

**ADDIS ABABA UNIVERSITY
COLLEGE OF HEALTH SCIENCE
SCHOOL OF VETERINARY MEDICINE**

**PATHOLOGICAL, BACTERIOLOGICAL AND SEROLOGICAL STUDY ON
PNEUMONIC SHEEP AND GOATS WITH SPECIAL EMPHASIS ON *MANNHEIMIA*,
BABERSTEINIA, *PASTEURELLA* AND *MYCOPLASMA* SPECIES**

BY

TILAYE DEMISSIE

**JUNE, 2010
DEBRE ZEIT, ETHIOPIA**

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A thesis submitted to the School of Graduate Studies of Addis Ababa University, College of health science, and School of Veterinary Medicine in partial fulfillment of the requirements for the Degree of Master of Science in Tropical Veterinary Pathology

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LIST OF ABBREVIATIONS

CCPP	Contagious caprine pleuropneumonia
CFT	Complement Fixation test
CIRAD-EMVT	Centre de Cooperation Internationale en Recherche Agronomique pour le Developpement-Departement d'Elevage et de Medecine Veterinaire des Pays Tropicaux.
CSA	Central statistical authority
DNA	Deoxyribonucleic acid
DVM	Doctor of veterinary medicine
ELISA	Enzyme Linked Immunosorbent Assay
FOA	Food and Agricultural Organization of the United Nations
FVM	Faculty of veterinary medicine
IgG	Immunoglobulin
IHA	Indirect Haemagglutination
IL	Interleukin
LPS	Lipopolysaccharide
M.a	<i>Mycoplasma arginini</i>
M.ovp	<i>Mycoplasma ovipneumoniae</i>
Mcc	<i>Mycoplasma Capricolum</i> subspecies <i>capricolum</i>
Mccp	<i>Mycoplasma Capricolum</i> subspecies <i>capripneumoniae</i>
Mmc	<i>Mycoplasma mycoides capri</i>
MmmLC	<i>Mycoplasma mycoides</i> subspecies <i>mycoides</i> Large colony
MmmSC	<i>Mycoplasma mycoides</i> subspecies <i>mycoides</i> small colony
MOA	Ministry of agriculture
NAHDIC	National Animal Health Diagnostic and Investigation Centre
NH ₃	Ammonia
NVI	National Veterinary Institute
°C	Degree centigrade
OF	Oxidation Fermentation

OIE	Office Internationale Des Epizooties
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PPLO	Pleuro Pneumonia like Organism
SPSS	Statistical program for social science
SRBC	Sheep red blood cells
TNF	Tumor Necrosis Factor
TSI	Triple Sugar Iron Agar
URT	Upper Respiratory Tract
WHO	World Health Organization of the united nation

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ABSTRACT

A prospective, non-probability and purposive type study was conducted from October to April on pneumonic sheep and goats to isolate and characterize *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species involved in pneumonia of sheep and goats. The concurrent occurrence of *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species together were studied and *Mannheimia haemolytica* isolates were strain serotyped. Comparative isolation of *Mannheimia*, *Babersteinia* and *Pasteurella* species from the same samples of lung was performed. Conventional bacteriological isolation and identification procedures (Quinn *et al.*, 2002; Sisay and Zerihun, 2003 and OIE, 2004) were used for isolation and identification of *Mannheimia*, *Babersteinia* and *Pasteurella* species. Isolation and identification of *Mycoplasma* species was conducted according to Miles and Nicholas (1998) and OIE (2008). Growth inhibition by monospecific hyperimmune sera and PCR techniques were used for *Mycoplasma* species identification. The total isolation rate from pneumonic lung from both sheep and goats were 28 % (78/279) for *Mannheimia haemolytica*, 3.9 % (11/279) for *Babersteinia trehalose* and 2.2% (6/279) for *Pasteurella multocida*. Total isolation rate of *Mannheimia*, *Babersteinia* and *Pasteurella* species together was 40.7% from pneumonic sheep and 24.1% from pneumonic goats. Serotyping of *Mannheimia haemolytica* isolates was carried out using IHA against 10 reference antisera. Serotype A2 (21.5%), A8 (20.5%), and A1(17.7%) were the three dominant serotypes from total isolates of both species. Serotype A2 (26.3%), A1(17.5%), A 8 (15.8%) were dominant serotypes from sheep isolates and serotypes A8 (33.3%), A1 (19%), and A11(14%) were the dominant serotypes isolated from goat. Serotype A13 was not isolated both from sheep and from goats and serotypes A5, A7, and A12 were not isolated from goats in the present study. Thirty-five mycoplasmas were isolated and characterized from pneumonic lungs of sheep and goats in the present study. Four *Mycoplasma* species namely, *Mycoplasma ovipneumoniae* (9 isolates), *MmmLC* (2 isolates), *Mmc* (1 isolate) and *Mycoplasma arginini* (2 isolates) were identified from sheep. Five *Mycoplasma* species namely, *MmmLC* (9 isolates), *Mcc* (3 isolates), *Mccp* (3isoaltes), *Mmc* (4 isolates) and *M. arginini* (2 isolates) were isolated and identified from goats. The concurrent isolation rate of *Mannheimia haemolytica* and *Mycoplasma* species was 24.6% in sheep and 9.5% in goats. *Mycoplasma ovipneumoniae* was isolated at 15.8% rate with *Mannheimia haemolytica* in sheep. This was the highest concurrent occurrence observed while

compared to other *Mycoplasma* species isolated from *Mannheimia haemolytica* positive lungs. *Mmm*LC, *Mmc* and *Mycoplasma arginini* were also isolated from *Mannheimia haemolytica* positive sheep. Generally, the isolation rate of *Mycoplasma* and *Mannheimia haemolytica* together was significantly lower in goats than in sheep and the only *Mycoplasma* species isolated together with *Mannheimia haemolytica* from goats was *Mmc*.

Finally, the results are discussed and compared with previous work. Some points on control strategies and areas of further research are also suggested.

Keywords: Antisera/*Babersteinia*/DebreZeit/Ethiopia/*Mannheimia*/*Mycoplasma*/*Pasteurella*/Pathological/ Pneumonic lung / Rate/ Serotype.

1. INTRODUCTION

Ethiopia owns huge number of small ruminants, estimated to be 42 million heads of sheep and goats. Small ruminants are found mainly in the lowland agro-ecology, which constitutes 65% of the total area of the country, where 25% sheep and close to 100% goat populations exist (Anonymous 2000; PACE-Ethiopia, 2003). Sheep and goats play a significant role in the nation's economy. Meat and milk are major sources of protein and hides, live animals, and carcasses account for a significant proportion of exports (CSA, 2006). Hence, an increase in small ruminant production could contribute to the attainment of food self-sufficiency in the country particularly in response to protein requirement for the growing human population as well as to enhance the export earnings. This rich potential, however, is not properly exploited due to constraints of which diseases come in the forefront. Among these, respiratory diseases represent a serious threat to small ruminant production (PACE-Ethiopia, 2003).

Pneumonia refers to the inflammation of the pulmonary parenchyma usually accompanied by the inflammation of bronchioles and often pleurisy and, it is characterized by respiratory embarrassment or sometimes toxemia. Upper respiratory infections are accompanied with respiratory abnormalities and very often, they descend to pneumonia. Lambs and kids are particularly susceptible to pneumonia (Lopez, 2001; Kusiluka and Kambarage, 2005).

Pneumonia is usually preceded by some kind of stress. Stressful conditions include weaning, long distance transport, sudden change in weather, poor ventilation, overcrowding, and malnutrition, sudden change in feed, and parasitism. The microorganisms that cause pneumonia are usually, normal inhabitants of the upper respiratory tract. Under normal conditions, the body's natural defense inhibits the growth of the germs and keeps the balance between the microbes and the body. Stressful conditions may break the balance in favor of microorganisms and then cause disease. Animals maintained on a good plane of nutrition are both protected and more resistant to stress (Donachie, 2009).

Bacterial, viral, and parasitic infections break down tissue defense barriers. Loss of this natural protection increases a host susceptibility to secondary bacterial infections. Animals whose lungs

are already weakened from previous diseases will suffer from leukotoxins and lipopolysaccharides, both potent toxins that, in high levels, promote inflammation and severe lung damage. It has been suggested that *Mycoplasma* interaction with the host's cilia prevents normal ciliary activity, facilitating the invasion of the lower respiratory tract by other organisms, including *Mannheimia haemolytica* and *Pasteurella multocida* (Jones *et al.*, 1985; Martin and Aitken, 2000).

In Ethiopia, pneumonic pastuerellosis and manheimiosis have been investigated extensively in sheep particularly in highland areas. Much is known regarding about contagious caprine pleuropneumonia(CCPP) in goats but little is known about the role of other *Mycoplasmas* as an etiology of pneumonia in small ruminants in Ethiopia. There are also no well written documents about the concurrent occurrence of *Mycoplasma*, *Pasteurella* and *Mannheimia* in Ethiopia.

Therefore, the objectives of this work are:

To study the concurrent occurrence of *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species involved in pneumonia of sheep and goats

To isolate and identify *Mycoplasma* species involved in small ruminants' pneumonia and compare the isolation rate of *Mycoplasma* species between sheep and goats

Serotyping of *Mannheimia haemolytica* isolated from pneumonic lungs and comparing them with *Mannheimia haemolytica* serotypes distributed in sera of the same animals

To study on the pathological changes induced by single organism and/or combination of *Mycoplasma*, *Pasteurella*, *Babersteinia* and *Mannheimia* species

2. LITERATURE REVIEW

2.1. Revision on the reclassification of the genus *Pasteurella*

The term pasteurellosis was broadly used to designate a number of infections in domestic animals caused by Gram-negative non-motile facultative anaerobic rods or coccobacilli formerly grouped under the genus *Pasteurella* (after Louis Pasteur). For several decades, the genus *Pasteurella* was believed to be only one single genus with numerous species responsible for or associated with a wide range of systemic, pulmonary and septicaemic infections in various species of farm animals, particularly ruminants. However, with more recent advancements in molecular biology involving DNA hybridization studies and 16S rRNA sequencing, most of the formerly recognized species were found to share a number of uncommon features and became the subject of intensive revision and reclassification. In this respect, *Pasteurella haemolytica* biotype A was allocated to a new genus and renamed as *Mannheimia*. This new genus now contains several species including *M. haemolytica*, *M. granulomatis*, *M. glucosida*, *M. ruminalis* and *M. varigena* (Angen *et al.*, 1999a; Highlander, 2001; Zecchinon *et al.*, 2005). The name *Mannheimia* was given in tribute to the German scientist Walter Mannheim for his significant contributions in the recent taxonomy of the family *Pasteurellaceae*. On the other hand, *Pasteurella haemolytica* biotype T was first reclassified as *P. trehalose*. However, this organism was recently revised and removed to a new separate genus by the name of *Bibersteinia trehalose* (Blackall *et al.*, 2007). In addition, avian *Pasteurella* species including *Pasteurella gallinarum*, *Pasteurella paragallinarum* and *Pasteurella volantinum* were similarly removed to a new separate genus named as *Avibacterium* (Blackall *et al.*, 2005).

Before the establishment of this newly revised classification, *Pasteurella haemolytica* was known to comprise two biotypes: A and T, based on fermentation of arabinose and trehalose, respectively. Within these two biotypes, 17 serotypes were further identified on basis of soluble or extractable surface antigens by indirect haemagglutination procedure or rapid plate agglutination test (Carter and Chengappa, 1991). Serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16 and 17 belong to biotype A which was reclassified as *M. haemolytica*. However, serotype A11

was later reclassified as *Mannheimia glucosida* (Younan and Fodor, 1995). The rest serotypes (3, 4, 10 and 15) belong to biotype T, which was initially reclassified as *P. trehalose* before being finally moved to a separate new genus as already mentioned (Angen *et al.*, 1999a; Angen *et al.*, 1999b; Blackall *et al.*, 2007).

Pasteurella multocida is characterized serologically by capsular serogroups A, B, D, E, F and as well as 16 somatic serotypes (Carter, 1990; Odugbo *et al.*, 2003; Boyce *et al.*, 2004; Sleim, 2005; Odugbo *et al.*, 2006).

It is worth mentioning that *M. haemolytica*, *P. multocida* and *Bibersteinia trehalose* (*P. trehalose*) constitute the most important members of the family *Pasteurellaceae* that pose serious hazards in livestock industry. Although *Pasteurella*, *Bibersteinia* and *Mannheimia* have long been considered primarily animal pathogens they were also reported to produce serious systemic and/or localized infections in human beings (Klein and Cunha, 1997; Marinella, 2004; Ashley *et al.*, 2004).

2.2. Pneumonic pasteurellosis and mannheimiosis of sheep and goats

2.2.1. Etiology

Pneumonic Pasteurellosis and Mannheimiosis are caused by *Pasteurella multocida* or *Bibersteinia trehalose* and *Mannheimia haemolytica*, respectively and affect various species of animals (Gilmour *et al.*, 1991; Links *et al.*, 1992; Odugbo *et al.*, 2003; Odugbo *et al.*, 2006). Because *Mannheimia haemolytica* is frequently isolated from the lungs of affected animals, most investigations have focused on this species. However, *Pasteurella multocida* types A and D have occasionally been associated with the disease (Links *et al.*, 1992; Zamri-Saad *et al.*, 1996).

Strains of *Mannheimia haemolytica* are differentiated by a combination of biotypes and serotypes; and apparent association between biotypes, serotypes and the disease caused are described in table 1.

Table 1: *Mannheimia haemolytica* and *Baberssteinia trehalose* biotypes, serotype and the diseases they cause.

Biotype	Serotype	Designation	diseases
A	1,2,5-9,11,13,14,16	A1	Septicemia (lambs)
		A2	Pneumonia (cattle)
		A5-A9	Pneumonia (sheep and goats)
		A11-A14, A16	Pneumonia
			Inhabitants of URT of normal sheep, goats and cattle
T	3,4,10,15	T ₃ , T ₄ , T ₁₀ , T ₁₅	Septicemia (sheep)

Source: De Alwis (1993)

The infectious serogroups of *P. multocida* associated with outbreaks of pneumonic pasteurellosis in sheep and goats are A and D (particularly 1A and 4D) as shown in table 2 and these have been incriminated both as secondary and primary agents of pneumonia (Chandrasekhar *et al.*, 1991; Zamri-Saad *et al.*, 1996; Odugbo *et al.*, 2003).

Table 2:- Designation of *Pasteurella multocida* serotypes by Nomioka-Carter method.

Capsular type	Somatic type	Serotype	Diseases
A	1,3,5,7,9	1A	Pneumonia (cattle, sheep, pig)
		3A	Pneumonia (pig)
		5A	Fowl cholera
		7A	Septicemia (cattle)
		8A,9A	Fowl cholera
B	6,11	6B	Hemorrhagic septicemia
		11B	Wound infection (bovine)
D	1,2,3,4,10	1D,4D,10D	Pneumonia (pig)
		3D	Pneumonia (cats)
		4D	Pneumonia (sheep and pig)
E	6	6E	Hemorrhagic septicemia

Source: De Alwis (1993)

2.2.2. Virulence factors of *Pasteurella* and *Mannheimia*

It is established that the ability of pathogenic bacteria to cause infection is greatly influenced by certain endogenous factors, which can enhance the pathogenicity of the organism and facilitate rapid invasion and destruction of target tissues of the susceptible host. These factors are generally designated as virulence factors and constitute parts of the surface components of the bacterial cell and cellular products. Virulence factors are, in fact, capable of promoting adhesion, colonization and proliferation of the organism within the animal tissues. In other words, virulence factors are actively involved in conversion of the organism from commensal into pathogen (Confer *et al.*, 1990; Gonzales and Maheswaran, 1993; Quinn *et al.*, 2002). The role of virulence factors in the pathogenicity of *M. haemolytica* has been extensively investigated (Gonzales and Maheswaran, 1993; Zecchinon *et al.*, 2005).

The cell capsule constitutes an important virulence factor which plays vital roles in the pathogenicity of pathogenic bacteria and establishment of infection. The virulence mechanism of

the cell capsule is mostly attributed to its ability to protect the invading organism against cellular and humoral defense mechanisms of the host. The capsular materials of *M. haemolytica* and other *Pasteurella* species were identified as polysaccharide basic structures produced during the logarithmic phase of growth of the bacteria. Each serotype of *M. haemolytica* produces a characteristic polysaccharide capsule in order to avoid phagocytosis by macrophages and polymorphonuclear leukocytes and to protect the organism against complement-mediated destruction of the outer membrane in serum. The capsular material of *M. haemolytica* can also interact with the pulmonary surfactant and thereby facilitates the adhesion of the invading organism to the respiratory tract epithelium of susceptible animals (Whiteley *et al.*, 1990; Ackermann and Brogden, 2000; Jaramilo *et al.*, 2000).

Fimbriae are smaller appendages present on the surface of many Gram-negative bacteria. They are specific surface structures of the bacterial cell wall that permit or enhance adherence to and colonization of the target epithelium of the susceptible animals. Fimbriae are present in various strains of *Pasteurella* and *Mannheimia* species. Fimbriae produced by *M. haemolytica* are capable of enhancing mucosal attachment of the organism and colonization of the lower respiratory tract epithelium. Successful colonization will thus enable considerable increase in the number of bacteria seeded in the lung tissue beyond the level that normal lung capacity could efficiently resolve (Gonzales and Maheswaran, 1993).

Similar to all other Gram-negative bacteria, the cell wall of *M. haemolytica* contains lipopolysaccharides (LPS) endotoxin. This endotoxin is one of the most important virulence factors involved in the pathogenesis of pneumonic pasteurellosis. It has been shown that serotypes A2 and A8 of *M. haemolytica* possess a rough LPS while the other 14 serotypes have characteristic smooth LPS. Experimental evidence indicated that *M. haemolytica* endotoxin is directly toxic to endothelial cells and capable of altering leukocyte functions and causing lysis of blood platelets. However, the precise mechanism of endothelial cell injury by endotoxin has not yet been fully clarified but the toxic effect of endotoxin could be reduced by neutrophils (Breider *et al.*, 1990; Lacroix *et al.*, 1993; Jaramillo *et al.*, 2000).

It has also been shown that *M. haemolytica* produces a soluble heat labile exotoxin known as leukotoxin because of its high specificity for leukocytes of ruminants. The leukotoxin is a 102 KDa protein secreted at the logarithmic phase of growth of the bacteria and considered as a main weapon or virulence factor for *M. haemolytica*. In fact, the leukotoxin is pore-forming cytotoxin that can produce several biological effects on leukocytes and blood platelets of ruminants. The most susceptible cells are macrophages and neutrophils from most ruminant species, lymphocytes and cultured lymphoma cells. At low concentration, leukotoxin impairs phagocytosis and lymphocyte proliferation while at higher concentration it has a cytotoxic effect resulting in cell death due to lysis. The lysis of cells is attributed to the formation of transmembrane pores in the target cell, and thereby allowing the movement of potassium, sodium and calcium ions through transmembrane gradients (Clinkenbeard and Upton, 1991; Majury and Shewen, 1991; Zecchinon *et al.*, 2005). Leukotoxin also causes stimulation of polymorphonuclear leukocytes and activation of macrophages with consequent release of proinflammatory cytokines such as interleukin-1 (IL-1), interleukin-8 (IL-8), leukotrienes and tumour necrosis factor (TNF). This action would further lead to the release of H₂O₂ which, in turn, is converted into hydroxyl radicals by alveolar endothelial cells. The free hydroxyl radicals cause considerable damage and necrosis of the pulmonary alveolar epithelium resulting in accumulation of edema fluid and fibrin inside alveoli and interstitial spaces (Biberstein and Hirsh, 1999; Lopez, 2001).

The leukotoxin and enzymes released following cytolysis are both chemotactic for various types of inflammatory cells causing more damage to the lung tissue due to increased cell recruitment into the area (Zecchinon *et al.*, 2005). Furthermore, *M. haemolytica* leukotoxin was found to induce *in vitro* morphological alterations in neutrophils similar to those produced by the viable infective organism during the natural course of the disease in the living animal (Clarke *et al.*, 1998).

Some *M. haemolytica* serotypes including serotype A1 and serotype A2 were found to produce certain extracellular enzymes that are involved in the pathogenesis of the disease. One of them is neuraminidase, a unique neutral protease which specifically hydrolyses O-sialoglycoproteins (Abdullah *et al.*, 1992). This enzyme is produced *in vivo* during active *M. haemolytica* A1 lobar infection in goats (Straus and Purdy, 1994). Although the precise role of neuraminidase is not

fully understood, it was suggested to play an active role in the colonization of epithelial surfaces by removing terminal sialic acid residues from mucin, thereby modifying normal host innate immunity (Biberstein and Hirsh, 1999). In addition, *M. haemolytica* A1 was also found to produce an IgG1 specific protease, which was regarded as another virulence mechanism contributing to the pathogenesis of the disease (Lee and Shewen, 1996).

Another important antigen is iron-regulated outer membrane protein which promote iron acquisition by bacteria (Czuprynski *et al.*, 1991; Morck *et al.*, 1991; Pandher *et al.*, 1999).

2.2.3. Pathogenesis and pathology

The pathogenesis of pneumonic Pasteurellosis and Mannheimiosis remained a subject of considerable speculation and controversy due to the complex nature of the disease and the lack of consistency of the results obtained by experimental approach (Mohamed and Abdulsalam, 2008). In earlier literature, Yates (1982) reviewed several findings of field workers and researchers who demonstrated that *M. haemolytica* cannot act alone as the causative pathogen of the disease in the absence of a well-defined predisposing factor. Failure of induction of the disease by direct inoculation of the organism in healthy animals was attributed to the rapid clearance of the bacteria by pulmonary defense mechanisms. On the other hand, clinical signs of acute pneumonic pasteurellosis were successfully induced by intratracheal or endobronchial inoculation of a pure culture of *M. haemolytica* (Vestweber *et al.*, 1990; Foreyt and Silflow, 1996) without the involvement of any predisposing factor. Many other authors also believe that pneumonic pasteurellosis is a secondary bacterial complication of a previous viral infection of the respiratory system (Cutlip *et al.*, 1993; Cutlip *et al.*, 1996). However, the sequential development of the pulmonary lesions is highly mediated by complex interactions between the naturally existing causative organism in the upper respiratory tract, the immunological status of the animal and the role of predisposing factors in the initiation of infection (Mohamed and Abdulsalam, 2008).

Endotoxins produced by rapid growth and multiplication of the bacteria in infected lobules will cause extensive intravascular thrombosis of pulmonary veins, capillaries and lymphatics. These vascular disturbances eventually result in focal ischemic necrosis of the pulmonary parenchyma

accompanied by severe inflammatory reaction dominated by fibrinous exudates (Jones *et al.*, 1997; Lopez, 2001). Formation of antigen-antibody complexes may also contribute to the vascular permeability and chemotaxis of neutrophils with the subsequent release of lysozyme (Mohamed and Abdulsalam, 2008). The severity of lesions, however, depends on the rate and extent of bacterial proliferation and the amount of endotoxin released, which in turn depends on the virulence of the bacterial strain and the degree to which the defenses of the host are impaired (Dungworth, 1993).

The pathological alterations associated with pneumonic pasteurellosis and mannheimiosis have been extensively investigated (Dungworth, 1993; Jones *et al.*, 1997; Lopez, 2001). The gross lesions in affected lungs were generally described as a prototype of fibrinous (lobar) bronchopneumonia with prominent fibrinous pleurisy and pleural effusions. The inflammatory process is well dominated by fibrinous exudation in the pulmonary alveoli accompanied by interstitial edema and congestion, imparting a marbled appearance of the cut surface of the affected parts of the lung tissue. Lesions are always bilateral with cranioventral distribution, usually below a horizontal line through the tracheal bifurcation. The cranial and the middle lobes are the mostly affected parts, but in severe cases, infection may be more extensive involving substantial portions of the diaphragmatic lobe or dorsocaudal lobe (Mohamed and Abdulsalam, 2008).

