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GROSS AND HISTOPATHOLOGICAL STUDY ON PNEUMONIC LUNGS OF SMALL RUMINANTS SLAUGHTERED AT DIFFERENT RESTAURANTS AND MODJO EXPORT ABATTOIRS, ETHIOPIA

MSc THESIS

BY

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ADDIS ABABA UNIVERSITY

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DEPARTMENT OF PATHOLOGY AND PARASITOLOGY

MSC PROGRAM IN VETERINARY PATHOLOGY

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GROSS AND HISTOPATHOLOGICAL STUDY ON PNEUMONIC LUNGS OF SMALL RUMINANTS SLAUGHTERED AT DIFFERENT RESTAURANTS AND MODJO EXPORT ABATTOIRS, ETHIOPIA

A thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfilment of the requirements for the degree of Master of Science in Veterinary Pathology

By

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Bishoftu, Ethiopia

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As member of the examining board of the final MVSc open defense, we certify that we have read and evaluated the thesis prepared by: Tsegaye Wolde entitled **"Gross and histopathological study on pneumonic lungs in small ruminants slaughtered at different restaurants and Modjo export abattoirs, Ethiopia"** and recommended that it be accepted as fulfilling the thesis requirements for the degree of Masters of Veterinary Science in Veterinary Pathology.

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Co-advisor; Department chairperson		

DEDICATION

I declare that this thesis manuscript is dedicated to Almighty God Glory, Who has help, favors, and strengthens me at every aspects. I rejoice in all His works are done in truth; for He spake, and it was done. Blessed be the Lord for evermore.

AUTHER'S STATEMENT

Firstly, I declare that this thesis is my bonafide work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced MVSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at university/ College library to be made available to borrowers under rules of the library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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TABLE OF CONTENTS

Page

LIST OF ABBREVATIONS

AAU	Addis Ababa University
Ab	Antibody,
ALPI	Akililu Lema Pathobiology Institute
BALT	Bronchial Associated Lymphoid Tissue
CAE	Caprine Arthritis Encephalitis
CCPP	Contagious Caprine Pleuropneumonia
CD	Clusters of Differentiation;
CLA	Common Leucocyte Antigen
CSA	Central Statistical Agencies
CVMA	College of Veterinary Medicine and Agriculture
DAB	Diaminobenzidine
EMA	Ethiopian Meteorological Authority
ESGPIP	Ethiopia sheep and goat productivity improvement program
FAO	Food and Agriculture Organization of the United Nations
H & E	Haematoxylin and eosin
HIER	Heat Induced Epitope Retrieval
ICL	International Clinical Laboratory
IHC	Immunohistochemistry
ILCA	International Livestock Center for Africa
MoARD	Ethiopian Ministry of Agriculture and Rural Development
NAHDIC	National Animal Health Diagnostic and Investigation Center
NVI	National Veterinary Institute
OPP	Ovine Progressive Pneumonia
PI-3	*Parainfluenza*-3
RSV	Respiratory Syncytial Virus
spp	species
subsp	subspecies
TB	Tuberculosis

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXIS

ABSTRACT

Cross sectional study designed to attain the objectives to characterize gross and microscopic lesions in pneumonic lung of small ruminants was conducted from October, 2019 to May, 2020. Totally 155 lungs (sheep = 86) and (goats = 69) were examined from export abattoirs and different restaurants in Modjo town during the study period. Out of which 107 (69.03%); 60 (69.8%) and 47 (68.12%) in sheep and goats, respectively, had displayed different types of pneumonia. Suppurative bronchopneumonia (45%) was the most frequent followed by interistitial pneumonia (21.5%), fibrinous bronchopneumonia (10.3%), granulomatous pneumonia (9.35%), verminous pneumonia (6.54%) aspiration pneumonia (4.67%) and embolic pneumonia (2.8%). Bronchopneumonia, both suppurative and fibrinous were grossly characterized by lungs consolidation commonly on the cranioventral area, however; fibrineous type was characterized by typical fibrin deposits while the suppurative type was characterized by suppurative exudates. The common microscopic characteristics were fibrin strands in fibrinous bronchopneumonia and massive neutrophils in suppurative bronchopneumonia. Interistitial pneumonia was typically identified grossly by prints of coastal ribs at the surface of lung and its rubbery texture on palpation and by proliferation of smooth muscle and slightly presence of edema in the alveolar interistitium microscopically. The gross appearance of verminous pneumonia was similar to interistitial pneumonia however, the cut surface reveal parasites both grossly and microscopically. The granulomatous pneumonia was recognized by the presence of nodules grossly and cellular granulomatous rim, a zone of layers consists of various inflammatory cells and few fibrosis in outer layer microscopically. Immunohistochemistry results revealed that the alveolar and peribronchiolar surface infiltrated macrophages were expressed CD45 and CD68 immunopositive reaction. Aerobic bacterias commonly *Staphylococcus aureus*, *Streptococcus* spp., *Escherchia coli* and *Pasteurella* spp. were isolated; and parasites commonly *Dictyocaulus filarial*, *Mulleries capillaris* and *Protostrongylus rufescens* were identified from pneumonic lung tissues. It could be concluded that pneumonia was one of the problem of small ruminants at abattoirs. Large number of aerobic bacterias and parasites were identified. Although no doubts that the parasites isolated were the causes of pneumonia from which they isolated, and

it was exactly while the animals were at the field for the bacterias isolated causal relationship should be done at the field.

Key words: *Abattoirs, Ethiopia, Gross* and *histopathological lesions, Immunohistochemistry, Pneumonia, Restaurants, Sheep* and *goats.*

1. INTRODUCTION

Ethiopia‘s estimated livestock population is often said to be the largest in Africa. In the country, there are approximately 57.8 million cattle, 28 million sheep, 28.6 million goats, 1.23 million camels and 60.5 million poultry (CSA, 2016).

Small ruminants play an important role in nutrition and income of people around the world. They serve primarily as source of meat also provide milk, skin, and wool (Mbilu, 2007). Small ruminants provide 35% of meat and 14% of milk consumption in Ethiopia (FAO, 2004). In the central high lands where mixed crop-livestock production system is practiced, small ruminants account for 40% of cash income and 19% of the house hold meat consumption (Fletcher and Zelalem, 1993). Owing to their remarkable adaptability to adverse environments, goats assume important position in Ethiopian livestock economy. In combination with sheep, they generate income from exports of live animal, meat and skin (Aleme and Zemedu, 2015). That as it these species have received much less attention from research and development agencies (ILCA, 1990).

In spite of large livestock population in Ethiopia, the productivity and the economic benefits to the farmers remains marginal mainly due to malnutrition, poor animal production systems and management problems, poor genetic potentials of the local stock, marketing, social factors, structural constraints, prevalent diseases and general lack of Veterinary care (Biffa *et al*., 2006; Sissay *et al*., 2007). These animals are highly susceptible to respiratory diseases, which account for almost 50% mortality amongst them (Kumar *et al*., 2014). Respiratory diseases are common in all species of domestic animals, and they are appear due to the interaction of many of infectious agents like (bacteria, mycoplasma, viruses, fungi, parasite, host defense and environmental factors which are causes high mortality rate and economic losses associated with respiratory diseases of sheep and goats (Andrawis, 2001; Lacasta *et al*., 2008).

Furthermore, most of the infectious agents that cause the respiratory disease is ubiquitous in nature and are normal inhabitants of the nasopharynx (Sisay and Zerihun, 2003). Vulnerability of

the respiratory system to aerogenous (airborne) injury is primarily because of (i) the extensive area of the alveoli, which are the interface between the respiratory system and inspired air; (ii) the large volume of air passing continuously into the lungs; and (iii) the high concentration of noxious elements that can be present in the air (Zachary, 2017).

The infectious respiratory diseases of sheep and goats, irrespective of the etiology, contribute to 5.6 percent of the total diseases of small ruminants (Chakraborty *et al*., 2014). The infectious respiratory disorders are classified into two groups: the diseases of upper respiratory tract including sinusitis caused by the larvae of parasites, nasal foreign bodies, gaseous irritation, and enzootic nasal tumors and the diseases of lower respiratory tract comprising mainly pneumonia. Often these are of infectious origin (bacterial, viral, or fungal). Depending upon the environmental, physiological, and etiological factors, respiratory conditions might be acute, chronic, and/ or progressive in nature *(Kumar et al*., 2014).

The respiratory system is a major system in the animal body which communicates directly with the external environment. The respiratory tract of an adult goat comes into contact with approximately 7-8 liters of air per minute, that is, 11,000 liters of air in a day. Thus, the quality of inhaled air has major implications on the respiratory health of the animals (McGlone *et al*., 2010), the disruption of defensive mechanisms to get rid of inhaled material may occur if an individual is exposed to highly concentrated particles in certain situations or if an exposure occurs during strenuous labour. Airborne contaminants may then serve as a primary cause of respiratory disease or can exacerbate a preexisting respiratory conditions or pulmonary disease. Depending on the inhaled substance, acute or chronic reactions occur as particles are deposited on the alveolar surface. Acute reactions are characterized by swelling (oedema) and inflammation (Ricciotti and Fitzgerald, 2011), while chronic reactions are characterized by connective tissue scarring (fibrosis) and the formation of specific aggregates of immune cells (granulomas) (Wynn, 2008). Some of the effects of exposures may be immediate, whereas others such as lung disease related to asbestos deposits may not present for many decades (Braun and Kisting, 2006).

