THE POTENTIAL OF IMMUNOHISTOCHEMISTRY IN DIAGNOSIS OF CONTAGIOUS BOVINE PLEUROPNEUMONIA IN APPARENTLY HEALTHY ANIMALS

JOHN DIDAS MBERELIYE MLAY

A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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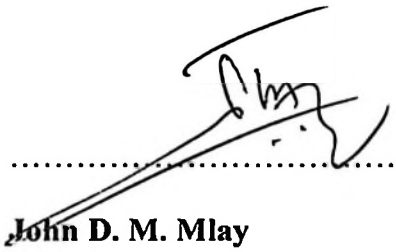
ABSTRACT

Diagnosis of contagious bovine pleuropneumonia (CBPP) in asymptomatic animals is a big challenge towards control of the disease. This is because most, if not all, of the available diagnostic methods are incapable of detecting the disease in such animals. In this study, an immunohistochemistry protocol was developed and employed to detect *Mycoplasma mycoides* subspecies *mycoides* Small Colony (*Mmm*SC) type, the causal agent of CBPP, in apparently healthy animals slaughtered at Morogoro urban abattoir. Lungs (n=13) with CBPP-like lesions were collected from the abattoir, kept in cool boxes, and transported to the veterinary pathology laboratory for investigation. After thorough gross examination, the lung samples were prepared for bacteriology, histology and immunohistochemistry using monoclonal antibodies 3H12 and 6E3. Grossly, the lungs were non-collapsing, marbled with widened interlobular septa, oedematous and fibrinous. They were also consolidated and had areas of sequestration. Cultures from two samples showed growth of small colonies with "fried egg" appearance on Mycoplasma Experience medium. Histological examination revealed expanded alveoli containing fibrin, oedema and inflammatory cells such as lymphocytes, plasma cells, macrophages and alveolar epithelial cells in all samples. In more severe cases the inflammatory cells occupied the lost architecture of the alveoli. There was also fibrinous vasculitis and bronchiolitis with peripheral leukocytic infiltration. In immunohistochemistry, *Mmm*SC antigens were detected from the sections prepared from all collected lung samples in the alveolar lumen and wall, bronchial and bronchiolar lumina, epithelium as well as bronchial glands and cartilage. It is concluded that immunohistochemistry is efficient in detecting *Mmm*SC antigens in apparently healthy slaughtered animals.

Its employment could be a sound option in diagnosis of CBPP in carrier asymptomatic animals and could thus aid in a long run, the control of the disease.

DECLARATION

I, John Didas Mbereliye Mlay, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.



..... 04.03.2013 .....

John D. M. Mlay **Date**

(MSc. Candidate)

The above declaration is confirmed

..... 04 March 2013 .....

Prof. Joshua J. Malago **Date**

(Research Supervisor)

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I owe a debt of gratitude to Municipal abattoir meat inspecting staffs that were always ready to help me in the collection of condemned lung samples.

Last but not least, I am grateful to all those not mentioned here, but in one way or another gave me the support to complete this study successfully. God bless you all.

DEDICATION

This work is dedicated to my beloved wife Dorothy, my son Denis-Mchomba, and my daughters Mai-Diana and Dina-Glory.

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LIST OF ABBREVIATIONS AND SYMBOLS

CBPP	Contagious Bovine Pleuropneumonia
CFT	Complement fixation Test
DAB	3-Diaminobenzidine
dH_2O	Distilled water
DPX	Distrene, Plasticizer and Xylene (components of resinous mounting medium)
ELISA	Enzyme-Linked Immunosorbent Assay
FDx	Final diagnosis
GBI Labs	Golden Bridge International Labs
H & E	Haematoxylin and Eosin
HRP	Horse Radish Peroxidase
ID	Identity
IHC	Immunohistochemistry
Kbp	kilo base pairs
MAb	Monoclonal Antibody
*Mmm*SC	*Mycoplasma mycoides* sub species *mycoides* Small Colonies type
mM	Millimolar
NBF	Neutral Buffered Formalin
NBS	Normal Blocking Serum
OIE	Office International des Epizooties
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PDx	Provisional diagnosis
pH	Hydrogen ion Concentration
Spp	Species
US$	United States dollar
µ/ µm	Microns, micrometers
µl	Microlitre
µg	Microgram
%	Percent
°C	Degree Celsius

CHAPTER ONE

1.0 INTRODUCTION

1.1 Overview of Contagious Bovine Pleuropneumonia

Contagious Bovine Pleuropneumonia (CBPP) is an infectious disease of cattle caused by small-colony type *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*SC) (Masiga and Domenech, 1995). The disease is endemic in Asia and Africa, particularly Eastern and Central part. Its annual economic loss is estimated at US$ 2-3 million due to morbidity, mortality, reduced animal productivity and quality of meat and hides, cost of intervention, and restriction to export trade (Masiga *et al.*, 1999). It is a major threat for cattle health and production; and also the most significant epidemic disease in Africa reported from 17 countries in 2001 to 27 countries in 2002 (Tambi *et al.*, 2006).

The disease manifests as an acute, sub acute or chronic disease. In its acute phase, CBPP is characterized by severe exudative inflammation of the lungs, serofibrinous pleuritis and heavy mortalities. The lung lesions may undergo sequestration in which case the *Mmm*SC is contained in circumscribed lesions (sequesters) and the infection becomes latent as the disease enters a chronic phase. Animals in chronic phase produce less milk and meat; and are asymptomatic potential sources of new infection (Mbulu *et al.*, 2004). These types of animals, also known as "lungers", are the most serious aspect for the spread of the disease in apparently healthy herd (Sewell and Brocklesby, 1990). They are the source of new infections following stress like drought and long distance trekking that results in rupture of sequesters and subsequent release of *Mmm*SC. This has led to extension of endemic, re-emerging or epidemic CBPP infections throughout the pastoral herds of Western, Central and

Eastern Africa (Windsor, 2000; Miltiadou *et al.*, 2009). In the 1990's, for instance, "lungers" were responsible for episodes that caused massive losses of cattle in East Africa, with Uganda and Tanzania being the most severely affected countries (Masiga *et al.*, 1996).

Effective control of CBPP relies on combined use of strict movement controls, slaughter of affected animals, vaccination and surveillance. In several countries, pastoralists like the Maasai (in Tanzania) are constantly moving in search of water and pasture. This has contributed to disease control failure with regard to restricted cattle movements. Indeed, animals kept under pastoral systems are continuously at risk of contracting CBPP (Schnier *et al.*, 2009).

Vaccines like combined Rinderpest and CBPP (Bisec vaccine), T_1-SR and T_1-44 that have been once used or are currently in use in some places, have proven ineffective in various countries (Melewas, 1999; Abdo *et al.*, 2000; Thiaucourt *et al.*, 2000; Mbulu *et al.*, 2004). Low efficacy of the T_1-44 vaccine with short duration of immunity has also been reported (OIE, 2004). Further, vaccination programs have been difficult to implement in many pastoral communities in Africa due to wide infected area, limited resources, failure of herdsmen to present the whole herd for vaccination or failure to follow vaccination regime and refusal to continue vaccination following previous vaccination reactions (Melewas, 1999). The highly challenged vaccination programs have resulted in patches of unvaccinated animals and more virulent field strains that have been potential source of outbreaks.

Cattle rustling among pastoral tribes in East Africa and other traditions like payment of dowry, clan rituals and inheritance of cattle were other means of spread of infections from apparently healthy animals (Kusiluka and Sudi, 2003).

1.2 Problem Statement and Justification

Efficient control of CBPP not only requires restricted movement of animals and effective vaccine strains, but also reliable diagnostic strategies (Mbulu *et al.*, 2004; Pilo *et al.*, 2007; Bischof *et al.*, 2009). In Africa, diagnosis of apparently healthy infected animals plays an important role in the control of the disease. The diagnosis could be followed by effective vaccination in quarantined area and surveillance. Unfortunately, diagnostic tests that permit detection of asymptomatic carriers as well as those applicable under field conditions are lacking (Barber *et al.*, 1970; Muuka *et al.*, 2011).

The World Organization for Animal Health (OIE) recommends complement fixation test (CFT) as an international diagnostic test for CBPP. Enzyme linked immunosorbent assay (ELISA) is another commonly used diagnostic method. However, both tests are expensive, need well trained personnel to perform, and are unsuitable for field application and mass screening in poor societies of Africa (Nicholas *et al.*, 2000). In this respect, meat inspection at slaughter houses and abattoirs to discover lung lesions is probably a promising diagnostic approach to asymptomatic carriers. Such identified lungs with CBPP lesions can further be studied grossly, microbiologically or histopathologically for confirmatory diagnosis (Muuka *et al.*, 2011). In this study, besides gross, bacteriological and histological examination of condemned lungs due to CBPP-like lesions during meat inspection,

immunohistochemistry which has been poorly utilised in CBPP diagnosis was put into practice. Elsewhere, this technique has proved to be a robust and useful assay in the diagnosis of CBPP, especially where the causative organism is not recoverable (e.g. following long transport distances), where an animal has died of acute disease, or where serology can not be performed or is inconclusive (Ferronha *et al.*, 1990a; Scanziani *et al.*, 1997).

However, this method has not been used in Tanzania where sporadic cases of CBPP occur and diagnosis is mistakenly for East Coast Fever or other pneumonic diseases (Kitalyi, 2005). This shortcoming can be solved by conclusive diagnostic procedures like IHC. In addition, contrary to previous studies, the use of 3H12 and 6E3 antibodies to diagnose CBPP has never been applied. By exploring the effectiveness and sensitivity of 3H12 and 6E3 antibodies to diagnose CBPP will widen the application of IHC for CBPP diagnosis in Tanzania and other countries where specific antibodies are lacking.

1.3 Objectives

1.3.1 Overall objective

To develop immunohistochemical technique for CBPP diagnosis.

1.3.2 Specific objectives

- To confirm the presence of CBPP in lungs of apparently healthy cattle slaughtered at Morogoro municipal abattoir.
- To apply immunohistochemical technique for diagnosing CBPP.

- To develop standard operating procedure for CBPP diagnosis using immunohistochemistry.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Actiology of Contagious Bovine Pleuropneumonia

Contagious Bovine Pleuropneumonia (CBPP) is a highly infectious respiratory disease of cattle caused by *Mycoplasma mycoides* subspecies *mycoides* small colony type (*Mmm*SC). The disease is of major economic importance in sub-Saharan Africa (FAO, 2003) with a constant risk of reintroduction into other parts of the world. The *Mmm*SC is a member of the class *Mollicutes* (trivial name, mollicutes), which evolved from Gram-positive bacteria that possess genomes with low guanine and cytosine content (Phylum *Firmicutes*), and belongs to the genus *Mycoplasma* (trivial name, mycoplasmas). They are characterised by small size (500-1100 kbp), lack of cell wall, extreme fastidious *in vitro* and tendency to form centred colonies on solid medium (Nicholas, 2004). They are also known as the smallest self-replicating organisms (Westberg *et al.*, 2004).

The genome of *Mmm*SC type strain PG1T has been sequenced to map all the genes in order to further studies regarding cell function of the organism and to better understand CBPP. The genome consists of a single circular chromosome of 1,211,703 bp (**Plate 1**).

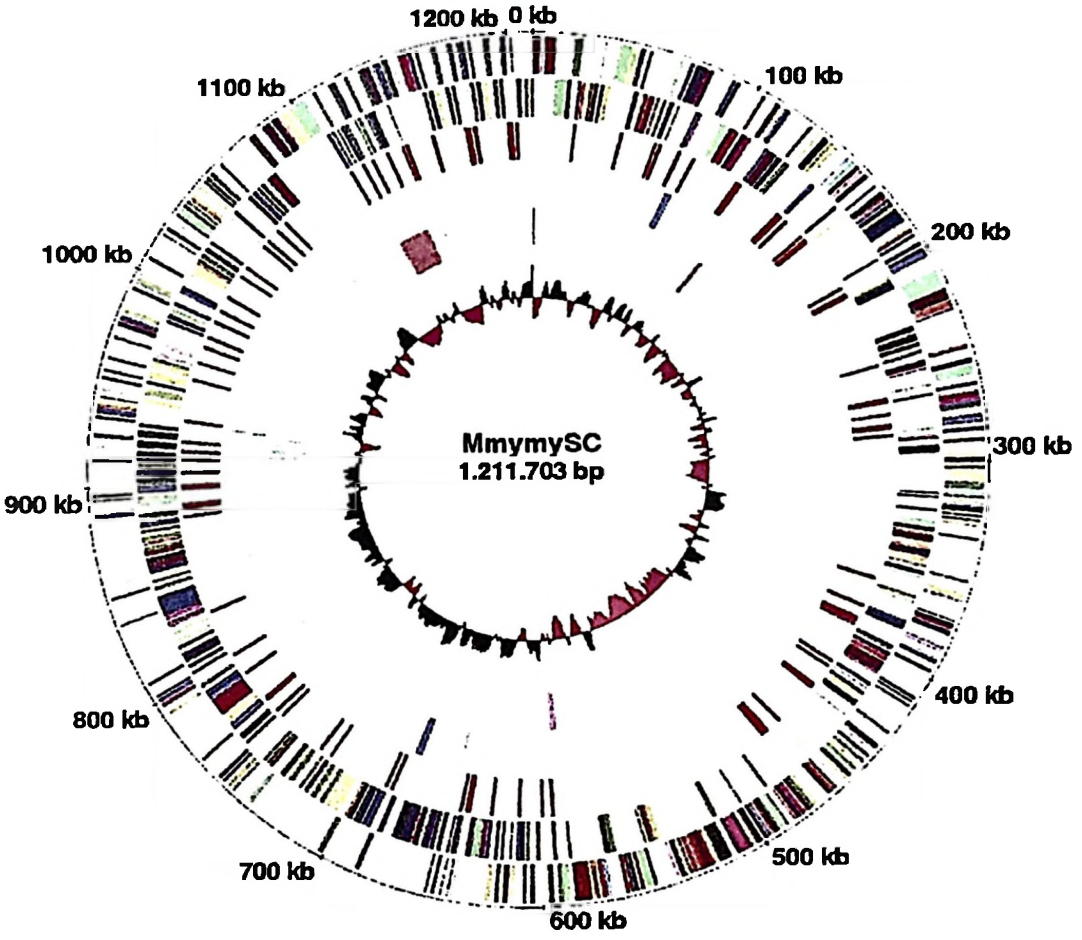

Plate 1: **Circular representation of the *Mycoplasma mycoides mycoides* Small Colony type genome (Source: Westberg *et al.*, 2004).**