The diseased portions of the lung become remarkably consolidated or hepatized, dark red in colour and covered with tangled masses of fibrinous strands. The interlobular septa are often distended with yellow gelatinous edema fluid or fibrin and the pleura are noticeably thickened and dull with focal adhesions to the thoracic wall. Multiple areas of coagulative necrosis are consistently observed within the pneumonic portions of lung parenchyma and they appear irregular in shape and sharply demarcated with thick white borders and deep red central zone. The mediastinal and bronchial lymph nodes are frequently congested, edematous and/or hemorrhagic and the trachea and major bronchi are occasionally flooded with conspicuous amounts of frothy fluid (Dungworth, 1993; Brogden *et al.*, 1998; Lopez, 2001; Mohamed and Abdulsalam, 2008).

The histological features of the affected lungs are dominated by diffuse capillary congestion, interstitial and alveolar edema together with vascular thrombosis of capillaries, small blood vessels and pulmonary lymphatics. Alveolar wall necrosis is commonly seen in many affected lobules with the presence of variable amounts of fibrinous exudates and inflammatory cells inside the alveoli. The necrotic areas are usually surrounded by a rim of elongated cells, often referred to as “swirling macrophages” or “oat cells” which are now thought to be degenerated neutrophils mixed with alveolar macrophages (Dungworth, 1993; Brogden *et al.*, 1998; Lopez, 2001).

Extensive and wide spread pulmonary vascular thrombosis is well observed as a consistent finding in natural and experimental infections with *M. haemolytica* in sheep and goats. However, the thrombi are usually limited to small blood vessels, capillaries and lymphatics of pneumonic lungs, but large vessels are not apparently affected. An unusual incidence of thrombotic occlusion of a large pulmonary vein of an adult Angora goat with an acute fibrinous bronchopneumonia due to *Mannheimia haemolytica* infection was further documented (Mohamed and Abdulsalam, 2008). The occurrence of such thrombi in large pulmonary blood vessels may well reflect the highly potent procoagulant effect of *M. haemolytica* endotoxin on the vascular endothelium of the lung of infected animals (Mohamed and Abdulsalam, 2008).

Bacterial colonies are frequently detected near the necrotic areas of the lung tissue. Fibrinocellular exudate is frequently observed inside the bronchi and bronchioles with accumulation of dark swirling macrophages inside alveoli and alveolar ducts. *M. haemolytica* antigen has been detected in the necrotic alveolar walls and also in fibrin, serous exudate and degenerating leukocytes by the use of immunoperoxidase technique. In addition, fibrinous pleurisy is also indicated by massive deposition of fibrinous exudates and diffuse infiltration of leukocytes in the plural surfaces (Brogden *et al.*, 1998 and Lopez, 2001).

It is worth mentioning that the gross and histopathological lesions in *M. haemolytica* infected lungs were apparently similar to those produced by *P. multocida*. However, some authors suggested that the most striking differences between them were the lack of focal necrosis of the lung tissue and the presence of large numbers of neutrophils in cases of *P. multocida* pneumonia (Mohamed and Abdulsalam, 2008). Dungworth (1993) stated that *M. haemolytica* tends to cause

fulminating fibrinous lobar pneumonia while *P. multocida* usually causes fibrinopurulent bronchopneumonia, also described other differential features between *M. haemolytica* and *P. multocida* pneumonias (Dungworth, 1993).

The pathological findings in the pneumonic lungs of spontaneous or experimental *M. haemolytica* infections in goats were also similar to those observed in sheep (Zamri-Saad *et al.*, 1991; Dungworth, 1993; Brogden *et al.*, 1998; Lopez, 2001). The most consistent findings in goats were also dominated by extensive bilateral cranioventral consolidation (hepatization) of the cranial and the middle lobes with fibrinous strands covering most of the affected parts of the lung. Fibrinous pleurisy with or without adhesions was observed in most cases, while some other cases showed focal abscesses from which pure cultures of *M. haemolytica* were isolated (Hassan, 1999; Mohamed, 2002).

2.2.4. Clinical signs and diagnosis

Observable clinical signs of acute respiratory distress usually develop within 10 to 14 days in adult animals after being exposed to stress but a much earlier onset is more typical (Radostitis *et al.*, 2000). Nevertheless, infected animals in severe cases may die because of toxemia even before the development of significant pulmonary lesions. In this case, sudden death may be the first sign of acute outbreaks particularly in young animals. After the onset of respiratory disturbances, infected animals appear extremely dull with reduced appetite and remarkable depression. They soon develop high fever, anorexia and rapid shallow respiration accompanied with profuse mucopurulent nasal and ocular discharges. Later on, productive cough, which is accentuated by physical effort or movement, usually develop in most of the infected animals. Marked dyspnoea with an expiratory grunt may be observed in very advanced stages of the disease (Dungworth, 1993; Radostits *et al.*, 2000; Lopez, 2001). In acute outbreaks, the clinical course of the disease is relatively short terminating in death or recovery in either treated or non-treated animals. However, a number of sick animals that survive the acute phase may become chronically infected. The severity of the disease is rather variable under field conditions and serious

economic losses would ultimately result from massive fatalities in acute outbreaks or from poor productivity in chronically infected animal (Radostitis *et al.*, 2000).

Diagnosis is based on clinical signs and herd history. Dead animals can be used for a definitive diagnosis. Isolation and culture of the bacteria harvested from a tracheal wash and from pulmonary secretions can be used to isolate and identify the causal agent. A necropsy of lobes from the lungs will show hemorrhagic (bloody) secretion, and possibly pus and dead tissue lesions. Diagnostic laboratories also use the polymerase chain reaction (PCR) technique, which can be costly, and immunohistochemical techniques. These analyses are valuable in identifying causal agents and determining treatment and preventive methods (Browning, 2007).

2.2.5. Status of sheep and goat mannheimiosis and pasteurellosis in Ethiopia

In Ethiopia, a number of attempts have been made so far to identify the different serotypes of both *Mannheimia* and *Pasteurella* species in small ruminants. Recent works on the Mannheimiosis and pasteurellosis have demonstrated the presence of serotypes A1, A2, A5 to A9, A11, A12, A13 A14 and T3, T4, T10 and T15 (Gelagay, 1996; Tesfaye, 1997; Aschalew, 1998; Mekonnen, 2000; Sisay and Zerihun, 2003 and Belay, 2007). Data on status of small ruminants *Pasteurella multocida* is scant.

2.3. Pneumonic mycoplasmosis in sheep and goats

2.3.1. General view and characteristics of *Mycoplasma*

Mycoplasmas are grouped in the class *Mollicutes*; Order: *Mycoplasmatales*; Family; *Mycoplasmataceae* and genera *Mycoplasma* (Nicolas, 2002). *Mycoplasma* grows slowly and generally requires 3 to 6 days incubation before colonies are apparent. Growth is best at 37°C in atmosphere of CO₂ and sterols are required by all species of *Mycoplasma*. Colony size varies from 0.1mm to 1mm. When observing with dissecting electron microscope, many species exhibit

“fried egg” morphology (except *M. ovipneumoniae*). This appearance is the result of the central portion of the colony embedding into the agar with peripheral zone of surface growth. Some species produce film or spot, which are composed of cholesterol and phospholipids and seen as a wrinkled film on the media surface (Adehana *et al.*, 2006).

Mycoplasma ovipneumoniae displays an uncharacteristic morphology on solid medium containing the usual concentration of agar (1.5-2.0%). Colonies do not have the “fried egg” appearance because they lack the central down growth that gives most *Mycoplasma* colonies that morphology. Rather, the colonies on such agar appear granular. On medium with lower agar concentrations, the normal appearance is reestablished (Da Massa, 1992).

Mycoplasmas of the mycoides clusters are pathogens of the ruminants and comprise six closely related species that are subdivided into two subgroups based on genetic similarity. The capricolum subgroup includes *Mycoplasma capricolum* subspecies *capricolum*, *Mycoplasma capricolum* subspecies *capripneumoniae*, and *Mycoplasma* subspecies *bovine group7*(BG7). The mycoides subgroup consists of *Mycoplasma mycoides* subspecies *capri*, *Mycoplasma mycoides* subspecies *mycoides small colony*, and *Mycoplasma mycoides* subspecies *mycoides large colony*(Nicholas, 2002).

Generally mycoplasmas exhibit a greater degree of variability between species and strains and causes different disease syndromes, however, clonal lineages have been observed for a number of species (McAuliffe *et al.*, 2004; Thiaucourt and Roger, 2005) and disease outbreaks have been attributed to single strains in most of the cases (Thiaucourt and Roger, 2005).

Infections due to *Mycoplasma* species impose serious constraints on animal production all over the world because of high mortalities. It has resulted in great direct economic losses because of the substantial reduction in milk yield, meat, skins and hair to many countries reputed for animal production in Africa, Europe, Middle East, Asia, Australia and North America (Belton, 1990; FAO/WHO/OIE, 2000; Ruffin, 2001). The indirect losses are due to the chronic nature of *Mycoplasma* infection, which results in emaciation, delayed marketing and reduced fertility (Cottew and Leach, 1996).

2.3.2. Etiology of pneumonic mycoplasmosis in sheep and goats

The etiological agent of mycoplasma pneumonia in goats include *MmmLC* (Vallalba *et al.*, 1992), *Mmc* (Cottew and Leach, 1996), *Mcc* (DaMassa *et al.*, 1992), and *Mccp* (Thiacourt and B"olske, 1996) but *Mccp* the etiology of CCPP, is the most virulent and induce fatal systemic disease in goats (Table 3).

Sheep mycoplasma pneumonia has been associated with *Mycoplasma ovipneumoniae*, *Mcc*, *MmmLC* and *Mycoplasma arginini* (Carmichael *et al.*, 1992; Chaturvedi *et al.*, 1992; Ikheloa *et al.*, 2001; Kusiluka and Kambarage, 2005). However, Brandao (1995) recovered two strains of *MmmSC*, generally believed to be cattle pathogen, from goats with pneumonia and three strains of same organism from the milk of sheep with the symptoms of mastitis

Table 3:- Diseases/syndromes of sheep and goats caused by *Mycoplasma* species.

organism	Sheep	Goat	Cattle	Sign
<i>Mccp</i>	No	Yes	No	Pleuropneumonia (Rurangirwa and Kinyili, 2004)
<i>Mcc</i>	Yes	Yes	No	Mastitis, arthritis, keratoconjunctivitis, pneumonia (Nicholas, 2004)
<i>MmmSC</i>	Rare	Rrare	Yes	Pleuropneumonia (Thiaucourt <i>et al.</i> , 2004)
<i>MmmLC</i>	Yes	Yes	Rare	Pneumonia , arthritis, mastitis (Nicholas, 2004; Rurangirwa and Kinyili, 2004)
<i>Mmc</i>	?	Yes	No	Pneumonia , arthritis, mastitis (Rurangirwa and Kinyili, 2004)
<i>Mycoplasma group 7</i>	No	No	Yes	Mastitis, poly arthritis and abortion (Hum <i>et al.</i> , 2000)
<i>Mycoplasma agalactiae</i>	Yes	Yes	No	Mastitis, arthritis, keratoconjunctivitis (Nicholas, 2004)

Source: - Nicholas (2002).

Mycoplasma ovipneumoniae plays a role in disease of goats and sheep. This *Mycoplasma* can be isolated frequently from the lung, trachea, and nose and occasionally from the eyes of sheep with pneumonia but can also be found in the respiratory tract of healthy sheep. Sheep inoculated intravenously and 1-day old lambs infected by aerosol developed an interstitial pneumonia. Studies have suggested that *Mycoplasma ovipneumoniae* and *Pasteurella haemolytica* serotype A

act synergistically to produce a chronic disease (atypical pneumonia) principally affecting lambs up to 12 months of age (Ruffin, 2001).

2.3.3. Pathogenesis, pathology and immunity

Modulation or activation of host immune response is critical in the pathogenesis of mycoplasmal disease. Some pathogenic mycoplasmas including those involved in the pulmonary diseases are mitogenic for B or T lymphocytes (Muhlrat and Schade, 1991). Activation of the macrophages and monocytes leads to the release of cytokines including tumour necrosis factor and interleukins, resulting in the initiation of inflammation. Pneumonia producing mycoplasmas that adhere to the ciliated respiratory epithelium can induce ciliostasis, loss of cilia and cytopathic changes (Quinn *et al.*, 2002). *Mycoplasma* can adhere to neutrophils and macrophages and can impair phagocytic function. In addition individual species damage cells by active penetration (Quinn *et al.*, 2002). It is recognized that some mycoplasmal antigens react with the antigens of the host tissue. This molecular mimicry has two possible outcomes, which are important in the pathogenesis. The antigenic similarity to the host tissue may allow the *Mycoplasma* to establish persistent infection by avoiding recognition by the host immune system. It may also lead to the development of autoimmune disease if an immune response to *Mycoplasma* antigen develops and cross-react with host cell antigen (Quinn *et al.*, 2002).

Tight adherence of most *Mycoplasma* species to the host cell membrane may afford protection against defense mechanisms like mucociliary clearance and phagocytosis. Thus, provide an environment for local toxicity by products exerted by the organism such as H_2O_2 and NH_3 to accumulate and cause tissue damage. The absence of cell wall segregating the *Mycoplasma* species membrane from the host cell favor the exchange of antigen between the cells. As a result, autoimmunity will be stimulated which cause tissue damage (Thiaucourt *et al.*, 1992; Ross and Young, 1996).

The lesions and clinical signs of contagious caprine pleuropneumonia (CCPP) have been extensively investigated when compared to other respiratory mycoplasmal infection and most

lesions and clinical signs of respiratory *Mycoplasma* are similar so that throughout this literature lesions and clinical signs of CCPP are discussed unless otherwise specified. The lesions of CCPP are limited to the respiratory system. Acute disease is characterized by unilateral or bilateral pneumonia and serofibrinous pleuritis with straw-colored fluid in the thorax. On cut surface, the lung is granular with copious straw-colored exudate. Pea-sized, yellow nodules may be found in the lungs. These nodules are surrounded by areas of congestion. Varying degrees of lung consolidation or necrosis can be seen, and the regional (bronchial) lymph nodes are enlarged. Some long-term survivors have chronic pleuropneumonia or chronic pleuritis, with encapsulation of acute lesions and numerous adhesions to the chest wall (CFSPH, 2008).

Congestion, consolidation and marbling of the lungs together with fibrinous pleuritis, pleural effusion and distension of the interlobular septae are prominent features. The chronic disease is characterised by thickening of visceral pleura and fibrinous adhesion to the chest wall. No sequesterae are formed as in the case of contagious bovine pleuropneumonia. Histopathologically, *Mycoplasma* F 38 infection is characterized by intralobular edema while *Mycoplasma mycoides* subspecies *capri* is characterized by interlobular edema (Kusiluka and Kambarage, 2005).

Histopathological examination of the CCPP affected lungs often shows interlobular acute serofibrinous to fibrino-necrotic pleuropneumonia with infiltrates of serofibrinous fluid and inflammatory cells, mainly polymorphonuclear leucocytes, in the alveolar spaces, bronchioles, interstitial septae and subpleural connective tissues (Kibor, 1990; Ojo and Obi, 1996).

Two main classes of antigens stimulate the immune response to the *Mycoplasma* infection. These are membrane glycolipid and lipid free proteins. Glycolipid antigens elicit most of the common serological reactions and also serve as receptors for complement mediated lysis and growth inhibition by antibodies. Protein antigens are reactive in eliciting delayed hypersensitivity and macrophage migration inhibition, both examples of cell-mediated immunity (Ross and Young, 1996).

In *Mycoplasma* infections, serological secretory and cellular immune mechanisms are stimulated. Antibodies produced locally by plasma cells in the lamina propria in the lung protect the host by

aiding the clearance of infectious organisms and blocking their deleterious effect. Systemic cellular and humoral immune responses can be detected by various serological tests and by assessment of antigens stimulatory of lymphocytes *in vitro*. These systemic reactions are the extraneous manifestation of the local immune response of *Mycoplasma* species and appear not directly responsible for combating the infective organism. The level of antibodies detected by direct serological tests, therefore, may not correspond to the immunity of the host (Thiaucourt *et al.*, 1992; Ross and Young, 1996).

2.3.4. Epidemiology

Infections due to *Mycoplasma* species are distributed all over the world. It imposes serious constraints on animal production in Africa, Europe, Middle East, Asia, Australia and North America (Belton, 1990; FAO/WHO/OIE, 2000; Ruffin, 2001) but CCPP is a serious disease and showed limited epidemiological distribution in countries in Africa, Asia (including India and Pakistan) and the Middle East (CFSPH, 2008). *Mccp* is difficult to isolate from clinical material, and its presence has not been confirmed in all affected countries. Countries known to have isolated *Mccp* include Ethiopia, Kenya, the Sudan, Tunisia, Oman, Turkey, Chad, Uganda, Niger, Tanzania, Eritrea, Nigeria, Benin, Cameroon and the United Arab Emirates (CFSPH, 2008).

Contagious caprine pleuropneumonia is highly contagious and is transmitted during close contact by the inhalation of respiratory droplets. Some outbreaks have occurred in endemic areas when apparently healthy goats were introduced into flocks, and in one experiment, a goat developed clinical CCPP nearly three months after contact with infected goats and a month after all other animals had recovered (CFSPH, 2008).

2.3.5. Isolation, biochemical characterization and diagnosis of *Mycoplasma*

Wild strains should be passed and preferably cloned three times before identification is attempted. Colony morphology and growth characteristics may be of some help for the differentiation of *Mycoplasma* species. Biochemical characterization and growth inhibition with

known antisera are the main tools to identify the species of *Mycoplasma* although techniques that are more modern like PCR and enzyme restriction analysis could reveal the confirmation.

Contagious caprine pleuropneumonia should be suspected when severe respiratory disease, with a high morbidity and mortality rate, is seen in goats. The typical necropsy lesions can aid diagnosis. Polymerase Chain Reaction (PCR) assays are used to identify cultures of *M. capripneumoniae*, as well as to identify this organism directly in tissue samples. Immunohistochemistry can identify *M. capripneumoniae* antigens in tissue samples, but it is not routinely used in diagnostic laboratories. Antigens can also be detected with gel immunoprecipitin tests (Nicholas, 2002).

Biochemical tests cannot identify an isolate unequivocally, which at present can only be done by serological or genetic means. Sensitivity to digitonin, phosphatase activity, protein digestion, arginine hydrolysis, tetrazolium reduction, glucose fermentation, and film and spot formation are biochemical tests performed routinely in isolation and characterization of *Mycoplasma* species (Bolske *et al.*, 1995; Nicholas, 2002). The members of the mycoides clusters are typically glucose fermenters and unable to hydrolyze arginine except *Mcc* which can sometimes hydrolyze arginine (+/-) but serum digestion by their proteolytic activity exclusively distinguishes members of ruminants *Mycoplasma* species belonging to the mycoides clusters with the exception of *MmmSc*, which cannot digest inspissated serum. This feature is the most important for the practical differentiation of *MmmLc* and *MmmSc* types of the mycoides subspecies mycoides in diagnostic works (Miles and Nicholas, 1998).

Serological tests that are used to diagnose *Mycoplasma* include complement fixation, latex agglutination, indirect haemagglutination and enzyme linked immunosorbent assays (ELISA). Serological tests are generally used on a herd basis and not for individual diagnosis. These tests do not identify all reactors, and cross-reactions occur with other species in the *M. mycoides* cluster. In addition, animals with acute CCPP rarely develop measurable titers before death (Pettersson *et al.*, 1998).

2.3.6. Status of small ruminants' respiratory mycoplasmosis in Ethiopia

Little is known about the role of mycoplasmas as an etiology of pneumonia in small ruminants in Ethiopia and, if present, is that of *Mycoplasma capricolum subspecies Capripneumoniae* or the disease CCPP in goats, which is well studied and documented, so that this literature also deals on what is present. In Ethiopia, the disease CCPP was first observed in 1983 in the region bordering Kenya and Sudan both of which are known for a high prevalence of CCPP (Anonymous, 1992; Mebratu, 1998). It was confirmed later in 1990 by isolation and identification of *Mccp* (Thiaucourt *et al.*, 1992). Since then the disease has become endemic in different regions of the country and a total of 72 outbreaks have been reported for the year between 1999-2008 in six different regions of the country. It should also be noted that there are considerable number of unreported cases each year (MOA, 2008). It is one of the most severe disease on account of its morbidity and mortality (Rurangirwa and McGuire, 1996) and is a major threat to goat production in developing countries (Lorenzone *et al.*, 2006).

Mccp induced CCPP-like clinical disease was not observed in sheep while antibody was quite frequently detected (Mekonnen, 1996; Yigezu *et al.*, 2004; Solomon, 2005). Sheep are naturally resistant to CCPP disease. The isolation of *Mccp*, however, proves the role of sheep as a reservoir of infection (Yigezu *et al.*, 2004).

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted at Hashim Nur Ethiopian Livestock and Meat Export (HELMEX) abattoir, which is located in Debre Zeit. Debre zeit is located in Oromiya National Regional State about 47 Kms South East of Addis Ababa at an altitude of 1850 meters above sea level. It is found at 9⁰N latitude and 40⁰E longitude. The area experiences a bimodal rainfall pattern with a short rainy season from March to May and a long one from June to October. The area has an average annual rain fall of 800 mm and an average minimum and maximum temperatures of 12.3⁰C and 27.7 ⁰C, respectively (CSA, 2006).

3.2. Study animals

Animals were selected as candidates for this study based on observation of respiratory signs (coughing, nasal discharge) and a total of 600 animals (sheep, n=300 and goat, n=300) exhibiting respiratory disease signs were tagged, their age recorded and submitted for complete necropsy observation and sampling during slaughter.

3.3. Study protocol

Purposive (non-probability) sampling was carried out on sheep and goats with respiratory signs (coughing, nasal discharge).

3.4. Sample size

The study was purposive and nonprobability type but as a base line number to start with, the method used for sample size calculation for randomized study was calculated using 50% prevalence (Thrusfield, 2001) as follows.

$$N = \frac{1.96^2 P_{exp} (1-P_{exp})}{d^2}$$

$$d^2$$

Where: N = required sample size, P_{exp} = expected prevalence, d = desired absolute precision

Using 95%, confidence interval, 5% precision and 50%, expected prevalence 384 animals per species were needed but this number was reduced to 300 animals per species in this study because of time shortage.

3.5. Sample collection and processing

3.5.1. Sample collection

Blood samples were collected from the jugular vein using sterile plain vacutainer tubes and needles. Each tube was then labeled according to the animal's ear tag, kept inclined for some times to allow clotting and finally the sera were separated and kept at -20°C until tested.

Lung lesion scoring was conducted using a system modified from Bryant *et al.* (1999). The lungs were examined grossly and the extent of involvement was determined by visual observation, palpation and measurement of the lesion while it was in the thoracic cavity. The proportion of the lung surface area that showed consolidation was measured (Length × Width) by ruler. The extent of pneumonia was evaluated based on the area of the consolidated lobe expressed as a percent of the total area of the lobe and animals were determined to have severe lung lesions if over 50% of any lobe was consolidated. Animals were considered to have moderate lung lesions if greater than 5% but less than or equal to 50% of a lobe was consolidated. Animals were considered to have mild lung lesion if less than 5% of any lobe was consolidated (Bryant *et al.*, 1999).

In addition, a pleural adhesion score was recorded for each set of lungs as follows. No adhesions or pleurisy if not any, moderate adhesions or pleurisy if involving <50% of the lung/pleural surface area and severe adhesions or pleurisy if involving >50% of the lung/pleural surface area. Any other lesion occurring elsewhere in the lung was recorded (Modified from Bryant *et al.*, 1999).