The lung, the important organ of respiratory system, is vulnerable to many infectious and non-infectious agents causing various pathological conditions in farm animals. Among the

inflammatory and non-inflammatory disease conditions, pneumonia either acute or chronic causes debility and death leading to great economic loss to the farmers and distress (Alam *et al.*, 2001; Ferdausi *et al.*, 2008). Pneumonia is a major respiratory disease of domestic animals worldwide. Pneumonia in sheep and goats is classified according to the involvement of different pulmonary regions and anatomical sites, and nature of the inflammatory exudate and reaction present. The alteration in the homeostatic environment of the lung parenchyma due to stressors like physiological/ environmental stress, decreased immunity, infectious pathogens and the environmental pollutants lead to the development of the pneumonia (Jubb *et al*., 2016).

Pneumonia, inflammation of the lung tissues, the main respiratory disease, occur widespread among sheep and goat all over the world and it is considered to be one of the most important causes of losses in the small ruminants (Woldemeskel *et al*., 2002; Bell, 2008a; Garedew *et al*., 2010), especially in countries where livestock management and husbandry are yet to be developed. The incidence of pneumonia is usually very high in developing countries including Ethiopia and causes serious financial losses to the livestock industry (Raji *et al*., 2000). Despite the high economic effect, there are very few detailed studies on the gross and histopathological pneumonic lung lesions in small ruminants in Ethiopia. Information on various causes of lung damage that lead to pathological changes/ lesions, which might directly or indirectly affect the public health are very important. Therefore, the present study was designed to attain the following objectives:

- Characterization of gross and microscopic lesion of pneumonia and other lungs injuries in sheep and goats.
- Demonstration of the infiltrated inflammatory cells in the pneumonic lung lesions by immunohistochemistry method using the monoclonal antibody.
- Identification of aerobic bacteria and parasites from pneumonic lungs.
- To assess the concurrent occurrence of bacteria and parasite from pneumonic lesions in sheep and goats in the study area.

2. LITERATURE REVIEW

2.1. Etiology of Pneumonia

Most causes of pneumonia in small ruminants are bacterial, viral, and parasitic infections. It can be also caused by *Mycoplasma*, and *Chlamydia*. Viral agents such as *Parainfluenza*-3 (*PI*-3) are common in sheep and goats and can increase susceptibility to infection by causing inflammation of the respiratory tract. Viral pneumonias generally are associated with fairly mild disease and clinical signs but can act as a predisposing factor for bacterial pneumonias. A number of viral agents have been identified as potential causes of viral pneumonia in sheep and goats. Certain infectious of ovine progressive pneumonia (OPP) and caprine arthritis encephalitis (CAE) can cause pneumonia in sheep and goats. Other organisms, including *Mycoplasma*, *Dictyocaulus* (lung worms), and *Eiemeria* can also cause lung problems (Chakraborty *et al*., 2014).

The etiological classification of bacterial pneumonia is complicated by the fact that many types of bacteria may be isolated from the same pneumonic lesions. However, the *Pasteurella spp*. are the most common bacteria isolated from cases of clinical pneumonia in sheep and goats. Other bacteria isolated from pneumonic lungs of sheep and goats *are Corynebacterium pyogens*, *Streptococcus spp*., *Staphylococcus aureus*, *Proteus mirabils*, *Pseudomonas aeuroginosa* and *Escherchia coli*. Bacteria and *Mycoplasmas* are commonly involved together in the pathogenesis of pneumonia in goats and sheep (Yatoo *et al*., 2018). There are many species in genus *Mycoplasma* associated with pneumonic and respiratory conditions in small ruminants, namely, *Mycoplasma agalactiae*, *Mycoplasma mycoides subspecies mycoides*, *Mycoplasma bovis*, *Mycoplasma capri*, *Mycoplasma capripneumoniae*, *Mycoplasma capricolum*, *Mycoplasma putrefaciens*, and many others (Kumar *et al*., 2011; Rahal *et al* ., 2014; Al-Momani *et al*., 2011). The *Pasteurella* spp., *Mycoplasma* spp., *Chlamydia* spp., *Haemophilus* spp., and *Salmonella* spp., are associated with causing either primary or secondary bronchopneumonia in sheep and goats, Both *Pasteurella hemolytica and Pasteurella multocida are bacterial organisms carried in the respiratory tract of many normal animals.* Most newborns are exposed to these organisms, but do not develop the disease because of natural resistance, a healthy environment, and ingestion of

antibodies in colostrum that help control the infection. *Pasteurella multocida* and *Pasteurella haemolytica* can be cultured from the upper respiratory tract of normal sheep and goats. A confirmed synergism is an initial infection with *PI*-3 virus or A*denovirus* followed by invasion of *P. haemolytica*, biotype A (The Merck Veterinary Manual, 1991).

Bacterial infections in a sheep and goat farm are a common clinical and subclinical finding (Hoek *et al*., 2011; Hogerwerf *et al*., 2013; Kumar *et al*., 2013). Some common respiratory commensal bacteria include *Pasteurella spp*. (Mazengia and Chanie, 2012), *Staphylococcus spp*., *Streptococcus pneumonia* (Kumar *et al*., 2012), *Arcanobacterium pyogenes*, *Haemophilus spp*., and *Klebsiella* pneumonia while the common *mycoplasmas* isolated from sheep and goats are *Mycoplasma capricolum subsp*. *capripneumoniae* (a causal agent of caprine contagious pleuropneumonia), *M. mycoides subsp. capri* (involved in contagious agalactia syndrome), *M. bovis* (Kumar *et al*., 2012), and *M. ovipneumoniae* (Nicholas *et al*., 2008). Tuberculosis is one of the important bacterial zoonosis in many parts of the world. Its diagnosis and reporting in meat animals is important for the understanding of the disease situation in a given area and hence taking the necessary legal and Veterinary sanitary measures. Pulmonary tuberculosis has been diagnosed in slaughtered meat animals in different countries such as USA (Rhyan *et al*., 1992), India (Chowdhury *et al*., 2001), Nigeria (Igbokwe *et al*., 2001), Zambia (Lofgren, 2001), Korea (Kim- JaeHoon *et al*., 2002) and Czech Republic (Pavlik *et al*., 2002a).

A variety of fungi characterized by the presence of chronic granulomatous lesions in lungs. *Aspergillus spp*., *Cryptococcus neoformans, Pseudoallescheria boydii, Emmonsia crescens*, *Mortierella wolfii*, *Pneumocystis carinii*, *Candida albicans, and Rhodotorula rubra* have been recorded or identified as the main causative agents of mycotic pneumonia sheep and goats (Pawaiya *et al*., 2015; Refai *et al*., 2017). The parasitic pathogens responsible to cause pneumonia in small ruminants are *Dictyocaulus filaria*, *Muellerius capillaris*, and *Protostrongylus rufescens*, *Bicaulus spp*., *Cystocaulus ocreatus and Cystocaulus nigrescens*, *Neostrongylus linearis*, *Spirocaulus spp*. and others (Adem, 2016).

In many cases, high humidity, dust, damp bedding, excessive heat, tight buildings with inadequate ventilation, and irritating gases such as ammonia compromise disease resistance and

natural defense mechanisms in the sheep or goat, allowing pneumonia to develop. Weakness from a difficult birth, inadequate intake of colostrum, and other stresses contribute to the development of pneumonia in nursing animals. Often, a mild viral infection will occur, compromising the animal and allowing secondary bacterial infections to take place (Bell, 2008b).

2.2. Epidemiology

In addition to the infectious agents which cause the pneumonia, there are risk factors which contribute to the susceptibility of the animal. The three risk factors (Animal, Environmental and management and Pathogen) interact in the pathogenesis of specific pneumonias. These are of paramount importance in any consideration of pneumonia and the details of the epidemiology of pneumonia. Susceptibility to pneumonia is determined by the animal's resistance to infection by agents that cause or predispose to pneumonia. Factors that impair innate resistance or adaptive resistance (immunity) increase the animal's susceptibility to pneumonia. For instance, shipping not only increases the risk of exposure of animals to pathogens to which they have not been exposed but also can impair innate resistance through damage to the respiratory tract by airborne irritants, dehydration, food deprivation and the effects of stress (Lonergan *et al*., 2001).

2.3. Disease Transmission and Pathogenesis

The main route of transmission of pneumonia and other respiratory infections is by inhalation of infective aerosols. Most infectious organisms are spread by direct contact with body fluids (saliva, nasal discharge, etc.) and fecal material. These problems can also be transmitted from one animal to another by contaminated hands, buckets, feeders, troughs, and equipment. The disease transmission in lambs most likely occurs through contact with ewes, other lambs, and/or juveniles in the herd; therefore ewes that survive pneumonia may act as carriers and serve as subsequent sources of the pneumonia-causing pathogens for susceptible lambs (Raghavan *et al*., 2016).

The pathogenesis of the disease depends on the presence of virulence factors in the infectious agents, host immunity and presence or absence and severity of the predisposing factors. The *P. hemolytica* possess adhesive fimbriae, secrete proteolytic enzymes which break down the mucosal barrier and impair the mucociliary function of the respiratory tract thus facilitating colonization and, the cytotoxin cause lysis of respiratory tract cells. The presence of other pathogens in the respiratory tract such as *Para-influenza*-3 virus and *Adenoviruses* disrupt the phagocytic mechanisms and the host immunity thus favoring proliferation of *Pasteurella* (Panciera and Confer, 2010).

