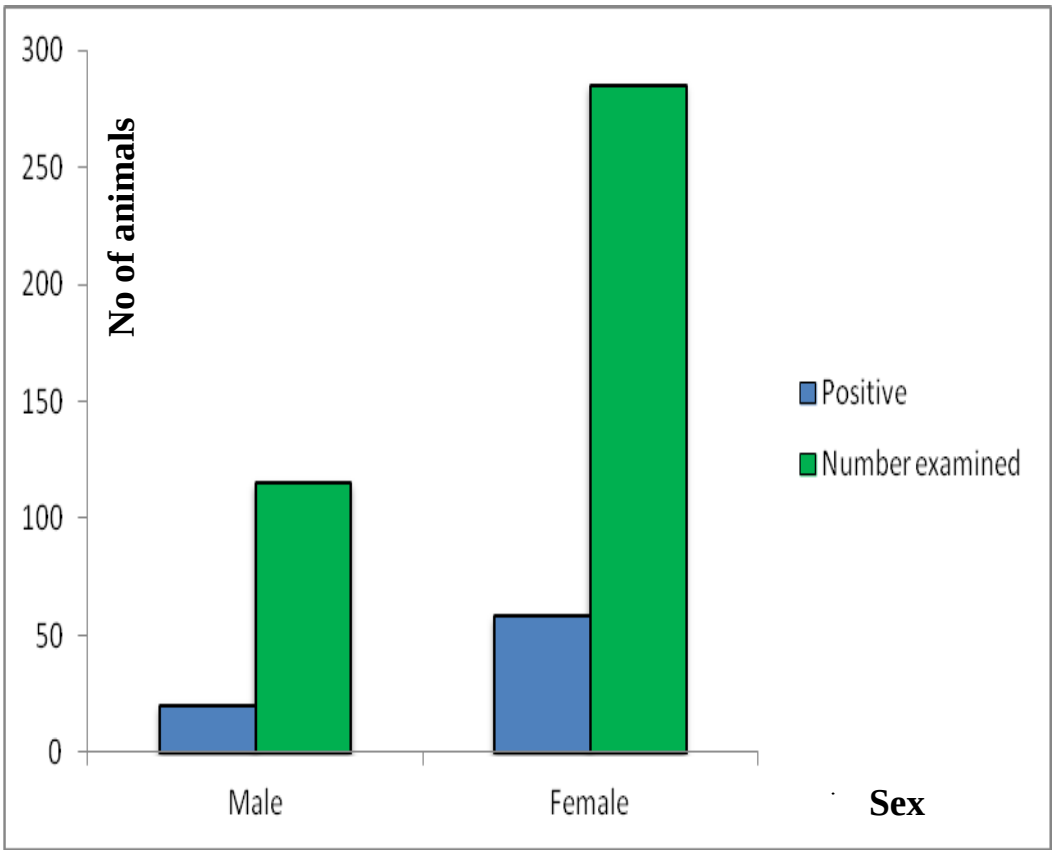


Figure 4.2: Seroprevalence of *Mycoplasma bovis* in cattle based on age in three Local Government Areas of Adamawa State

Table 4.5: Sex specific Seroprevalence of *Mycoplasma bovis* in Cattle in three Local Government Areas of Adamawa State

Sex	Number examined	Number positive (Prevalence)
Male	115	20 (17.4)
Female	285	58 (20.4)

X^2=0.457 df=1 p=0.499 p>0.05



X^2=0.457 df=1 p=0.499 p>0.05

Figure 4.3: Sex specific seroprevalence of *Mycoplasma bovis* in Cattle in three Local Government Areas of Adamawa State.

Table 4.6: Breed distribution of antibodies to *Mycoplasma bovis* in Cattle in three Local Government Areas of Adamawa State.

Breed	Number examined	Number positive (Prevalence %)
White Fulani (Bunaji)	243	53 (21.8)
Sokoto Gudali (Bokoloji)	42	5 (11.9)
Red Bororo (Rahaji)	61	10 (16.4)
Cross	54	10 (18.5)