The whole lung affected by pneumonia was collected. Each animal's lung was collected separately, in sterile plastic bag (sheep, n=167 and goats n=112), labeled according to the corresponding tag on the live animals and transported to the laboratory in an icebox. For histopathology, the tissue samples (about 6cm) were collected in 10% buffered formalin. Of 300 sheep and 300 goats with respiratory signs and tagged during the ante mortem, only 167 sheep and 112 goats showed mild to severe pneumonia at postmortem and a total of 279 lungs were collected.

3.5.2. Sample processing

Inorder to isolate and identify *Mannheimia*, *Babersinia* and *Pasteurella* species the outer surface of the pneumonic lungs was first seared with a heated spatula before the cut inner surface of the lungs were minced by sterile scissors and forceps. The minced tissues were inoculated into tryptic soya broth and aerobically incubated at 37°C for 24hrs. After 24hrs aerobic incubation a loopful of bacterial growth was streaked on 7% sheep blood agar and incubated aerobically at 37°C for another 24hrs. The plates were then examined for presence of growth and colonies were examined for colony morphology, size, shape, and presence or absence of haemolysis. The suspected colony was subcultured onto blood agar and MacConkey agar, and 24hrs later the suspected colonies were Gram stained and subcultured on tryptic soya agar for further biochemical tests (Quinn *et al.*, 2002; Sisay and Zerihun, 2003; OIE, 2004). The lists of the biochemical tests, medias and chemicals used for isolations and identifications are indicated in annex part.

Inorder to isolate and identify *Mycoplasma* species, approximately 1 g of lung tissue from interface between consolidated and normal part of a lesion was minced in a sterile mortar containing 10 ml of hayflick's broth (Oxoid), vigorously grinded, strained using wire mesh, the suspension was centrifuged and the supernatant was filtered using a 0.45µm membrane filter. Then, 0.5ml of the filtered suspension was inoculated into *Mycoplasma* broth base containing *Mycoplasma* supplements (annex-5) and an equal amount was dispended on solid medium plates;

and the broths were incubated aerobically at 37°C but the plates were incubated in a humid atmosphere containing 5% CO₂ at 37°C (Miles and Nicholas, 1998; OIE, 2008).

The broths were checked daily for turbidity, as evidence of *Mycoplasma* growth in comparison with the medium without inoculums, but the plates were examined every one to three days using a stereomicroscope and viewed for the presence of 'fried egg' colonies (Miles and Nicholas, 1998; OIE, 2008).

Broth cultures suspected of contamination, by their gross turbidity within two days, were filtered through a 0.45µm membrane filter and subcultured again. Passages were made 7-10 days of incubation, whether the broths became turbid or not (blind passages) and a sample was considered negative when no growth was observed after three blind passages (Miles and Nicholas, 1998; OIE, 2008).

Subculturing from broth was made by taking loopful of growth and inoculating into *Mycoplasma* Agar (PPLO) and /or broth and subculturing from solid media was made by transferring excised (cut) agar block and pushing it on to *Mycoplasma* agar. The cloning (passage) of the isolates was performed by repeated transferring of a single colony with specific morphological characteristics. In order to differentiate colonies of *Mycoplasma* species from L forms of microorganisms, subcultures were made onto agar base without penicillin (Miles and Nicholas, 1998; OIE, 2008).

Isolates serotyping and serotypes distribution of *Mannheimia haemolytica* was performed by the IHA technique described by Baberistein (1978) and modified by Kawamoto *et al.* (1990).

Monospecific antisera (hyperimmune sera) against specific capsular antigens of *Mannheimia haemolytica* strains were used for serotyping of the isolates and capsular antigens of known strains against sera were used for evaluation of serotype distribution. Agglutinations with titer over 1/40 were taken as positive (Kawamoto *et al.*, 1990). A positive and a negative control were included in each plate.

Briefly, capsular antigen was extracted from a 24hr culture of *Mannheimia haemolytica* isolated for strain isolation (but from known serotypes of *Mannheimia haemolytica* for serotype distribution in sheep sera) in tryptose soya broth. The antigen was incubated in a water bath at 60°C for 30 minutes to kill the bacteria. Centrifuged at 3000rpm for 30minutes and supernatant was removed. 10ml PBS was added and centrifuged at 5000rpm for 30 minutes to disintegrate the bacterial cell. The clear supernatant was collected into sterile test tubes to be used as a capsular antigen.

For sensitization of sheep red blood cells (SRBC), fresh male sheep blood was collected in Alsever's solution at a proportion of 3:8. The suspension was centrifuged at 2500 rpm for 5 minutes, washed twice with phosphate buffered saline solution (PBS) by centrifuging at 2500 rpm for 5 minutes per washing. One hundred µl of washed and packed SRBC, were added to 10ml of capsular antigen after which 50µl of 50% glutaraldehyde was added and homogenized by gentle shaking. This mixture was incubated at 37°C with agitator for an hour and then the suspension was centrifuged at 2500 rpm for 5 minutes and washed twice with PBS. Finally, the sediment was adjusted to 1% using PBS.

As a test proper in V-bottomed microplates 50µl of PBS were distributed to all wells and 50µl of test sera diluted 1:10 in PBS (but known monospecific antiserum of *Mannheimia haemolytica* serotype for strain serotyping of the isolate) were added to the first column of the plate. A serial two fold dilution (1:20 to 1:160) was made by transferring 50µl to the penultimate well and the final 50µl diluted sera were kept at 4°C for possible continuation of the assay. Finally, 50µl of antigen sensitized RBC was added to respective wells, covered with parafilm and incubated for an hour at 37°C (Kawamoto *et al.*, 1990). Sensitized and unsensitized RBCs were added to the respective positive serum as positive control and negative control, respectively.

The test results were interpreted in such a way that 50% or more complete and /or coarse agglutination of RBC indicated positive reaction, whereas small button like cell deposit was considered as negative.

Biochemical characterization of *Mycoplasma* isolates was done according to Miles and Nicholas (1998) and OIE (2008). Briefly, 2ml of the biochemical identification medium was dispensed into four test tubes for one test sample (annex-5). After thorough agitation of the broth culture to be tested, 0.1ml was inoculated into each tube with biochemical media. For tetrazolium reduction test double tubes of the substrate were needed and one was sealed with sterile paraffin after inoculated deep into the substrate to identify the multiplication in anaerobic condition. These tubes were incubated at 37°C and examined daily for colour changes of the medium; positive or negative reaction were noted by colour changes as indicated on table 4.

Table 4: Interpretation of biochemical colour changes after *Mycoplasma* inoculation

Biochemical substrate	First colour	Positive reaction	Negative reaction
Glucose fermentation	Pink	Yellow	First colour
Arginine hydrolysis	Red	Deep red	First colour
Phosphate activity	Yellow	Red	First colour
Tetrazolium Reduction			
Aerobic			
Anaerobic	Yellow	Pink	First colour
	Yellow	Pink	First colour

Source: Miles and Nicholas (1998)

Mycoplasma species identification by growth inhibition test was done according to Miles and Nicholas (1998) and OIE 2008. Briefly, a clear broth culture was made from triple- cloned (passed) and biochemically tested *Mycoplasma* under test over 48hr incubation. Fifty µl of two dilutions of the broth culture (10^{-1} and 10^{-3}) was allowed to run down against tilted agar plates separately using the running drop technique and allowed to dry (3 well separated drops were used per concentration) after which 6mm well were bored on the dried running drops. Carefully, these wells were filled by 60µl of specific hyperimmune sera and incubated in a humid atmosphere containing 5% CO₂ at 37°C. The plates were examined for zone of growth inhibition starting from 48hr and onward. A growth inhibition was indicated when no growth around 3mm of the edge of the well was observed (Miles and Nicholas, 1998; OIE, 2008).

The OIE standard test procedure (OIE, 2008) was adopted for Complement fixation test (CFT). Test sera including positive and negative controls were de complemented in hot water at 60°C for 30 minutes. Twenty five µl of serially two fold diluted test sera were pipetted to each well except the first two columns left for positive and negative controls. Twenty five µl of 1:20 diluted antigen was added into each well except the column with 1:20 diluted serum, which was left to check serum anticomplementary effect. This combination was incubated at 37°C for 30min in incubator with agitator. Finally, 25µl of titrated complement were added to each well and incubated at 37°C under constant agitation on orbital shaker for 30min and this above-mentioned procedure is termed as complement system.

The haemolytic system was prepared as follow; sheep RBCs collected in Alsever's (at 3:8 ratio) solution was washed 3 times in Veronal solution with calcium and magnesium (VCM) by centrifuging at 2500 rpm per washing for 5min and concentrated RBCs were measured and equal volume of diluted Amboceptor (rabbit anti sheep RBC antibody 1:1000 diluted) was added to sensitize the RBCs. This combination brings the final solution to 1% and termed as haemolytic system. Finally, 25µl of sensitized RBCs (indicator) was pipetted into each well and the plates were sealed to avoid evaporation, and then incubated at 37°C for 30min with constant agitation. The plates were then kept at refrigerator at +4°C over night to allow non-haemolyzed RBCs to settle.

Polymerase chain reaction (PCR) technique was performed according to Miles and Nicholas (1998) and Viljoen *et al.* (2005). Briefly, DNA was extracted from the *Mycoplasma* cultures and master mixture was prepared (Details of DNA extraction and master mixture preparation on annex7). Fifty µl of the master mixture and 5µl of the DNA extract were added into microcentrifuge tubes and placed in thermocycler. Finally, the thermocycler was programmed to perform the following. DNA denaturing at 94°C for 5 minutes followed by 30 cycles with each of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds and with a final extension step of 72°C for 5 minutes (Miles and Nicholas, 1998).

A 1.5% agarose gel was prepared by mixing 120ml of 1x TAE and 1.8g agarose in a beaker. To melt the agarose to a homogenous solution the mixture was heated in microwave oven and cooled to 45°C after which 1µl ethidium bromide was added. The gel was poured into clean gel tray with comp and any air bubble was removed by disposable pipette tip. After the gel has been set, the comp was removed and the gel was placed into gel electrophoresis tank with 1X TAE. 5µl of gel loading buffer was mixed with 10µl of each sample (PCR product) and filled to the gel wells. The DNA marker, positive and negative controls were also filled to gel wells. After covering the safety cover, the gel electrophoresis was run at 220V and 8A power for 45 minutes. Finally, the gel was taken to transilluminator, DNA bands were viewed and photographed (Miles and Nicholas, 1998; Viljoen *et al.*, 2005).

For histopathology, techniques developed by Bancroft and Cook (1994) were followed. Briefly, the tissue samples were trimmed properly and fixed in 10% buffered formalin. The tissues were dehydrated in ascending grades of alcohol. The tissues were cleared by two changes in xylene and then tissues were impregnated with molten paraffin wax. Paraffin blocks containing tissue pieces were made (embedding). The tissues were sectioned in 5µm thickness. Then the sections were allowed to spread on warm water bath (45°C) and taken on oil and grease free glass slide. A small amount of gelatin was added to the water bath for better adhesion of the sections to the slide. The sectioned tissues were deparaffinized in three changes of xylene. Then the tissues were rehydrated through descending grades of alcohol. The sections were stained with hematoxylin and eosin. Finally, the sections were mounted with cover slip using DPX after dehydration and clearing (Bancroft and Cook, 1994). The tissues were examined under microscope and photomicrograph was taken using compound microscope and photomicrographic camera (Olympus at NAHDIC and sonny at NVI).

3.5.3. Data analysis

The data were entered into Microsoft Excel spreadsheet and analyzed using SPSS software version 15. As data are not randomized, descriptive statistics, (rates) were mainly used. However, statistical analysis using chi-square test has been performed to analyze significance of

isolation rates between species, and to measure association between lung lesion severity and frequencies of isolation. Percentages and geometric mean were used to compare sero-distribution of *Mannheimia haemolytica* in sheep sera.

1.1.1. Morphology, staining and cultural characteristics of the bacteria

Gram-negative pleomorphic coccoid or bacillary organisms that may be arranged in pairs or in chains were observed. Stains from freshly isolated colonies showed pleomorphic Gram-negative small coccobacilli. However, pleomorphic bacteria were reported after 24 hours and from smears of old cultures.

The growth in primary broth cultures was not characteristic in this study even if mentioned in many literatures, and this may be due to the presence of other putative contaminants but no variability with pH or no bacterial deposit at the bottom of the test tube was observed. *Mannheimia haemolytica* usually revealed after 48 hours in moderate sized, round, raised, moist, translucent colonies on primary blood agar after 24 hours of incubation at 37°C aerobically. Small bacterial colonies beyond the colony size was observed. *Polysiphonia* resembles highly the same colony characteristics, except the hemolytic activity is weaker and the colony size is a little bit larger in the solid media. The hemolytic activity appeared to decrease on repeated subculturing and non-hemolytic development following many passages.

After 24 hours of incubation at 37°C on blood agar, *Mannheimia* colonies looked as small and pink points (soluble hemolysis) but colonies of *Actinobacillus pleuropneumoniae* (*Actinobacillus pleuropneumoniae* subsp. *pleuropneumoniae*) and *Actinobacillus pleuropneumoniae* (*Actinobacillus pleuropneumoniae* subsp. *pleuropneumoniae*) with the exception that none of *Actinobacillus pleuropneumoniae* showed indurated larger hemolysis.

Mannheimia colonies showed more round, pinkish, moist, (soluble hemolysis) and spreading, but no elongated hemolysis and indurated hemolysis. The characteristic hemolysis of *Mannheimia* is usually defined as moderate hemolysis but not spreading. The hemolysis of *Mannheimia* is usually defined as moderate hemolysis but not spreading.

4. RESULT

4.1. Isolation and identification of *Mannheimia*, *Babersteinia* and *Pasteurella* species

4.1.1. Morphology, staining and cultural characteristics of the isolates

Gram-negative pleomorphic coccoid or coccobacillary organisms that may be arranged in pairs or in chains were observed. Smears from freshly isolated colonies usually revealed Gram-negative small coccobacilli. However, pleomorphism increased after repeated subculturing and from smears of old cultures.

The growth in primary broth cultures was not characteristics in this study even if mentioned in many literatures and this may be due to the presence of other possible contaminants but an even turbidity with little or no bacterial deposit at the bottom of the test tube was observed. *Mannheimia haemolytica* mostly revealed small (droplet) to moderate sized, round, raised, moist, translucent colonies on primary blood agar after 24hrs of incubation at 37°C aerobically. Small haemolysis beyond the colony size was observed. *Babersteinia trehalose* holds the same colony characteristic except the haemolytic activity is weaker and the colony size is a little bit larger in the latter case. The haemolytic activity appeared to decrease on repeated subculturing and sometimes disappeared following many passages.

After 24 hours of aerobic incubation at 37°C on MacConkey agar *Mannheimia* colonies looked as pink and pin point (lactose fermenters) but colonies of *Babersteinia trehalose* (*Pasteurella trehalose*) looked as pale and pin point (non lactose fermenters) with the exception that some of *Babersteinia trehalose* ferment lactose on longer incubation.

Pasteurella multocida colonies were round, grayish, non- haemolytic, mucoid (sometimes) and larger than that of *Mannheimia haemolytica* and *Babersteinia trehalose*. The characteristic "Sweetish" odour was not observed in this study. *Pasteurella multocida* isolates did not grow on MacConkey agar and were non-haemolytic on blood agar.

Pasteurella multocida isolates revealed less morphological pleomorphism on smear made from fresh cultures when compared to *Mannheimia haemolytica* and *Babersteinia trehalose*. They were Gram-negative, uniformly short coccoid rods (coccobacillary).

4.1.2. Biochemical characteristics of the isolates

Mannheimia haemolytica isolates (57 from sheep and 21 from goats) were non-motile, positive to cytochrome-oxidase production and to catalase; they fermented D-manitol, D-glucose, and succurose; but were negative for ornithine decarboxylase, urease, indole, H₂S production and trehalose. They all produce acid but not gas from glucose, sucrose, lactose, maltose and manitol. All these isolates fermented D-arabinose but not trehalose and salicine. Biochemical test variation was not observed between sheep and goat isolates of *Mannheimia haemolytica* in this study.

Babersteinia trehalose (isolated from sheep only and n=11) isolates were non-motile, positive to cytochrome-oxidase production but variable to catalase production unlike that of *Mannheimia haemolytica*, which were uniformly catalase positive. They fermented D-manitol, D-glucose, and succurose; but were negative for ornithine decarboxylase, urease, indole, and H₂S production. They fermented trehalose, salicine and variable in lactose. They did not ferment D-arabinose at all. No *Babersteinia trehalose* was isolated from goats.

Six isolates from goats were Gram- negative coccobacilli, non haemolytic on blood agar, non motile, positive to cytochrome-oxidase and catalase, reduced nitrate to nitrite, positive to D-manitol, D-glucose, D-succurose and trehalose fermentation, produce indole and ornithine decarboxylase positive and negative to urease production and did not grow on MacConkey agar and these isolates were identified as *Pasteurella multocida*.

4.1.3. Isolation rate of *Mannheimia*, *Babersteinia* and *Pasteurella*

Comparative isolation of *Mannheimia*, *Babersteinia* and *Pasteurella* species from the same samples of lung was performed. The total isolation rate of *Mannheimia*, *Babersteinia* and *Pasteurella* from pneumonic lungs of both sheep and goats was 34.1% (95/279). The isolation rate from pneumonic lungs of both sheep and goats was 28% (78/279) for *Mannheimia haemolytica*, 3.9% (11/279) for *Babersteinia trehalose* and 2.2% (6/279) for *Pasteurella multocida*. Total isolation rate of *Mannheimia*, *Babersteinia* and *Pasteurella* species together was 40.7% from pneumonic sheep and 24.1% from pneumonic goats (table 5).

Table 5:- Isolation rates of *Mannheimia haemolytica*, *Babersteinia trehalose* and *Pasteurella multocida* from pneumonic lungs of sheep and goats.

Animal Species	Pneumonic lung	<i>Mannheimia Haemolytica</i> No. positive, (%)	<i>Babersteinia Trehalose</i> No. positive, (%)	<i>Pasteurella Multocida</i> No. positive, (%)	Total, (%)
Sheep	167	57, (34.1%)	11, (6.6 %)	0, (0 %)	68, (40.7 %)
Goats	112	21, (18.8 %)	0, (0 %)	6, (5.4 %)	27, (24.1%)
Total	279	78, (28 %)	11, (3.9 %)	6, (2.2 %)	95, (34.1%)

Mannheimia haemolytica was isolated at 34.1% (57/167) rate from sheep and at 18.8% (21/112) rate from goats with statistically significant difference rate of isolation ($X^2=7.87$ and $P=0.005$). *Babersteinia trehalose* was also isolated at statistically significant difference ($X^2=7.68$, $P=0.002$) from sheep (6.6%) and goats (0%) pneumonic lungs. *Pasteurella multocida* isolation rate was 0% from pneumonic sheep and 5.4 % from goats; the difference was statistically significant ($X^2=9.14$, $P=0.002$).

When comparing isolation rate between age groups, out of 279 animals with pneumonic lungs 57.3% (160/279) were at young age and 42.6 % (119/279) were adult. As indicated in table 6, the isolation rate from pneumonic lungs for *Mannheimia haemolytica* was 27.5% from young and

28.6% from adult sheep and goats, respectively. This difference was not statistically significant ($X^2=0.4$ and $P=0.084$). *Babersteinia trehalose* was isolated at rate of 3.8% from young and 4.2% from adult animals, and this difference was still statistically insignificant ($X^2=0.037$ and $p=0.08$) but *Pasteurella multocida* was isolated at statistically significant difference ($X^2=9.14$ and $P=0.002$) from the two age groups of goats (3.8% from young and 0% from adult). *Pasteurella multocida* was not isolated from sheep in this study.

Table 6: Isolation rates of *Mannheimia haemolytica*, *Babersteinia trehalose* and *Pasteurella multocida* from pneumonic lungs of sheep and goats of different age groups.

Animal age group	No. of animals	<i>Mannheimia haemolytica</i> isolation rate	<i>Babersteinia trehalose</i> isolation rate	<i>Pasteurella multocida</i> isolation rate
Young	160	27.5%	3.8%	3.8%
Adult	119	28.6%	4.2%	0%
Total	279	15.8%	2.2%	2.2%

4.1.4. Serotyping (Strain identification) of *Mannheimia haemolytica* isolates

Because of the absence of monospecific antibodies for *Babersteinia trehalose* serotypes and *Pasteurella multocida* biotypes these two species were not serotyped in this study. Monospecific antibodies for serotypes A14, A16 and A17 were also not available. Serotyping was done only for *Mannheimia haemolytica* isolates using 10 monospecific antibodies (table 7).

Table 7: *Mannheimia haemolytica* serotypes isolated from pneumonic lung of sheep and goats.

		A1	A2	A5	A6	A7	A8	A9	A11	A12	A13
Spp	total	No, (%)	No, (%)	No, (%)	No, (%)	No, (%)	No, (%)	No (%)	No, (%)	No, (%)	N, (%)
Sheep	57	10, (17.5)	15, (26.3)	2, (3.5)	8, (14)	2, (3.5)	9, (15.8)	0, (0)	5, (8.8)	6, (10.5)	0, (0)
Goat	21	4, (19)	2, (9.5)	0, (0)	3, (14)	0, (0)	7, (33.3)	2, (9.5)	3, (14)	0, (0)	0, (0)
Total	78	14, (17.9)	17, (21.8)	2, (2.6)	11, (14)	2, (2.6)	16, (20.5)	2, (2.6)	8, (10.2)	6, (7.7)	0, (0)

Out of the total 78 *Mannheimia haemolytica* isolated from both sheep and goats, 17(21.8%), 16 (20.5%), and 14(17.9%) corresponds to serotypes A2, A8 and A1, respectively. These three serotypes were the dominant serotypes isolated followed by serotypes A6 (14%), A11(10.2%) and A12(7.7%). The least isolated serotypes were serotypes A5, A7 and A9 with equal 2.6% rate of isolation. No isolate was belonged to serotype A13 (0%).

Out of the 57 *Mannheimia haemolytica* isolated from sheep, the dominant serotypes were serotypes A2 (26.3%), A1(17.5%), and A8 (15.8%) and moderately isolated serotypes were A6 (14%), A12 (10.5%), and A11 (8.8%). The least isolated serotypes from sheep were serotypes A5 (3.5%) and A7 (3.5%). Monospecific antibody for serotypes A9 and A13 were not related with any *Mannheimia haemolytica* isolates from sheep in this observation.

Out of 21 total *Mannheimia haemolytica* isolated from goats, serotypes A8 (33.3%) and A1 (19%) were the dominant serotypes whereas serotypes A11(14%) and A6 (14%) were moderately isolated. The least isolated serotypes were serotypes A2 (9.5%), and A9 (9.5%). Serotypes A5, A7 and A13 were not isolated from goats in this study. As indicated in table 7, serotype A13 was isolated neither from sheep nor from goats in the present study.

4.1.5. Comparison of lung isolated serotypes with serotypes detected in sheep sera

Total of 167 sheep sera for which all pneumonic lungs were cultured for bacterial isolation were tested for serotype specific antibodies by IHA test against 10 known *Mannheimia haemolytica* antigens at the dilutions of 1/20, 1/40, 1/80 and 1/160. Antigens of serotypes A14, A16 and biotypeT were not available and then not tested. All the 10 known *Mannheimia haemolytica* antigens reacts to the number of sera with variable titer results and no serum was found negative to atleast one of these antigens (table 8). Those sheep sera showing haemagglutination reaction at 1/40 dilution and above were taken as positive (Kawamoto *et al.*, 1990), but dilution at 1/20 was not taken as positive to minimize mistakes due to serotype cross reaction. Goats sera were not tested for *Mannheimia haemolytica* serodistribution because of lack of species specific known strain.