2.2 Pathogenicity of Contagious Bovine Pleuropneumonia

The disease is characterised by a relatively long incubation period and a highly variable clinical course. Animals dying of acute severe cases have pathognomonic pulmonary lesions that include sero-fibrinous pleurisy with copious yellowish exudates accompanied by unilateral broncho-pneumonia in which adjacent lobules may show differing developmental stages of the lesion and are separated by thickened interlobular septa (Thomson, 2005). Recovered animals may harbour the infection in necrotic areas of lung tissue separated from the surrounding normal

tissue by a fibrous capsule (sequestrum). These sequesters may or may not contain live *Mmm*SC organisms. When they do, the animals are carriers or "lungers" and are apparently healthy but potentially able to excrete *Mmm*SC and infect susceptible animals. The *Mmm*SC are often localized in the focal necrotic micro abscesses of the lung, in the dense fibrotic pyogranulomatous areas, and in the infiltrations of macrophages around bronchioles and perivascular cellular cuffs (Bashiruddin *et al.*, 1999; FAO, 2002; Coetzer and Tustin, 2004). The *Mmm*SC-macrophage interaction usually determines the subsequent progression of the disease (Marshal *et al.*, 1995).

2.3 Clinical findings of Contagious Bovine Pleuropneumonia

Clinical diagnosis of CBPP is hard as initial signs may be slight or non-existent and are indistinguishable from any severe pneumonia with pleuritis (Scudamore, 1995; OIE, 2008). Diagnosis of sub clinical infection in a living animal is also difficult due to inadequacies of the current diagnostic tests in identifying sub clinical cases (Centikaya *et al.*, 2003). In practice, CBPP is best diagnosed by pathological, microbiological, molecular, or serological procedures.

Since the pathological lesions of CBPP e.g. the "organizing centres" observed in the interlobular septa of lungs with lesions are distinctive (Ferronha *et al.*, 1990; Di Francesco *et al.*, 1998), abattoir surveillance of CBPP involving lung examination is a practical method for disease monitoring (OIE, 2008). This is an important factor in identifying cattle infected sub-clinically.

2.4 Pathological findings of Contagious Bovine Pleuropneumonia

2.4.1 Gross lesions

The pathological lesions of CBPP are confined to the thoracic cavity and lungs, and the lesions are usually unilateral (Nunes Petisca *et al.*, 1990). The affected portion of the lung shows obvious consolidation. On incision, it reveals interlobular septa distended with amber-coloured fluid and typical marbled appearance. The lungs are swollen; the colour varies from pink to dark red, has a moderate firm consistency, and exudes clear fluid and sometimes blood from cut surfaces. The interlobular septa are grossly thickened (Geering and Amanfu, 2002).

2.4.2 Histopathology

Routine haematoxylin and eosin staining (H&E) is the cornerstone of tissue-based diagnosis. The process stains thin tissue sections so that pathologist can visualize tissue/cellular morphology. The process uses a haematoxylin dye to stain cell nuclei blue and an eosin dye to stain other structures pink or red. Properly applied, this technique provides exceptional detail of tissue structures and the makeup of the cells. This detail is required for tissue-based diagnosis, particularly in the detection and classification of pathogens. The standard paraffin processing moves specimens through a series of steps so the soft tissue is supported in a medium (paraffin wax) that allows sectioning. The standard steps are fixation, dehydration, clearing and paraffin wax impregnation. Embedding in paraffin wax is followed by sectioning using a microtome to produce very thin (4-5 μm) sections that are placed on a microscope slide ready for staining (Gordon and Bradbury, 1990).

Microscopically, the earliest pulmonary lesions of CBPP consist of foci of catarrhal bronchiolitis, with distension of the lymphatics in the interlobular septa and thickened alveolar walls. Alveoli are filled with fluid and cells (macrophages and polymorphonuclear leucocytes) as a result of thrombosis on blood and lymphatic vessels (Geering and Amanfu, 2002). Hyperemia of septa and fibrinous exudates in the alveoli are evidenced by the presence of erythrocytes and pink colour in the alveoli. Walls of alveoli are also congested as well as engorgement of capillaries. Another feature is collapsing bronchioles surrounded by fibrinous tissue with few inflammatory cells of mononuclear type and also polymorphonuclear cells especially degenerating neutrophils. Collapsing alveoli consolidated with oedema and fibrin and surrounding fibrinous tissue is indicated by spindle shaped cells (Geering and Amanfu, 2002).

2.5 Isolation of *Mycoplasma mycoides* subspecies *mycoides* Small Colony type

Culture, which requires some expertise, is an important diagnostic method because it demonstrates the presence of *Mmm*SC in tissue lesions so as to confirm an outbreak of the disease (Centikaya *et al.*, 2003). Several media like Hayflick's, PRM and Mycoplasma Experience have been described to enable growth and yield of *Mmm*SC (Hudson, 1971; Freundt, 1983; Nicholas and Baker, 1998; Nicholas *et al.*, 2000). Despite these media, it has been demonstrated that culture may not achieve the accuracy desired. Instead, detection systems that do not rely on the viability of *Mmm*SC should be considered favourably (Bashiruddin *et al.*, 2005).

2.6 Immunohistochemistry Assay

Immunohistochemistry (IHC) being based on the binding of antibodies to a specific antigen in tissue sections, has been established as a solid and reliable methodology for both routine diagnostic and research activities in veterinary medicine (Ramos-Vara, 2005). Immunohistochemistry is a technique in which the specific interaction between an immunoglobulin and its homologous antigen is visualized on histological sections by a microscopically detectable label. Generally, the label consists of an enzyme, such as peroxidase, that reacts with an appropriate substrate-chromogen solution to produce a specific colour at the site of reaction (Scanziani, 1998). The *Mmm*SC or their antigens can be demonstrated in clinical specimens by the IHC techniques (Bashiruddin *et al.*, 1999; Ferronha *et al.*, 1990a).

2.6.1 Immunohistochemical staining of fixed tissues

The sensitivity of immunoperoxidase techniques is central to wide variety of specific antigen localization in IHC. Using monoclonal antibodies like M92/20, IHC has proved to be a robust assay in the diagnosis of CBPP, particularly where the causative organism, *Mmm*SC, is not recoverable (e.g. following long transport distances), where the animal has died of acute disease, or where serology cannot be performed or is inconclusive (Ferronha *et al.*, 1990; Scanziani *et al.*, 1997). In an examination of 11 CBPP affected lungs from Portuguese cattle, IHC detected all, while polymerase chain reaction (PCR) and culture detected 5 and 4 cases respectively (Ayling *et al.*, 1998). The ability to detect the causative agent at specific sites in a tissue is a major attribute of IHC technique compared to other methods like PCR that merely confirm the presence or absence of that particular agent.

Immunohistochemistry may also detect the agent in decomposing tissue from which isolation may prove difficult (Adegboye *et al.*, 1995).

2.7 Contagious Bovine Pleuropneumonia diagnosis and control

In Tanzania where maintenance of an efficient surveillance system is challenging, abattoir could be a good source of information about CBPP. Lungs with CBPP-like lesions have been spotted in rural slaughter houses regularly, indicating that CBPP is present in those areas where most of the cattle for slaughter in urban areas come from (Swai *et al.*, 2012).

Abattoir monitoring is a cheap and highly effective method for surveillance for CBPP. Involvement of trained meat inspectors facilitates the collection of diagnostic samples from suspected lungs. The samples will be processed in the laboratory for IHC technique and in less than 7 days the results will be known. Trace back will be carried out whenever positive cases are encountered to identify the origin of the animals. Once the origins are identified, herds at these points will be sampled for further laboratory tests.

In this study, lungs with CBPP-lesions from abattoir will be a clue towards confirmation of the presence of the disease and will be a foundation to the establishment of IHC as diagnostic test for CBPP. In addition, the study intends to develop standard operating procedures (SOP) on this technique which will lead to a regular use of the technique in our laboratories.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Location and Duration of Study

The study was conducted in Morogoro Municipality involving the municipal abattoir and Microbiology and Parasitology department as well as Veterinary Pathology department of the Sokoine University of Agriculture from November 2011 to April 2012. The Morogoro town lies at latitude of 6° 50' 20" South and longitude of 37° 39' 210" East at an attitude of 500 – 600 meters above sea level (Komba *et al.*, 2012). Animal slaughtering in this abattoir start everyday as early as 4 am and the municipal meat inspectors are fully involved. Apart from meat inspection, the meat inspectors usually do the antemortem examination of the animals to ensure that they are healthy before they are slaughtered. This abattoir receives animals from livestock markets within and outside the municipality.

3.2 Sample Collection and Preservation

During the study period, 13 plucks of condemned lungs with CBPP-like lesions (consolidated, fibrinous, oedematous and with enlarged interlobular septa), were collected from the abattoir during routine meat inspection. All these were from apparently healthy slaughtered cattle. The lungs and their trachio-bronchial lymph nodes were then taken to the laboratory (Faculty of Veterinary Medicine, Sokoine University of Agriculture, Morogoro) in cooler boxes for further examination and study.

3.3 Laboratory examination

3.3.1 Macroscopic examination

Collected lungs were thoroughly examined for macroscopic lesions. Two portions were then made from each sample. The first portion was immediately frozen (-20°C) for *Mmm*SC isolation and the second portion was fixed in 10% neutral buffered formalin (NBF) for histopathology (**Appendix I**) and IHC.

3.3.2 *Mycoplasma mycoides* subspecies *mycoides* Small Colony isolation

With the aid of a sterilised (by Bunsen burner flame) pair of forceps and a sterile scalpel blade, a piece of frozen lung tissue was carefully trimmed and smeared on one angle of plates containing Mycoplasma Experience Media (Reigate, UK. **Appendix II**). Using a Bunsen burner sterilised wire loop, the smear was streaked along the other area of the plates. The plates were incubated at 37°C in a humid atmosphere containing 95% air - 5% CO_2. The plates were inspected from day 2 to day 14 (Awan *et al.*, 2004).

3.3.3 Tissue processing and histopathology

The lung tissues were fixed in 10% NBF for at least 48 hours, processed routinely, and embedded in paraffin wax. The standard paraffin process (tissue processing) implemented in this work moves specimens through a series of steps so the soft tissue is supported in a medium that allows sectioning. The standard steps starts with fixation that preserves the tissue. This was followed by dehydration through graded ethanol (70%, 90% and absolute), clearing in chloroform and finally infiltration of the tissue with molten paraffin wax. It was followed by embedding that allows the orientation of the specimen in a block that can be easily sectioned and also makes it

easy to handle and to store. Then sectioning was done using a rotary microtome (Baird & Tatlock) to produce 4 μm thin paraffin sections that were placed on mounting bath (Electrothermal) before being picked by microscope slides. Two sets of sections were made; one set for histopathology routine H&E and the other set attached on pre coated (subbed) slides for IHC. These sections were dried overnight in paraffin oven (Electrolux) at 50°C (Gordon and Bradbury, 1990).

3.3.4 Routine haematoxylin and eosin staining

Routine H&E staining is the foundation of tissue-based diagnosis. The process uses a haematoxylin dye to stain the cell nuclei blue and eosin dye to stain cytoplasm and other structures pink or red. When properly applied, this technique provides exceptional detail of tissue structures and the make up of the cells. Regressive method of H&E staining was applied where the sections were sent to water by three changes of xylene dewaxing and then treated with descending strengths of ethanol and finally hydrated by water. The sections were treated with Harris's haematoxylin, differentiated with 1% acid alcohol then blued in alkaline water made by saturated lithium carbonate. Eosin counter stain was applied then dehydration through ascending strengths of ethanol. Finally the sections were cleared in three changes of xylene for two minutes each and mounted with cover slips using DPX resinous mounting medium (Talukder, 2007). The sections were left to dry before they were examined under light microscope (Olympus BX41, Japan).

3.3.5 Immunohistochemistry

3.3.5.1 Deparaffinization

The 4 μm sections of formalin-fixed, paraffin embedded tissues on coated slides (**appendix III**) were heated at 55°C for 10 minutes in an oven to melt the paraffin. Deparaffinization was done by immersing sections in three xylene changes (5 minutes each). This was followed by rehydration involving two changes of 100% ethanol rinses (10 minutes each), then two changes of 95% ethanol for 10 minutes each, and washing in distilled water for 1 minute. Excess liquid was carefully aspirated from the sections before the next step (Haines *et al.*, 1992).