X^2=2.778 df=3 p=0.427 p>0.05

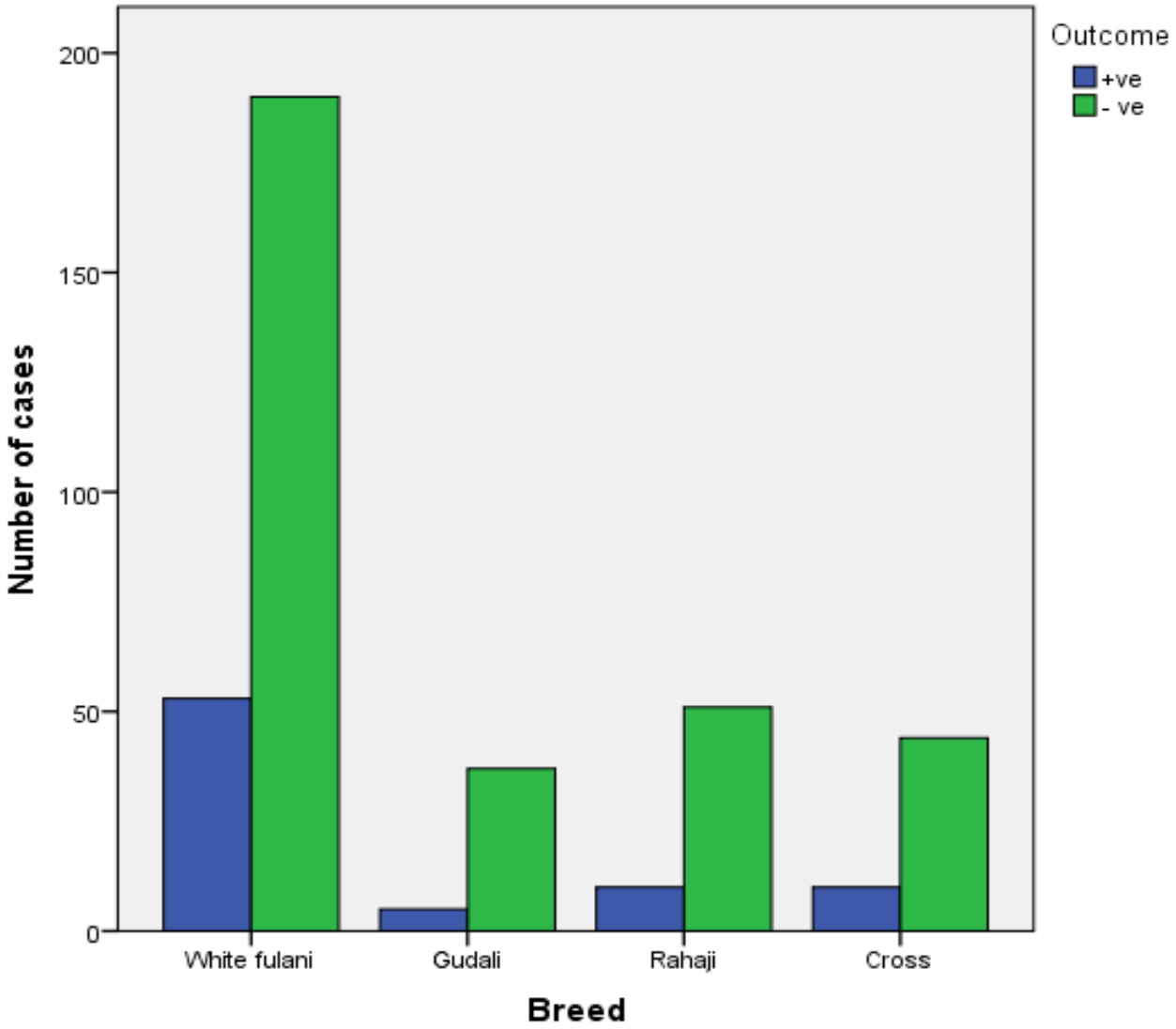


Figure 4.4: Seroprevalence of *Mycoplasma bovis* in cattle based on breed in three Local Government Areas of Adamawa State

CHAPTER FIVE

DISCUSSIONS

The result of the study on isolation has shown that *Mycoplasma bovis (M. bovis)* was present in cattle in the selected Local Government Areas of Adamawa State with an isolation rate of 2.0% (2/100). The isolation rate of 2.0% for *M. bovis* recorded in this study is of clinical importance because these infected cattle could be a point source of infection to many naive herds, farms and areas. Similarly, the isolation of *M. bovis* organism from pneumonic lungs of cattle originating from Adamawa State evidently shows that *M. bovis* related infections are still very active in the area. Similar observations were made in Sokoto, Kebbi, Kaduna and Maiduguri (Egwu *et al*., 1996; Ajuwape *et al*., 2003; Tambuwal, 2009).