Table 8:- Distribution of the 10 known *Mannheimia haemolytica* serotypes in the sheep sera.

Dilutions	Serotypes tested and positive results detected at different titers									
	A1	A2	A5	A6	A7	A8	A9	A11	A12	A13
1/20	46	14	10	33	31	33	9	16	26	24
1/40	26	12	6	18	22	29	4	6	16	11
1/80	12	5	4	3	14	10	3	0	12	2
1/160	12	5	0	3	6	10	0	0	4	2
No. pos. $\geq$ 1/20	96	36	20	57	63	82	16	22	58	39
No. pos. $\geq$ 1/40	50	22	10	24	32	49	7	6	32	15
%pos. $\geq$ 1/20	57	21.5	11.9	34.1	37.7	49.1	9.6	13.2	34.7	23.4
%pos. $\geq$ 1/40	30**	13.2	6	14.4*	19.2*	29.3**	4.2	3.6	19.2*	9
GM.Pos. $\geq$ 1/40	48	74	50	55	63	64	52	45	50	40

**= the most dominant serotypes, *= moderately dominant serotypes and GM=geometric mean of antibody titer.

The predominant serotypes were serotypes A1(30%) and A8 (29.3%) whereas the least prevalent serotypes were serotypes A9 (4.2%) and A11(3.6%). Of the 167 sera tested, 96 showed multiple reactions to more than one *Mannheimia haemolytica* antigens particularly to serotypes A8 and A1. Among the 10 serotypes, serotype A2 had shown the highest mean antibody titer 1/74

followed by serotypes A8 (1/64) and A7(1/63). Whereas serotypes, A11 and A13 were with the least mean positive antibody titers 1/45 and 1/40, respectively (table 8).

When comparing the dominance of *Mannheimia haemolytica* serotypes in sheep sera with that of lung isolates of the same sheep great variation was observed. For instance, serotypes A9 and A13 were not isolated from pneumonic lung but from the same sheep sera serotype A9 was positive at 4.2% and serotype A13 at 9%. Serotype A11 was the moderately isolated serotype from the pneumonic lungs but it was the least positive in sera of the same animals. The dominance of serotypes A1, A2 and A8 were almost similar both from pneumonic lung and in the sera of the same sheep in this study. These 3 serotypes might be the main causes of pneumonia in sheep.

4.2. Isolation and identification of *Mycoplasma*

For isolation and identification of *Mycoplasma* species and to assess their concurrent occurrence with *Mannheimia haemolytica* 126 pneumonic lungs were cultured. Of these 126 pneumonic lungs cultured, 57 were from sheep (all previously positive for *Mannheimia haemolytica*) and 69 were from goats (21 of which were previously positive for *Mannheimia haemolytica* and the rest 48 negative).

4.2.1. Morphological characterization of the isolates

All the species isolated were grown aerobically with 5% CO₂ at 37°C and were observed critically using morphological characteristics of *Mycoplasma*. Colonies of *Mycoplasma* from sheep and goats exhibited the characteristic "fried egg" appearance with an elevated central spot except the colonies of *M. ovipneumoniae* (fig 1). *M. ovipneumoniae* colonies were small, center less and with "rough" surface. In early growth, some *Mycoplasma* colonies produced different morphologies, often small, centre less, and of irregular shape but with successive passages the isolates demonstrate their conventional 'fried egg' colony morphology except *M. ovipneumoniae*,

with” rough” surface. In early growth, some *Mycoplasma* colonies produced different morphologies, often small, centre less, and of irregular shape but with successive passages the isolates demonstrate their conventional ‘fried egg’ colony morphology except *M. ovipneumoniae*, which remains centre less. Colonies of *Mmm*LC and *Mcc* were the larger colonies observed in this study (fig 2).

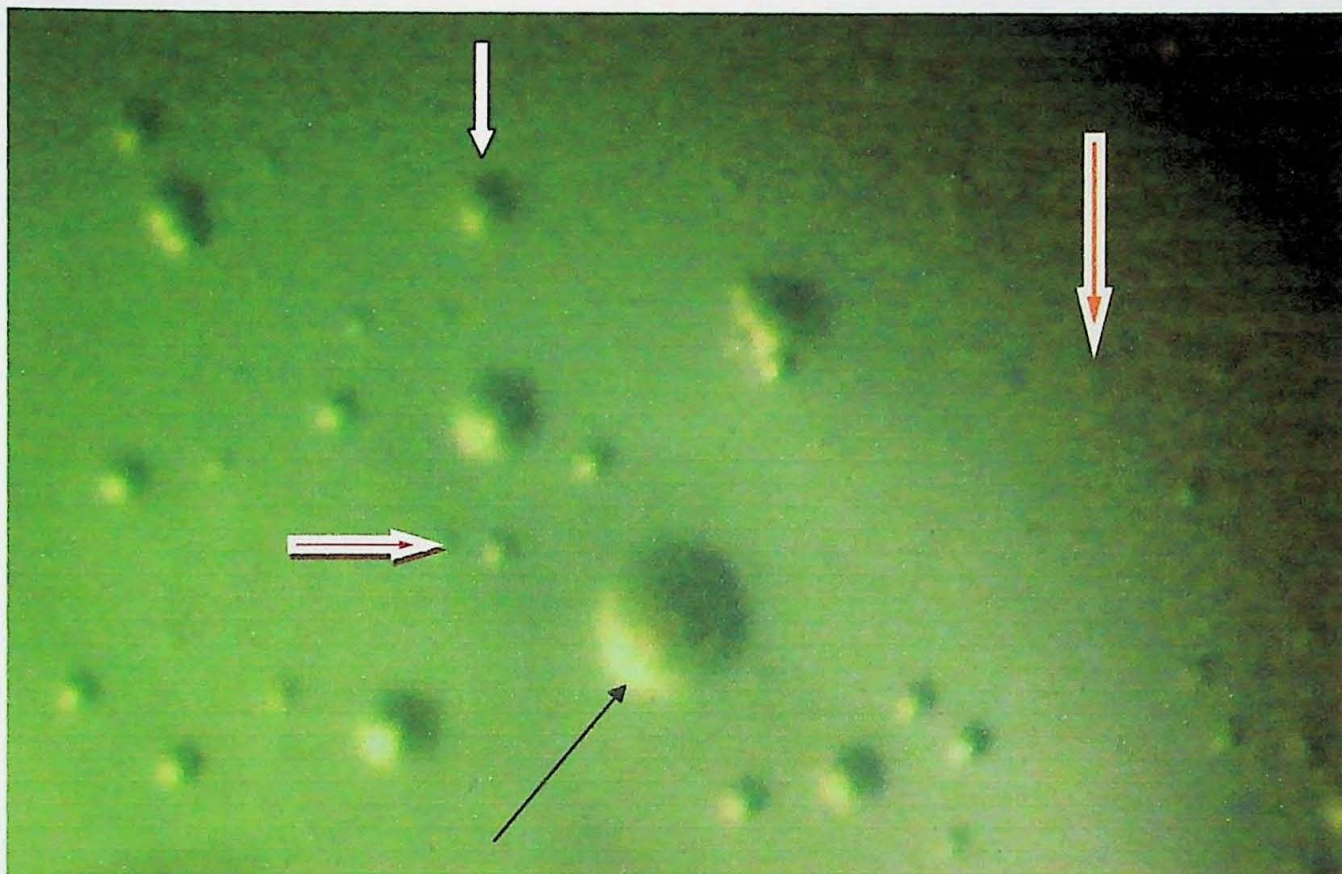


Figure 1:- *Mycoplasma* colonies with the typical characteristic “fried egg” appearance (dense center or nipple shaped morphology) but with mixed colonies of different sizes (large sized black arrow; medium, white arrow and small red arrow) and after repeated subculturing pure colonies were obtained (See fig-2).

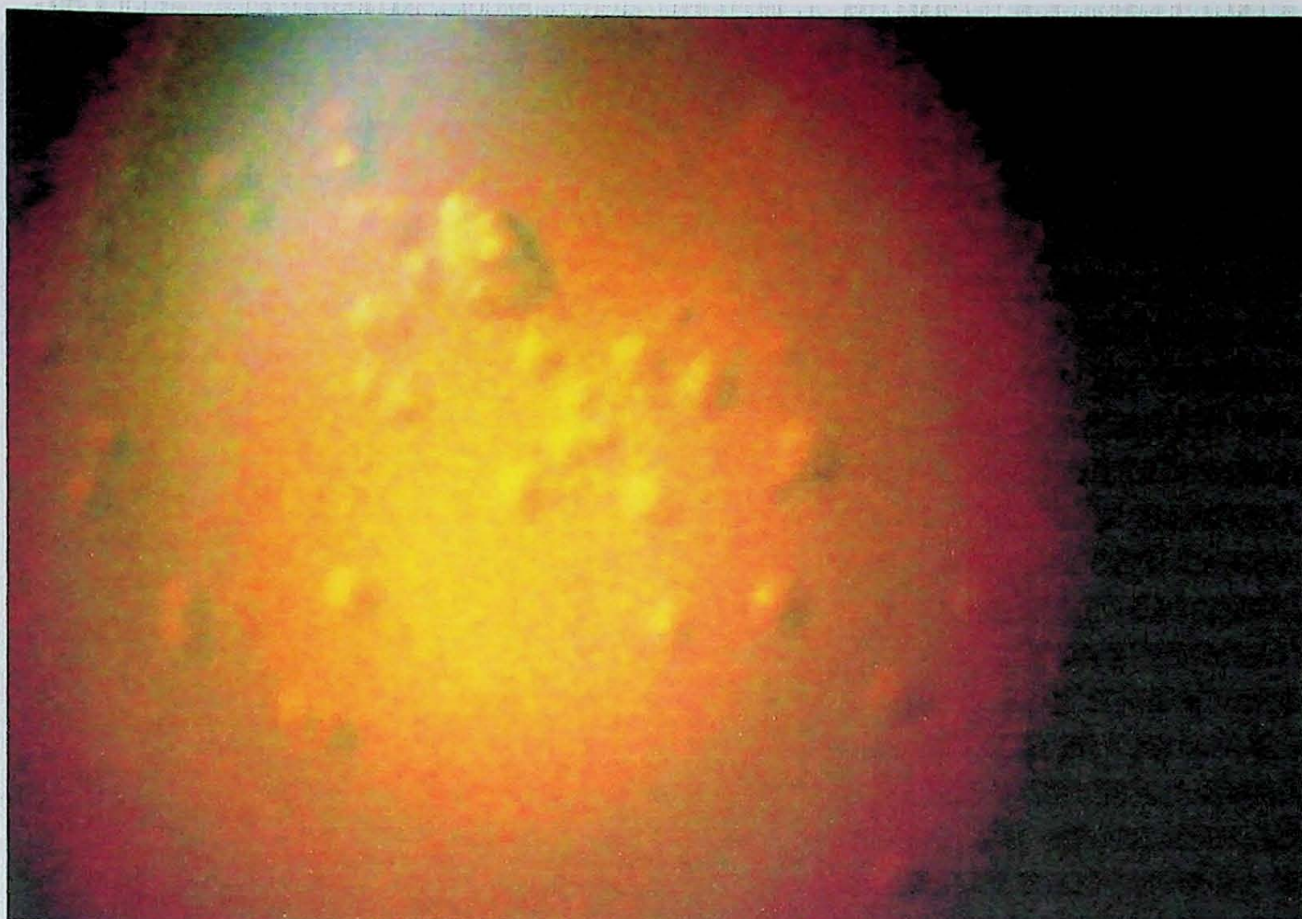


Figure 2:- Three times cloned (passed) and uniform sized *Mycoplasma* colonies (*MmmLC*) with the typical characteristic “fried egg” appearance (cloned from fig 1).

4.2.2. Biochemical characterization, growth inhibition and species identification

Sensitivity to digitonin test for cholesterol requirement, phosphatase activity, and test for proteolytic activity, catabolism of arginine, tetrazolium reduction, and glucose hydrolysis were performed for species identification (Miles and Nicholas, 1998; OIE, 2008). All the strains were found to be digitonin sensitive, indicating that they require cholesterol for their growth (fig 3). Summary of the result of biochemical tests for *Mycoplasma* isolates of sheep and goats was indicated in table 9.

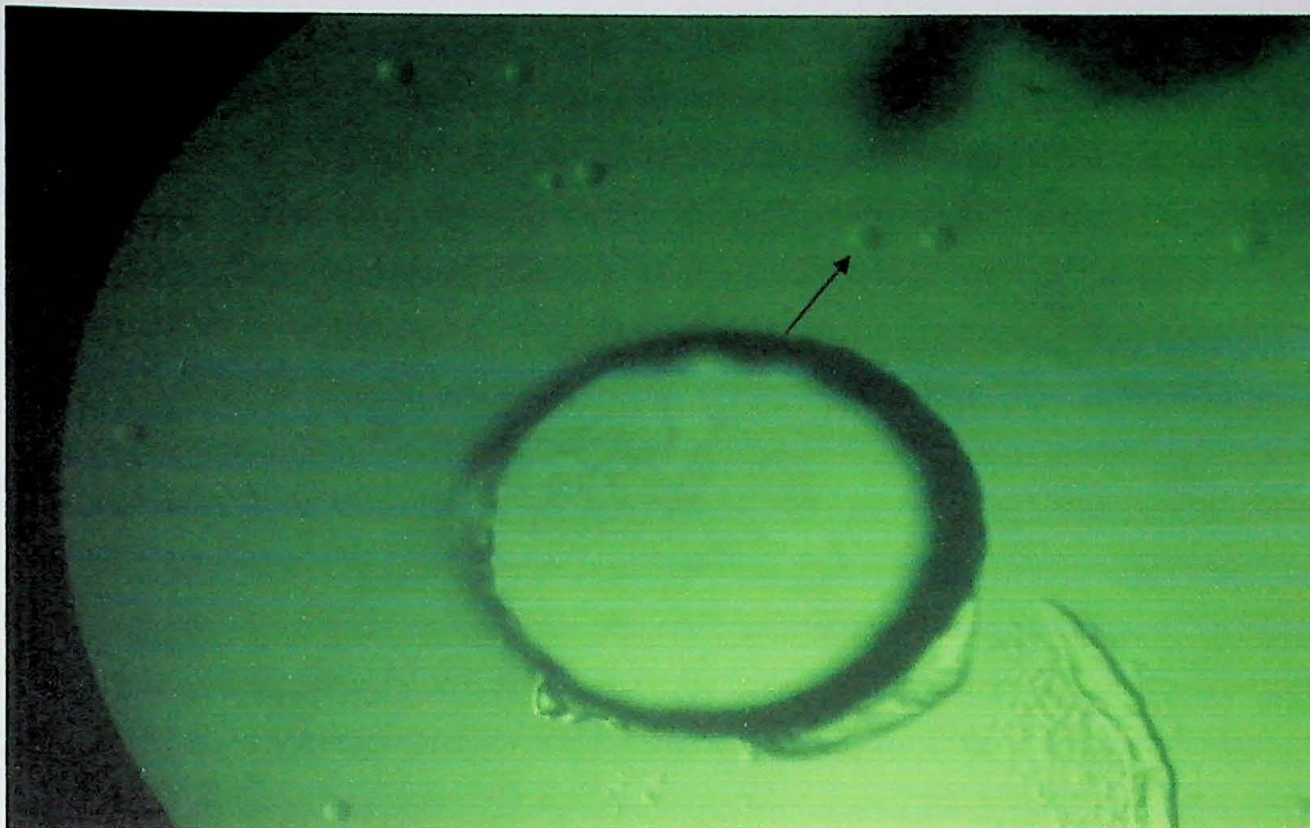


Figure 3: Digitonin sensitivity test. *M. ovipneumoniae* colonies grow at considerable distance from the edge of digitonin disk. *M. ovipneumoniae* colonies are flat and center less unlike other colonies seen at fig 1 and fig 2.

Four *Mycoplasma* species namely, *MmmLC*, *Mmc*, *Mcc* and *Mccp* digested inspissated serum with *MmmLc* the most digester and *Mccp* the least (fig 4). *M. ovipneumoniae* and *M. arginini* did not digested inspissated serum at all. *M. arginini* and *Mcc* were positive for arginine hydrolysis but while *Mcc* is positive for phosphatase production, *M. arginini* was negative.

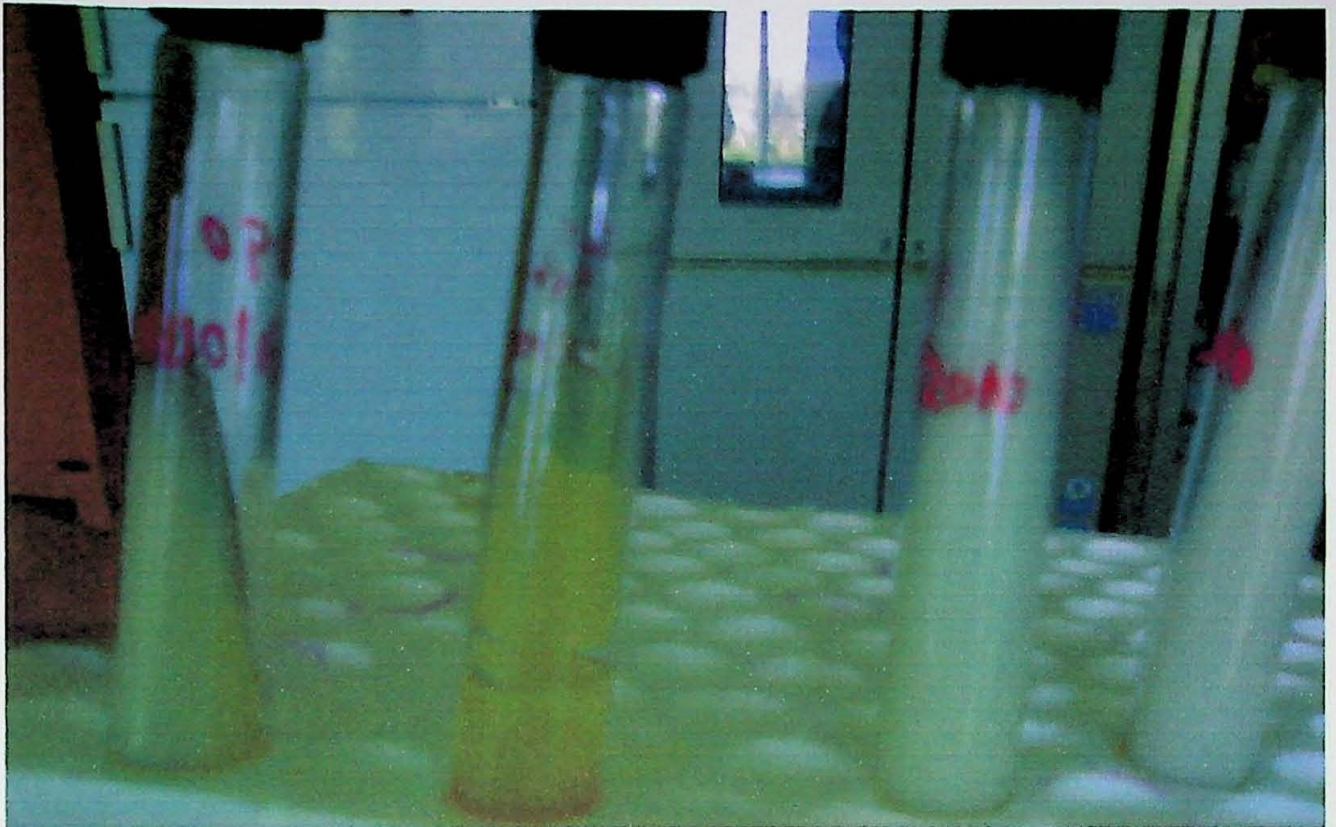


Figure 4: Serum digestion test. Left to right, *Mmc*, *MmmLC*, *Mcc* and *Mccp* inoculated serum. Note that *MmmLC* almost liquefied the serum. *Mccp* digested the least (only on long incubation).

Table 9: Biochemical characteristics of *Mycoplasma* species isolated from goats and sheep.

<i>Mycoplasma</i> species isolated	Biochemical tests					
	Glucose	Arginine	Tetrazolium aerobic/anaerobic	Phosphatase	Serum digestion	Digitonin sensitivity
<i>MmmLC</i>	+	-	-/v	-	+	+
<i>Mcc</i>	+	+/v	+/+	+	+	+
<i>Mccp</i>	+	-	+/+	-	+	+
<i>Mmc</i>	+	-	+/+	-	+	+
<i>M.ovp</i>	+	-	+/+	-	-	+
<i>M.a</i>	-	+	-/-	-	-	+

+ = Positive, - = negative and v = variable

Mycoplasma species from sheep were characterized and identified both biochemically and by growth inhibition test (table 10). Nine isolates which hydrolyzed glucose, reduced tetrazolium chloride anaerobically and anaerobically but which were negative for arginine hydrolysis, phosphatase activity and serum digestions were identified as *Mycoplasma ovipneumoniae* by growth inhibition test. Two isolates, which fermented glucose, digested serum, reduced tetrazolium chloride (both aerobically and anaerobically) and negative for phosphatase activity were identified as *MmmLC*. One isolate, which fermented glucose, reduced tetrazolium chloride, negative for arginine hydrolysis, and phosphatase activity but digested serum was identified as *Mmc*. Two species which hydrolyzed arginine, were positive for tetrazolium and digitonin but negative for other biochemical tests were biochemically identified as *Mycoplasma arginini*.

Table 10:- Ovine *Mycoplasma* species identification by growth inhibition against specific hyper immune serum.

<i>Mycoplasma</i> species isolated	Hyper immune sera used for growth inhibition					
	<i>MmmLC</i>	<i>Mmc</i>	<i>Mcc</i>	<i>Mccp</i>	<i>M.ovp</i>	<i>M.a</i>
<i>MmmLC</i>	<u>2/14</u>	0/14	0/14	0/14	0/14	0/14
<i>Mmc</i>	0/14	<u>1/14</u>	0/14	0/14	0/14	0/14
<i>Mcc</i>	0/14	0/14	<u>0/14</u>	0/14	0/14	0/14
<i>Mccp</i>	0/14	0/14	0/14	<u>0/14</u>	0/14	0/14
<i>M.ovp</i>	0/14	0/14	0/14	0/14	<u>9/14</u>	0/14
<i>M.a</i>	0/14	0/14	0/14	0/14	0/14	<u>2/14*</u>

*=biochemically isolated species

Twenty-one *Mycoplasma* isolates were identified and characterized from goats (table 11). Nine isolates, which fermented glucose, digested serum, reduced tetrazolium chloride (both aerobically and anaerobically), and negative for phosphatase activity were identified as *MmmLC* by growth inhibition test. Three isolates that fermented glucose, reduced tetrazolium chloride, hydrolyzed arginine, variable for phosphatase activity and weakly digested serum were identified as *Mcc* by growth inhibition test. Three isolates that fermented glucose, variable for tetrazolium chloride, digested serum and negative for phosphatase activity were identified as *Mccp* by growth inhibition test (fig 5). Four isolates which fermented glucose, reduced tetrazolium chloride but which were negative for arginine hydrolysis and phosphatase activity and digested serum were identified as *Mmc*. Two isolates which hydrolyzed arginine, positive for tetrazolium and digitonin but negative for glucose were biochemically identified as *M. arginini*.