3.3.5.2 Antigen retrieval (heat treatment antigen unmasking)

This is a heat treatment of the sections aimed at rectification of immunoreactivity loss by many antigens as a result of formalin fixation. In this process the slides were put in staining dish containing antigen retrieval solution (10 mM sodium citrate buffer [**appendix IV**]) and heated at 95°C (in water bath) for 5 minutes. The dish was topped up with fresh citrate buffer and heated again in water bath at 95°C for another 5 minutes. The staining dish was then removed from the water bath and placed on a bench to cool without cover for about 20 minutes. After cooling, the sections were washed in three changes of distilled water for 2 minutes each. Excess fluid was then carefully aspirated from the sections (Tang *et al.*, 2007).

3.3.5.3 Inactivation of endogenous peroxidase

The process is also known as quenching of endogenous peroxidase activity. This was done by treating the sections with 3% H_2O_2 in distilled water and incubated for 5 - 10

minutes in humid chamber at room temperature. The sections were then rinsed in three changes of PBS pH 7.4 (**appendix V**) for 5 minutes each.

3.3.5.4 Blocking

Sections were incubated in pre-blocking solution using SPlink HRP detection kit (GBI Labs, Mukilteo, WA, USA; Cat No D01-60) for 10 minutes at room temperature to block non-specific immunoglobulin binding. The slides were then drained and a ring around the tissue was applied using colourless nail polish to prevent spreading of solutions from the slides and concentrating the reagents only over the tissue (Rogers *et al.*, 2006).

3.3.5.5 Primary antibody

Subsequently, the slides were incubated with either 6E3 or 3H12 mouse anti-*Mmm*SC antibodies (kind donation from Dr. C. Brooks of Agri-Food and Biosciences Institute, Ireland) for 60 minutes at room temperature in a humidified chamber. Monoclonal antibody 6E3 is a caprylic acid purified tissue culture fluid specific for *Mmm*SC while 3H12 is a mouse ascites fluid which detects both small and large colony *Mmm*SC. The MAb 6E3 was diluted at 1:2000 and MAb 3H12 at 1:10000 using 2% goat serum in PBS. After the one hour incubation, the sections were rinsed with 3 changes of PBS for 2 minutes each (Rogers *et al.*, 2006).

3.3.5.6 Secondary antibody

Still in humidified chamber, the sections were incubated with ready to use broad spectrum biotinylated secondary antibody (Lot #: KT 125608F from GBI Labs -

Mukilteo, WA, USA) for 10 minutes at room temperature. The sections were rinsed with 3 changes of PBS for 2 minutes each.

3.3.5.7 Detection

Incubation with ready to use HRP-Streptavidin (Lot #: KT 125608F from GBI Labs – Mukilteo, WA, USA; Cat no D01-60) followed for 10 minutes at room temperature. The sections were then subjected to 3 changes of PBS rinse for 5 minutes each. The next step was incubation of the sections with DAB substrate/DAB chromogen (DAB+ Substrate kit Lot #: K126929A from GBI Labs – USA; C09 - 12). One drop of DAB chromogen (20x concentrate) was added to 1 ml of DAB substrate buffer. This was well mixed and applied on the sections for 5 minutes. When desired colour was developed, the sections were gently rinsed in tap water for about 2 minutes.

3.3.5.8 Counterstaining

Though optional, counterstaining was done by immersing the sections in Harris's haematoxylin for 20 seconds. This was followed by thorough rinsing under tap water for about 2 minutes. The sections were then blued in PBS for about 60 seconds and then rinsed in distilled water. Dehydration and clearing in alcohol and xylene, respectively followed by soaking the sections in 95% ethanol twice for 10 seconds each, then 100% ethanol twice for 10 seconds each, then 3 changes of xylene for 10 seconds each. Excess xylene was wiped out and immediately the sections were mounted with cover slips using DPX. Sections were then left to dry ready for examination using light microscope (Olympus BX41, Japan). Photographs were taken by DP21 camera (Olympus, Japan).

3.3.5.9 Controls

Immunohistochemistry should include a known positive and negative control to assure the proper functioning of staining system as well as for the valid interpretation of the results. For a positive control, lung tissues confirmed to have *Mmm*SC antigens through isolation of the micro-organisms were used. A lung tissue from grossly normal lungs and from whose cultures did not grow *Mmm*SC was used as a negative control.

CHAPTER FOUR

4.0 RESULTS

4.1 Gross Lesions

All the samples had severe unilateral lesions (**Plate 2a, 2b**) and in some cases, only a portion of a lobe was affected. The affected areas showed varying stages of grey or red marbling and thickening of the tissue. These lobes were also covered with fibrin layer of differing thickness and colour intensity (**Plate 2b**). An incision through the affected lobe showed hepatisation, oedema, thickening of the interlobular septa, a marbled appearance (**Plate 3**) and copious amounts of clear, yellowish fluid exuding from cut surface. Some lungs had areas of sequestration where coagulative necrosis was vivid.

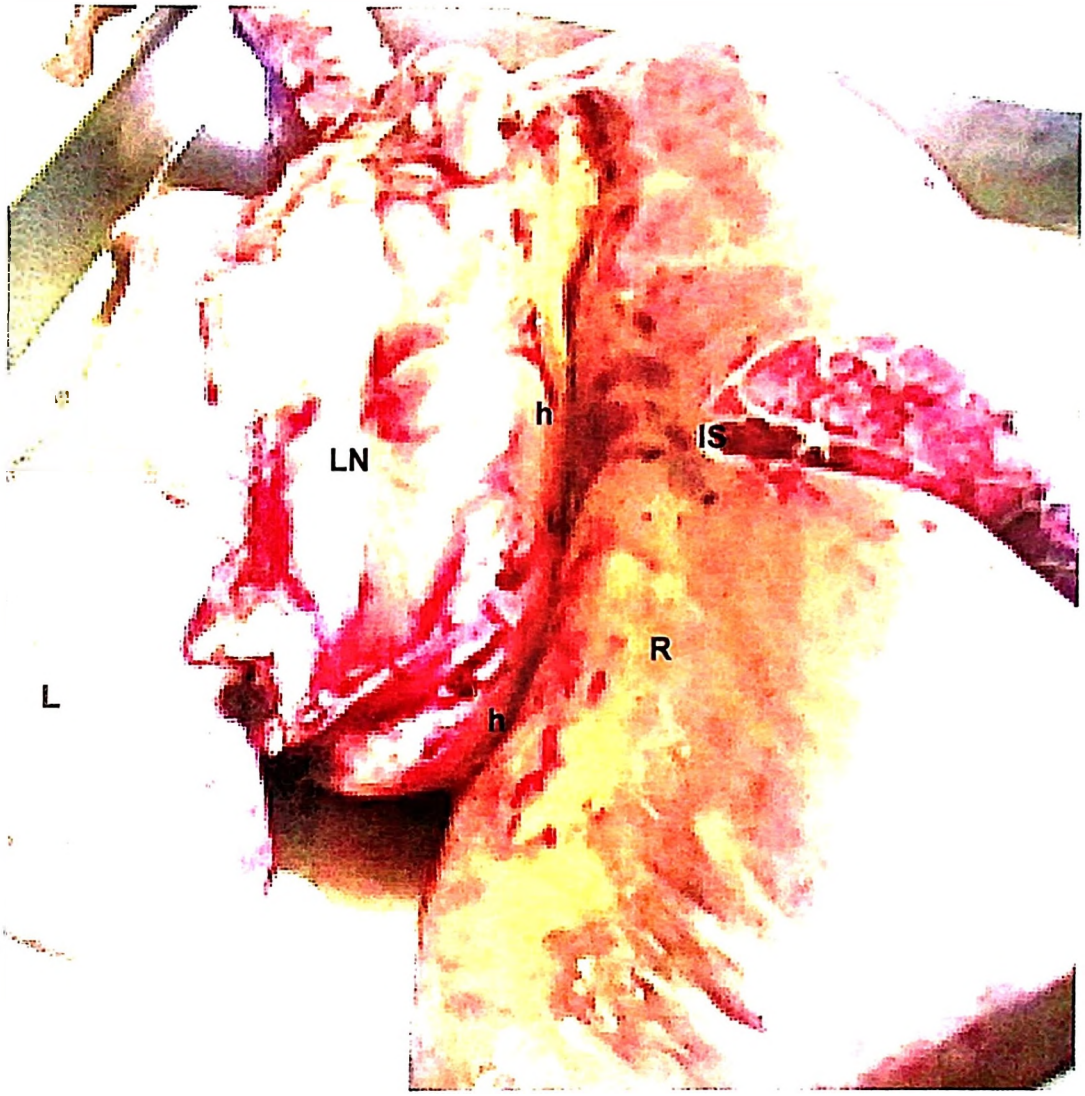

Plate 2a: Unilateral involvement of CBPP lesion in a lung lobe. The affected right lobe (R) is extremely enlarged, firm and fleshy, non-collapsing compared to the left unaffected one (L). On incision, prominent interlobular septa (IS) were observed. The affected lung also shows haemorrhagic (h) areas. The tracheo-bronchial mediastinal lymph nodes (LN) are enlarged.

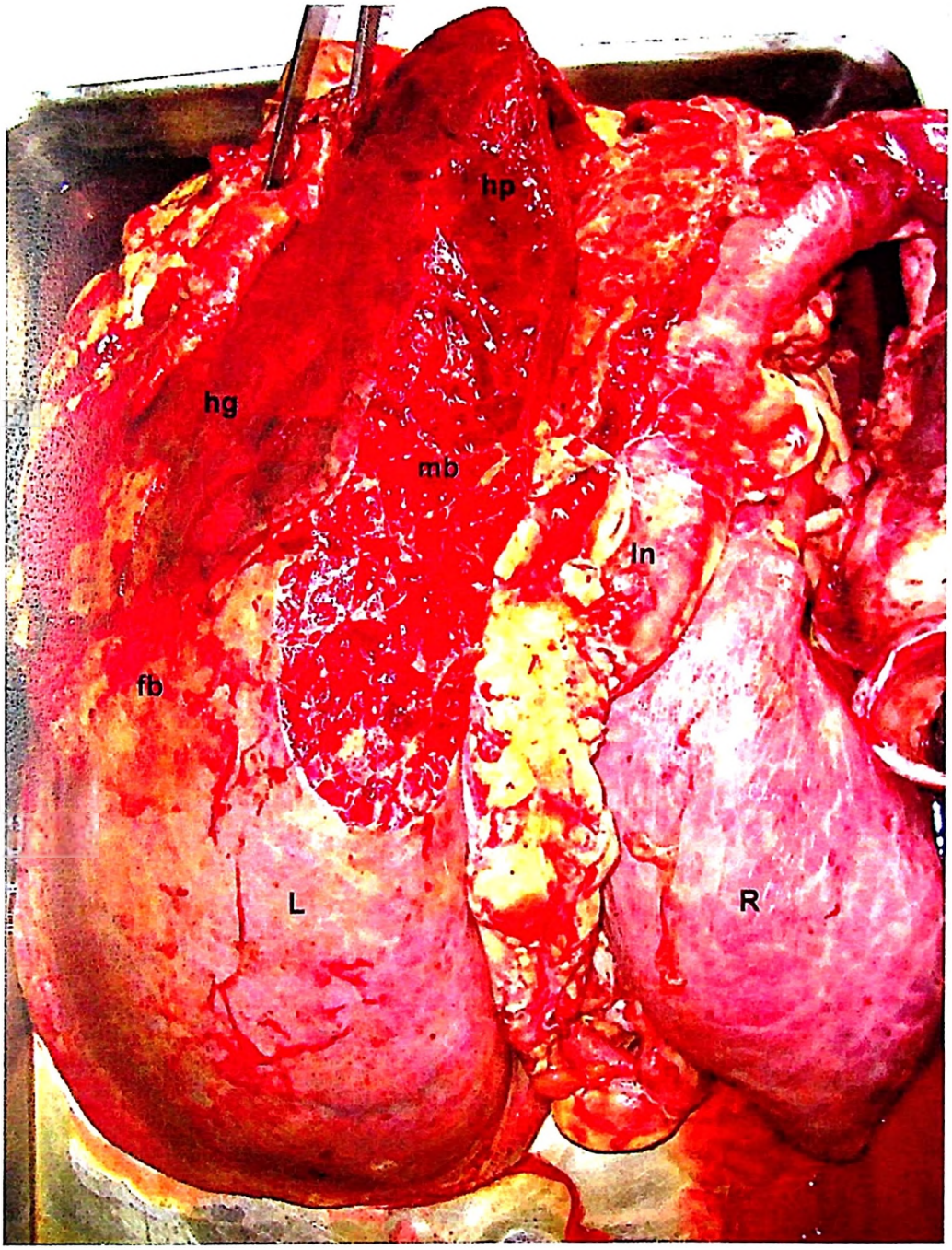

Plate 2b: The affected left (L) lobe is hepatised (hp), has prominent interlobular septa, marbling (mb), haemorrhages (hg) and covered with fibrin (fb). The lymph nodes (ln) are enlarged. The right (R) lobe is not affected.