2.4. Types of Pneumonia and Lesion Characteristics

The word pneumonitis has been used by some as a synonym for pneumonia; however, others have restricted this term to chronic proliferative inflammation generally involving the alveolar interstitial and with little or no evidence of exudate. However, the word pneumonia is used for any inflammatory lesion in the lungs, regardless of whether it is exudative or proliferative, alveolar, or interstitial. Pneumonias in domestic animals can be classified based on morphologic changes including, texture, distribution, color, general appearance and exudation of the affected lungs into four morphologically distinct types: bronchopneumonia, interstitial pneumonia, granulomatous pneumonia, and embolic pneumonia. Changes in the gross appearance of pneumonic lungs include abnormal color, the presence of nodules or exudate, fibrinous or fibrous adhesions, and the presence of rib imprints on serosal surfaces. On cut surfaces, pneumonic lungs may have exudate, hemorrhage, edema, necrosis, abscesses, bronchiectasis, granulomas or pyogranulomas, and fibrosis, depending on the stage (Zachary, 2017).

2.4.1. Bronchopneumonia

In bronchopneumonia, the inflammatory exudates collect in the bronchial, bronchiolar, and alveolar lumens. The severe inflammatory response around the bronchi and bronchiole, especially bronchio-alveolar junctions has been regarded as hallmark of bronchopneumonia (Jubb *et al*., 2006). Bronchopneumonias are generally caused by bacteria and *Mycoplasmas*,

by bronchoaspiration of feed or gastric contents, or by improper tubing. Authors (kumar, 2005; Oruc, 2006), reported that acute bronchopneumonia to be the most frequent type of lung inflammation. It can be suppurative bronchopneumonia if the exudate is predominantly composed of neutrophils and fibrinous bronchopneumonia if fibrin is the predominant component of the exudates.

2.4.1.1. Suppurative bronchopneumonia

Suppurative bronchopneumonia is characterized by cranioventral consolidation of lungs, with typically purulent or mucopurulent exudate present in the airways, which is caused by bacteria and *Mycoplasma*. The lungs were red to gray in color, single small to large multifocal variable sized discrete abscesses were seen and sometimes caeseous upon incision. The inflammatory process in suppurative bronchopneumonia is generally confined to individual lobules, and as a result of this distribution, the lobular pattern of the lung becomes notably emphasized. Suppurative foci revealed intact to degenerate neutrophils. Histopathologically, the suppurative areas were characterized by infiltration of inflammatory cells (mainly neutrophils) in the alveoli, bronchi, and bronchioles. Sloughed epithelial cells and necrotic debris were also noted in the bronchi and bronchioles. Abscesses containing colonies of bacteria surrounded by neutrophils were observed (Ettore *et al*., 2007; Mugale *et al*., 2015).

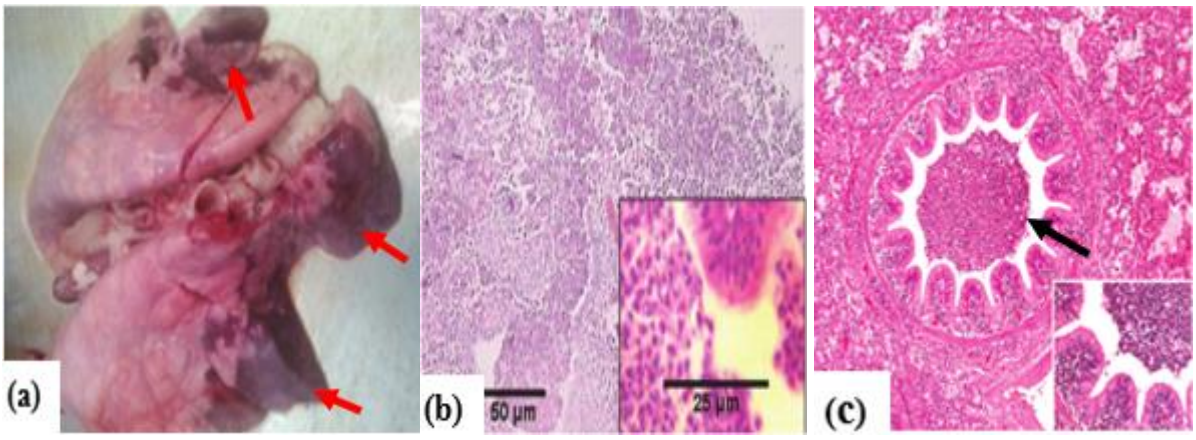


Figure 1: Suppurative bronchopneumonia, gross and histopathological lesion: red to gray cranioventral lobes (arrows) (**a**) with characteristic polymorphonuclear cells and few mononuclear cells infiltration in the alveoli, bronchi, and bronchioles (**b**) and bronchiole in the

center of the figure plugged with purulent exudate (arrow). The adjacent alveoli are filled with leukocytes and edema fluid (**c**). Sources: (Ettore *et al*., 2007; Mugale *et al*., 2015; Mekibib *et al*., 2019).

2.4.1.2. Fibrinous bronchopneumonia

In fibrinous bronchopneumonia (lobar pneumonias or pleuropneumonias), the inflammatory process involves numerous contiguous lobules and the exudate moves quickly through pulmonary tissue until the entire pulmonary lobe is rapidly affected. Pathogens causing fibrinous bronchopneumonia in domestic animals include *Mannheimia* (*Pasteurella*) *haemolytica* (*pneumonic mannheimiosis*), *Histophilus somni* (formerly *Haemophilus somnus*), *Actinobacillus pleuropneumoniae* (*porcine pleuropneumonia*), *Mycoplasma ovipneumoniae*, and *Mycoplasma mycoides subsp*. *mycoides* small colony type *(contagious caprine pleuropneumonia* (Zachery, 2017). The lesions were grossly characterized by fibrin deposition on the pleural surface, areas of consolidation mostly in the lobes (cranial, cardiac, and accessory), lungs were hard to cut and marbling with thickening pleura. In the serofibrinous pleuropneumonia at early stage severe congestion and hemorrhage, giving the affected lungs a characteristical intense red discolouration, and later the pleura was thickened with the formation of fibrinous capsule (Farooq *et al.*, 2017; Mallu *et al.*, 2017).

Histopathologically, the lesions were characterized by exudation in the bronchi, bronchiols, and alveoli majorly composed of fibrin and neutrophils, denuded epithelial cells, and necrotic debris (Thannon, 2017). Interlobular septa were thickened due to proliferation of alveolar septal cells and infiltration of fibrinocellular exudates comprising predominantly of neutrophils with occasionally mononuclear cells (Kumar, 2005; Oruc, 2006; Ettore *et al*., 2007).

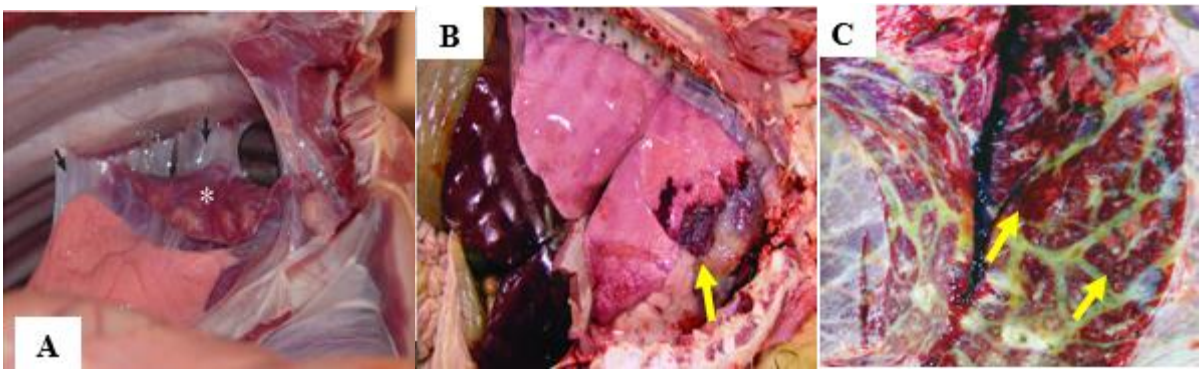


Figure 2: Fibrinous bronchopneumonia, gross lesion: **A**, Thick bands (arrows) of connective tissue between the visceral and parietal pleura. The cranial lobe (asterisk) appears consolidated and dark red. **B,** Cranioventral consolidation of the lung with fibrin on the pleural surface (arrow). **C,** Cut surface, affected parenchyma appears dark and hyperemic (arrows). Source: (Zachery, 2017).

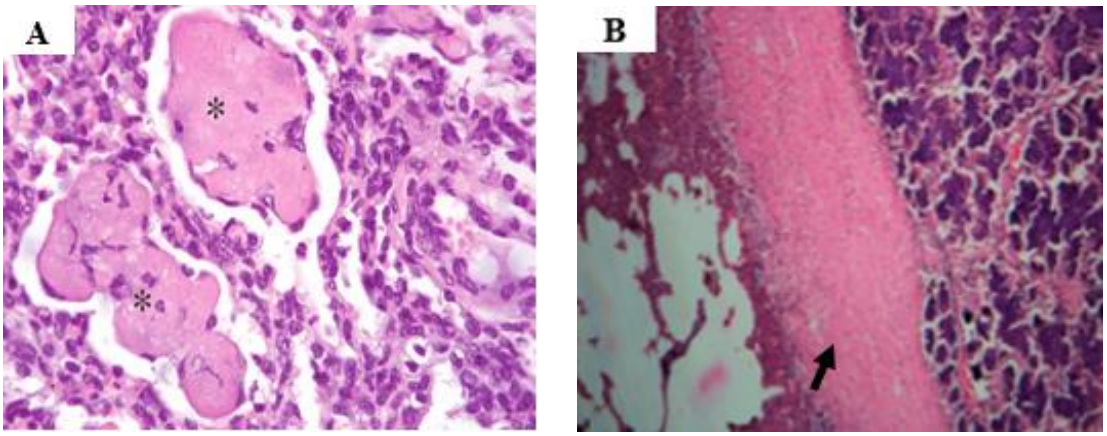


Figure 3: Fibrinous bronchopneumonia, microscopic lesion: **A,** Large aggregates of condensed fibrin (asterisks) surrounded and infiltrated by phagocytic cells. **B,** Section of lung showing thickening of interlobular septa (arrow) with infiltration of fibrinocellular exudates. Sources: (Rather *et al*., 2014; Zachery, 2017)

2.4.2. Interstitial pneumonia

In interstitial pneumonia the inflammatory process takes place primarily in any of the three layers of the alveolar walls (endothelium, basement membrane and alveolar epithelium) and contiguous

bronchiolar interistitium. Virus and parasitic infections are most commonly known to cause interstitial pneumonias. Presence of rib impressions on the pleural surface and failure of lungs to collapse are the typical gross features. Lungs with interstitial pneumonia were considered by severe congestion of blood vessels, hemorrhage in the alveoli, inflammatory cells in the lumen of bronchus, reactive cells in and around the bronchial wall, and sometimes inter-alveolar septa were thickened due to the accumulation of mononuclear cells and proliferation of fibrous connective tissue, lungs were voluminous and enlarged (Chowdhury, 2018).