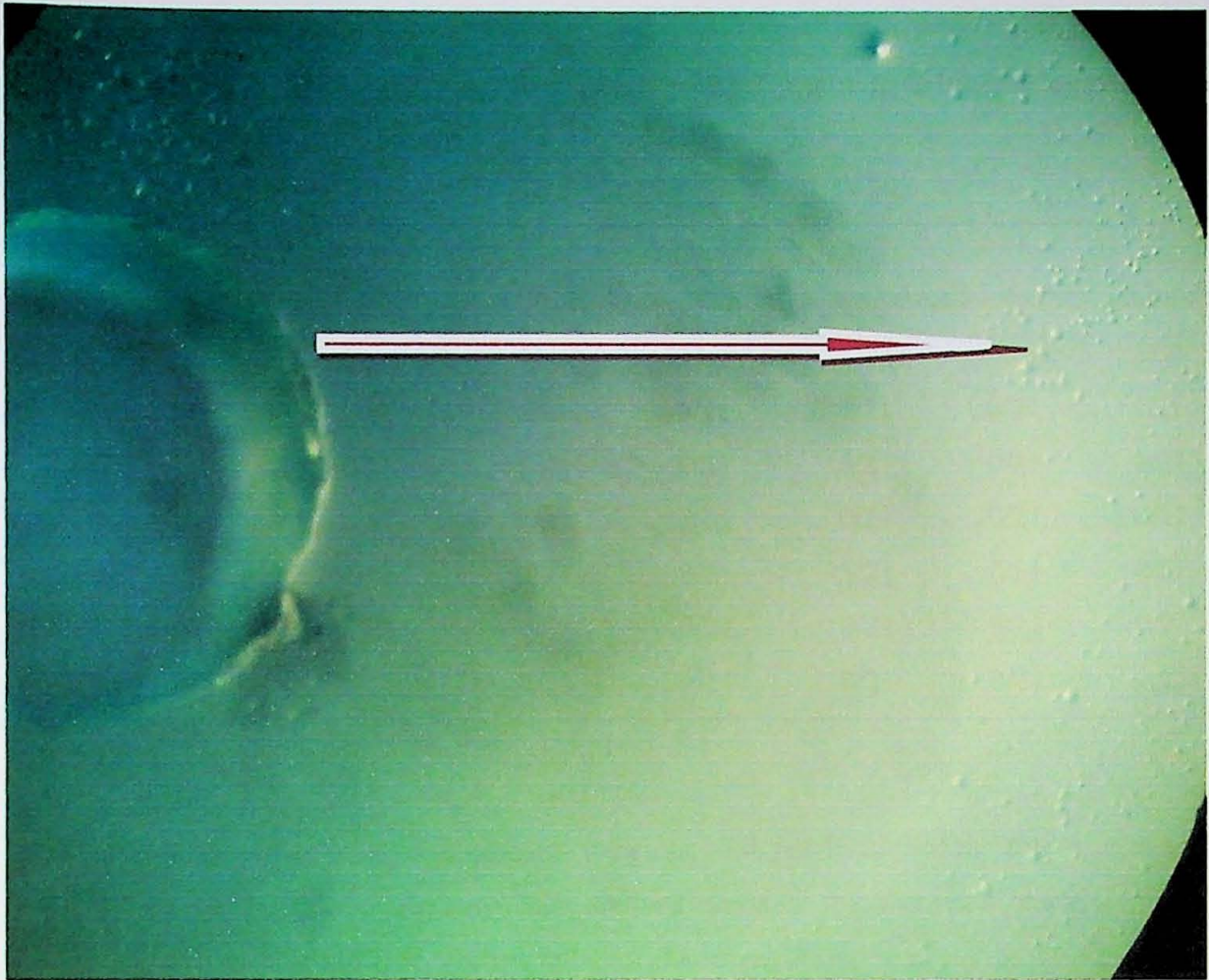


Figure 5: Growth inhibition test. *Mccp* specific hyperimmune sera inhibited the growth of the isolated *Mccp* colonies over a large distance. Note the free space (gap) between the well and the colonies (arrow) and note very small colonies characteristic of *Mccp* species.

Table 11:- Caprine *Mycoplasma* species identification by growth inhibition against specific hyper immune sera.

<i>Mycoplasma</i> species isolated	Specific hyperimmune sera used for growth inhibition					
	<i>MmmLC</i>	<i>Mmc</i>	<i>Mcc</i>	<i>Mccp</i>	<i>M.ovp</i>	<i>M.a</i>
<i>MmmLC</i>	<u>9/21</u>	0/21	0/21	0/21	0/21	0/21
<i>Mmc</i>	0/21	<u>4/21</u>	0/21	0/21	0/21	0/21
<i>Mcc</i>	0/21	0/21	<u>3/21</u>	0/21	0/21	0/21
<i>Mccp</i>	0/21	0/21	0/21	<u>3/21</u>	0/21	0/21
<i>M.ovp</i>	0/21	0/21	0/21	0/21	<u>0/21</u>	0/21
<i>M.a</i>	0/21	0/21	0/21	0/21	0/21	<u>2/21</u> *

*=biochemically isolated species.

NB: Seven *Mycoplasma* isolated from goats were not inhibited by any of the five species-specific hyperimmune sera. These isolates also showed variable biochemical reaction and were lyophilized and kept at -86°C for further detailed characterization.

4.2.3. Comparison of isolation rates of *Mycoplasma* between age and species of animals

Total *Mycoplasma* isolation rate was 27.8 % (35/126) for both species, 24.6 % (14/57) for sheep and 30.4 % (21/69) for goats. Three *Mycoplasma* species namely, *MmmLC*, *Mmc* and *M. arginin* were isolated both from sheep and from goats. *Mcc* and *Mccp* were isolated from goats only and *Mycoplasma ovipneumoniae* were isolated from sheep only.

When comparing the isolation rate of each *Mycoplasma* species between sheep and goats, *MmmLC* was isolated at 3.5 % (2/57) from sheep and 13% (9/69) from goats which was a highly statistically significant difference between the two species ($X^2=8.3$ and $p<0.001$). *Mmc* was isolated at 1.8% from sheep and 5.8% from goat and *Mycoplasma ovipneumoniae* was isolated at 15.8% from sheep and at 0% from goats. *Mcc* and *Mccp* were both isolated at 4.3% from goats and not from sheep at all. All these differences in isolation rate were highly statistically significant between the two species ($p<0.001$).

When comparing the isolation rate of each *Mycoplasma* species between young and adult age groups *Mmc* was isolated at 1.4 % (1/72) from young and at 7.4% (4/54) from adult animals and the isolation rate was statistically significant ($p=0.015$). *MmmLC* was isolated at 12.5 % from young animals and 3.7% from adult animals and the difference was statistically significant ($P=0.008$). The rest *Mycoplasma* species were isolated at statistically insignificant difference between young and adult age groups.

4.2.4. Comparison of cultural isolation of *Mycoplasma* with CFT results

Of the several serological tests used for serodiagnosis of *Mycoplasma* infections, CFT remains the most widely used particularly for the detection of *Mccp* infection (OIE, 2008). Due to lack of known specific antigens for *Mycoplasma* species other than *Mccp*, sera were not tested against other *Mycoplasma* species. Sera of 279 animals with pneumonia and cultured for bacterial isolation (167 from sheep and 112 from goats) were examined for the presence of specific antibodies against *Mccp* by CFT at NVI. Out of the total 279 sera tested, 109 (39%) were found positive for *Mccp* antibodies. When seropositivity was compare per species of animals out of 112 goats, 91(81.3%) were found positive whereas of 167 sheep tested only 18(10.8%) were found seropositive. The differences were highly statistically significant ($p< 0.001$).

When comparing CFT result with cultural result, of 69 pneumonic goats *Mccp* was isolated at 4.3% (3/69) only; however, 84% (58/ 69) of these goats were serologically positive for *Mccp*. *Mccp* was not isolated from pneumonic sheep lungs but 10.8% (17/167) of these sheep were

serologically positive to *Mccp*. In general, the isolation rate of *Mccp* from pneumonic lungs was very low or even nil as seen in sheep when compared to serological results.

4.2.5. PCR confirmation of *Mccp* isolates

A polymerase chain reaction (PCR) techniques developed by Miles and Nicholas (1998) and Viljoen *et al.* (2005) was followed. Three isolates were identified as *Mccp* by growth inhibition and biochemical test. Two isolates that were identified as *Mccp* by biochemical and growth inhibition tests were also confirmed as *Mccp* by PCR technique (fig 6). Unfortunately, one *Mccp* isolate was missed and not tested by PCR. Species specific primers were not available for rest *Mycoplasma* species so that they were not confirmed by PCR.

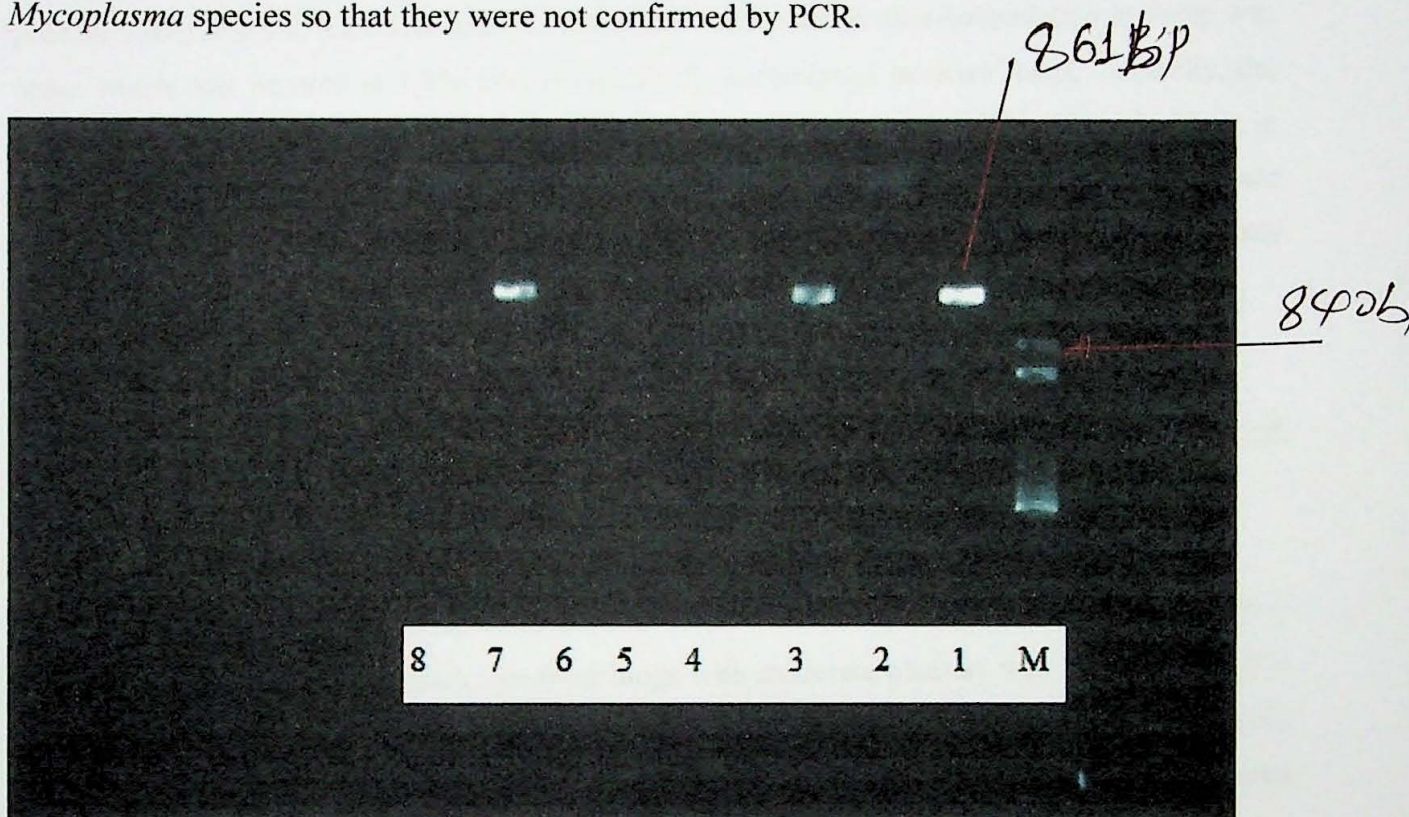


Figure 6: PCR result of *Mccp* isolates. Lanes 1 positive control, lane 2 negative control and lane 3 and 7 were *Mccp* positive samples.

4.2.6. Concurrent occurrence of *Mannheimia haemolytica* and *Mycoplasma* species

The concurrent isolation rate of *Mannheimia haemolytica* and *Mycoplasma* species was 24.6% in sheep and 9.5% in goats. *Mycoplasma ovipneumoniae* was isolated at 15.8% (9/57) rate with *Mannheimia haemolytica* in sheep. This was the highest concurrent occurrence observed when compared to other *Mycoplasma* species isolated from *Mannheimia haemolytica* positive lungs. *MmmLC* and *Mycoplasma arginini* were both isolated at 3.5% from *Mannheimia haemolytica* positive sheep and the least *Mycoplasma* associated with *Mannheimia haemolytica* in sheep was *Mmc*, which was isolated at 1.8% from *Mannheimia haemolytica* positive lungs. Generally, the isolation rate of *Mycoplasma* and *Mannheimia haemolytica* together was significantly lower in goats than in sheep. The only *Mycoplasma* species isolated together with *Mannheimia haemolytica* from goats was *Mmc*, which was isolated at 9.5% (2/21) from *Mannheimia haemolytica* positive goats' pneumonic lung.

4.2.7. Comparison of the isolation rate of *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species with lesion grades and severity of pleurisy

As indicated in table 12 the isolation rate of *Mannheimia haemolytica* from lung lesion without pleurisy was 24.6%. The isolation rate from lungs with moderate pleurisy was 38.5% and 76.9% from the lung with sever pleurisy. Rate of isolation of *Mannheimia haemolytica* was highly statistically associated with the severity of pleurisy ($X^2=17.9$ and $p<0.001$). *Mccp* was isolated at 1.8% rate from pneumonic lung with out pleurisy and at 16.7% rate from lung with sever pleurisy and the isolation rate of *Mccp* was highly statistically associated with degree of pleurisy ($X^2=9.36$, $p<0.001$). All the rest *Mycoplasma* species as well as *Babersteinia trehalose* and *Pasteurella multocida* were isolated at statistically insignificant difference from pneumonic lungs with or with out pleurisy ($P>0.05$).

The isolation rate of *Mannheimia haemolytica* from lung with mild lesions was 28.3%. It was 29.3% from lungs with moderate lesion and 26.2% from lungs with severe lesion. The isolation rate of *Mannheimia haemolytica* was statistically insignificantly associated with lesion severity ($X^2=0.6$ and $p=0.72$). Likewise the isolation rate was insignificantly associated with lesion grade for all the rest bacterial species isolated in current study ($P>0.05$).

Species	Sheep			Goats		
	Mild	Moderate	Severe	Mild	Moderate	Severe
<i>Mannheimia haemolytica</i>	28.3%	29.3%	26.2%	28.3%	29.3%	26.2%
<i>Actinobaculum</i>	1.7%	2%	1.4%	1.7%	2%	1.4%
<i>Pasteurella</i>	1.7%	0.7%	1.4%	1.7%	0%	0%
<i>Moraxella</i>	1.7%	1.4%	1.4%	1.7%	1.4%	0%
<i>Streptococcus</i>	1.7%	1.4%	1.4%	1.7%	1.4%	0%
<i>Corynebacterium</i>	1.7%	1.4%	1.4%	1.7%	1.4%	0%
<i>Haemophilus</i>	0%	0.7%	1.4%	1.7%	0%	0.7%
<i>Mycoplasma</i>	1.7%	1.4%	0%	1.7%	1.4%	0.7%
<i>M. fortuitum</i>	0%	1.4%	1.4%	1.7%	1.4%	0%

4.3. Pathological changes

Out of 600 animals with respiratory disease signs and tagged during intervention examination, gross lung lesions were present in 279 (sheep, n=167 and goats, n=112) animals at slaughter. Out of the 279 pneumonic lungs, 15.4% (54/279) had mild lung lesions, 19.3% (141/279) had moderate lung lesions and 14.3% (54/279) had severe lung lesions. When comparing lesion severity between sheep and goats, out of 167 sheep pneumonic lungs 12.6% (21/167) had mild lung lesions, 14.93% (25/167) had moderate lung lesions and 20.3% (34/167) had severe lesions. 43.2% (49/112) of goats had mild lung lesions, 19% (56/112) had moderate lesions and 7.1% (8/112) had severe lung lesions.

Out of the 279 pneumonic lungs, 28.7% (249/279) had pleuropneumonia lesions only, 5.3% (18/279) lungs had moderate pneumonia in addition to pleuropneumonia lesions and 4.7% (13/279) had severe pneumonia that result in infarction of the lung with disintegration and necrosis.

Table 12: Isolation rates (%) of *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species from different grades of lesions and severity of pleurisy.

Bacterial Species	Lesion grade and rate of isolation			Pleurisy grades and rate of isolation		
	Mild lesion	Moderate lesion	Sever lesion	No pleurisy	Moderate pleurisy	Sever pleurisy
<i>Mannheimia haemolytica</i>	28.3%	29.3%	26.2%	24.6%	38.5%	76.9%
<i>Babersteinia trehalose</i>	3.8%	5%	2.4%	3.6%	0%	15.4%
<i>Pasteurella multocida</i>	1.9%	0.7%	4.8%	2.4%	0%	0%
<i>MmmLC</i>	5.7%	3.6%	3.6%	3.6%	11%	0%
<i>Mmc</i>	1.9%	1.4%	1.2%	1.6%	0%	0%
<i>Mcc</i>	1.9%	1.4%	1.2%	0.6%	0%	7.9%
<i>Mccp</i>	0%	0.7%	1.2%	1.78 %	0%	16.7%
<i>M.ovp</i>	5.7%	2.9%	6%	2.8%	5.6%	30.8%
<i>M. a</i>	0%	2.1%	2.4%	1.2%	11.1%	0%

4.3. Pathological changes

Out of 600 animals with respiratory disease signs and tagged during antemortem examination, gross lung lesions were present in 279 (sheep, n=167 and goats, n=112) animals at slaughter. Out of the 279 pneumonic lungs, 19.4% (54/279) had mild lung lesions, 50.5% (141/279) had moderate lung lesions and 18.3% (51/279) had severe lung lesions. When comparing lesion severity between sheep and goats, out of 167 sheep pneumonic lungs 18.6% (31/167) had mild lung lesion, 50.95% (85/167) had moderate lung lesions and 30.5 % (51/167) had sever lesions. 48.2 % (54/112) of goats had mild lung lesions, 50% (56/112) had moderate lesions and 75% (84/112) had severe lung lesions.

Out of the 279 pneumonic lungs, 88.9% (248/279) had parenchymal lesion only, 6.5% (18/279) lungs had moderate pleurisies in addition to parenchymal lesions and 4.7% (13/279) had severe pleurisies that result in adhesion of the lung with diaphragm and heart.

Out of 167 pneumonic lungs of sheep, 87.4% (146/167) had parenchymal lesions only, 7.2% (12/167) had moderate pleurisies in addition to parenchymal pneumonia and 5.4% (9/167) had severe pleurisies that result in adhesions of lung with neighboring organs. Out of the 112 goats pneumonic lungs 91% (102/112) showed parenchymal lesion only, 5.3% (6/112) showed moderate pleurisies in addition to parenchymal lesion and 3.5% (4/112) showed severe pleurisies and adhesions.

It was found that 58% (163/279) lungs had lesions that colonized the right lobes (fig 7), 30% (84/279) lungs had lesions that colonized the left lobes, and the lesions had bilateral colonization in 11.5% (32/279) lungs (Fig 8).

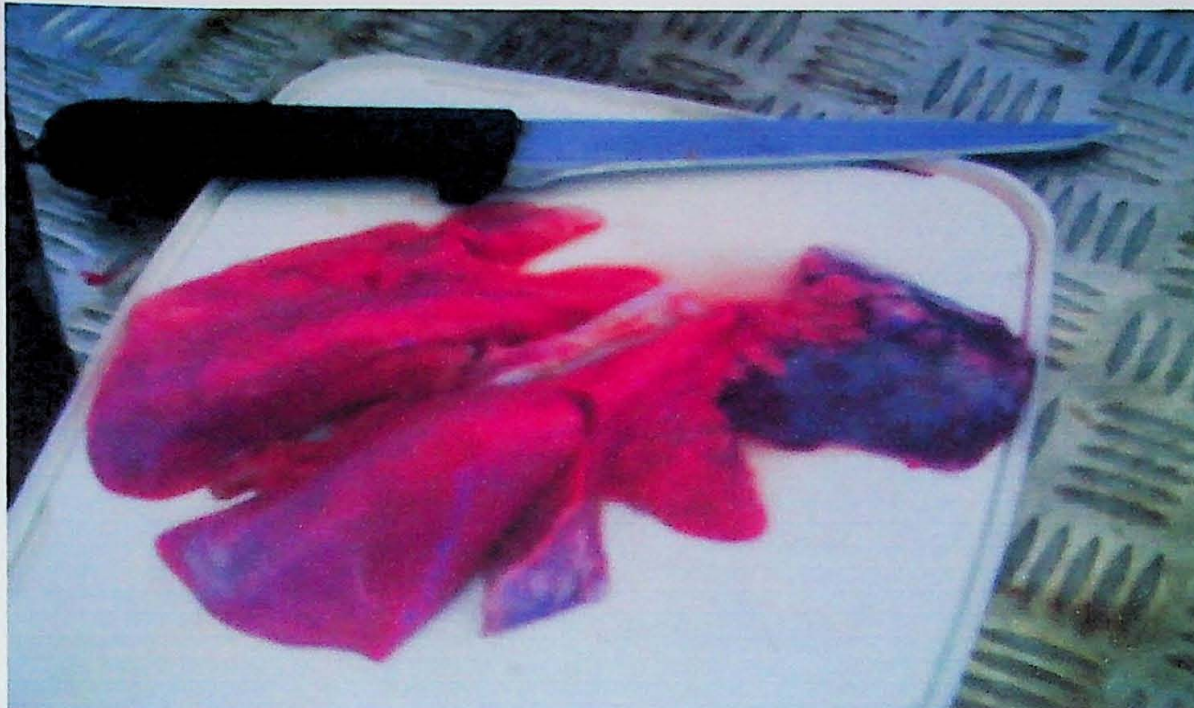


Figure 7: Consolidation of cranial lobe of the right lung with congestion and edema on caudodorsalr part of the lung



Figure 8: Multifocal hepatization of the lung with multifocal haemorrhages. The affected area looked dark red to purple, firm, and consolidated.

In most *Mycoplasma* positive lesions, the affected areas of lung were deep red and consolidated with numerous white pea sized nodules that protruded above the pleural surface. There was accumulation of fibrin on the pleural surface and pleural fibrosis with adhesions was present in these cases. Yellow pea sized nodules on the surface of the lung was surrounded by congested areas (fig 9).