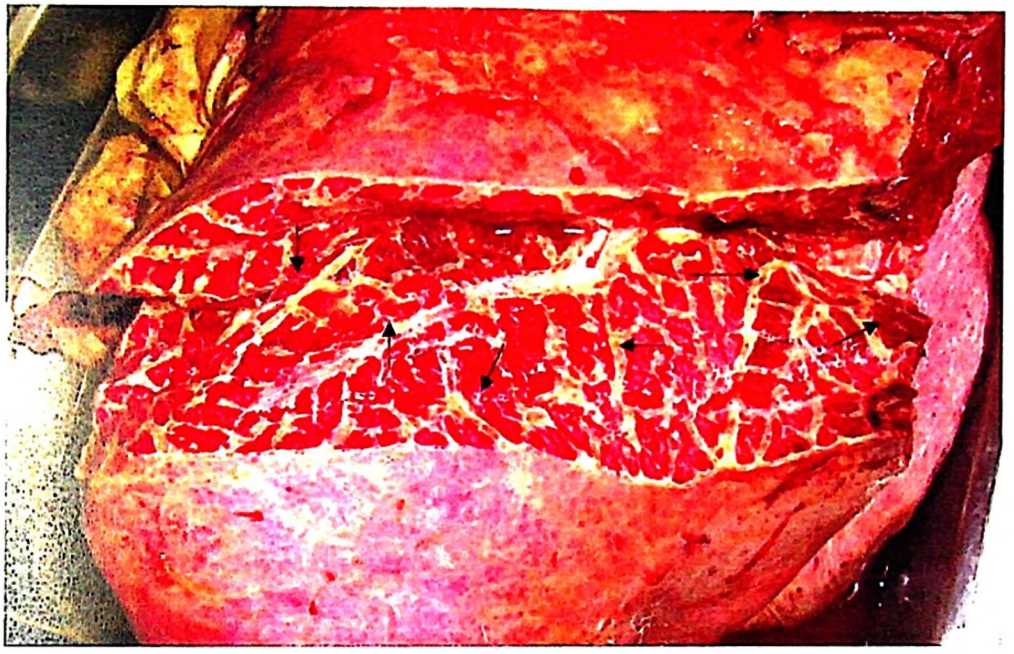

Plate 3: The parenchyma is consolidated, non-collapsing and the cut surface reveals marbling (arrows), the most known and prominent macroscopic lesion of CBPP. It is a pathognomonic lesion of the disease.

4.2 Isolation

To ensure the presence of CBPP, isolation of the causative organism was important. After incubation, the cultures were examined everyday under stereo microscope from day 2 for Mycoplasma growth. Of all the 13 lung samples, only 2 samples showed the small brownish red colonies typical of *Mmm*SC on day 6 (**Plate 4**). Further incubation of these two samples revealed the colonies changing morphology from discrete colonies to crystal-like and colour changing to deep red.

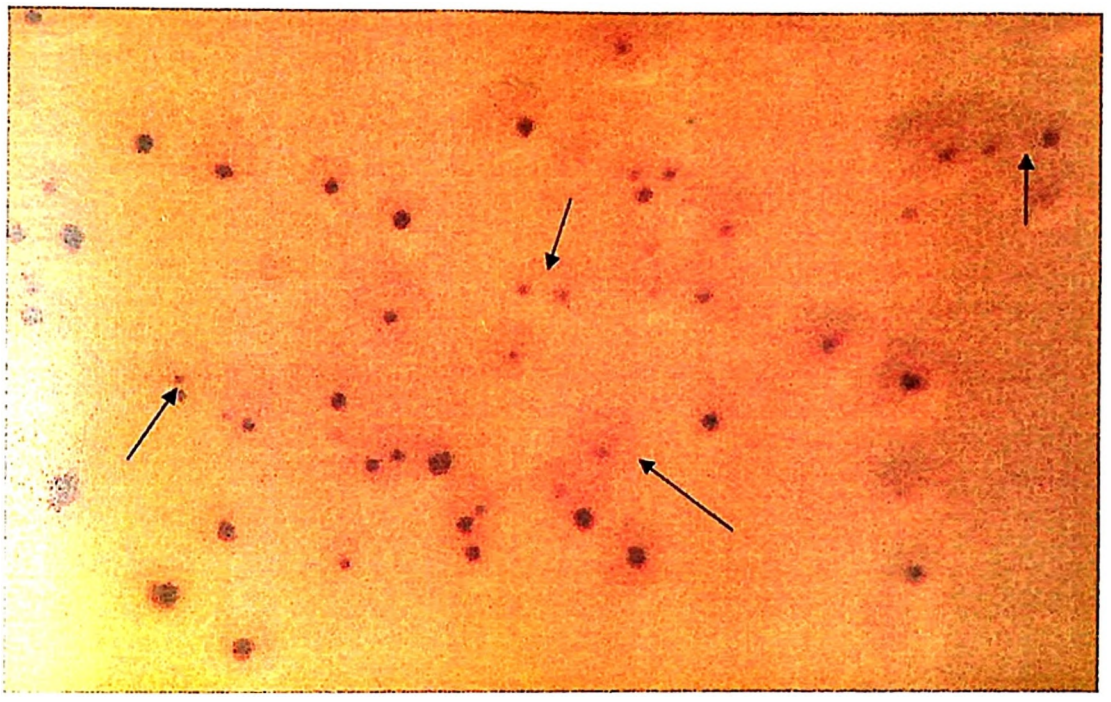

Plate 4: ***Mycoplasma mycoides mycoides*** **Small Colony type on Mycoplasma Experience medium after six days of incubation exhibiting the typical appearance of "fried eggs" small colonies (arrows) as they were viewed under stereo microscope (x 40).**

4.3 Histopathology

Microscopically, alveoli were diffusely expanded with fibrin, oedema and necrotic cells, some of which could be recognized as lymphocytes, plasma cells, macrophages and alveolar epithelial cells desquamated from the alveolar wall. There was marked enlargement of interalveolar septa. (**Plate 5**).

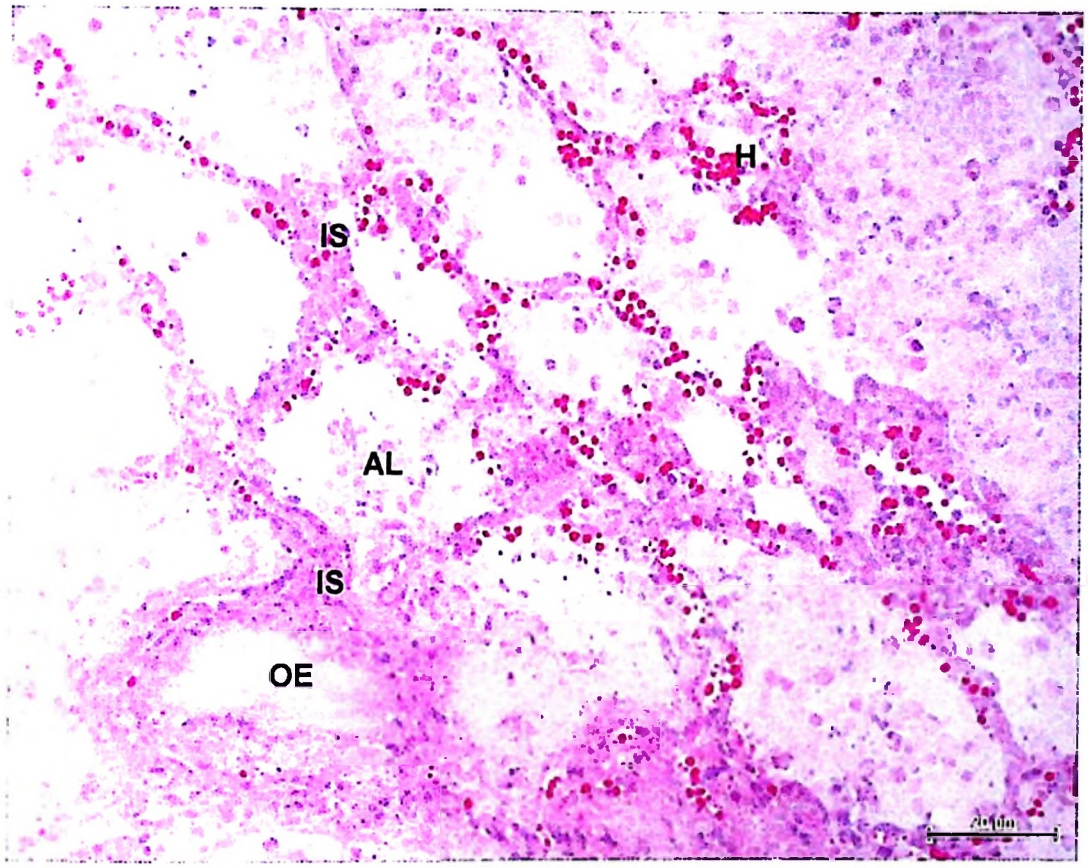

Plate 5: Expanding interalveolar septa (IS), hyperemic and haemorrhagic (H) in some places with desquamating epithelial cells. Alveolar lumina are filled with fibrin and necrotic cells (AL). There is also oedema (OE). (HE)

In more severe cases the architecture of the alveoli was lost and massive lymphocytes, plasma cells and macrophages occupied the original alveolar areas (**Plate 6a**).

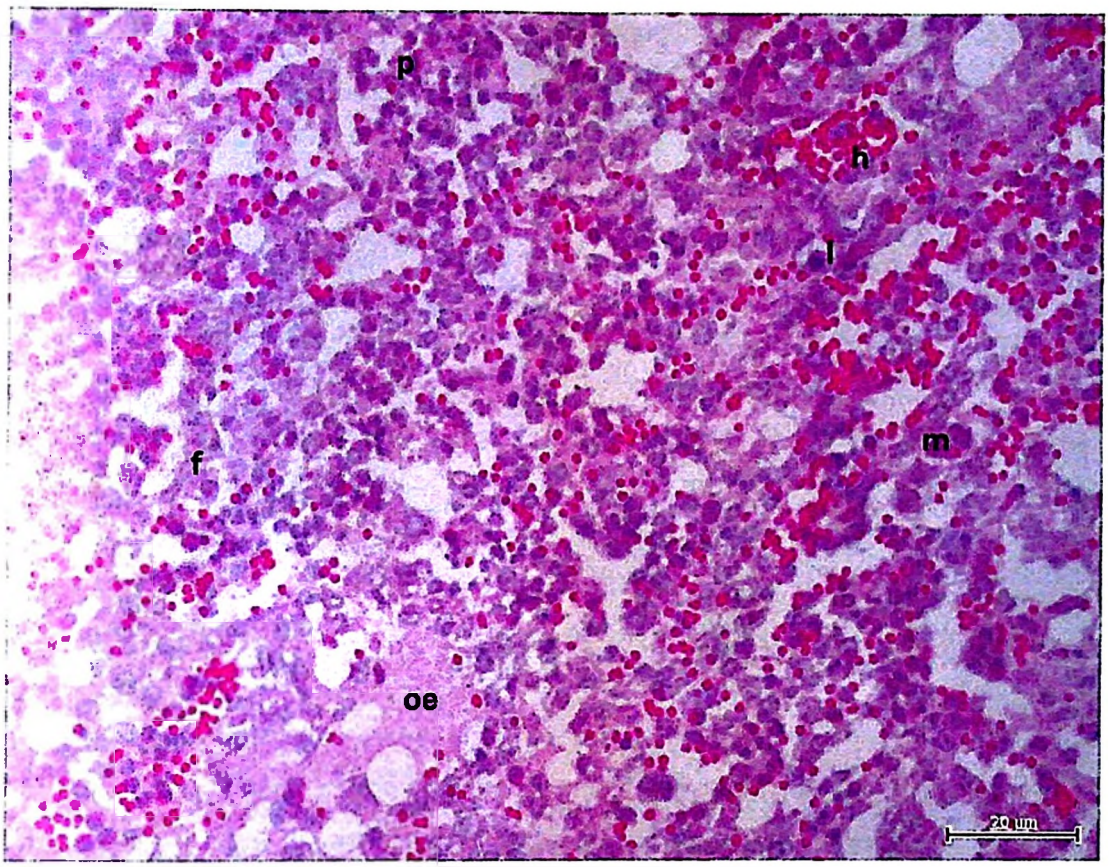

Plate 6a: **Lung tissue showing damaged alveoli, loss of architecture, oedema (oe), fibrin (f) and haemorrhage (h). There is also infiltration of plasma cells (p), lymphocytes (l) and macrophages (m).** (HE)

The alveolar wall (interalveolar septa) was diffusely expanded and moderately to severely hyperemic and haemorrhagic with desquamating epithelial cells. In more severe cases, lymphocytes, plasma cells and some macrophages infiltrate the area (**Plate 6b**). The architecture is lost in some areas where both the alveolar lumen and the wall are heavily infiltrated with lymphocytes, plasma cells and some macrophages.