Histologically, lesions were characterized by a marked increase in mononuclear cells in the interalveolar septa and presence of varying numbers of macrophages within the alveolar lumina (Oruc, 2006). Demissie *et al*. (2014) reported as, it can be characterized by focal epithelial hyperplasia of secondary and tertiary bronchi, smooth muscle hypertrophy in the wall of bronchi and connective tissue proliferation in the interstitial spaces. The thickened alveolar septae due to accumulation of macrophages containing hemosiderin pigments and lymphocytic cells and proliferation of fibrous connective tissue also characterize interstitial pneumonia. Exudates and haemorrhage was sometimes seen within the alveoli. Peribronchial and peribronchiolar proliferation of lymphocytes was detected in many interstitial pneumonitis, mononuclear cell and fibrous tissue proliferations were more prominent in interalveolar septa (Ahamad *et al*., 2016).

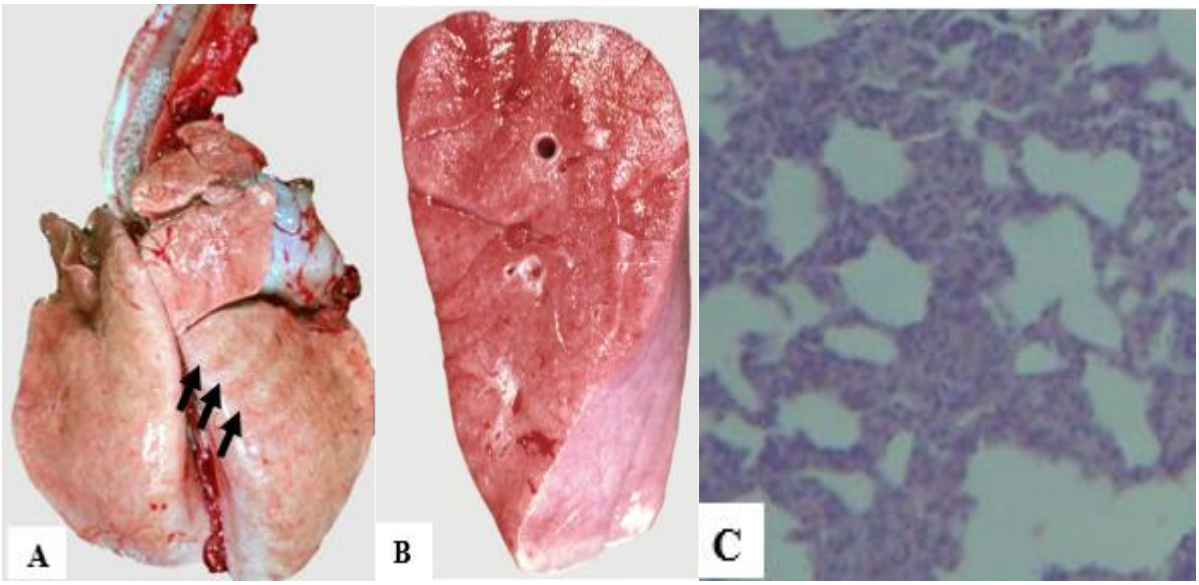


Figure 4: Interstitial pneumonia, gross and microscopic lesion: **A**, The heavy, rubbery, and costal (rib) imprints (arrows) on the visceral pleural surface of lung. The diffuse distribution is typical of interstitial pneumonia. The trachea contains froth (edema fluid). **B**, Transverse section, "meaty" appearance of pulmonary parenchyma. **C**, Interstitial pneumonia with epithelial hyperplasia smooth muscle hypertrophy and connective tissue proliferation in the interstitial spaces. Sources: (Demissie *et al*., 2014; Zachary, 2017)

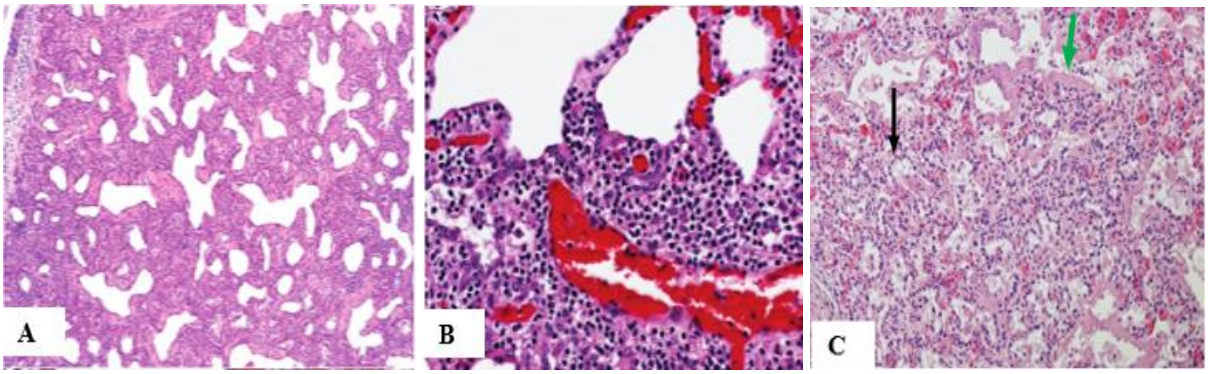


Figure 5: Interstitial pneumonia, histopathological lesion: **A**, The alveolar septa are notably thickened by severe interstitial infiltration of inflammatory cells. **B**, Higher magnification of **A** showing large numbers of lymphocytes and other mononuclear cells infiltrating the alveolar septal interstitium. **C**, Lung with acute diffuse interstitial pneumonia, with edema, hyaline membranes (green arrow), type II pneumocyte hyperplasia (black arrow), congestion, and expansion of the alveolar walls by a mild infiltrate of mononuclear inflammatory cells. Source: (Zachary, 2017).

2.4.3. Granulomatous pneumonia

Granulomatous pneumonia is characterized by the presence of variable numbers of caseous or noncaseous granulomas randomly distributed in the lungs. The most common causes of granulomatous pneumonia are systemic fungal diseases, such as *Cryptococcosis*, *Coccidioido mycosis*, *Histoplasmosis*, *Aspergillus spp*. or *Mucor spp*. and *Blastomycosis*; bacterial diseases, such as tuberculosis (*Mycobacterium bovis*) in all species; sporadically, aberrant parasites such as *Fasciola* and aspiration of foreign bodies. The affected lungs of sheep and goat grossly appeared as hard multifocal nodules of different size distributed throughout the lung surface. These foci

characterized by white color, well-circumscribed, variably sized nodules, which are surrounded by a clear discrete, red and hemorrhagic area (Figure 6: **A**). Microscopically, theses nodules showed central necrotic area appeared as a cheesy material and surrounded by a zone of layers which consist of the different inflammatory cells with zone of the fibrous connective tissue (Figure 7: **B**) (Mahdi *et al*., 2015).

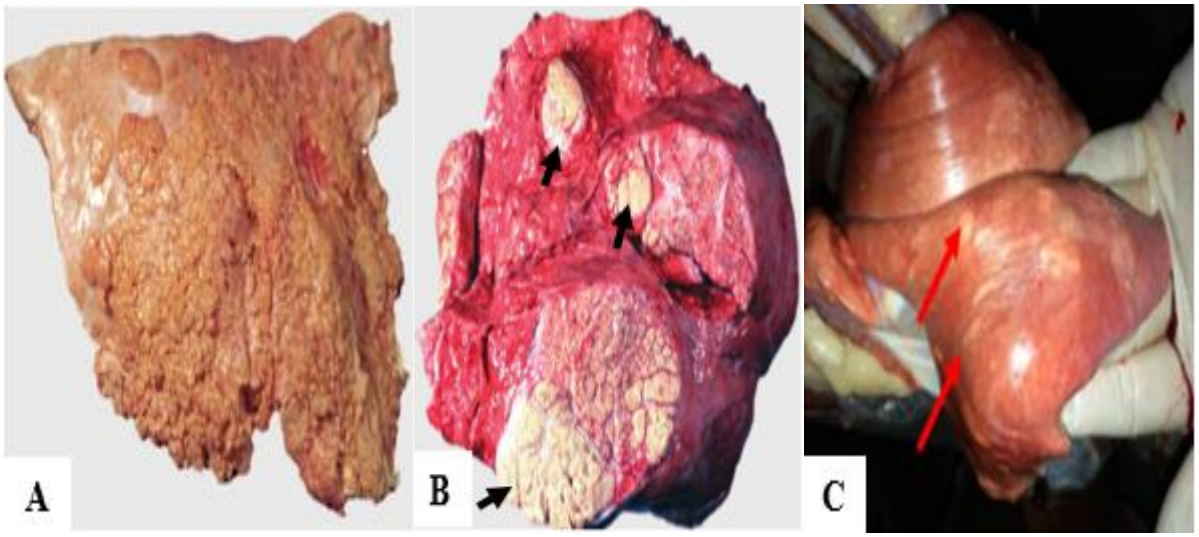


Figure 6: Granulomatous pneumonia, gross lesion: **A**, Multifocal, coalescing granuloma involves most of the lung lobe. **B**, Transverse section, large multifocal to confluent caseating granulomas ("cheesy," pale yellow-white (arrows) appearance) in the pulmonary parenchyma. **C**, Hard multifocal nodules with different size distributed throughout the lung surface. Source: (Mahdi *et al*., 2015)