The presence of *Mycoplasma bovis* infection in the State and its recovery in pneumonic cattle might have been due to introduction of carrier animals as a replacement or through contact with infected animals in common drinking points, night shades or natural infection as earlier observed by Nicholas, (2004). As commonly pointed by the author, the introduction of *M. bovis* in new areas is always linked to cattle movement.

This result however is lower than the earlier report of Ajuwape *et al*., (2003) who reported a prevalence of 23.0% in pneumonic lungs collected from cattle at abattoirs in Maiduguri, Kaduna, Sokoto and Ibadan. The low isolation rate recorded is as result of inadequate sample storage and lapse of time between sample collection and laboratory examination. *M. bovis* isolation has been reported to be higher if samples arrived laboratory and are processed immediately within 2 to 3 hours of collection (Nicholas and Ayling, 2003). The result is also lower than findings in other countries such as the Netherland 20.0% (Ter Laak *et al*., 1993), France 30.0% (Le Grand *et al*., 2001), in Israel 26 to 54.0% (Nicholas and Ayling, 2003), Turkey 23.0% (Karahan *et al*., 2010), and in Lithuania 14.3% (Gabinaitiene *et al*., 2011).

This could be because all authors sampled from pneumonic lungs. This may be attributed to the fact that they sampled during an outbreak of *M. bovis* infection in the farms. This difference may also be due to management practices. A number of researchers reported that *M. bovis* is an important agent of pneumonia and clinical mastitis in the USA, Australia and Europe and infection among the cows was influenced by management practices (Dahlia *et al*., 2011). The finding in this study is lower due to the age of cattle sampled. Since *M. bovis* is more prevalent in calves thereby causing calf pneumonia and calves can become infected by drinking milk from cows that have mastitis (Tenk, 2005). The findings of this study are in agreement with the earlier report of Marouf *et al*., (2011) in Egypt who also examined the respiratory tract (lungs) of cattle and buffalos and reported a prevalence of 1.7% and 2.7%, respectively. This study established the occurrence of *M. bovis* as an important pathogenic agent responsible for severe pneumonia in cattle. The organism is being reported for the first time in Adamawa State.

The findings from the seroprevalence indicate that *Mycoplasma bovis* antibodies were detected in 78 (19.5%) of the 400 cattle examined. The overall seroprevalence was lower than the earlier report of Tambuwal *et al*., (2011) who reported a prevalence of 66%. The cattle sampled in this study are extensively managed (free range), a practice which predisposes them to nutritional deficiencies and diseases leading to immunosuppression and vulnerability to infection. The lower seroprevalence reported in this study can be attributed to inadequate storage of samples and distance in transporting the sample. This probably explains the low rate of *M. bovis* infection recorded. The finding in this study is in agreement with the previous report of Byrne *et al*., (2000) in Ireland and Ayling *et al*., (2004) in Great Britain who reported a prevalence of 13- 23% and 22%, respectively, eventhough cattle sampled by both authors were intensively managed. The seroprevalence obtained is higher than the report

of Fu *et al*., (2011) and Zhao *et al*., (2012) both in China who reported a prevalalence of 7.69% and 5.95%, respectively. This may be attributed to samples from different region and adequate management and biosecurity practices.

The seroprevalence of *M. bovis* varied in different age groups (2.9 to 25.8%), with cattle of less than 1 year old having the highest seroprevalence of 25.8% followed by cattle of 4 years old (22.4%). This agreed with the earlier report of Zhao *et al*., (2012) who also reported highest seroprevalence in dairy cattle of less than 1 year old with no statistical difference between the age groups. The varied seroprevalence in different age groups suggests the possibility of horizontal transmission. The highest *M. bovis* seroprevalence in calves less than 1 year old may be due to ingestion of infected milk which is an important means of *M. bovis* transmission. Cows can become intermittent shedders of *M. bovis* as it has been shown that as many as 40% of cows can shed <100 cfu/ml *M. bovis* in the milk (Biddle *et al*., 2003), since contaminated milk could be the source of infection to young calves. Higher *M. bovis* seroprevalence in cattle of 4 years old may increase the risk of milk contamination with *M. bovis*. This is because cattle 3 to 4 years old cattle are the bedrock of herds that are of breeding age. They are in their first or second stage of gestation (calving), they have potential of the spread of *M. bovis* should they habour the organism subclinically or chronically. The milking system of herdsmen also has a potential for contaminating udders as hand milking encourages mastitis.Therefore, carrier cows are the most likely source for the infection for naive calves in herds.