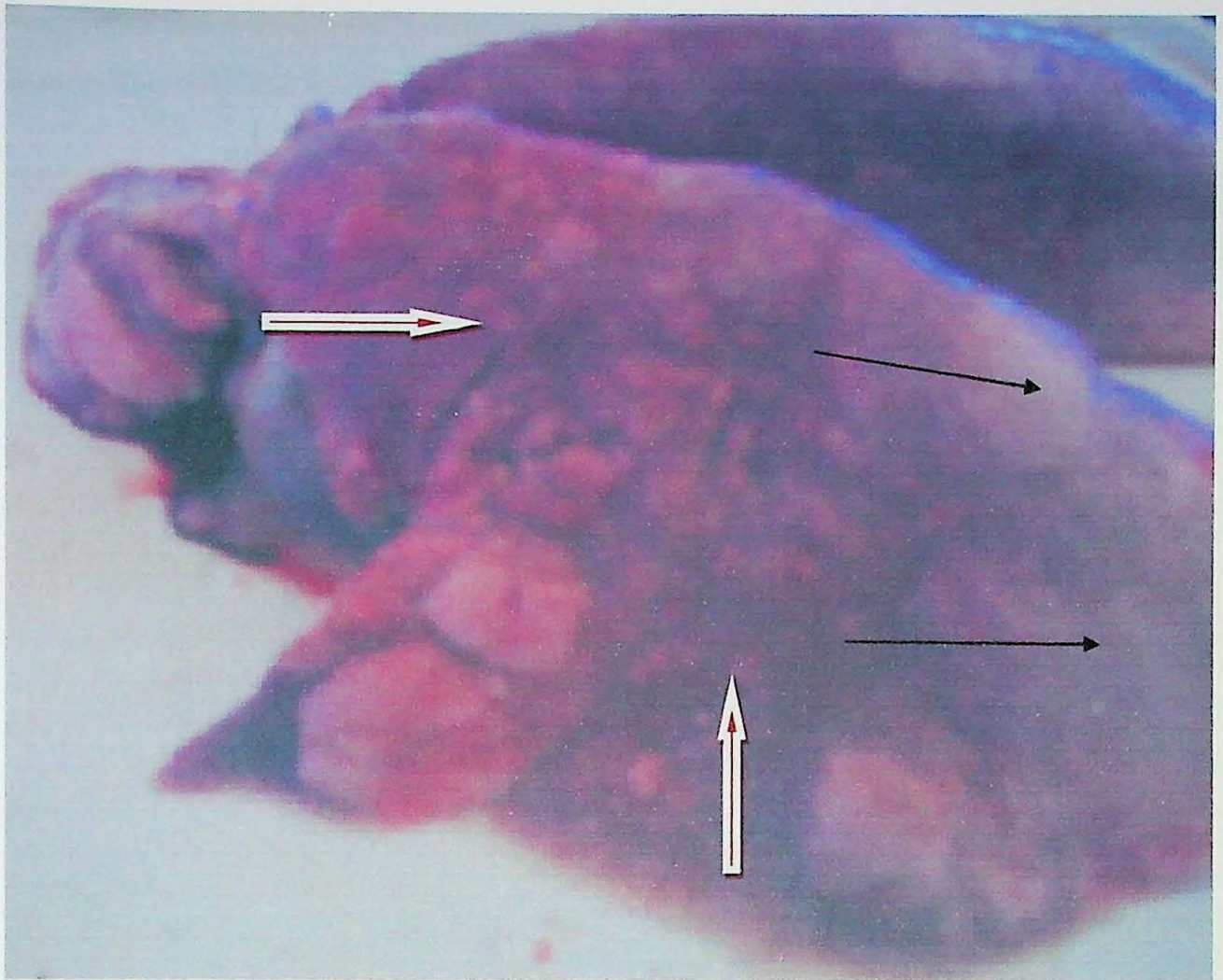


Figure 9: Generalized pneumonia. Note multifocal necrotic pea-sized yellow nodules (red arrows) surrounded by areas of congestion. There are heavy fibrin deposits (black arrows) on the caudo dorsal lobes of this lung. The histological lesion of this lung was show as fig 14.

Histologically all the lesions detected were of inflammatory nature and the detected lesions were categorized as exudative, proliferative and exudative-proliferative (broncho-interstitial bronchopneumonia) according to the predominant feature in inflammatory areas. Among all the histologic findings, exudative bronchopneumonia constituted the majority of the lesions. In some of bronchopneumonia peribronchial and perivascular lymphoid hyperplasia and haemorrhages were observed.

Suppurative broncho-pneumonia that was characterized by the presence of inflammatory cells consisting mainly neutrophils within the lumen of bronchial and in the bronchiolar wall as well were also detected. Adjacent to the bronchioles, there were focal infiltration of large number of

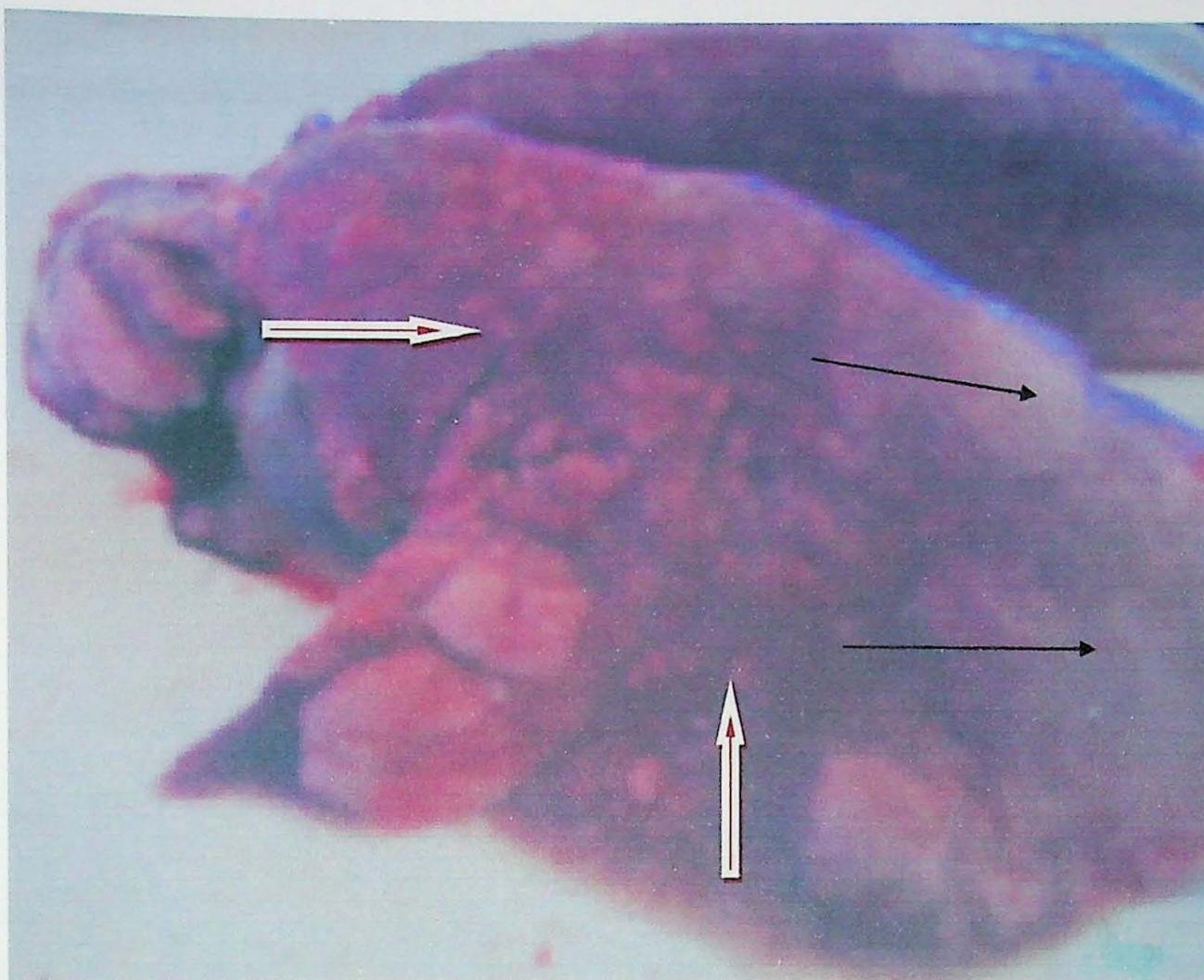


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Suppurative broncho-pneumonia that was characterized by the presence of inflammatory cells consisting mainly neutrophils within the lumen of bronchial and in the bronchiolar wall as well were also detected. Adjacent to the bronchioles, there were focal infiltration of large number of

neutrophils and few lymphocytes (Fig 10). *Mannheimia haemolytica* was predominantly isolated from such lesions and few mycoplasmas were also isolated from similar lesions.



Figure 10: Section of lung showing suppurative bronchopneumonia that was characterized by the presence of inflammatory cells mainly neutrophils within the lumen of bronchioles (arrows). (H & E, $\times 180$).

Interstitial pneumonia with histologic findings characterized by haemorrhagic and fibrinous inflammations were also repeatedly detected in animals that were positive for *Mannheimia haemolytica*. There were exudates containing degenerating and necrotic epithelial cells, neutrophils and mucus in the lumen of the airways (fig-11). In haemorrhagic broncho-interstitial pneumonia, severe haemorrhage and necrosis were prominent features and acute inflammatory cells infiltration and tissue debris were often present (fig-11). In other areas of lung tissue, there was presence of haemorrhage in the alveoli and congestion of blood vessels.

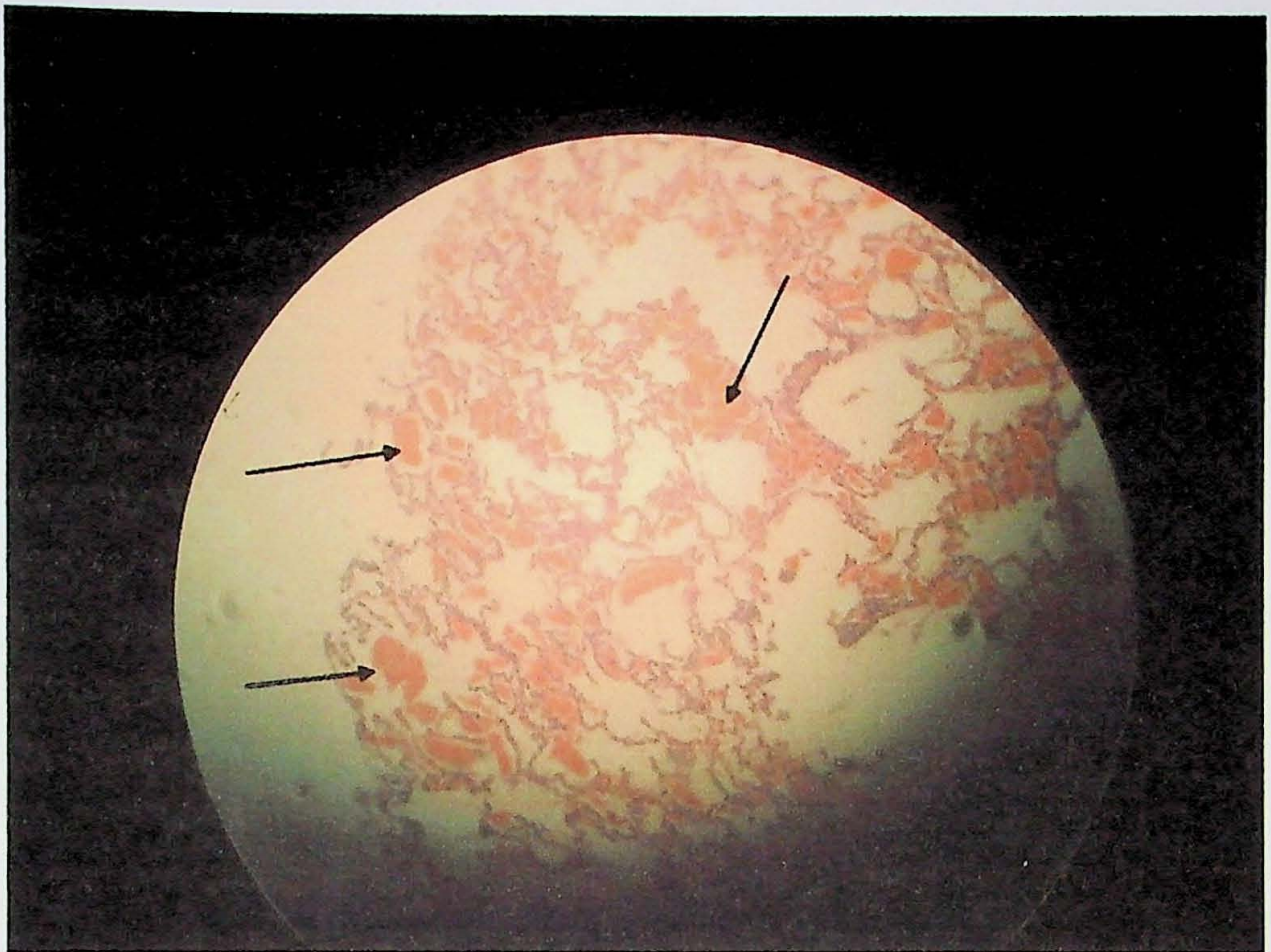


Figure 11: Interstitial pneumonia with sever haemorrhages (arrows) and lymphocytes infiltrating the interstitial area (H & E, 180 x).

Bronchitis was observed and it was characterized by the presence of exudates in most of the lumen of the bronchus. Infiltration of inflammatory cells was found in and around the bronchial wall. There was presence of haemorrhage in some alveoli and congestion of blood vessels. Bronchiolitis that was characterized by the infiltration of reactive cells in and around the bronchiolar wall and necrotic lesions in mucosal epithelia was recorded in some cases.

The other frequent and consistent histologic findings from animals positive for *Mannheimia haemolytica* was fibrino-suppurative bronchopneumonia that was characterized by fibrin mass admixed with number of neutrophils and macrophages deposit in the tertiary bronchus and airways (fig-12).

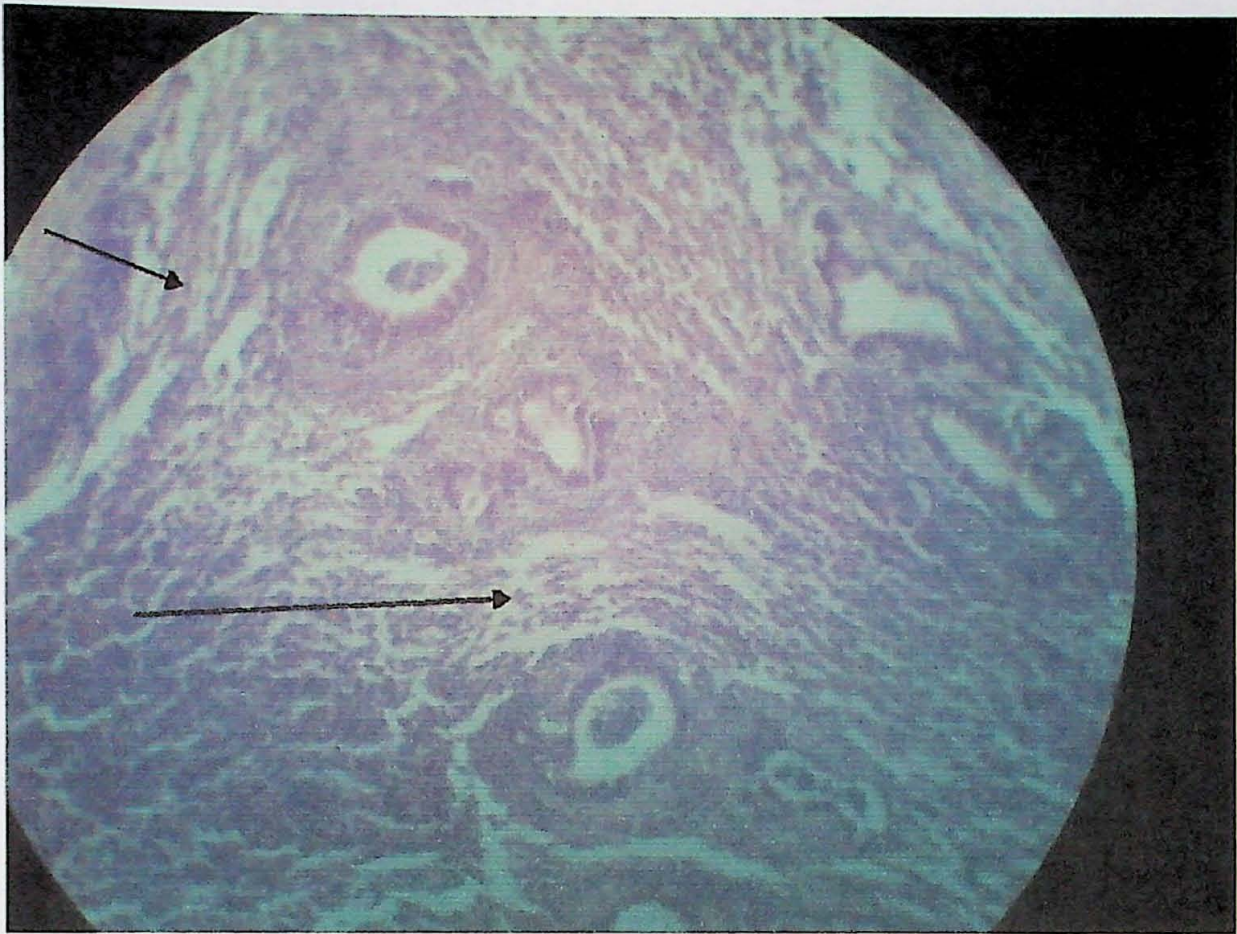


Figure 12: Fibrino-suppurative pneumonia with collagen fibers (arrow) associated with few reactive cells in addition to heavy cellular infiltration

The characteristic histologic findings of proliferative interstitial pneumonic lesion were focal epithelial hyperplasia of secondary and tertiary bronchi and smooth muscle hypertrophy in the wall of bronchi. Macrophages, lymphocytes, few neutrophils and connective tissue proliferation caused thickening of interstitial area (fig-13 arrows). Emphysematous findings, which are characterized by collapsed and few ruptured air vesicles were detected.

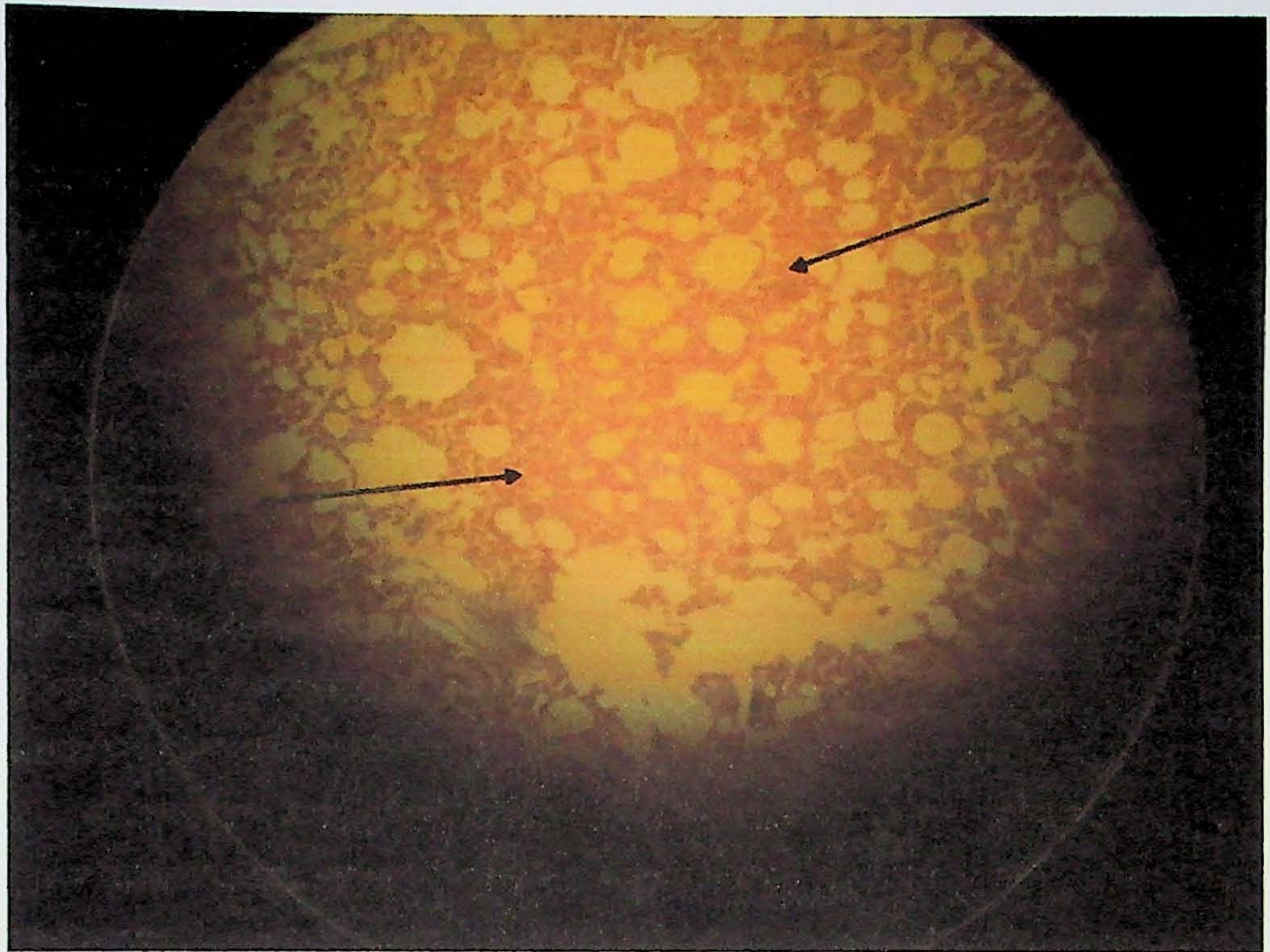


Figure 13: Proliferative interstitial pneumonia with connective tissue proliferation in the interstitial area of the lung and resulting in narrowing of the alveolar lumen.