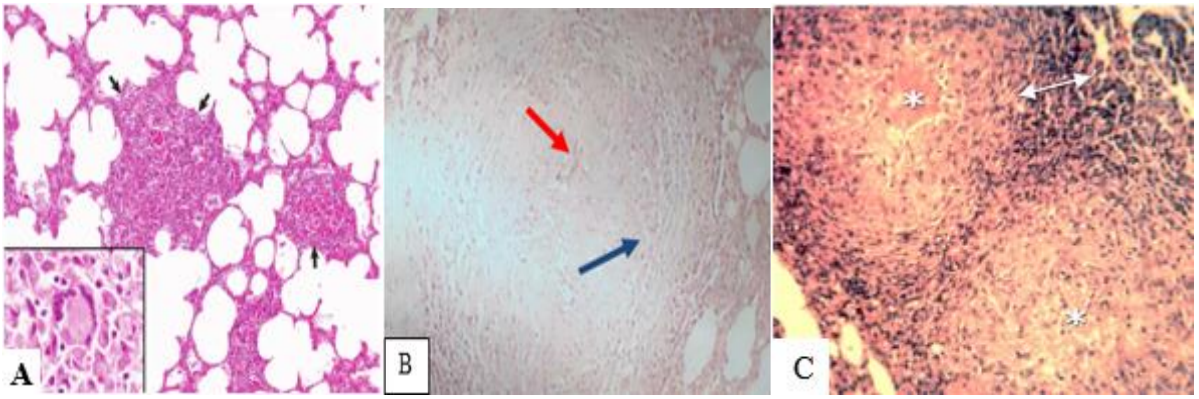


Figure 7: Granulomatous pneumonia, microscopic lesion: **A**, Confluent noncaseous granulomas (arrows) with a small necrotic center filled with neutrophils, surrounded by histiocytes and

mononuclear cells, and with an outer rim of connective tissue. Inset, Epithelioid macrophages and a large multinucleated giant cell. **B**, Granulomatous reaction consists of central area of caseous necrosis surrounded by a zone of layers consist of different inflammatory cells and fibrous connective tissue. **C**, Typical granuloma formed by a necrotic center (asterisks) surrounded by macrophages and then by an external band of fibrous connective tissue (double arrows). Sources: (Mahdi *et al*., 2015; Zachary, 2017)

2.4.4. Embolic pneumonia

Embolic pneumonia refers to a particular type of pneumonia in which gross and microscopic lesions are multifocally distributed in all pulmonary lobes. The lung injury is hematogenous, and the inflammatory response is typically centered in pulmonary arterioles and alveolar capillaries. The most common etiologies are vegetative endocarditis (right side of the heart), jugular thrombosis, rupture of hepatic abscesses into the vena cava, embolic foreign body (hair, septic emboli, etc). The gross characterization of affected lungs with embolic pneumonia was by multifocal nodules of the same size distributed randomly throughout the pulmonary lobes. The gross lesions were white foci, small in size (4-6 mm), surrounded by a discrete, red, hemorrhagic halo. Microscopically, these nodules showed multifocal neutrophilic aggregations that were randomly scattered throughout the pulmonary lobes (Azizi *et al*., 2013).

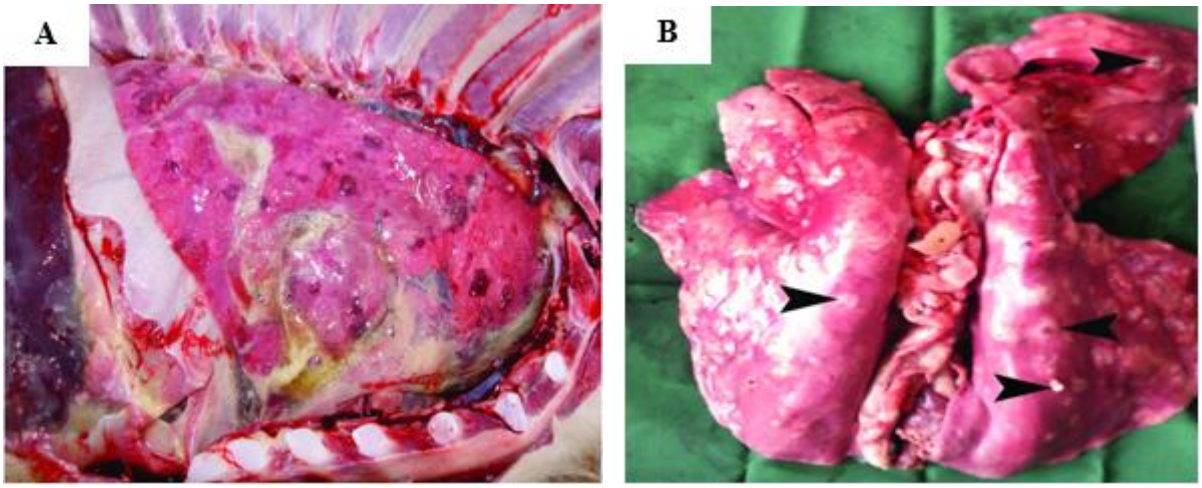


Figure 8: Embolic pneumonia, gross lesion: **A**, The lungs fail to collapse and show numerous hemorrhagic nodules distributed randomly throughout all lung lobes originated from a severe

septic omphalophlebitis. **B**, Macroscopic appearance of embolic pneumonia caused by *Staphylococcus aureus*. White, raised foci of 4-6 mm in size are observed throughout pulmonary lobes (arrow heads). Source: (Azizi *et al*., 2013).

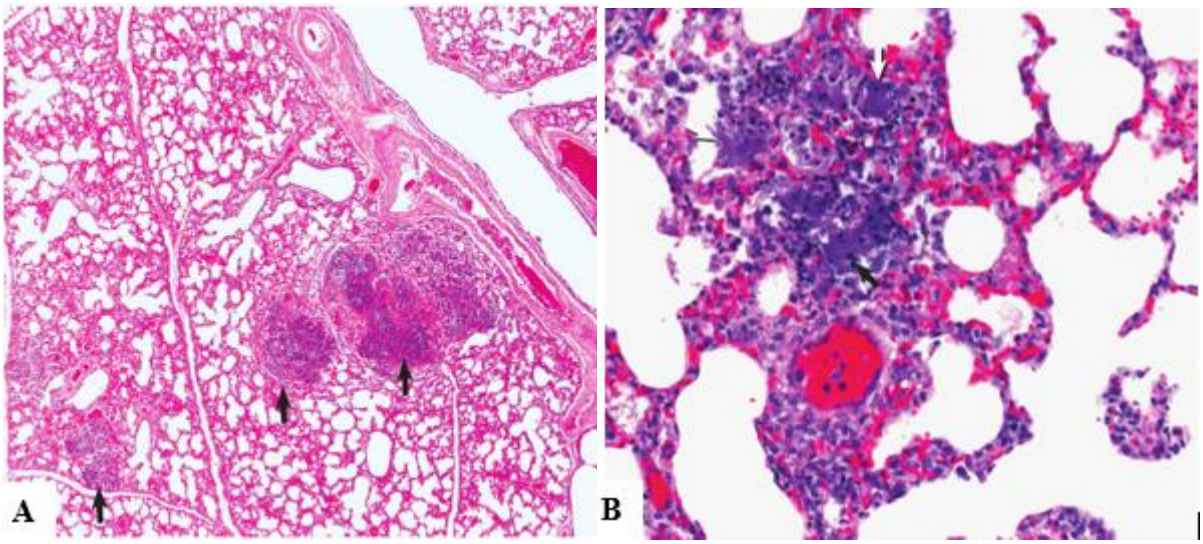


Figure 9: Embolic pneumonia, microscopic lesion: **A**, Foci of necrosis and infiltration of neutrophils (arrows) resulting from septic emboli. The multifocal distribution of the lesion, which is typical of embolic pneumonia. **B**, Embolic focus in the lung, bacterial colonies (arrows) mixed with neutrophils and cellular debris. Sources: (Azizi *et al*., 2013; Zachary, 2017)

Table 1: Gross pathological changes observed in various types of pneumonia

Type	Portal of Entry	Distribution	Texture/Consistency
Bronchopneumonia	Aerogenous	Cranioventral consolidation	Firm/ Hard
Interstitial pneumonia	Aerogenous/or hematogenous	Diffuse	Elastic/ rubbery with rib imprint
Granulomatous pneumonia	Aerogenous/or hematogenous	Multifocal	Nodular
Embolic pneumonia	Hematogenous (septic emboli)	Multifocal	Nodular

Sources: (Zachary, 2017; Mugale and Balachandran, 2018)