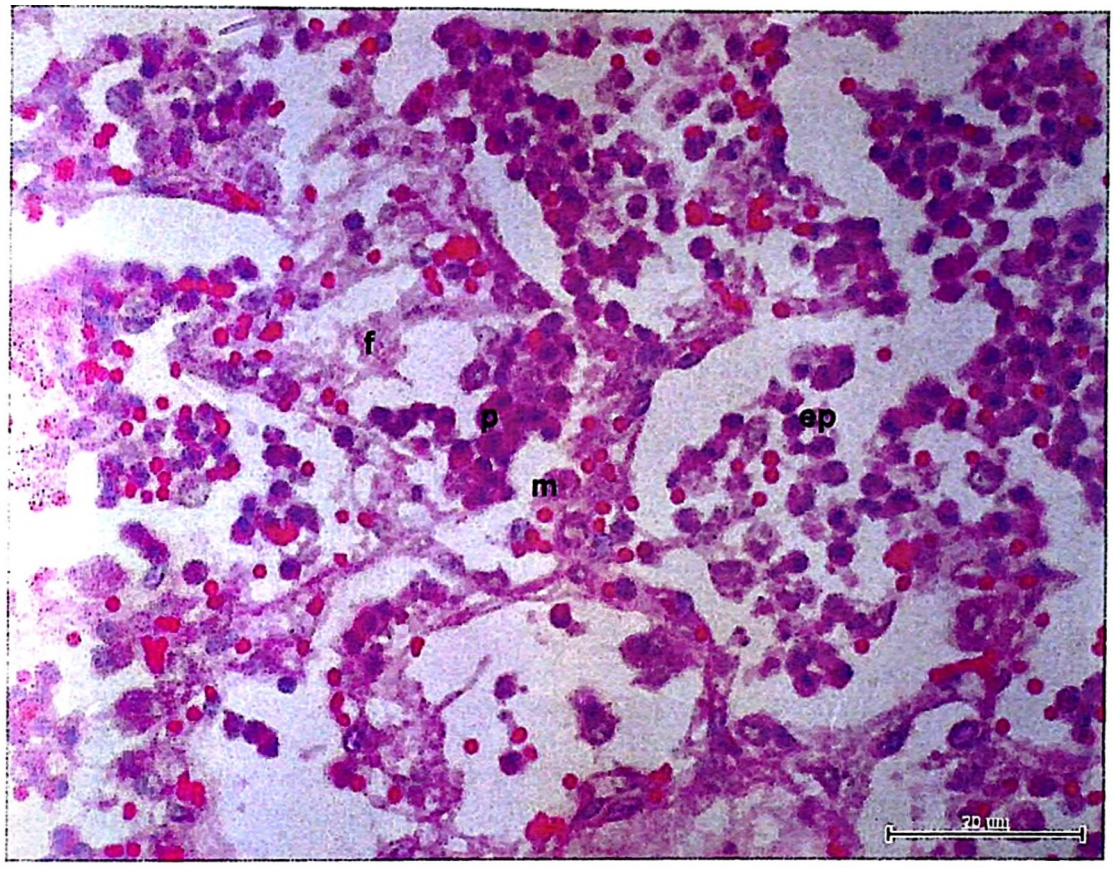

Plate 6b: Alveolar lumen with abundant plasma cells (p), strands of fibrin (f), desquamating epithelial cells (ep) and few macrophages (m). (HE)

Blood vessels in affected areas were peripherally infiltrated with lymphocytes and plasma cells. The vessels were inflamed (vasculitis) whereby the tunica media and adventitia were partly disrupted by fibrin i.e. fibrinous vasculitis, inflammatory cells and necrotic cells leading to rupture and haemorrhage. Endothelial hypertrophy was also observed (**Plate 7**).

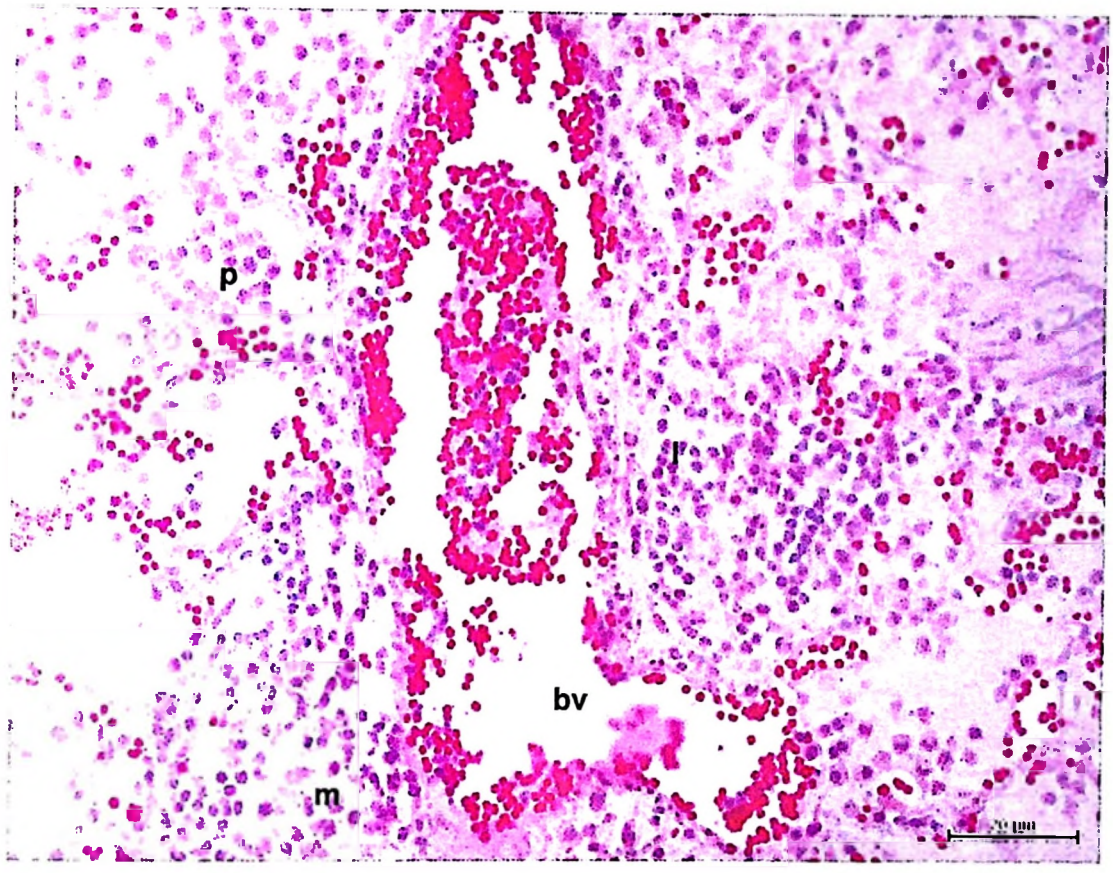

Plate 7: Ruptured blood vessel (bv) and perivasular leukocytic infiltration dominated by plasma cells (p), lymphocytes (l) and few macrophages (m). (HE)

In another case, affected lung sections revealed inflamed blood vessel (vasculitis) with marked infiltration with lymphocytes and plasma cells (perivascular cuffing), severe haemorrhage and marked oedema in disintegrating alveoli (**Plate 8**).

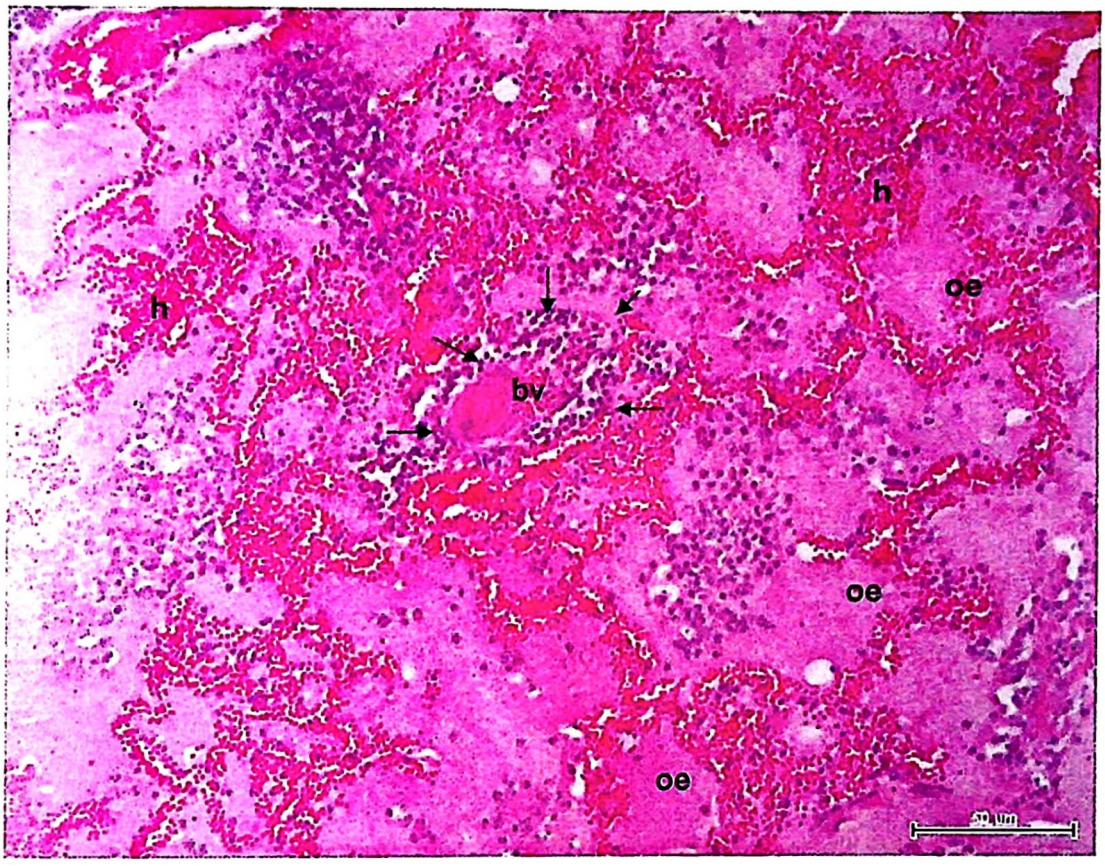

Plate 8: Inflamed blood vessel (vasculitis) in affected area (bv) with marked infiltration with lymphocytes and plasma cells (perivascular cuffing shown by arrows). Haemorrhage (h) and oedema (oe) are also prominent features in this section. (HE)

There was also lymphoplasmacytic infiltration and some macrophages peripheral to bronchi and bronchioles as well as in their lamina propria, submucosa and the bronchial cartilage; disrupted bronchial and bronchiolar muscularis; fibrinonecrotic exudates and oedema within the bronchi, bronchioles and peribronchial interstitium; disintegrating bronchiolar epithelial cells; and collapsing bronchioles (**Plate 9**).

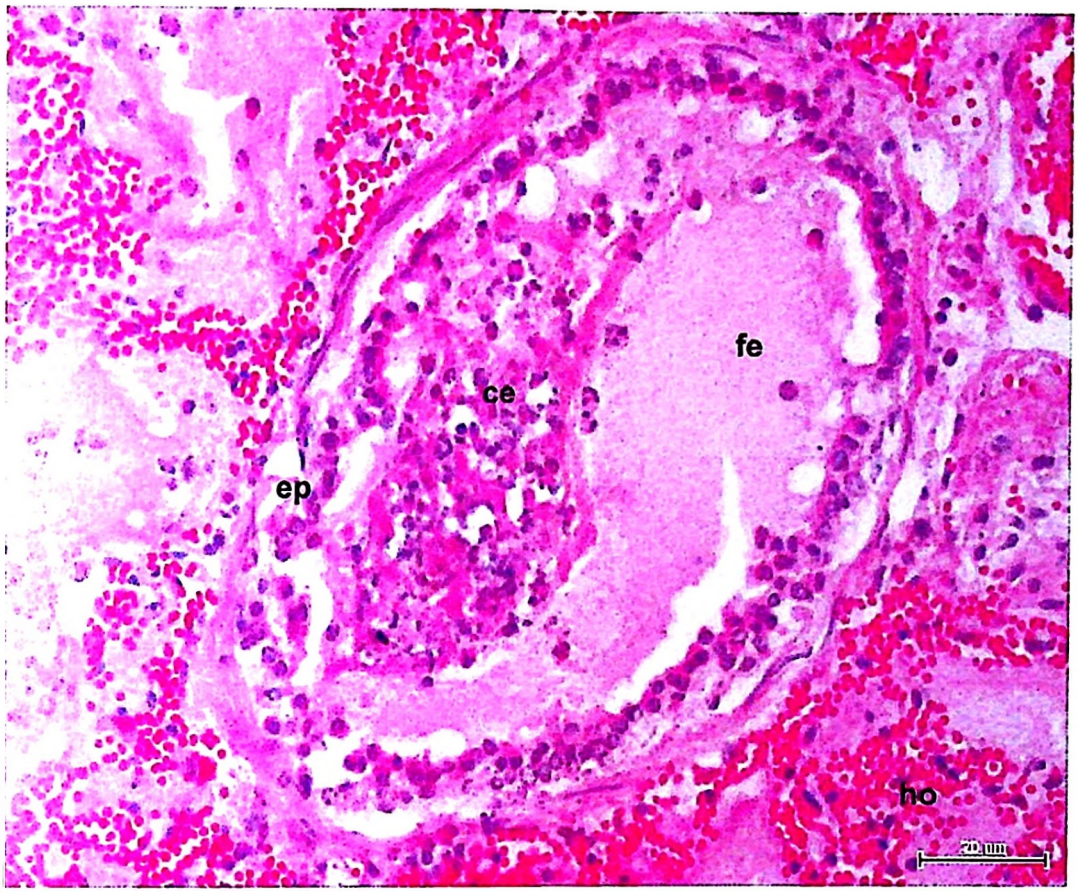

Plate 9: **Bronchiole with fibrinous exudates (fe), cells (ce) and desquamating epithelium (ep). Also severe haemorrhage and oedema (ho) in alveolar septa.** (HE)

4.4 Immunohistochemistry

Immunohistochemistry reaction was characterized by the presence of light to dark brown granules in cells and tissues. Using the monoclonal antibodies (MAbs) 3H12 and 6E3, which have previously been shown to react with antigens on all Mycoplasma of the mycoides cluster, demonstrated *Mycoplasma mycoides* subspecies *mycoides* Small Colony antigens throughout the affected lungs (**Plate 10a**). The antigens were detected in the alveolar lumen and wall, bronchiolar lumen and epithelium, glands and cartilage. *Mycoplasma mycoides miscodes* Small Colony antigens were not detected in control normal lung tissues.