Most of the lesions from animals positive for mycoplasmal isolation were characterized by subacute or chronic bronchopneumonia with foci of caseous necrosis and these lesions had more advanced necrosis and fibroplasias in chronic cases (characteristics for mycoplasmal lesions (fig-14). Histologically, the milder and probably earliest histologic lesions of caseonecrotic bronchopneumonia affected primarily the smaller bronchioles, with accumulation of necrotic leukocytes in the bronchiolar lumen (Fig-14). Necrotized cells had altered cellular outlines with homogeneously eosinophilic cytoplasm (red arrows, fig-14), and lysed or pyknotic nuclei (black arrows). Neutrophils and macrophages were recognizable at the periphery of the necrotic material, adjacent to intact bronchiolar epithelium (fig-14, blue arrow). In some bronchioles, the epithelium was discontinuous, and necrotic epithelial cells sloughed into the lumen. The bronchiolar walls were thickened by edema and infiltrates of lymphocytes with fewer neutrophils

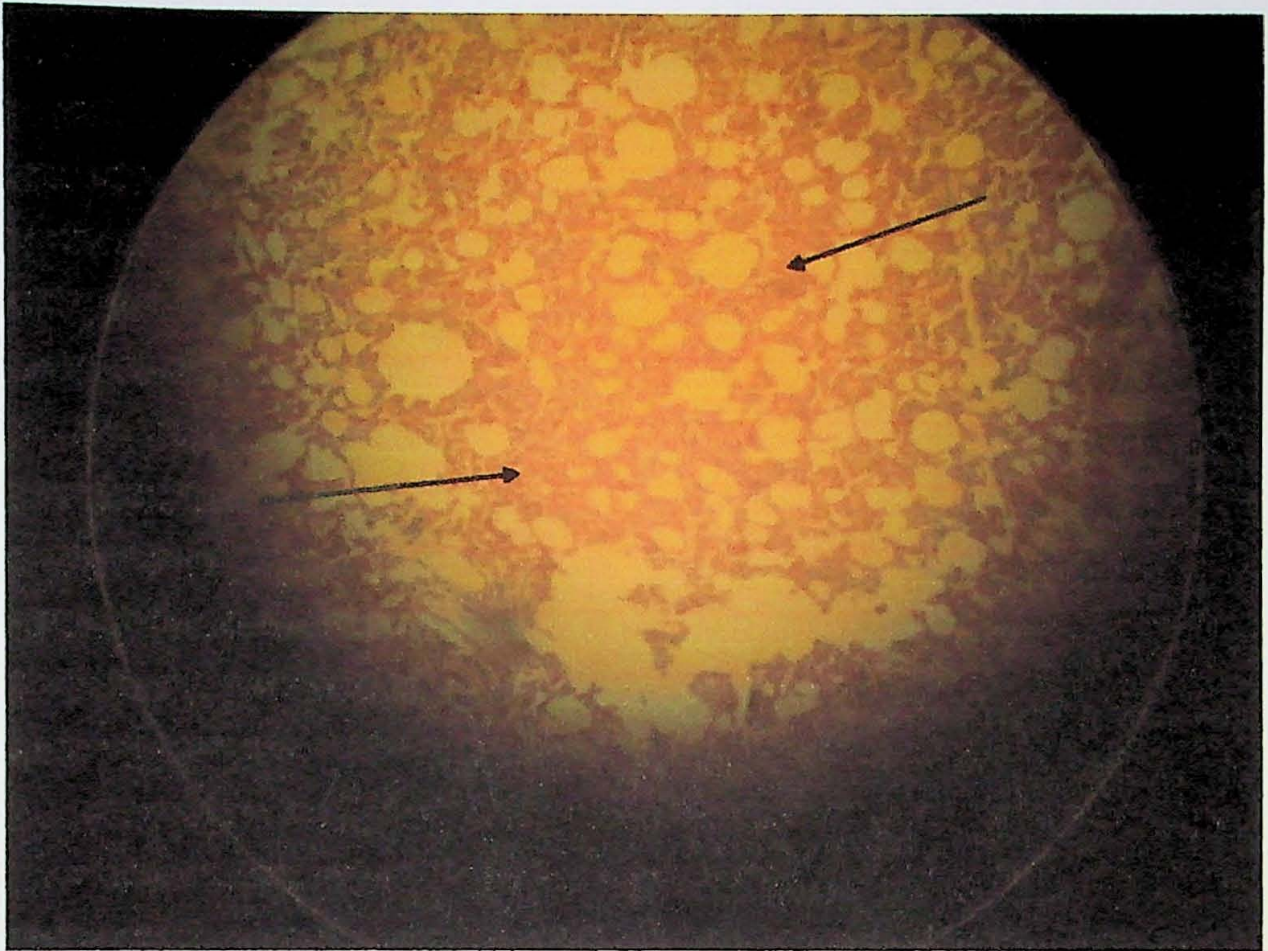


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Most of the lesions from animals positive for mycoplasmal isolation were characterized by subacute or chronic bronchopneumonia with foci of caseous necrosis and these lesions had more advanced necrosis and fibroplasias in chronic cases (characteristics for mycoplasmal lesions (fig-14). Histologically, the milder and probably earliest histologic lesions of caseonecrotic bronchopneumonia affected primarily the smaller bronchioles, with accumulation of necrotic leukocytes in the bronchiolar lumen (Fig-14). Necrotized cells had altered cellular outlines with homogeneously eosinophilic cytoplasm (red arrows, fig-14), and lysed or pyknotic nuclei (black arrows). Neutrophils and macrophages were recognizable at the periphery of the necrotic material, adjacent to intact bronchiolar epithelium (fig-14, blue arrow). In some bronchioles, the epithelium was discontinuous, and necrotic epithelial cells sloughed into the lumen. The bronchiolar walls were thickened by edema and infiltrates of lymphocytes with fewer neutrophils

and macrophages. Similar foci of caseous necrosis that contained recognizable necrotic leukocytes were occasionally present in the alveoli or the interlobular septa in more advanced lesion.

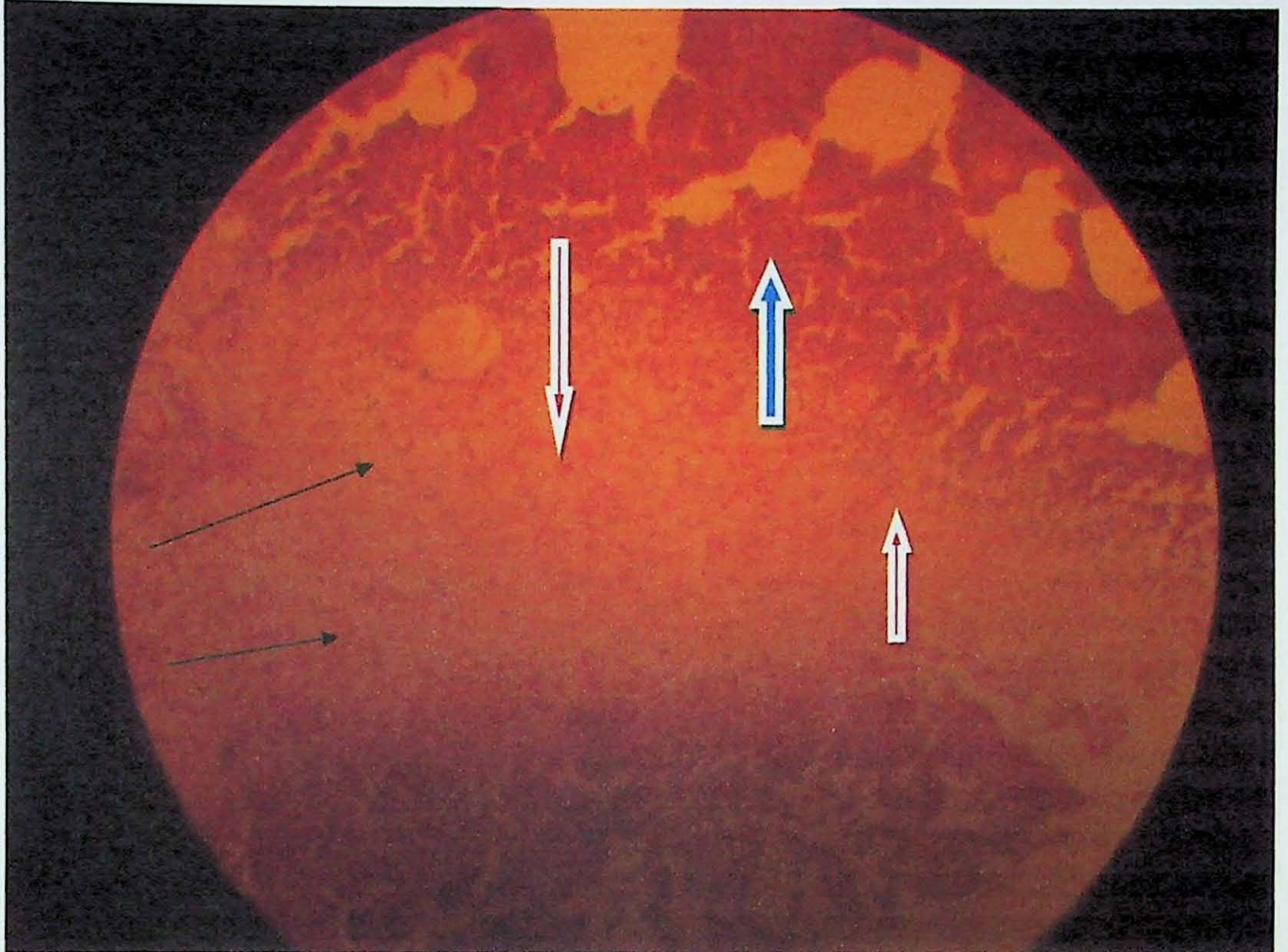


Figure 14: Caseonecrotic-broncho pneumonia with foci of caseous necrosis (red arrows). There are lysed or pyknotic nuclei at the centre (black arrows). Neutrophils and macrophages were recognizable at the periphery of the necrotic material and adjacent to intact bronchiolar epithelium (blue arrow) (H & E, 180x).

5. DISCUSSIONS

Respiratory problems are common and relevant diseases of sheep and goats in all major small ruminants-rearing countries. They cause mortality in lambs and kids, reduced growth and an important economic impact in drug costs and condemnations in abattoirs (Goodwin *et al.*, 2004).

Respiratory diseases of small ruminants are considered as the most important diseases of small ruminants in Ethiopia causing a serious economic impact on livestock (Gelagay *et al.*, 2004). In Ethiopia, sheep and goats are kept as mixed flocks, which may facilitate the transmission of diseases from one animal species to the other.

In Ethiopia, pasteurellosis in sheep caused by *Mannheimia haemolytica* and *Babersheinia trehalose* have posed health problem in most regions due to the significant economic loss they caused through mortality, morbidity and high cost of treatment (Gelagay, 1996; Tesfaye, 1997; Aschalew, 1998; Mekonnen, 2000; Gelagay *et al.*, 2004; Belay, 2007). According to Mekonnen (2000) questionnaire survey in Arsi high lands, of the diseases affecting sheep, the one manifested by nasal discharge and coughing with the occurrence of sudden death in some, was mentioned by more than 90% of the respondents. Previously, Takleye *et al.* (1992) observed that pneumonia accounted for 38.2% of the total cases with 80% of the overall mortality of sheep occurring in the central highlands of the country but information in goats were scant.

Mannheimia, *Babersheinia* and *Pasteurella* species are normal inhabitants of upper respiratory tract of animals. *Mannheimia haemolytica* can be isolated from 95% of the tonsils and 64% of nasopharyngeal swabs from normal sheep (Gilmour, 1990). They considered to be the final cause of the pneumonia but the mechanism by which they get in to the lung and produce the lesion has not yet fully understood. Other agents like virus and *Mycoplasma* may act synergistically. Experimentally these synergistic effects were observed by many researchers including Jones *et al.* (1986) who reported the association of *Mannheimia haemolytica* with *Mycoplasma ovipneumoniae*.

In this study, the isolations and identifications of small ruminants' mannheimiosis and pasteurellosis were achieved from clinically affected small ruminants. This isolation is significant and strengthens aforementioned findings of different authors in Ethiopia.

According to Welsh *et al.* (2004) throughout 9 years study from sheep pneumonic lung samples carried out in a diagnostic laboratory in UK, the isolation frequencies varied from 30.6% to 64.1% for *Mannheimia haemolytica* and 20.5% to 47.4% for *Pasteurella multocida*. The 28.4% isolation rate for *Mannheimia haemolytica* in the present study was slightly lower than 9 years study of Welsh *et al.* (2004). The difference could be due cold and foggy temperature of UK as opposed to hot tropical temperature of Ethiopia.

The isolation rate for *Mannheimia haemolytica* in the present study (28.4%) was also lower than 67.6% (Tesfaye, 1997) and 63.8% (Ascalew, 1998). This difference could most probably be due to agroclimatic factors as these authors sampled highland sheep where cold stress usually exist. In the present study animals were pooled together from different agroclimatic zones.

The isolation rate of *Pasteurella multocida* was very low in the present study when compared to that of Welsh *et al.* (2004). This could partially be due to low real prevalence of *Pasteurella multocida* in Ethiopia or it could be due to any other reasons. Jaramillo *et al.* (2007) indicated great variation in frequency of isolation of *Pasteurella multocida* from pneumonic lung in Mexico.

The highest frequency of isolation of *Mannheimia haemolytica* when compared to *Babersinia trehalose* and *Pasteurella multocida* in this work coincides with the data reported by other national and foreign studies. Most of the studies carried out in sheep pneumonic lungs in Ethiopia reported *Mannheimia haemolytica* with higher rate of isolation than *Pasteurella multocida*. Tesfaye (1997) reported 67.6% *Mannheimia haemolytica* vs. 2.2% *Pasteurella multocida*; Ascalew (1998) reported 63.8% *Mannheimia haemolytica* vs. 1% *Pasteurella multocida* and Mekonnen (2000) reported 56% *Mannheimia haemolytica* vs. 3% *Pasteurella multocida*. Likewise when foreign studies were observed still the rate of isolation of *Mannheimia haemolytica* was relatively higher. For example, Allan *et al.* (1995) in lamb pneumonia reported

55.5% *Mannheimia haemolytica* vs. 44.5% *Pasteurella multocida* in England; Haziroglu *et al.* (1999) reported 42% *Mannheimia haemolytica* vs. 8% *Pasteurella multocida* in Turkey and Welsh *et al.* (2004) reported 46.3% *Mannheimia haemolytica* vs. 34.7% *Pasteurella multocida* in UK.

The isolation rate of *Babbersteinia trehalose* was very low when compared to previous work in Ethiopia (Tesfaye, 1997; Ascalew, 1998 and Mekonnen, 2000) and this could most probably be that these authors isolated it from tonsils in addition to lungs. *Babbersteinia trehalose* is generally isolated at higher rate from tonsil than from lung (Gilmour *et al.*, 1998).

Out of the total 78 *Mannheimia haemolytica* isolated from both sheep and goats 17(21.8%) corresponds to serotype A2, 16 (20.5%) to serotype A8, and 14 (17.9%) to serotype A1. These were the three dominant serotypes isolated followed by serotypes A6 (14%), A11(12.7%) and A12 (9%). The least isolated serotypes were A5, A7 and A9 with equal isolation rate of 2.6%. No isolate was belonged to serotype A13 (0%). The results of this study almost agree with report of Aschalew (1998) who reported serotypes A2 (27%), A1 (22.7%) and A8 (9.1%) as the dominant serotypes isolated from pneumonic lung. Tesfaye (1997) reported serotype A1 as the most dominant serotype isolated both from lung and tonsil followed by serotypes A2, A5, A6 and A8 as moderate serotypes isolated from pneumonic lung. Inline with this Mekonnen (2000) in the conclusion part of his report also stated that serotype A2 and A8 are likely to be the cause of pneumonic pasteurellosis in sheep in Arsi as he continuously isolated these serotypes from sick and incontact sheep. The present result together with the findings of Tesfaye (1997), Aschalew (1998) and Mekonnen (2000) indicated serotypes A1, A2 and A8 were the dominant causes of sheep pneumonia in Ethiopia with little variation in percent (rates) of isolation.

In the work done to measure the distribution of *Mannheimia haemolytica* serotypes in sheep sera in this study, the predominant serotypes were serotypes A8 (30%), A1(29.3%), and A7 and A12 both with 19.2% whereas the least prevalent serotypes were A5(6%) and A11(3.6%) . Mekonnen (2000) reported serotype A11 as most dominant serotype in sheep sera but in the present study serotypes A11 and A5 were the least dominant serotypes. He also reported serotypes A2, A7 and A8 as the dominant serotypes and this was in agreement with the present

finding. Tesfaye (1997) reported serotype A1 followed closely by serotypes A5, A8, and A9 respectively as the dominant serotypes and serotypes A11 and A13 as the least serotypes. This report is strongly approaching to the present finding in that serotype A11 was the least observed, while serotype A13 was not observed in this study. Gelagay (1996) reported serotypes A2 and A8 as the dominant serotypes and serotypes A5 and A12 as the least dominant serotypes. Belay (2007) also reported serotypes A2, A5, A11, and A6 as the dominant serotypes while serotypes A9, A13 and A14 as the least serotypes. When summing up all these data, serotype dominance was in no case isolated constantly in the similar and accurate manner in each and every studies. Variations of serotype distribution in the serum can even occur in different districts of the same zone. As an evidence, Tesfaye (1997) reported serotypes A1, A5, and A8 as the dominant serotypes in Gimba district of Wollo, Ethiopia whereas serotypes A9, A14, and A1 as the dominant serotypes in Quanna district of Wollo, Ethiopia which showed variations of serotype distribution even within the nearby areas. Belay (2007) reported better consistency of serotypes isolation in nearby areas. He isolated serotypes A2, A5 and A6 as the most prevalent serotypes in Dessie zuria woreda; serotypes A11, A2, A5 in Tuhuludere woreda and serotypes A2, A6 and A5 in Kutaber woredas of the same zone.

The variation in serotype distribution also holds true anywhere else in the world. Foreexample, Fraser *et al.* (1982) in UK reported 80% of the serotypes were A1, A2, A6, A7 and A9 whereas Gilmour (1990) in UK also reported serotype A2 as the dominant serotype and A5, A8, A13 and A14 as the least dominant serotypes. These two reports showed great variation of serotypes in the same country.

The serotypes of *Mannheimia haemolytica* differs in their geographic distribution and this may be of significance in the formulation of vaccines. All the serotypes involved in pneumonic manheimiosis varies from year to year, area to area and flock to flock. The difference could be attributed to the season of sampling, specific flock to be sampled and the area or the type of stresser involved (Gilmour, 1993). This variation in serotype could be due to any other reason as for instance, Angen *et al.* (1999b) and Angen *et al.* (2002) stated that serotyping of *Mannheimia* species by IHA is not specific enough for a *Mannheimia haemolytica* reliable identification because the genus *Mannheimia* are phenotypically and genotypically very heterogeneous. Angen

et al. (2002) isolated *Mannheimia*-like organism from animals with respiratory disease and this showing that reclassification of the genus *Mannheimia* might be still unclosed.

Casamitjan (1994) showed that serotype A1 is certainly the only serotype having a primary pathogenic effect considering its toxin. A vaccine against mannheimiosis should include this serotype.

For prevention and control of mannheimiosis and pasteurellosis, the vaccines against these agents should be administered to the animals. Animals should be vaccinated at an early stage in their lives, though it may be desirable to allow a minimum period to elapse after birth and before vaccination to allow the immunological system of the animal to develop more fully. The ability to respond to the vaccine, for example, in the case of lambs may be desirable to allow a minimum period of four weeks to elapse between birth and vaccination. In addition, vaccination may be carried out at particular periods of the year to provide protection against seasonal outbreaks of pasteurellosis (Gilmour *et al.*, 1998).

Sheep vaccines which are intended specifically to combat only the pneumonic form of the disease in adult sheep should comprise antigenic material derived from the A serotypes only of *Mannheimia haemolytica*. Alternatively sheep vaccines intended for combating both the septicaemic and pneumonic forms of the disease typically comprise antigenic material derived from a combination of A and T serotypes (Gilmour *et al.*, 1998).

Gilmour *et al.* (1998) stated preferably, sheep vaccines should comprise antigenic material derived from serotypes A6, A1, and A2 (a trivalent vaccine) for use in combating pneumonias in adult sheep. In addition to what mentioned above antigenic materials derived from all the dominant serotypes like serotypes A1, A2, A6, A7 and A8 (multivalent vaccine) should be comprised in sheep vaccine as these serotypes were repeatedly isolated in Ethiopia at different times but the protection potential of the multivalent vaccine should also be proved experimentally. In line with this, Odugbo *et al.* (2004) inferred from experimental work that serotypes A2, A7 and A9 have a greater tropism than the other serotypes for lung and causing

lung injury in lambs. Lambs infected with serotypes A1, A6 and A11 generally showed less lung consolidation (Odugbo *et al.*, 2004).

Furthermore, sheep vaccines for combating both pneumonic mannheimiosis and septicaemic pasteurellosis, in addition to serotype A antigenic material should comprise antigenic material of serotype T, preferably a combination of T3, T4 and T10 serotype antigenic materials as Tesfaye (1997) and Aschalew (1998) isolated these serotypes from pneumonic sheep in Ethiopian highlands. The type of vaccine and time of vaccination of goats may be similar to that of sheep but further study is needed about serotype distribution and status of mannheimiosis and pasteurellosis in goats.

Pasteurellosis and mannheimiosis in domestic sheep are often described as a disease complex (Clarke, 1998; Alley and Ionas, 1999 and Donachie, 2009) and generally are thought to result from invasion of the lung by bacteria belonging to *Pasteurellaceae* family following a compromise of the respiratory tract. The initiating insult can be from respiratory infection by pathogenic bacterial or viral agents one of which is *Mycoplasma ovipneumoniae* (Clarke, 1998; Alley and Ionas, 1999; Brogden *et al.*, 1998 and Donachie, 2009).

In this study, the isolation and identification of small ruminant mycoplasmas was achieved from clinically affected small ruminants. This isolation is significant because mycoplasmas are one of the major causative agents of small ruminant diseases and have gone unnoticed or attributed to other causes due to the difficulties in their diagnosis. The existence of mycoplasmas in sheep confirms their importance in the disease process.

This is the first in Ethiopia to isolate and identify a range of *Mycoplasma* species from sheep and goats presenting clinical signs of respiratory disease. A total of 35(28%) *Mycoplasma* species were isolated from 126 lung samples (sheep, n=57 and goats, n=69). This indicates and confirms the endemicity of mycoplasmal pneumonia in sheep and goats in Ethiopia. Out of the 35 isolates, 40% (14/35) were from sheep and 48.6% (17/35) were from goats. The higher rate of isolation of *Mycoplasma* species from lungs of goats than from sheep suggested goats are more susceptible to mycoplasmal infections.

In the present study, out of 57 *Mannheimia haemolytica* positive sheep lungs 16 % (9/57) were positive for *Mycoplasma ovipneumoniae* and this was in agreement with works of Clarke (1998) and Donachie (2009) who isolated *Mannheimia haemolytica* and *Mycoplasma ovipneumoniae*. Other reports also indicated isolation of *Mycoplasma ovipneumoniae* from sheep pneumonia in association with *Mannheimia haemolytica*, *Babersinia trehalose* and *Pasteurella multocida* (Hunter *et al.*, 1997; Schoeb *et al.*, 1997 and Weiser *et al.*, 2003). Low numbers of *Mycoplasma ovipneumoniae* are often isolated from the lungs of healthy sheep, but during times of stress or inclement weather, subclinical infection may result in acute fibrinous pneumonia, pulmonary abscessation or pleurisy (McAuliffe *et al.*, 2003a). A coughing syndrome in sheep in which there is persistent and long-term coughing, is believed to be caused by a combination of *Mycoplasma ovipneumoniae* and *Mycoplasma arginini* (Nicolas *et al.*, 2008).

Mycoplasma ovipneumoniae was not isolated from goats' pneumonic lung in this study but goats can also harbor *Mycoplasma ovipneumoniae*, and growing evidence incriminates this *Mycoplasma* in goat disease. In an experimental study, young goats infected with this agent developed pneumonic signs (Goltz *et al.*, 1986) with similar disease patterns to that of sheep (Mohan and Obwolo, 1990). *Mycoplasma ovipneumoniae* can predispose animals to Mannheimiosis, pasteurellosis and viral infections (Nicholas *et al.*, 20008).

Martin and Aitkin (2000) had suggested that *Mycoplasma* interaction with the host prevents normal ciliary activity, facilitating the invasion of the lower respiratory tract by other organisms including *Mannheimia haemolytica* and *Pasteurella multocida*. Therefore, for those sheep, which were positive both for *Mannheimia* and for *Mycoplasma* species in the present study, it could most probably be the *Mycoplasma* that leads these animals to mannheimiosis. In line with this, Jones *et al.* (1985) indicated ciliostasis and loss of cilia in tracheal organ cultures infected with *Mycoplasma* species *in vitro* which may lead to inability to efficiently clear infectious agents from mucosal linings, thereby promoting infection by other pathogens.

In addition to *Mycoplasma ovipneumoniae*, in present study *MmmLC*, *Mmc* and *Mycoplasma arginini* were isolated at 4% (2/57), 2% (1/57) and 4% (2/57) respectively from the 57 *Mannheimia haemolytica* positive pneumonic sheep.

Mycoplasma mycoides clusters, which are the most pathogenic *Mycoplasma* of ruminants' particularly of goats, were also isolated from sheep and this is in agreement with previous findings that isolated the mycoides clusters either from sheep or goats or from both (Chaturvedi *et al.*, 1992; Ikheloa *et al.*, 2001 and Adehana *et al.*, 2006).