2.5. Other Types of Pneumonia

2.5.1. Enzootic Pneumonia or Shipping Fever

Enzootic pneumonia also is referred to as atypical pneumonia or *Mycoplasma* pneumonia of sheep. It is a chronic non progressive pneumonia of sheep. The list of infectious agents involved in ovine enzootic pneumonia includes *Mannheimia haemolytica*, *Pasteurella multocida*, *Parainfluenza virus* 3 *(PI*-3*), Adenovirus*, *Reovirus, Respiratory Syncytial Virus (RSV)*, *Chlamydiae*, and *Mycoplasmas* (*Mycoplasma ovipneumoniae*) *(*Ayling *et al*., 2004; Lin *et al*., 2008). Other *Mycoplasma* species and *Chlamydophila psittaci ovis* all can act as secondary invaders after a *Mycoplasmal* infection. Such infection in animals caused by a bacterial species related to genus *Pasteurella* is known as pasteurellosis. *Pasteurella multocida* (*P. septica*) is carried in mouth and respiratory tract of several animals, notably cats (Verma and Kamil, 2005; Verma, 2005). Environmental conditions and various stress factors such as transportation, housing deficiency, and bad weather also play a role to further aggravate the clinical conditions. Among the various diseases considered to be caused by *P. multocida*, alone or in association with other pathogens, most important is shipping fever in cattle and sheep, which may also be caused by *Mannheimia haemolytica*, in the absence of *P. multocida* (Chaturvedi and Minakshi, 2000; Minakshi *et al*., 2000).

Lesions are variable and depend largely on the agents involved and on the duration of the inflammatory process. The lesions similar to those of bronchopneumonia, characterized by a severe fibrinous bronchopneumonia (cranioventral) with pleuritis (Fig., 10: **A**). Subacute to chronic cases progress to purulent bronchopneumonia, and sequelae include abscesses and fibrous pleural adhesions. Microscopic lesions are characterized by an influx of macrophages and neutrophils into the bronchi, bronchioles, and alveoli, and with time there is also notable BALT hyperplasia (Fig., 10: **B**) and mild interstitial and alveolar edema. In some cases, accumulation of exudate can be severe enough to cause occlusion of bronchioles and atelectasis of the corresponding lobules (McGavin and James, 2007; Dusty and Pugh, 2012: Zachary, 2017).

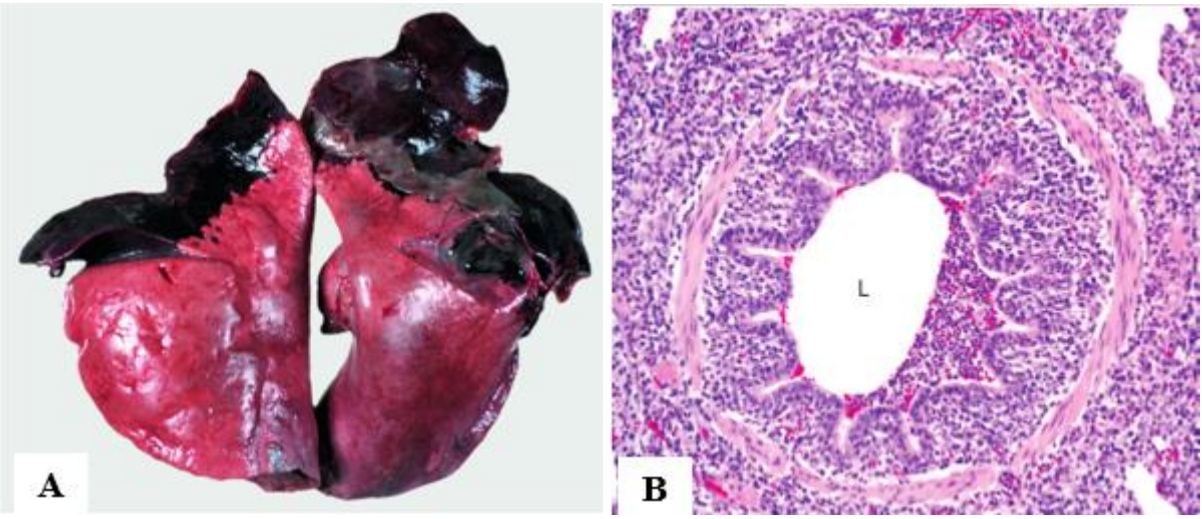


Figure 10: Enzootic pneumonia, gross and microscopic lesion: **A**, Pneumonic mannheimiosis (*Mannheimia haemolytica*), the cranioventral aspects of the lungs are red, swollen, and very firm (consolidated), with some fibrin on the pleural surface. The consolidated lung resembles liver, referred to as "hepatization." **B**, Lymphocytes and histiocytes infiltrate the bronchiolar lamina propria and peribronchiolar and alveolar interstitium. The bronchiolar lumen also contains neutrophils and erythrocytes. L, lumen of the affected bronchiole. Sources: (McGavin and James, 2007; Zachary, 2017).

2.5.2. Verminous pneumonia

The three primary lungworms of small ruminants are of clinical and economic importance: *Dictyocaulus filaria*, *Muellerius capillaris*, and *Protostrongylus rufescens*. Of these, *M. capillaris* seems to be the most prevalent and goats appear to be more likely than sheep to demonstrate clinical disease after infection with *M. capillaris*, and the lesions more typically are interstitial in goats, whereas they more often are subpleural in sheep (Berrag, 1997; Smith, 2015). Diagnosis is made at necropsy; the diaphragmatic lung lobes are seen to be most affected, and nodular (*M. capillaris*) or lobular lesions that contain the worm may be present (Berrag, 1997; McKenna, 1999).

Grossly, lesions appear as dark or gray, depressed, wedge-shaped areas of atelectasis involving few or many lobules usually along the dorsocaudal aspect of the lungs. On cut surface,

edematous foam and mucus mixed with white, slender (up to 80-mm long) nematodes are visible in the bronchi. In the most severe cases, massive numbers of nematodes fill the bronchial tree. Microscopically, the bronchial lumens are filled with parasites admixed with mucus because of goblet cell hyperplasia, and there is squamous metaplasia of the bronchial and bronchiolar epithelium because of chronic irritation. There are also inflammatory infiltrates in the bronchial mucosa; alveolar edema; hyperplasia of BALT caused by persistent immunologic stimuli; hypertrophy and hyperplasia of bronchiolar smooth muscle because of increased contraction and decreased muscle relaxation; and a few eosinophilic granulomas around the eggs and dead larvae. These granulomas, grossly, are gray, non caseated nodules (2 to 4 mm in diameter) and may be confused with those seen at the early stages of tuberculosis (Jubb *et al*., 2016; Zachary, 2017).

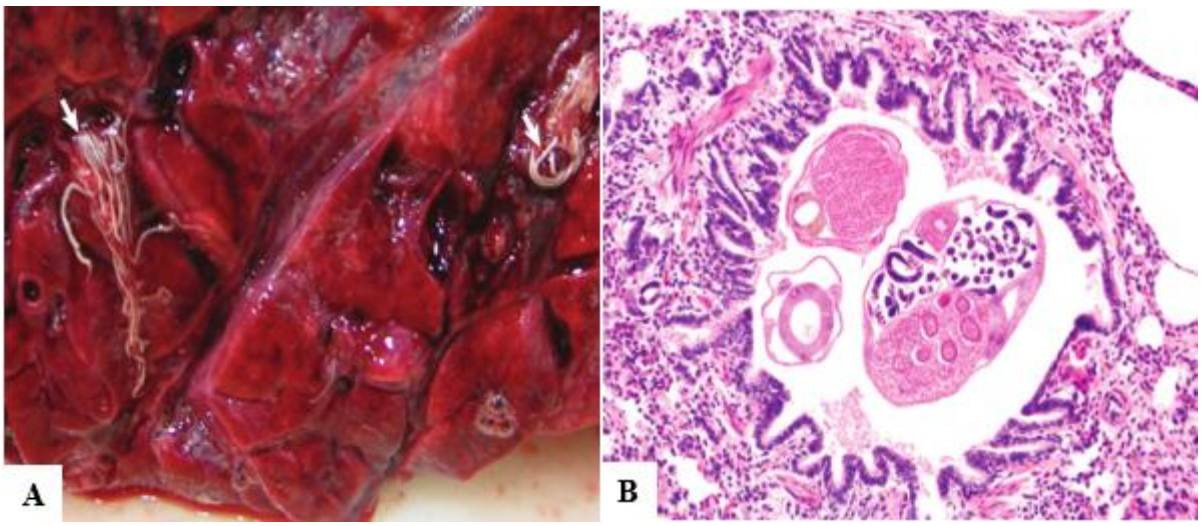


Figure 11: Verminous pneumonia, gross and microscopic lesion, bronchus: **A**, The bronchi contain numerous slender white lungworms (arrows) and large amounts of clear foamy fluid, indicative of pulmonary edema. **B**, Cross section of bronchus containing nematodes. Sources: (Jubb *et al*., 2016; Zachary, 2017).