From this study, female cattle have higher prevalence (20.4%) in comparison with the males (17.4%) eventhough there has not been any statistical significant difference. This agreed with the earlier report of Tambuwal *et al*., (2011) who reported more females showing antibodies to *M. bovis* than males. This is because more females are kept in a herd in comparism to the

males for the purpose of reproduction and milk production. The fact that female cattle predominates pastoral herds means that any maintainance of *M. bovis* in such hers would be done by these female cattle. They can play important roles in the spread/epidemiology of *M. bovis* because they remained for longer periods in herds, pass through more stress of reproduction, calving and nursing, have greater chance of coming in contact with contaminated water, pasture, formites and environment. For this reason, they can transmit the infection from one generation to another.

Breed distribution showed White Fulani (21.8%), Red Bororo (16.4%), Adamawa Gudali (11.9%), and Cross breed (18.5) eventhough there is no statistical significant difference. The higher prevalence observed in White Fulani suggests more of this breed was sampled thus the reason for the breed predisposition. *Mycoplasma bovis* infection had not been reported to have breed specificity. Therefore, all cattle irrespective of the breed had equal chance of contracting the infection.

The presence of *M. bovis* infection as immunomodulator and/or co-factor may have an important role in the development of contagious bovine pleuropneumonia (CBPP) due to *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm*SC), being very much endemic in Nigeria (Egwu *et al*., 2001). Certain similarity in pathogenesis of *M. bovis* infection (Stipkovits *et al*., 2001) with that of *Mmm*SC infection may contribute to inefficiency of vaccination against CBPP of cattle in the study area. Thus, the presence of *M. bovis* in these animals might account for the endemicity of CBPP in Nigeria especially that no vaccination campaigns are vigorously pursued in Nigeria.

The result obtained from this study may indicate the importance of *M. bovis* in cattle in the study area as a respiratory disease (Le Grand *et al*., 2001). Other workers have demonstrated

the organism to be involved in arthritis, mastitis and reproductive diseases (Adegboye *et al*., 1996; Abbas, 1996; Le Grand *et al*., 2001) as frequently observed in cattle herds in Nigeria. Therefore, it is likely that some of the previously diagnosed cases of bovine respiratory, reproductive problems as well as other pathologic condition of the udder (mastitis), otitis and arthritis may have been associated with *M. bovis* either singly or in combination with other infectious agents incriminated with the disease.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, both cultural and serological studies established the occurrence of *Mycoplasma bovis* infection in cattle in three Local Government Areas of Adamawa State of Nigeria. The

isolation rate was 2.0% (2/100) and seroprevalence rate of 19.5% was obtained. *Mycoplasma bovis* as a primary aetiological agent was more frequently recovered in Yola LGA of Adamawa State. Cattle of all ages, sexes and breeds were observed to stand the chance of being infected with the organism.

To the best of our knowledge, this is the first time serological survey of *M. bovis* was conducted in this transboundary State of Nigeria. Therefore providing a serological and isolation baseline data for *M. bovis*. From this study it was concluded that:

1. *Mycoplasma bovis* has been demonstrated in cattle in the three Local Government Area,
2. Possible effect of the agent in bovine respiratory disease, arthritis, mastitis and reproductive disorders, and
3. Possible economic effects on production and productivity.

6.2 Recommendations

1. Aggressive extension Veterinary Services by State Ministry for Livestock Production to educate/enlighten the farmers/herders on the dangers of living with mycoplasma disease (*M. bovis* infections and CBPP).

2. The need for regular routine serological surveillance to ascertain the prevalence of the disease in the study area.

3. Since there is no vaccine currently against *M. bovis* infections, control should be aimed at/towards reducing stress, provision of adequate housing with good ventilation.

4. Bilateral cooperation between Adamawa State Government and Republic of Cameroun to regulate the movement of animals across the porous borders via establishment of more control posts.

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APPENDIX I: PREPERATION OF MYCOPLASMA AGAR AND BROTH

NATIONAL VETERINARY RESEARCH INSTITUTE,
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

c) 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.

Document from CIRAD, EMVT, UPR15
Author: Lucía 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°C

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

PROCEDURE FOR BIOCHEMICAL IDENTIFICATION OF MYCOPLASMA (Poveda, 1998)

1. PREPERATION OF PPLO BROTH (500ml)

A. -Broth base 10.5g)

-Distilled water 350ml) sterilized by autoclaving.