The isolation rate for *MmmLC* was 4% (2/57) and 13% (9/69) for sheep and goats, respectively. This isolation rate was lower than that of Adehana *et al.* (2006) who reported the isolation rates of 22.2% (4/18) and 43.8% (14/32) for *MmmLC* from pneumonic sheep and goats, respectively, in Benin. The low isolation rate in the current study could be due to low prevalence of *MmmLC* in Ethiopia, or it could be due to any other reason, as for instance, Ikheloa *et al.* (2001) reported negative result of *MmmLC* from pneumonic lungs of sheep and goat in Nigeria but Ojo and Obi (1996) recovered this organism from sheep in Nigeria. In addition, earlier report confirms the isolation of *MmmLC* from sheep (Chaturvedi *et al.*, 1992). It can be concluded that *MmmLC* is pathogenic to sheep and goats and is not host specific (OIE, 2002). *MmmLC* has been isolated in several countries throughout the world. It represents one of the most important causes of disease in herds of goats in US (Da Massa *et al.*, 1992) and France (Thiaucourt and Roger, 2005). This economically important *Mycoplasma* was isolated in this study indicating that Ethiopian also sharing similar problem with these aforementioned countries.

Mmc was also isolated from sheep and goats in this study. *Mmc* has also been isolated in Spain in one outbreak of caprine pleuropneumonia (Villalba *et al.*, 1992). *Mmc* together with *MmmLC* produced a pleuropneumonia in small ruminant that resembled CCPP (Alafiatayo *et al.*, 1990; Nicholas, 2002).

Mcc was isolated from pneumonic goats but not from sheep in this work. This was in agreement with finding of Adehana *et al.* (2006), who isolated *Mcc* from pneumonic goats and not from sheep in Benin. The present finding did not agree with Ikheloa *et al.* (2001) who isolated *Mcc*

from both pneumonic goats and sheep in Nigeria. In goats, *Mcc* is highly destructive, causing high morbidity and mortality (DaMasa *et al.*, 1992). *Mcc* has been associated with pneumonia resembling CCPP in goats (Kusiluka *et al.*, 2005; Madanat *et al.*, 2001).

Although *Mcc* is primarily a goat pathogen but has also been encountered in sheep. Outbreaks of natural disease attributable to *Mcc* include disease in sheep in Zimbabwe and a septicemic disease in lambs in the United States (DaMasa *et al.*, 1992).

MmmLC, *Mmc* and *Mcc* can sometimes be associated with a respiratory disease syndrome similar to that caused by *Mccp* but they may also cause mastitis and arthritis. Generally, the diseases caused by these organisms are not severe as those caused by *Mccp* (Houshaymi *et al.*, 2002; Rurangirwa and Kinyili, 2004).

Mycoplasma arginini was isolated both from sheep and from goats in this study. *Mycoplasma arginini* with *Mycoplasma ovipneumoniae* have been reported to produce fatal pneumonia in sheep (Pasic *et al.*, 1990; Carmichael *et al.*, 1992; Niang *et al.*, 1998 and Alley *et al.*, 1999).

Mccp was not isolated from sheep in this study. The isolation rate of this organism in goats was 4.3%. The low isolation rate in this study is in agreement with that of Ikheola *et al.* (2001), who reported lower incidence of 3.7% (1/27) in goats in Nigeria. Adehana *et al.* (2006) isolated *Mccp* both from sheep and from goats at the isolation rates of 22.2% (4/18) and 18.8% (6/32), respectively, in Benin. *Mccp* was not isolated from *Mannheimia haemolytica* positive lung in this study. Shiferaw *et al.* (2006) isolated *Mccp* from *Mannheimia haemolytica* positive goats from respiratory disease outbreak of sheep and goats in Afar region. Contagious caprine pleuropneumonia (CCPP) is one of the most severe diseases of goats. This disease, which affects the respiratory tract, is extremely contagious and frequently fatal; in naive flocks, the morbidity rate may reach 100% and the mortality rate can be as high as 80% (CFSPH, 2008).

Totally 32.6% goats and 6.5% sheep were found positive for *Mccp* antibodies using CFT. The difference was statistically highly significant between sheep and goats ($X^2=6.21$, $p<0.001$). This result was comparable with work of Birhanu *et al.* (2009), who reported the total seroprevalence

of 33% for goats and 18.25% for sheep. Eshetu *et al.* (2007) reported 31% seroprevalence in goats, which was lower than 81.3% of the present study. Relatively lower seroprevalence of 0.56% (Zenebe, 2004) and 16.5% (Solonon, 2005) were also reported. Beyene, (2003) reported significant difference in distribution of the disease CCPP among different agro ecological zones. The 10.8% prevalence in sheep in this study is lower than the 18.25% report of Birhanu *et al.* (2009) but higher than the 5% report of Mekonnen (1996) and the difference could be due to special factor or due to sampling methods used. OIE (2008) described that goats are only species affected by *Mccp* clinically and sheep are reservoirs for the disease and the seropositivity in sheep in present study indicated sheep might play role in dissemination of the disease.

Daniel *et al.* (2006) isolated *Mannheimia haemolytica* and/or *Pasteurella multocida* from lambs with moderate and severe lung lesions than in lambs with mild lesion and not in normal lung tissue. In present study *Mannheimia*, *Babersteinia*, *Pasteurella* and *Mycoplasma* species were isolated regardless of lesion severity. *Mannheimia haemolytica* and/or *Pasteurella multocida* are pathogens known to be capable of producing consolidative lesions and mild illness without severe clinical signs (Daniel *et al.*, 2006).

Rate of isolation of *Mannheimia haemolytica* and *Mccp* was highly associated with the severity of pleurisy. These bacterial species were isolated frequently from pneumonic lungs with severe pleurisy than from pneumonic lung with moderate and no pleurisy. Interestingly this was in agreement with the experimental works of Jaramillo *et al.* (2007) and Mohamed and Abdulsalam (2008), who showed acute mannhemiosis that induced unilateral or bilateral pneumonia with serofibrinous pleurisies. The lesions of CCPP affected lungs were generally described as a prototype of fibrinous (lobar) bronchopneumonia with prominent fibrinous pleurisy and pleural effusions (CFSPH, 2008).

Histopathologically, different types of inflammatory lesions and some other lesions were found in the lungs. Broncho-pneumonia with the presence of exudates and infiltration of inflammatory cells in the bronchiolar wall as well as in the lumen of bronchiole were the frequent lesions observed. There was also presence of exudates within the alveoli and showed slight necrosis of the alveoli.

Chronic interstitial pneumonia, which was characterized by the thickened alveolar septae due to accumulation of inflammatory cells and proliferation of fibrous connective tissue were another lesion type detected. Another frequently observed histologic lesion was caseonecrotic bronchopneumonia with necrotized cells, homogeneously eosinophilic cytoplasm, lysed or pyknotic nuclei, and with neutrophils and macrophages at the periphery of the necrotic area.

Even if histologic lesion of *Mycoplasma* positive animals were repeatable and indicative than that of *Mannheimia* and *Pasteurella* positive animals, considerable overlap of the different lesion types were observed and generally, it was difficult to conclude the relationship between the causative agent isolated and the lesion type detected.

6. CONCLUSIONS AND RECOMMENDATIONS

The finding here showed that the highest isolates from pneumonic lung, in both and each species of animals corresponded to *Mannheimia haemolytica*. This indicated that *Mannheimia haemolytica* might be the most pathogenic and caused pneumonia to small ruminants. Moreover, the highest frequencies of isolation of serotypes A1, A2 and A8 from pneumonic lung and from the serum of the same sheep verify that these serotypes are the most probable cause of pneumonia in sheep. *Babersinia trehalose* and *Pasteurella multocida* were also isolated but at lower frequencies and this indicated they involved in occasional pneumonia of sheep and goats.

To our knowledge, this report is the first for the isolation and identification of wide *Mycoplasma* species from sheep and goats respiratory disease in Ethiopia. The *Mycoplasma* species encountered in the current study from the pneumonic lungs of sheep and goats are indicative of mycoplasmal threat to small ruminant population in the country. The finding of these *Mycoplasma* species from sheep and goats probably reflects the fact that small ruminants are kept closely together in Ethiopia so that they share disease causing agents and diseases.

Mycoplasma ovipneumoniae was frequently isolated together with *Mannheimia haemolytica* in sheep. Reports obtained from literatures associated this microorganism with ovine chronic non-progressive pneumonia. Therefore, *Mycoplasma ovipneumoniae* infection could be an important and probably one of the main pathogenic microorganisms involved in the ovine chronic respiratory disease complex in Ethiopia particularly in highland sheep where pneumonia is real threat.

Significant variation was observed between CFT result and isolation of *Mccp* from goats naturally infected with *Mccp*. Isolation of *Mccp* from clinical disease is taken as gold standard (OIE, 2008). The comparable result of isolation and identification of *Mccp* by growth inhibition against monospecific hyperimmune sera and PCR indicated that the two techniques might be used interchangeably but wide study is required to confirm.

In line with aforementioned findings, the following recommendations are forwarded:

All the isolates of *Mannheimia*, *Pasteurella*, *Babersinia* and *Mycoplasma* species were lyophilized and kept so molecular study should be done on these isolates for further characterization by molecular techniques to know the genotypes of the isolates involved in sheep and goats pneumonia in Ethiopia.

The occurrence of multiple serotype of *Mannheimia haemolytica* with no cross protection among them is one of the reason why monovalent vaccines are not effective. Thus, the most frequently isolated serotypes like A1, A2 and A8 should be incorporated in polyvalent vaccine production in Ethiopia.

Sheep vaccines that are intended specifically to combat only the pneumonic form of the disease in sheep should comprise antigenic material derived from the A serotypes only of *Mannheimia haemolytica*. Alternatively, sheep vaccines intended for combating both the septicaemic and pneumonic forms of the disease typically should comprise antigenic material derived from a combination of A and T serotypes.

Mannheimia haemolytica and *Pasteurella multocida* were isolated from goats but not serotyped in this study, so that further study should be under taken and isolates from pneumonic lung of goats should be serotyped and the serum distribution of *Mannheimia haemolytica* in goats should be investigated.

There is an urgent need to combat mycoplasmal pneumonia in Ethiopia. In general, to earn from small ruminant combating mycoplasmal disease should be emphasized because of the serious threat that *Mycoplasma* constitutes to small ruminants production. Efforts should be made to produce vaccine to protect sheep and goats against *Mycoplasma* infections that can cause pneumonia in these animals. The vaccine would control infection, prevent death, economic losses to farmers and the leather industry in Ethiopia.

7. REFERENCES

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8. ANNEXES

Annex 1: Primary biochemical identification test

Grams stain (carter, 1984)

Procedure:

Make smear or film

Allow the film to dry in air

Fix the film by passing through the Bunsen flame several times.

Stain by Crystal violet

Rinse by water

Stain by iodine

Rinse off the iodine solution

Decolorize with 95% alcohol

Counter stain with safranin for 30 to 60 seconds and rinse

Blot with filter paper and examine at 100x under oil immersion.

Catalase test (Quinn *et al.*, 2002)

Principle: The breakdown of hydrogen peroxide into oxygen and water is mediated by the enzyme catalase.

Procedure:-A loop full of bacterial growth from the tryptose soya agar was placed on a clean slide and a drop of 3% H_2O_2 is added. An effervescence of oxygen gas with in few seconds indicates a positive reaction.

Oxidase test (Quinn *et al.*, 2002)

Principle: The cyto-chrome oxidase enzyme is able to oxidize the substrate tetramethyl-p-Phenylene diamine dihydro chloride, forming coloured end product Indophenols.

Procedure: Prepare a 1% aqueous solution of tetra metryl-p-phenylene diamine dihydrochloride, then a piece of filter paper is moistened in a Petridish with fresh reagent and the test bacterium is

streaked firmly across the filter paper with a glass rod. A dark purple colour a long streak line with in 10 seconds indicates a positive reaction.

O-F test (Quinn *et al.*, 2002)

Principle: the test is used to determine the oxidative or fermentative metabolism of a carbohydrate by the bacterium.

Procedure: Prepare O-F basal medium and sterilize by autoclaving at 121°C for 15 minute. The O-F basal medium has cooled to 50°C and 10% sterile glucose, sucrose and dextrose are added to the basal medium to make the final concentration of each sugar 1% and dispense into tubes. Two tubes of the O-F medium with sterile sugars are heated in a beaker of boiling water immediately before use to drive off any dissolved oxygen and the tubes are then cooled rapidly under cold running water. Both tubes are stab inoculated with the bacterium and a layer of sterile paraffin oil is layered on top of one of the tubes (sealed tube) and the tubes are incubated at 37°C and examined after 24 hours and then daily for up to 14 days.

Motility tests (Quinn *et al.*, 2002).

SIM mediums (sulfide Indole motility) mediums will be used to detect the motility. The medium will stab inoculated using a sterile wire. Then the tube is examined for motility 24hr to 48 hrs. If there is a diffuse growth through out the medium, the bacterium is motile. The growth of a non motile bacterium is confined to stab line. To interpret the results, hold the tube against a good light and compare the inoculated tubes with un inoculated one.

Annex 2: Secondary biochemical identification tests.

Indole tests (Quinn *et al.*, 2002).

Principle: Indole positive bacteria posses an enzyme tryptophanase, which splits tryptophan to indole.

Stab inoculates SIM medium with test bacterium and incubate at 37°C for 18 to 24 hrs and then add Kovac's reagent (0.2ml) to tube and stand for 10 minutes. Indole positive bacteria form a dark red ring while negative forms a yellow ring.

Urease tests (Quinn *et al.*, 2002). The surface of the urease media slant is streaked with the test organism. Incubated at 37°C for 18 to 24 hrs. Organism that hydrolyses urea rapidly may produce positive reaction within one or two hours. Red (pink) colour throughout medium indicates positive reaction.

Urease test (Quinn *et al.*, 2002)

Principle: Urease is an enzyme that can hydrolyze urea with the formation of ammonia (alkaline).

Procedure: Appropriate amount of urea agar base is dissolved in sterile water and sterilized in autoclave at 115°C and cooled down to 45-50°C in water bath. 40% sterile urea is added. The medium is dispensed into sterile tubes to form slant. The surface of the agar slant is streaked with the test organism and incubated at 37°C for 18 to 24 hours.

Interpretation: Red (pink) colour throughout medium indicates positive reaction.

Nitrate reduction test (Quinn *et al.*, 2002)

Principle: Nitrate reducing bacteria possess an enzyme nitrate reductase, which reduces nitrate to nitrite.

Procedure: The pure culture of organism to be tested is inoculated into nitrate broth (sterilized by autoclave) after cooling it. The inoculated test tubes are incubated at 37°C for 12-24 hours. Then add 5 drops of each reagent "A" and "B" (A = 0.5 % α -naphthylamine in 5N acetic acid, B = 0.8% sulphanilic acid in 5N acetic acid)

Interpretation: The formation of red color after addition of the reagent A and B indicates positive reaction while in negative reaction there is no color change.

Ornithine decarboxylase test (Quinn *et al.*, 2002)

Principle: Decarboxylase is an enzyme that can decarboxylize amino acid ornithine to putrescine.

Procedure: The pure culture of organism to be tested is inoculated into 0.5% L-Ornithine decarboxylase broth with and without amino acid ornithine and incubated at 37°C up to 4 days.

Interpretation: The formation of yellow colour from purple in test tubes with and without ornithine shows fermentative action of the organism to glucose, while only a tube with ornithine changed back to purple shows positive reaction.

Carbohydrate fermentation tests (Quinn *et al.*, 2002)

Principle: Many microorganisms possess enzymes which can ferment sugars and alcohols by producing acid with and without gas.

Procedure: The pure culture of organism to be tested is inoculated into phenol red broth with sterile 1% sugars and inverted Durham tubes are put. The tubes are incubated for 24-48 hours at 37°C.

Interpretation: The change of the colour of phenol red broth from red to yellow without formation of free space on the top of inverted Durham tube indicates the bacterium ferments the sugar without gas production. Formation of free space on the top of inverted Durham tube indicates production of acid and gas.

Annex 3: Media used for *Mannheimia*, *Bacteroides* and *Pasteurella*

Tryptose soyabroth (Oxoid, Hampshire, England)

Composition(g/l): pancreatic digest of soyabean meal 2.0., sodium chloride 5.0., Di-basic potassium phosphate 2.5 and glucose 2.5.

Preparation: Dissolve 30.0gm in one liter of distilled water and distributed into final containers, sterilize by autoclaving at 121°C for 15 minutes.

Tryptic soya agar (Difco, USA)

Composition (gm/liter): pancreatic digest of casein 15.0., enzymatic digest of soyabean meal 5.0., agar 15.0.

Preparation: Fourty grams of the powder was suspended in one liter of distilced water and mixed thoroughly. The medium also heated with frequent agitation and boiled for one minute to dissolve completely. Then auto claued at 121 °C for 15 minutes, cooled to 45-50°C at waterebath and dispensed to sterilized petridishes and test tubes.

Blood agar base (Titan Biotech limited, Bhiued)

Composition gm/950 ml: Heart muscle solids from infusion 2.0., Agar 15.0., Yeast extract 5.0., pancreatic digest of casein 13.0., sodium Chloride 5.0.

Preparation: Add 40.gm of the poucder in liter of distilled water. Dissolve the powder by heating. Sterilize by auto claving for 15 minutes at 121°C. Cool the base to 45 to 50 °C and add 50-70ml defibrinated blood in a liter of base and mix properly and pour to sterile petridishes.

MacConkey agar (oxoid, Hampshire, England)

Composition (gm/liter): peptone 20.0., lactose 10.0., bilesalts 1.5., Sodium Chloride 5.0., neutral red 0.03., Crystal violet 0.001., agar 15.0.

Preparation: Suspend 51.5 gm in 1 liter of distilled water. Bring to boil completerly. Sterilize by autoclaving at 121 °C for 15 minutes.

SIM medium (BBL, Becton Dickinson, USA)

Comeposition (gm/liter): Pancreatic digest of casein 20.0., Peptic digest of animal tissue6.1., ferrous ammonium sulfate 0.2., agar 3.5.

Preparation: Suspend 30 gm of the poulder in one liter of distilled water. Mix thoroughly. Heat with frequent agitation and boil for one minute. Autoclau at 121 °C for 15 minutes.

O-F basal medium (Merck, Darmstadt, Germany)

Composition (gm/liter): Peptone from casein 2.0., yeast extract 1.0., Sodium chloride 5.0., Dipotassium hydrogen phosphate 0.2., bromw thymol blve 0.08., agar-agar 2.5.

Preparation: Suspend llgm in one liter of distilled water by heating, auto claue at 121°C for 15 minutes: at approximately 50°C mix 900 ml o basal medium with 10% of D® glucose, sucrose and dextrose., disperse

Urea Agar Base (BBL, Becton Dickinson, USA)

Composition (gm/liter):: Pancreatic digest of gelatin 1.0., dextrose 1.0., sodium chloride 5.0., potassium prospbate 2.0., urea 20.0., Phenol red 0.012.

Prepatation: Suspend 40gm of urea powder in 1000ml of distilled water. Mix thoroughly and sterilize by filtration. Suspend 15gm of urea agar base in 900ml distilled water, autoclaue at 121 °C for 15 minutes, cool to 50°C and add 100 ml of filter urea. Mix thoroughly and dispense aseptically in sterile tubes. Cool tubed medium in slanted position so that deep butts are formed.

Ornithin decarboxylase broth (Merck, Germany)

Composition (gm/l): Peptone from meat 5.0, yeast extract 3.0., D(t) Glucose 1.0, Brome cresol purple 0.016.

Prepatation: Dissolve 9gm/liter together with 59gm/liter of ornithin in demineralized water. Prepare pure medium base as control too. Fill in to tubes upto about 3 cm: autoclave at 121 °C for 15 minutes.

Phenol-red broth base (Merck, Germany)

Composition (gm/l): Peptone from case4in 5.0., peptone from meat 5.0., sodium chloride 5.0, pherol red 0.018.

Preparation: suspend 15 gm in 1 liter of distilled water., mix thoroughly and sterilized by autoclave at 121°C for 15 minutes. Cool it down in water bath., at 45-50°C add the test sugars (make the final concentration of sugars at 0.5 %-1.0%).

Annex 4: Reagents used

Phosphate buffered saline solution

NaCl 8.0gm

KCl 0.2gm

NaH₂PO₄ 1.13 gm

CaCO₃ 10.0gm

MgSO₄ 7 H₂O 0.76 gm

Distilled water 1000ml

Adjust PH to 7.4 and auto clave at 121 °C for 15 minutes.

1. Alsever's Solution

Dextrose 18.6gm

NaCl 4.18 gm

Sodium Citrate 8.0gm

Distilled water 1000ml

Sterilized by Steaming

Annex 5: Mycoplasma Biochemical media with supplement

Glucose hydrolysis

Heart infusion broth (Difco).....180ml

Horse serum40ml

Yeast extract 25%.....20ml

Penicillin G.....200,000IU

Thallium acetate.....50IU

DNA (solution of 0.2%).....2.6ml

Glucose (solution of 50%).....2ml

Phenol red 0.1%.....5ml

PH of the media was adjusted to 7.8

Arginine hydrolysis

Heart infusion broth (Difco).....	180ml
Horse serum	40ml
Yeast extract25%.....	20ml
PenicillinG.....	200,000IU
Thallium acetate.....	50IU
DNA (solution of 0.2%).....	2.6ml
30% arginine.....	8.5ml (2.5gm)
Phenol red 0.1%.....	5ml

PH of the media was adjusted to 7.3

Tetrazolium reduction

Heart infusion broth (Difco).....	180ml
Horse serum	40ml
Yeast extract25%.....	20ml
PenicillinG.....	200,000IU
Thallium acetate.....	50IU
DNA (solution of 0.2%).....	2.6ml
2% chlorine Tri phenyl tetrazolium.....	5ml
Phenol red 0.1%.....	5ml

PH of the media was adjusted to 7.5

Phosphatase activity

Heart infusion broth (Difco).....	180ml
Horse serum	40ml
Yeast extract25%.....	20ml
PenicillinG.....	200,000IU
Thallium acetate.....	50IU

DNA (solution of 0.2%).....	2.6ml
Horse serum dephosphated at 60°C.....	20ml
Phenol red 0.1%.....	5ml

PH of the media was adjusted to 7.8

Annex 6: Determination of the age of animals (Mike, 1996)

<u>Age</u>	<u>Teeth condition</u>
1. Kids (<1 year)	8 Sharpe incisors
2. Yearlings (1-2 years)	central pairs replaced by permanent
3. Adult (3-5 years)	4-8 permanent teeth
4. Old (>5 years)	worn (missed) teeth

Annex 7: DNA extraction and Master mixture preparation

DNA extraction

1. Take 180µl of sample and add to 1.5ml microcentrifuge tube
2. Add 20 µl proteinase-k and mix by vortexing.
3. Incubate at 56°C for 1hrs
4. Mix for 1.5 second and add 200 µl buffer AL
5. Add 200 µl ethanol (96-100%) and mix thoroughly
6. Centrifuge at 800rpm for 1minute and discard the flow and change the spin column to 2ml tube
7. Add 500 µl buffer AW1 and centrifuge at 800rpm for 1min and change the spin column to 2ml tube
8. Add 500 µl AW1 buffer and centrifuge at 13,500rpm for 3min
9. Change new 1.5ml microcentrifuge tube and add 200µl buffer TAF for elution and centrifuge at 800rpm for 1min.

Master mixture preparation

1. DANase free water 200 µl

2. 1.5mM MgCl₂ 18 µl
3. 100 µl of dNTPs 6 µl
4. Buffer 1X 30 µl
5. 0.1 µM of primer 1 and primer 2, 6 µl from each primer
6. Taq DNA polymerase(5u/ µl) 3 µl

Finally, 45 µl of master mixture was added to 5 µl of DNA extract and taken to thermocycler (Viljeon, 2005).

9. DECLARATION

"I declared that this thesis is my original work, has not been presented for a degree in any other university and that all sources of materials used for the thesis have been duly acknowledged"

Name: Tilaye Demissie

Signature



Date of submission:

This thesis has been submitted for examination with our approval as university advisors

Name of advisors

Dr. Fufa Dawo (DVM, MSC)

Dr. Bolto Giro (DVM, MSC)

Signature