2.5.3. Aspiration pneumonia

Aspiration pneumonia is an obvious situation where pulmonary challenge with massive numbers of bacteria overcomes the lung defenses. Inhalation of significant amounts of feedstuffs or liquids leads to an intense inflammatory response and the development of aspiration pneumonia. This

clinical scenario may be secondary to dysphagia or laryngeal paralysis. Aspiration pneumonia also may occur as an iatrogenic disorder secondary to forced delivery of liquids or application of drenches. The severity of the inflammatory response depends on the material aspirated, the type of bacteria aspirated, and the distribution of aspirated material in the lungs (Dusty and Pugh, 2012; Ahsan *et al*., 2010). The main gross findings at postmortem examination were oesophageal dilation associated with non-collapsed enlarged lungs (Fig. 12: **A**) and foamy fluid within the trachea and bronchi, occasionally mixed with ruminal contents. Hydropericardium, pale areas in the myocardium, and haemorrhagic foci scattered in the endocardium, epicardium and at the base of the pulmonary artery were observed, as well as hyperaemia and erosions in the oral mucosa, and subcutaneous oedema of the face. Histopathologically, aspiration pneumonia characterized by diffuse neutrophilic infiltration within the alveoli, bronchi and bronchioles in association with vegetal material (Fig. 12: **B**) and granular basophilic structures (consistent with bacteria) within the bronchiolar lumen. The lungs also showed hyperaemia, occasional haemorrhagic foci, hyaline material lining the alveolar surfaces, emphysema (borders) and oedema (interlobular septa) (Antoniassi *et al*., 2010).

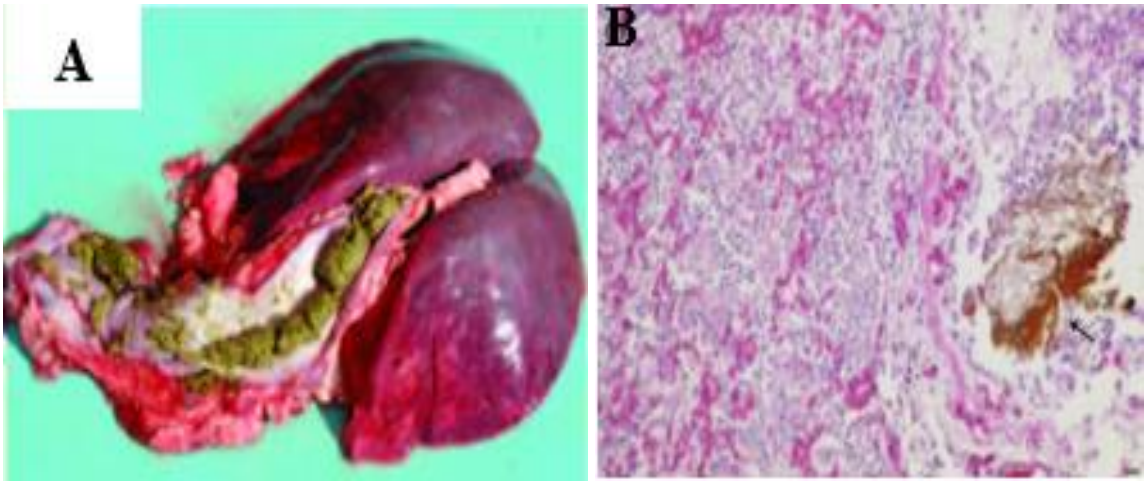


Figure 12: Aspiration pneumonia, gross and histopathologic lesion: **A,** Dilated oesophagus containing a large amount of food residue, and enlarged, diffuse, dark red lungs in a sheep. **B,** Vegetal material (arrow) within the bronchiolar lumen associated with diffuse neutrophilic infiltration in a sheep with aspiration pneumonia. Source: (Antoniassi *et al*., 2010).

2.6. Immunohistochemistry (IHC)

Immunohistochemistry is a laboratory technique combines immunological, chemical and histology in the study of constituents of tissue to detect specific antigens (i.e. proteins) in tissue or cells based on antigen antibody recognition; it seeks to exploit the specificity provided by the binding of an antibody with its antigen at the light microscopy level. It has been proven to be one of the most important ancillary techniques in the characterization of neoplastic disease in humans and has become equally important in Veterinary medicine, as oncologists demand more specific diagnosis (Ramos-vara *et al*., 2002a,b; 2008).

The advantage of the immunohistochemistry over most other methods of pathogen detection is being able to co-localize the pathogen of interest within the tissue lesions produced by the disease process and, depending up on the disease, even the specific cell types affected (Dagleish *et al*., 2010). The number of immunohistochemical tests available for diagnosis of infectious and neoplastic diseases, detection of cells and antigens with lesions. It enable to obtain reproducible and consistent results by using different immunohisochemical components including cellular markers and/ or primary antibody, secondary antibody, antigen retrieval, chromogens and different required equipment's. The most antibodies to cellular antigens used in veterinary immunohistochemistry laboratory have been developed against human or rodent antigens (Shi *et al*., 2001).

The different authors reported on immunohistochemical detection of different infectious agent from pneumonic lungs of small ruminants, for instance, Emikpe *et al*. (2019) described immunohistochemical assessments of pneumonia in sheep at Nigeria and Ghana; Jamshidi and Ozmen (2018) determined the persistance and prevalence of bovine herpesvirus 1 (BHV1) antigen from lung tissue of sheep with pneumonics by using immunohistochemistry staining technique. Similarliy, (Brown *et al*., 1991; Madboli and Eldebaky, 2016; Uma *et al*., 2018) detected different infectious agents such as viral and bacterial pathogens from small ruminants by using antibody. Thus, they exempliefied or evinced that this methodology is a highly specific which used in the confirmation of diagnosis of many diseases and enabling more accurate prognoses and targated treatments.

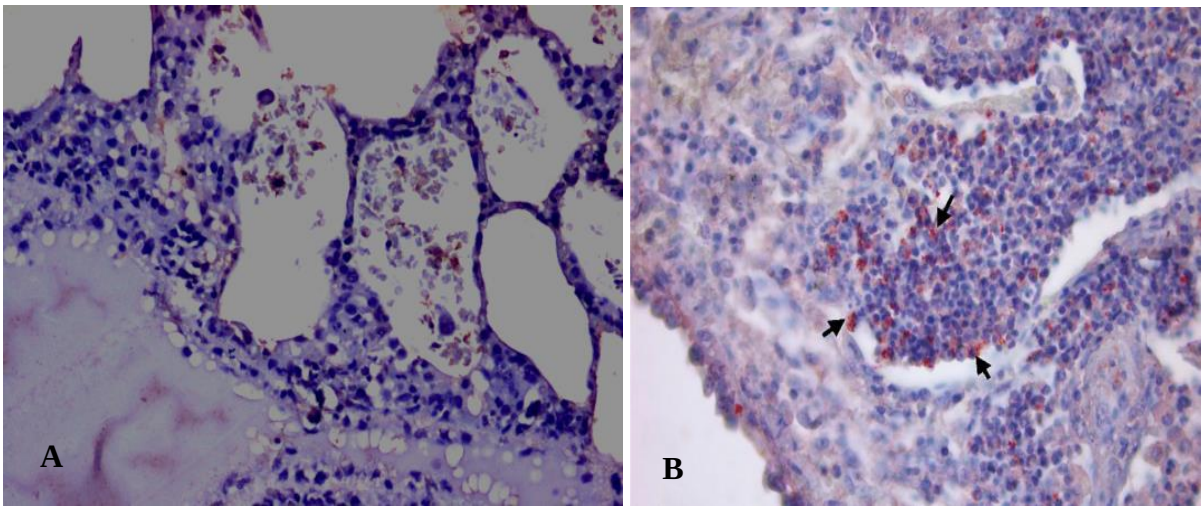


Figure 13: Lung showing the immunopositive staining in the exfoliated epithelium and inflammatory cells (A). The lung showed the CD68 primary antibody positive immunoreaction of inflammatory cells in the alveolar lumens in a free form (arrows, (B)). Source: (Uma *et al*., 2018; Yavuz and Dincel, 2019).

2.7. Diagnosis

Because lethargy and fever in sheep and goats may have several causes, a careful physical examination is required. In many cases, an exact (definitive) diagnosis is made by post-mortem (necropsy) examination. The initial diagnosis can be made from general appearance and physical examination. Palpation and careful observation of the lungs are essential in the diagnosis of pneumonia. Lung auscultation is a systematic approach that could be adopted to detect, identify and differentiate the degree of severity in ruminant pneumonia cases (Jesse *et al*., 2018). Respiratory problem is evident when abnormal respiratory sounds such as harsh, wheezes or crackles lung sound is heard during auscultation (Jesse *et al*., 2019). Culture and sensitivity performed on nasal secretions or on samples taken at necropsy can help identify the specific cause of the infection. To accurately identify an infectious cause of pneumonia, a transtracheal wash performed by a veterinarian, with culture and sensitivity, may be necessary. The diagnostic tests as well as procedures adopted in different parts of world incorporate combination of conventional and advanced diagnostic tests. However, the initial suggestive diagnosis involves the observation of clinical signs and postmortem findings followed by serological and molecular methods for the confirmation of etiological agents (Chakraborty *et al*., 2014).

2.8. Clinical Sign

Pneumonia is clinically characterized by labored breathing, coughing and/or rapid breathing, nasal discharges (clear to yellow, runny to thick), lacrimation, anorexia, depressions and sometimes pyrexia if there is systemic involvement. Exercised animals exhibit tachypnea and dyspnea. Young nursing animals that develop pneumonia commonly lose weight, become gaunt and lethargic, fail to nurse, and usually have a moderate fever. If the pneumonia remains undetected, serious lung damage will result and treatment will not be effective. Young animals that recover are susceptible to relapse during the feeding period and are more likely to suffer from heat stress and chronic cough. Coughing can lead to serious problems with rectal prolapse in feeder lambs. In bacterial pneumonia the major clinical findings are polypnea in the early stages and dyspnea later, abnormal lung sounds, and fever and toxemia. Some of acutely affected animals may die without showing any clinical signs. The subacute and chronic diseases are characterized by unthriftiness. The clinical signs will vary with the severity. Early in an outbreak there is more coughing in the group than previously; when moving the lambs some may lag behind and be breathing hard. There can be very sick animals which require individual treatments; there may be some deaths. There were sporadic cases of pneumonia in the survivors every time that they were handled (John Martin, 2012).