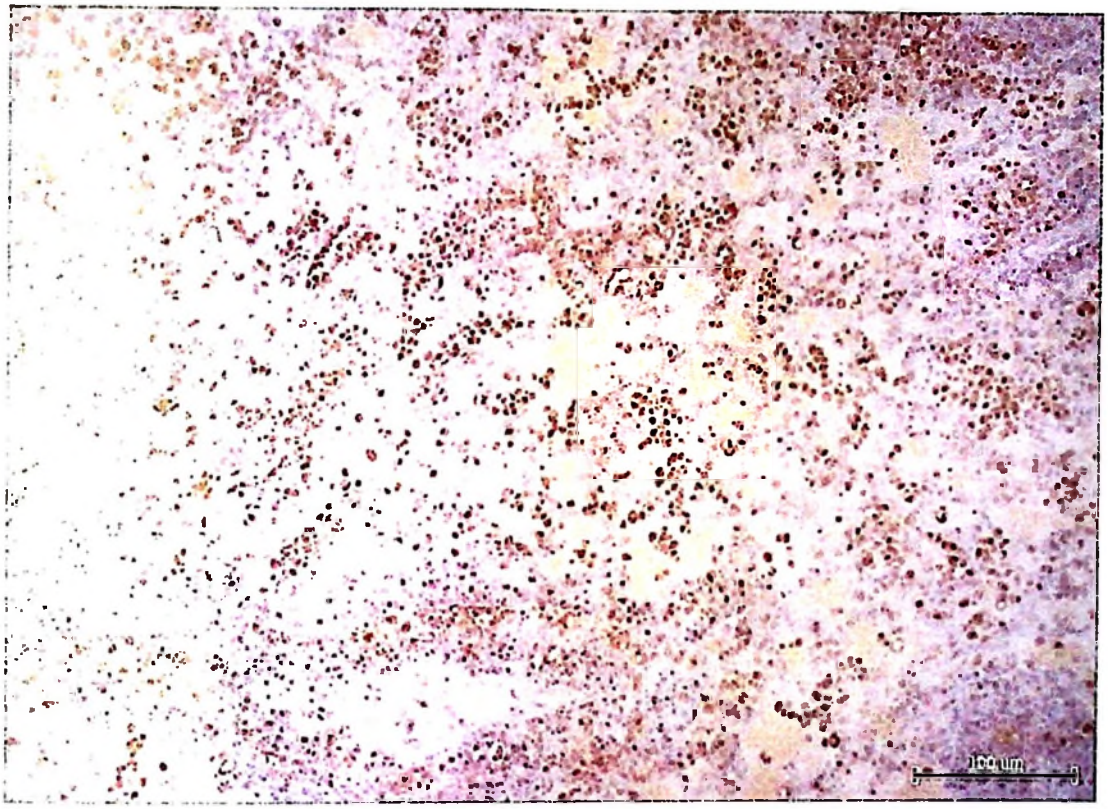

Plate 10a: Positive immunohistochemical staining diffused throughout the section (brown colour precipitates); in alveolar lumen, interalveolar septa, blood vessels, bronchiolar lumen and epithelium. (IHC)

In order to validate IHC results it was also important to include a section from a normal bovine lung as a negative control. The architecture of this section is well defined with clear empty alveoli and normal thin interlobular septa. There are no granular brown staining *Mmm*SC antigens (**Plate 10b**).

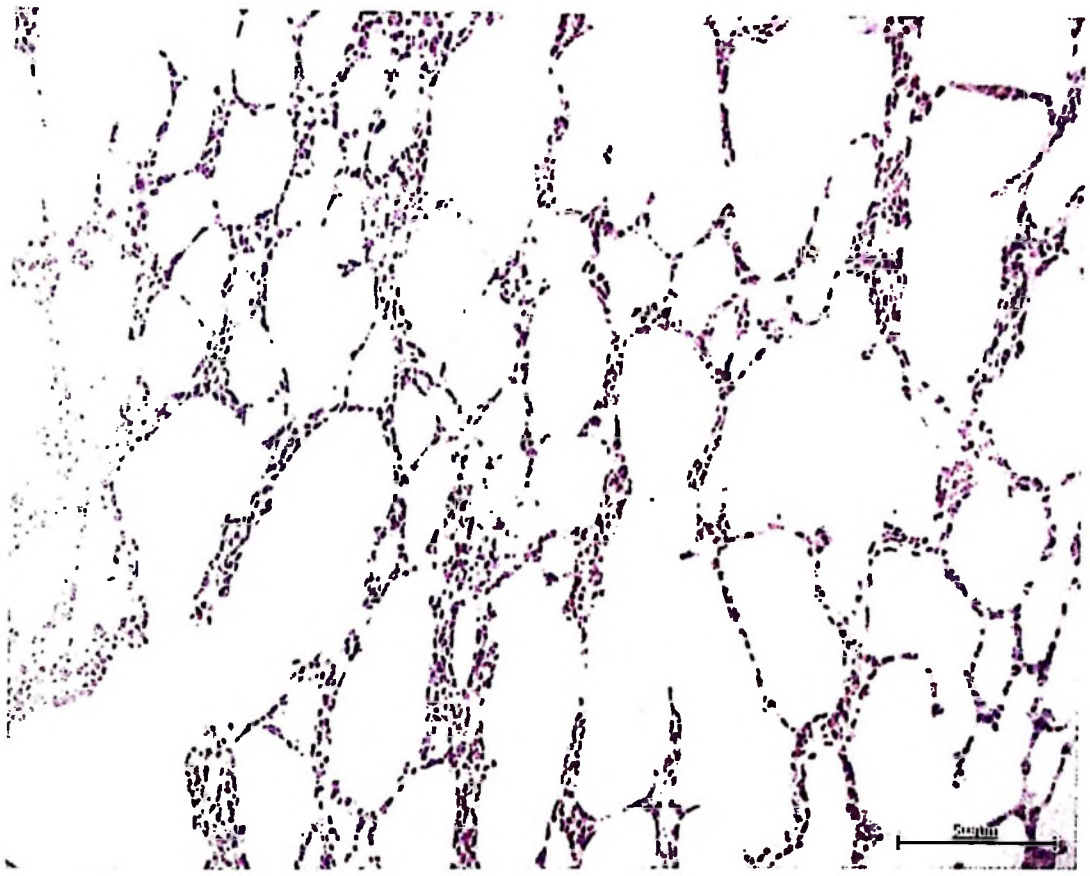

Plate 10b: Negative control. *Mycoplasma mycoides mycoides* Small Colony antigens were not detected in this section from normal lung tissue. (IHC)

In some lung samples positive reactivity with IHC staining was seen in the cytoplasm of inflammatory cells especially the macrophages and in the necrotic areas (**Plate 11**).

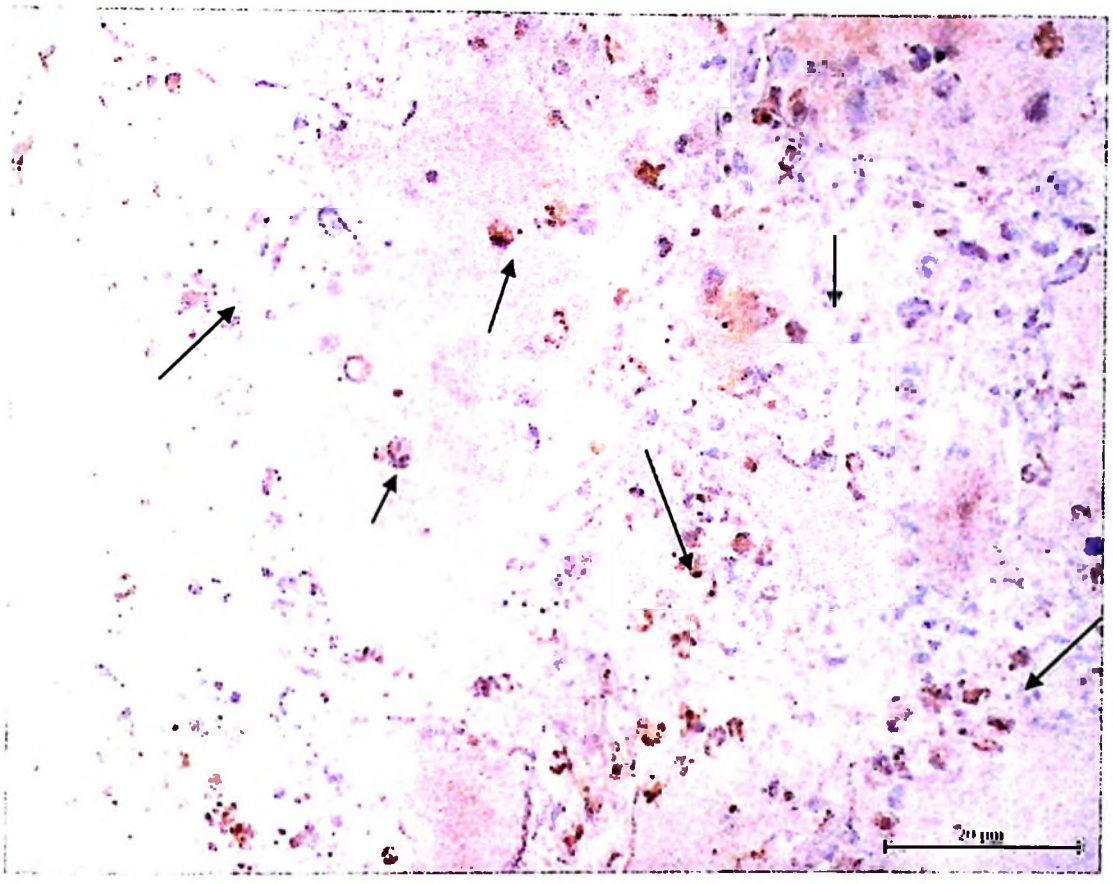

Plate 11: **Granular positivity of alveolar lumen, macrophage cytoplasm, desquamating cells and alveolar wall with 6E3 MAb (shown by arrows).** (IHC)

Other immunoreactive sites were detected in the small bronchioles and in the bronchiolar glands and epithelial cells (**Plate 12**).

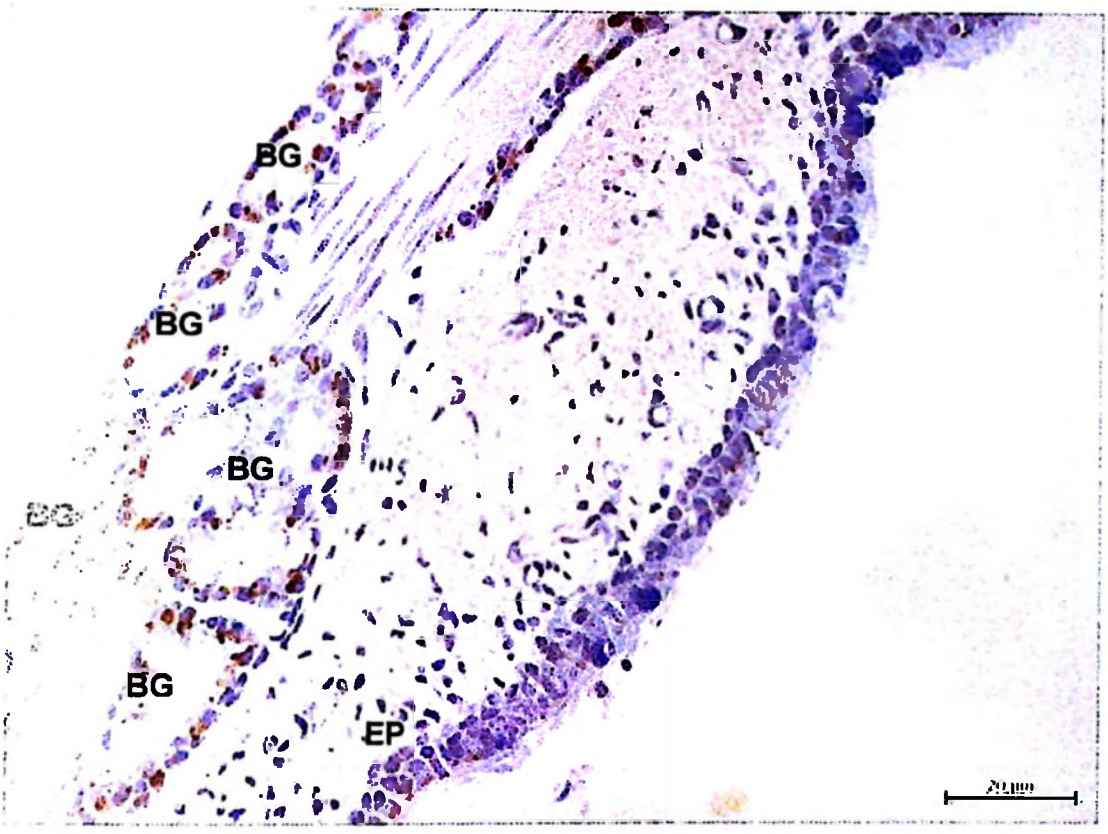

Plate 12: **Positive immunoreactivity (brown precipitation) in bronchiolar glands (BG) and epithelium (EP).** (IHC)

Bronchial cartilage was another site where the *Mmm*SC antigen were detected (**Plate 13**).