B. Supplement depends on the tests

i. Glucose test:

-yeast extract 17.5g)

-penicillin 3 vials)

-Amphotericin B 10ml) filter using 0.22µm millipore

ii. Phosphatase test:

-yeast extract 17.5g)

-penicillin 3 vials)

-Amphotericin B 10ml) filter using 0.22µm millipore

iii. Arginine test:

-yeast extract 17.5g)

-0.4% Phenol red 0.2ml)

-penicillin 3 vials)

-Amphotericin B 10ml) filter using 0.22µm millipore

iv. Tetrazolium reduction:

-yeast extract 17.5g)

-penicillin 3 vials)

-Amphotericin B 10ml) filter using 0.22µm Millipore

C. Horse serum:
   - Glucose, Arginine, and Tetrazolium = 10%
   - Phosphatase = 20%

The constitution of the PPLO broth should be 7:1:2 for Broth base, Supplement and Horse serum respectively.

2. BIOCHEMICAL REAGENTS

a. Glucose 1% solution:- Dissolve 1g of glucose into 100ml distilled water.

b. Arginine HCl 1% solution:- Dissolve 1g L- Arginine HCl into 100ml of distilled water.

c. 2,3,5 Tetrazolium HCl 2% solution: Dissolve 2g 2,3,5 tetrazolium HCl into 100ml of distilled water.

d. For Sodiun Phenolphthalein diphosphate stock solution:
   i. dibasic sodium phosphate stock solution = dissolve 12g of clear crystals of dibasic sodium phosphate in distilled water to make 100ml;
   ii. phenolphthalein stock solution = dissolve 1g of phenolphthalein in 100ml of alcohol.

   The Stock Solution a + b = Stock Solution of Sodium Phenolphthalein diphosphate.

   Therefore, 1ml of sodium phenolphthalein stock solution dissolve in 100ml distilled water will give: Sodium phenolphthalein diphosphate 1% solution.

PROCEDURE FOR THE TEST

A. Glucose fermentation

Add 2ml of 1% glucose to 10ml of Mycoplasma broth medium (MBM) without glucose, then inoculated with 300µl of culture medium containing the isolates and incubate.

Basal medium without sugar will also be inoculated as a growth and pH indicator control.

A positive result will be recorded when a colour change (red-yellow) between the inoculated test substrate and each of the control substrate

For negative reaction there is no colour change.

The test will be observed for 14 days.

B. Phosphatase test

1ml of 1% sodium phenolphthalein diphosphate solution will be added to 10ml MBM without phenol red and glucose, inoculated with 300µl of culture medium containing the isolated Mycoplasma and incubated.

Uninoculated medium will also be incubated as a negative control.

After 3- 4 days incubation, 5N NaOH will be added at final concentration of 5% to all the test tubes.

A positive reaction was determined by a red or pink colour change.

C. Arginine utilisation

2ml of 1% arginine hydrochloride will be added to 10ml MBM containing 0.4% phenol red and adjusted to pH 7.3. It will then be inoculated with 300µl of culture medium containing the isolated Mycoplasma.

Basal medium without arginine will also be inoculated and incubated as a growth and pH indicator control.

Positive reaction is indicated by arginine hydrolysis producing ammonia (NH_3) with an increase in pH which is indicated by pH indicator.

D. Tetrazolium reduction

5ml of 2% 2, 3, 5 triphenyltetrazolium hydrochloride will be incorporated into 15ml MBM containing no glucose in a vacutainer tubes. Then it will be inoculated with 300µl of culture medium containing isolated Mycoplasma and incubated. Adjust pH to 7.5.

Normal MBM containing 0.5% NaCl was also inoculated as a growth and pH indicator control.

Positive reaction is indicated by reduction of tetrazolium to an insoluble brick- red colour.

E. Film and Spot Production

Fresh cultures of the test and control organisms are prepared in a standard broth medium with 40% serum. The plates are placed in an incubator at 37^0C for 30 minutes to dry the surface.

The plates are inoculated with 200µL of culture medium containing isolated Mycoplasma, and incubated at an appropriate temperature in a moist atmosphere for 2 weeks. Also uninoculated plates were incubated as a negative control.

The plates are examined every 24 hours with a stereomicroscope for the appearance of spots on the colonies.

On the last day, the plates were flooded with distilled water, and observed the separation of the film from the agar's surface in the positive test.