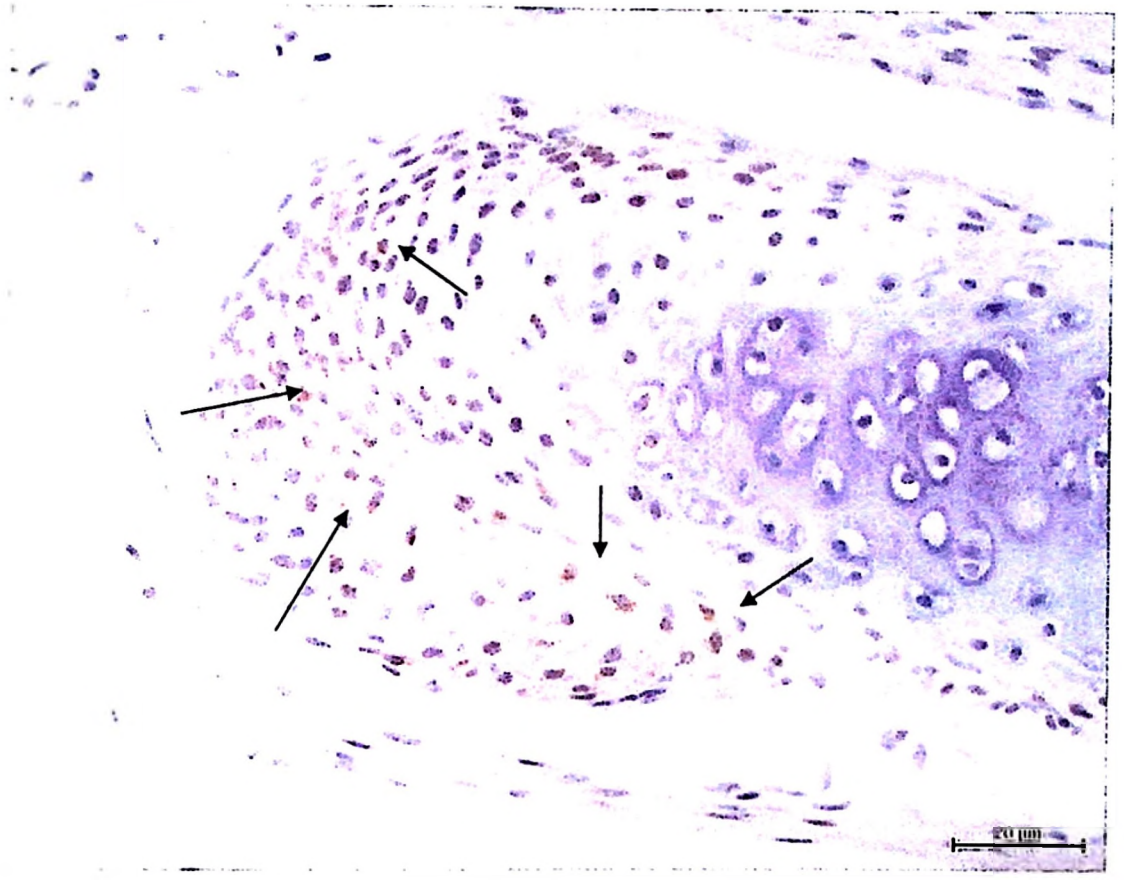

Plate 13: Positive immunohistochemical staining of cells in the bronchial cartilage (shown by arrows). (IHC)

The exudates in the bronchial lumen and the epithelial cells of the bronchioles also contained *Mmm*SC antigens and so were the cells in the alveolar walls (**Plate 14**).

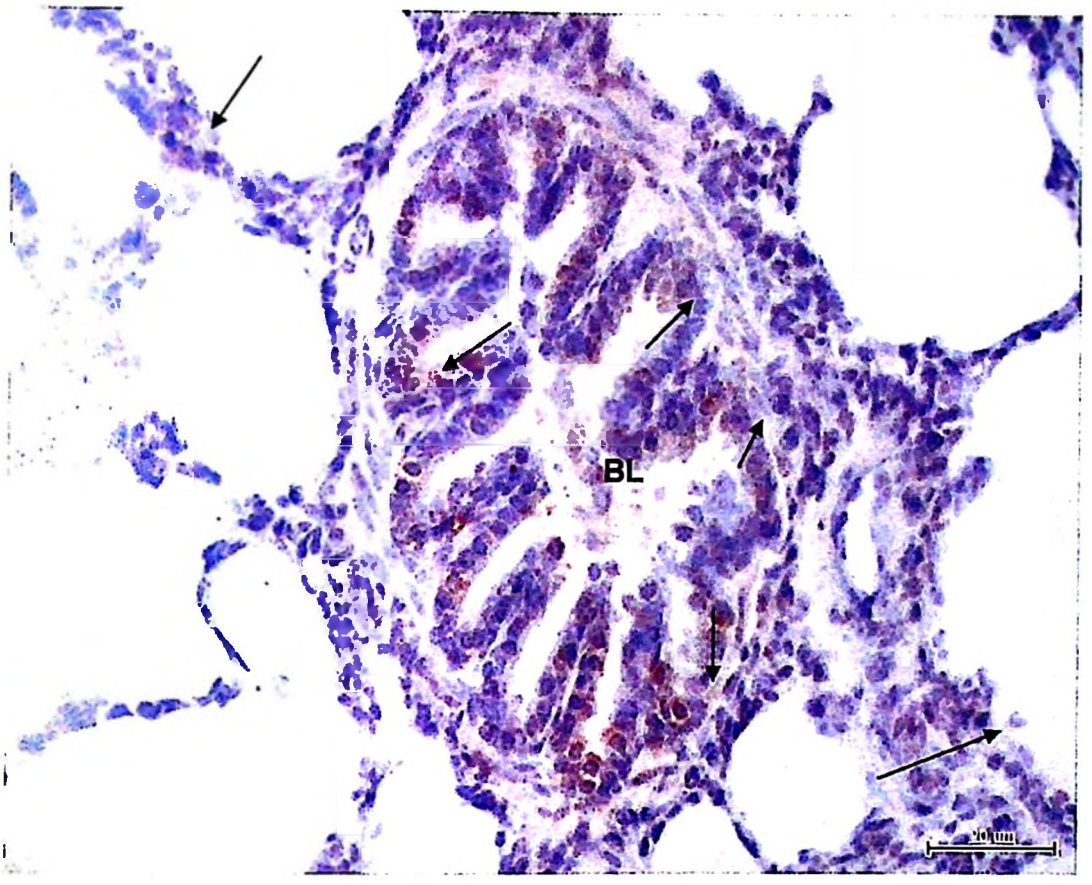

Plate 14: Positive immunohistochemical staining of cells in the exudates in the bronchial lumen (BL), epithelium and the cells in the interalveolar septa (arrows). (IHC)

The architecture of alveoli was lost and *Mmm*SC antigens were detected in the cells and the remnants of fibrotic alveolar walls (**Plate 15**).

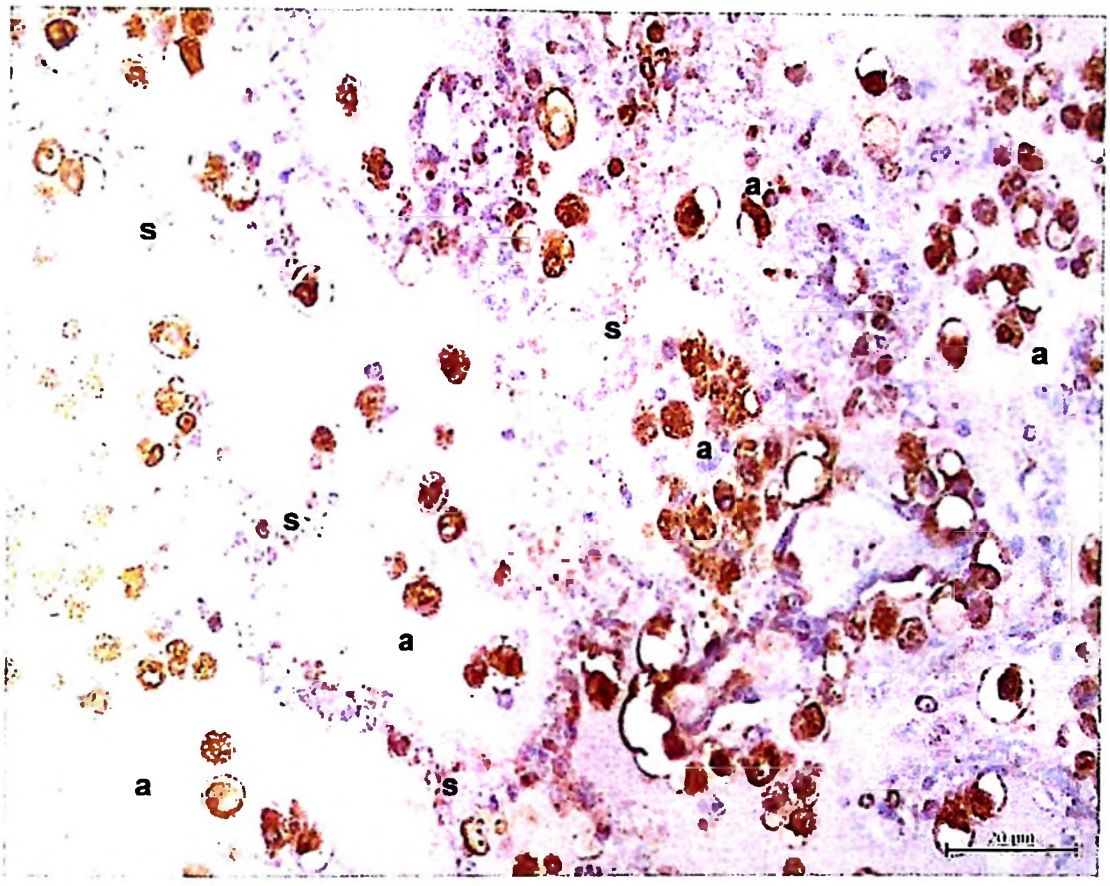

Plate 15: Positive immunohistochemical staining of cells in the alveolar lumen (a) and the fibrotic septa (s) with MAb 3H12. (IHC)

CHAPTER FIVE

5.0 DISCUSSION

Part of the problem of CBPP control has been the difficulty in the disease diagnosis. This is attributed to a number of factors including the fact that majority of animals infected with CBPP do not show clinical signs despite the presence of lung lesions (Petisca, 1990). The principal pathological consequence of *Mmm*SC is a massive inflammatory reaction mainly restricted to the lungs of the affected cattle, leading to death by respiratory distress caused by lung consolidation in up to 30% of cases (Provost *et al.*, 1987; FAO, 2003). Sub acute and symptomless forms of CBPP are very frequent and are characterised by mild signs or no clinical signs at all. Animals with this form transmit the infection and become the most dangerous of all (Masiga *et al.*, 1996). Chronic form of this disease which allows the host to recover and regain its normal healthy condition, remains a potential carrier as well as a threatening reservoir of *Mmm*SC (Provost *et al.*, 1987; FAO, 2003). This study focused on diagnosing the disease in these symptomless healthy animals slaughtered for human consumption at municipal abattoir. All slaughtered animals passed the antemortem examination as healthy and fit for slaughter. They had no signs of CBPP. However, during meat inspection their lungs were condemned for having CBPP-like lesions.

Macroscopic and microscopic findings observed in this study were consistent for all samples collected and were in harmony with previous observations on CBPP lesions (Petisca *et al.*, 1990; Geering and Amanfu, 2002; Thomson, 2005). In addition to gross and histologic observations, *Mmm*SC were isolated from 2 (n=13) samples.

Culture is an important method in diagnosing CBPP because it demonstrates the presence of *Mmm*SC in tissue lesions, and thereby confirm any outbreak of the disease. However, cultural methods are challenged by expertise and viability of the organisms after collection (Centikaya *et al.*, 2003). Further, sequester and marbling observed macroscopically during the disease's chronic stage pose difficulties in isolating the *Mmm*SC (Provost *et al.*, 1987). The success in isolation of *Mmm*SC heavily depends on proper collection, transportation and processing of appropriate samples. Also good quality media and adequately trained personnel are critical to effective laboratory isolation and identification of the causative agent of the disease. Centikaya *et al.*, (2003) reported that presence of antibacterial compounds, such as antibiotics, may contribute to the difficulty of *Mmm*SC isolation. Indeed, these factors could explain why out of 13 lungs with typical CBPP lesions (grossly and histologically and positive to *Mmm*SC antibodies) only 2 grew *Mmm*SC organisms on culture.

Contrary to isolation, results on immunohistochemistry demonstrated *Mmm*SC in all the 13 collected samples. The antigens were detected throughout the lung parenchyma, in blood vessels, bronchi, bronchioles, alveolar macrophage cytoplasms, and in the necrotic areas. The IHC localisation of *Mmm*SC antigens has revealed that the lung is the target organ. The ability of IHC to pick *Mmm*SC in all lung samples whereas isolation was positive only for 2 samples affirms the diagnostic potential of IHC in apparently healthy asymptomatic CBPP animals. Indeed, these very results affirm that it is difficult to isolate *Mmm*SC, the causative organism of CBPP. Thus culture as a diagnostic tool is too far to be used as a routine monitoring procedure for the disease. Instead, immunoperoxidase detection of the

*Mmm*SC antigens from the lungs which has proven reliability, efficiency, and consistency in this study is the sound diagnostic option. Generally, the detection of *Mmm*SC antigens in fixed tissues offers a number of advantages over other diagnostic techniques such as convenience of sample submission, rapidity, retrospective studies of stored specimens, and detection of non-viable organisms (Haines and Clark, 1991).

In a nutshell, IHC technique has proved to be sensitive for the diagnosis of CBPP. This diagnostic approach is less expensive and does not need very sophiscated equipment to perform as compared to CFT, ELISA and PCR. It nevertheless needs a well equipped laboratory, with properly laid down protocols and standard operating procedures to be performed. Upscaling and customizing the use of IHC will be a crucial step in monitoring re-emergence of CBPP in a particular geographical area.

Concisely, this very study strongly indicates that where isolation, is limited in application in the diagnosis of CBPP in Tanzania and many other African countries, IHC can be a good, cheap and handy method to confirm CBPP infection in apparently healthy animals slaughtered for human consumption. This diagnosis will then help surveillance of the disease from where the animals come from. For instance, animals slaughtered at Morogoro come from Kilosa, Mpwapwa, Ulanga, Dodoma and sometimes Mwanza. To control CBPP, thorough follow up investigation should be done in these areas to assess the magnitude of the disease and institute respective control measures.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

The aim of this study was to demonstrate the Mycoplasma (*Mmm*SC) organisms in the apparently healthy cattle slaughtered at the municipal abattoir. The abattoir, source of the samples proved to be an important point as far as the diagnosis of CBPP is concerned. In this area, skilled and qualified meat inspectors are needed as they play a big role in CBPP provisional diagnosis. These people are the ones who can tell beyond doubt the lesions seen in the pleural and on the lung of the slaughtered animal that are pathognomonic of CBPP.

The isolation of the organisms (*Mmm*SC) from the lung tissue using Mycoplasma Experience media though laborious and time consuming confirmed the presence of CBPP in the slaughtered cattle in Morogoro abattoir.

Histologically, all the samples revealed pathognomonic lesions of CBPP which were different from other types of pneumonia. Immunohistochemistry was even better, not only showing that the causative agent was present in the samples but also the technique was capable of locating in which part of the lung and even which types of cells contained the organisms. By using monoclonal antibodies, the technique demonstrated the ability of showing that the *Mmm*SC was at one time present in that tissue even if it was not there anymore due to its fragility behaviour or climatic or technical problems.

Now that IHC technique can be used as tools for laboratory diagnosis of CBPP, the next crucial step towards early detection of the disease is to design a field, on - the -

animal diagnostic kit easy to apply and user friendly for field personnel. This kind of a kit will help herd screening and for that matter facilitate detection of outbreaks in slaughter houses and abattoirs as a prelude to control spread of the disease to CBPP-free areas.

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APPENDICES

Appendix i: 10% Neutral Buffered Formalin

1. Sodium phosphate, monobasic dehydrates (NaH_2PO_4) ..... 18.0 g
2. Sodium phosphate, dibasic dehydrate ($Na_2HPO_4.2H_2O$) ..... 32.8 g
3. Formaldehyde 36% ..... 444.0 g
4. Distilled water ..... 4 000.0 g

Appendix ii: Preparation of Mycoplasma Experience media (Reigate, UK)

Agar diagnostic Medium – 25 ml.

1. The total volume of the medium when the components are mixed amounts to 25ml.
2. To reconstitute the medium, the agar was melted in boiling water at 100°C and allowed to cool slightly to 50°C water bath.
3. The diluent was added to the freeze-dried supplement, the mixture was agitated gently until the supplement was completely dissolved and placed in 50°C water bath for 15 minutes.
4. The reconstituted supplement was then added to the agar and mixed thoroughly, then 4 ml of the mixture was dispensed into Petri dishes then the medium was dried in an incubator at 37°C for 10 minutes and stored in the refrigerator till the day of use.

Appendix I: Preparation of gelatin-coated slides for histological tissue sections (R&D Systems®)

In order for tissue sections to be retained on histological slides during staining and washing steps, slides need to be coated with adhesive compounds. Although there are a variety of such compounds, gelatine was used, which is the most frequently used.

Procedure

1. Prepare the gelatin-coating solution by dissolving 5 g of gelatin in 1 L of heated, deionised H_2O (temperature should not exceed 45°C).

2. After the gelatin has dissolved, add 0.5 g of chromium potassium sulphate. Chromium potassium sulphate will positively charge the slides allowing them to attract negatively charged tissue sections.

3. Filter this solution and store at 2 - 8°C until use. It is recommended that this solution be filtered again immediately before use (adjust to room temperature before filtration).

4. Place the histological slides into metal racks.

Note: The slides should be cleaned by washing them in soapy water and rinsing them thoroughly, first in tap water and finally in deionised water.

5. Dip the racks containing the slides 3 to 5 times (~5 seconds each) into the gelatin-coating solution.

6. Remove the racks containing the slides and let them drain. Blot excess solution from the racks onto filter paper (gently tap the racks against the filter paper for better drainage).

7. Place the racks containing the slides on the bench and cover them with paper towels to protect them from dust.

8. Dry at room temperature for 48 hours.

9. Dried slides can be put back into the boxes that they arrived in and stored at room temperature until use.

Appendix iv: Antigen retrieval solution – 10mM citrate buffer (pH 6.0)

1. Add tri-sodium citrate dehydrate ..... 5.88 g

2. and 0.2M Hydrochloric acid solution ..... 44.0 g

3. to distilled water ..... 1 956 ml

4. Adjust pH to 6.0

Appendix v: Phosphate buffered saline (PBS)

Preparation

1. Measure a volume of 800 ml of dH_2O with a graduated cylinder and transfer to a flask.

2. Add a magnetic stir bar to the flask and place the flask on a magnetic stir plate. Adjust the speed of the magnetic stir bar so that oxygen is not introduced into the solution while it is rapidly mixed.

3. Transfer to the flask:

Sodium chloride (NaCl)	8.00 g
Potassium chloride (KCl)	0.20 g
di-Sodium hydrogen phosphate (Na_2HPO_4)	1.44 g
Potassium di-hydrogen phosphate (KH_2PO_4)	0.20 g

4. Allow the solutes to dissolve for 3 to 5 minutes.

5. Ensure that there are no remaining particles of undissolved salts in the solution before adjusting the pH. If particles are present, continue stirring vigorously.

6. Reduce the speed of the magnetic stir bar so that the solution is gently mixing.

7. Ensure that the pH meter has been properly calibrated and rinse the pH probe with dH_2O. Remove the excess water from the probe tip (without touching the probe tip) with a clean paper towel. Place the pH probe into the solution.

8. Slowly add 1 M HCl drop wise with a transfer pipette and allow the HCl to fully dissolve into the solution.

9. Stop stirring the solution.

10. Measure the pH with the pH meter.

11. Repeat Steps #8 through #10 until the pH of the solution is 7.4.

12. Pour the solution into a fresh graduated cylinder and adjust the final volume to 1

litre with dH_2O.

13. Transfer the solution to a container that is suitable for autoclaving at 15 lb/in^2.

14. Autoclave the solution on liquid cycle at 15 lb/in^2 for 20 minutes.

15. Allow the solution to cool to room temperature.

16. Store the PBS solution at room temperature. The PBS Solution is sterile; when using the PBS Solution, ensure that sterile techniques are employed.

Appendix vi: Immunohistochemistry protocol

Deparaffinization and sections to water

1. Deparaffinize sections in three changes of xylene each for 5 minutes.
2. Hydrate sections in descending grades of alcohol; two changes of 100% ethanol for 10 minutes each, two changes of 95% ethanol for 10 minutes each and finally in dH_2O for 1 minute with agitation.
3. Aspirate excess liquid from slides.

Antigen retrieval (unmasking of antigen)

1. Sections covered with 10mM Sodium citrate buffer (pH 6.0) and heat at 95°C for 5 minutes.
2. Top off with fresh buffer and heat at 95°C for 5 minutes.
3. Allow sections to cool in the buffer at room temperature for 20 minutes.
4. Wash in 3 changes of dH_2O for 2 minutes each.
5. Aspirate excess liquid from the slides.

Quenching (Inactivation of endogenous peroxidase)

1. Incubate in 3% hydrogen peroxide in dH_2O for 10 minutes.
2. Wash in 3 changes of PBS for 5 minutes each.

Immunoperoxidase Staining by HRP staining system

1. Incubate the sections for 10 minutes in ready for use pre-blocking solution (SPlink HRP detection kit). Dilute the primary antibody accordingly.
2. Remove excess pre-blocking solution from sections by blotting and make a ring around the tissue section using colourless nail polish.

3. Incubate sections in primary antibody (diluted in 2% NBS with PBS) for 60 minutes at room temperature.
4. Rinse in 3 changes of PBS for 2 minutes each.
5. Incubate sections for 10 minutes with ready to use broad spectrum biotinylated secondary antibody.
6. Rinse the sections in 3 changes of PBS for 2 minutes each.
7. Incubate with ready to use HRP-Streptavidin for 10 minutes at room temperature.
8. Prepare DAB by mixing 1 drop of DAB chromogen (20x concentrate) with 1 ml of substrate buffer.
9. Wash sections in 3 changes of PBS for 5 minutes each after 10 minutes incubation in HRP-Streptavidin.
10. Incubate sections in DAB enzyme for 5 minutes (till the desired colour is attained).
11. Counter stain in haematoxylin for 20 seconds.
12. Immediately rinse thoroughly in tap water for about 2 minutes and blue the sections in PBS for 60 seconds and rinse in dH_2O.
13. Dehydrate sections in ascending grades of ethanol; dip in 2 changes of 95% ethanol for 10 seconds each; dip in 2 changes of 100% ethanol for 10 seconds each; dip in 3 changes of xylene for 10 seconds each.
14. Mount sections with DPX and cover slip.
15. When dry, examine the sections by light microscope.

Appendix vii: Standard Operating Procedure for CBPP diagnosis

This is a flow of procedures of which if keenly followed there will be a uniform performance which will lead to results which are beyond doubt.

1. **Sample collection.**

Mycoplasma mycoides subspecies *mycoides* Small Colony biotype are delicate and do not survive much outside their host therefore sampling should be done at the time of slaughter. Cooler box with ice packs should be at hand to ensure that preservation is at considerable level before reaching the laboratory. Despite proper preservation, the samples should be transported fast to the laboratory.

In the laboratory, a portion of the sample meant for *Mmm*SC isolation should immediately be labelled and preserved in -20°C. After this, gross examination will be done, and then portion of the sample for histopathology/immunohistochemistry taken and preserved in a labelled container with 10% NBF. The remaining tissues can be disposed off or preserved in -20°C after thorough labelling.

2. ***Mycoplasma mycoides* subspecies *mycoides* Small Colony biotype isolation**

This work is done in bacteriology laboratory. All necessary arrangements should be done a day before. The preserved sample is then plated on solid media or broth aseptically following standard procedures. The plates of solid media and the tubes of broth should be well labelled. After inoculation these are incubated in a pre-set incubator (with temperature, humidity and CO_2). Follow up is done from day two whereby a homogenous cloudiness will appear for a positive growth in the broth and by using stereo microscope the small colonies (1 mm

diameter) with a classical appearance of "fried eggs" can be seen on solid medium. A growth in the broth will be followed by subculture on solid medium.

3. Tissue processing

Fixed tissues will be trimmed and put in labelled processing cassettes for standard histological processing and embedding in paraffin wax. Sectioning at 4 - 5 μ will be done with the aid of rotary microtome and attached onto labelled subbed glass slides for IHC and plain glass slides for routine H and E. All the slides will be dried in paraffin oven for overnight and from here they can be stored boxes for staining.

4. Routine H and E staining

This can be done progressively or regressively after which the sections are mounted and left to dry for microscopical examination.

5. Immunohistochemistry

Indirect IHC is done using monoclonal antibodies to detect *Mmm*SC antigens with the aid of conjugated secondary antibodies (**Appendix VI**).

6. Reporting

Laboratory results will be documented on a form (**Appendix VIII**) for reporting and further follow up.

SPE

Appendix viii: Laboratory reporting form

ANTE/POST-MORTEM EXAMINATION

Owner's Address		Animal's Particulars	
		ID	
		Date	
		Spp	
		Age	
		Sex	
		Breed	

History and Clinical Examination (Antemortem)

Examination of carcass/organs/tissues (Postmortem)							
Reporting date		Examiner/inspector					
Summary of lesions							
1							
2							
3							
4							
PDx							
Laboratory Requests							
	1	2	3	4	5	6	7
Bacteriology							
Virology							
Parasitology							
Histopathology							
Microscopy							
Other							
FDx							