2.9. Treatment

Treatment of pneumonia must be aimed at the cause of the secondary infection to attempt to save the animals, and to reduce lung damage. Because of the variety of agents that can be implicated, an accurate identification of the infectious agent is a must. Treatment of individual lambs is difficult except in the small flock. Treatment must be based on early identification of affected individuals and depends on whether the cause is bacterial, viral, or parasitic (Scott, 2011). Fluid therapy, if practical, often helps the recovery rate. Producers should be sure that sick newborns are nursing or that they are provided supplemental milk via stomach tube. In serious outbreaks, it is often advisable to treat all exposed animals with a therapeutic dose of antibiotics for several days. For the bacterial causes of pneumonia treatment with antibiotics such as penicillin,

tetracycline, oxytetracycline, ampicillin, tylosin, gallimycin, ceftiofur (naxcel), and florfenicol (nuflor) may be considered. Like most bacterial infections, culture and sensitivity testing is recommended. Treatment for all viruses involves treating the symptoms, not killing the virus. With this in mind, fluids, anti-inflammatory agents (banamine), and antibiotics for secondary bacterial infections are recommended. Most parasites can be treated using ivermectin or doramectin. Routine de-worming will also help prevent the parasitic causes of pneumonia (Fthenakis and Menzies, 2011; John Martin, 2012).

2.10. Control and Prevention

Control and prevention of respiratory disease in sheep and goats revolve primarily around environmental and stress management. Control measures of pneumonia can be achieved by good husbandry practices, isolation and treatment of the affected animals. Early diagnosis and proper treatment are critical in controlling this problem. Avoidance or minimization of predisposing factors such as overcrowding, long distance trekking and inclement weather can greatly reduce the incidence of pneumonia in a herd or farm. Animals should be housed in well ventilated but not drafty environments with an adequate number of air changes to prevent accumulation of noxious odors. Adequate transfer of passive immunity from dam to kid through the colostrum is of utmost importance in the prevention and control of respiratory disease in young small ruminants. On account of the lack of commercially available vaccines against all clinically important bacterial and viral small ruminant strains, most herd management programs do not include a vaccination plan for control of respiratory disease (Fthenakis and Menzies, 2011; Scott, 2011).

3. MATERIALS AND METHODS

3.1. Study Areas and Study Animals

The present study was conducted from October, 2019 to May, 2020 at Modjo export abattoirs and different restaurants found in Modjo town. The Modjo town is located at 8^0 32' to 8^0 36' N latitude and 39^0 7' to 39^0 9' E longitude with an altitude between 1788 and 1825 meters above sea level (m a.s.l.) in the central highlands of Ethiopia 73 Kilometers South East of Addis Ababa (CSA, 2005; Tageo.com; Kassie *et al*., 2014). It has annual rainfall of 901 mm of which 21.9% fall down during the main wet season extends from June to September and during the short/ a little rainy season (locally known as *Belg*) that extends from March to May. The average annual minimum and maximum temperature are 6.8°C and 31.8°C, respectively (Kassie *et al*., 2014; EMA, 2016).

The animals brought to the abattoirs for slaughter were raised around/ in the study area or originate from different parts of the country including Afar, Arsi, Bale, Borena, Meiso (West Hararghe), Jimma, East Wellega, Somali, Wollo, Wolaita Sodo, South Omo (or "Debub Omo"), and Arba Minch (Gamo Gofa) were included in the study. The animals from distant and near the study area was transport to the abattoir using vehicles and on their foot, respectively.

The Afar National Regional State is characterized by an arid and semi-arid climate with the altitude ranges from 120 meter below sea level to 1500 meters above sea level. The area located in northeast of Ethiopia between 39°34′ and 42°28′ E longitude and 8°49′ and 14°30′ N latitude at about 621 kilometers away from Addis Ababa. Temperatures vary from 20°C in higher elevations to 48°C in lower elevations. The area has mean annual rainfall below 500 mm in the semi-arid western escarpments and decreasing to 150 mm in the arid zones to the east. The rainfall is low, erratic and bimodal, the long rain usually occurs in the months of mid-June to mid-September, while the short rains usually come in March and April (MoARD, 2008).

Arsi and Bale highlands with their capital cities Asela and Robe are found in the Oromiya Regional State southeast of Ethiopia at about 175 and 430 Kilometers away from Addis Ababa respectively. Both zones has a common boundary and their average annual temperature ranges between 12°C -18°C. The area majorly represents the central plateau with an altitude of between 1500 to 4250 meters above sea level and receives a bimodal average annual rainfall of between 900-1400 mm occurring from July to October and April to May (Tolossa and Ashenafi, 2013).

Borena is one of zone in Oromia Regional State. The capital of the zone, Yabello is 575 Kilometers far from capital city Addis Ababa to south direction located in latitude and longitude of 5°00′ N 38°15′ E with an altitude ranges between 943 and 2,400 meters above sea level. The annual temperature varies between 19°C - 42°C. The average annual rain fall and the annual temperature of the area ranges between 400 to 1100 mm and 19°C - 42°C, respectively. The area receive a bimodal rainfall distribution of long rainy season extends from March to May and the short rainy season occurs from mid-September to the mid November (Wubishet *et al*., 2018).

Mieso is a town in eastern Ethiopia at about of 304 Kilometers away from Addis Ababa. Located in the West Hararghe Zone of the Oromia Region, it has a latitude and longitude of 9°14′ N 40° 45′ E with an elevation of 1394 meters above sea level (Wikipedia contributors, 2018). The annual rainfall at Mieso district ranged from a minimum of 500 mm to a maximum of 700 mm with minimum and maximum temperature of 25°C and 37°C, respectively (Degaga and Angasu, 2017).

Jimma is one of zone in Oromia Regional State. The Jimma town is capital and administrative center of the zone, is 350 Kilometers far from capital city Addis Ababa. The area located with an altitude ranges between 1689 and 3018 meters above sea level. It has the average annual rainfall ranged from a minimum of 1200 mm to a maximum of 2400 mm with minimum and maximum temperature of 11.8°C and 28.8°C, respectively (Aticho *et al*., 2018).

East Wollega is a zone of Oromia regional state far 328 kilometers from Addis Ababa to the West. Nekemte is the capital city of the zone located at latitude of 9° 06′ N and longitude of 36° 31′ E with an elevation of 2088 meters above sea level. The minimum and maximum annual rain

fall vary from 1200 to 2400mm and the mean annual temperature from 16.8°C to 29.1°C, respectively (Fita, 2014; Tilahun *et al*., 2014).

Somali occupied the second-largest area in Ethiopia and covers 350,000 km^2. Eighty percent of the area is arid and semi-arid. Somali pastoralists herd large numbers of sheep and goats. Jijiga is the capital of the Somali Regional State located at about 630 Kilometers east of Addis Ababa approximately 9° 20′ N latitude and 45° 56′ E longitude with altitude ranges from 1660 -1850 meters above sea level (Bekele *et al*., 2011). The climate of the area is a subtropical highland climate. The mean minimum and maximum temperature of around 17°C to 30°C, respectively. The rainfall distribution in the area is bimodal with a mean annual rainfall of 660 mm. There are two rainy seasons: the main *Meher* rains occur from July to September, and the short *Belg* rains in April and May. The dry season, known as *Bega*, is cooler by morning than the wet seasons due to lower cloud cover, but equally hot by afternoon though less humid (Kemal *et al*., 2019).

The North and South Wollo are zones of Amhara region. Dessie is the capital city of the zones, located North East Ethiopia at a distance of 400 km from Addis Ababa, at 11° 08′ North latitude and 39° 38′ East longitude and has an elevation of 2600 meters above sea level. The mean minimum and maximum temperature are 12.37°C and 26.27°C, respectively. The north and south zones of Wollo experience bimodal rain fall with a short rainy season occurs usually from March to May and long rainy season extends from June to September. The annual rain fall of the areas ranges from 800-1000 mm (DFEDB, 2015).

Wolaita Sodo is the administrative center of Wolaita zone in the Southern Nation Nationality and People Regional State, Ethiopia. It located at 390 Kilometers south of Addis Ababa and lies between latitude of 6° 4′ to 8° 5′ North and longitude of 37° 45′ to 38° 2′ East at an altitude of 700-2950 meters above sea level. An average annual rain fall ranging from 450-1446mm. The mean annual minimum and maximum temperature of the area is 11.4°C and 34.12°C, respectively (Dendana, 2014).

South Omo (or "*Debub Omo*") zone with its capital Jinka is located in Southern Nations, Nationalities and People's Regional state (SNNPRS) approximately 785 Kilometers away from

Addis Ababa. The zone is located in 4° 43′ - 6° 46′ N latitude and 35° 79′ -36° 06′ E longitude at an elevation of 1490 meters above sea level. The climatic condition ranges from *Dega* (humid) to *Kola* (semiarid) which constituted 34.4% of the zonal climatic condition. The temperature of the area falls between 15.7°C and 38°C, and the average annual rainfall was 492 mm (Molla, 2016; SOZAO, 2006).

Arba Minch is located at 5° 57′ N latitude and 37° 32′ E longitude with an altitude ranging from 1300 to 2600 meters above sea level, in the SNNPR state, Ethiopia at about 500 Kilometers from capital city, Addis Ababa. It has mean annual rain fall of 900-1000 mm and mean annual temperature of 23°C (Girma *et al*., 2014).

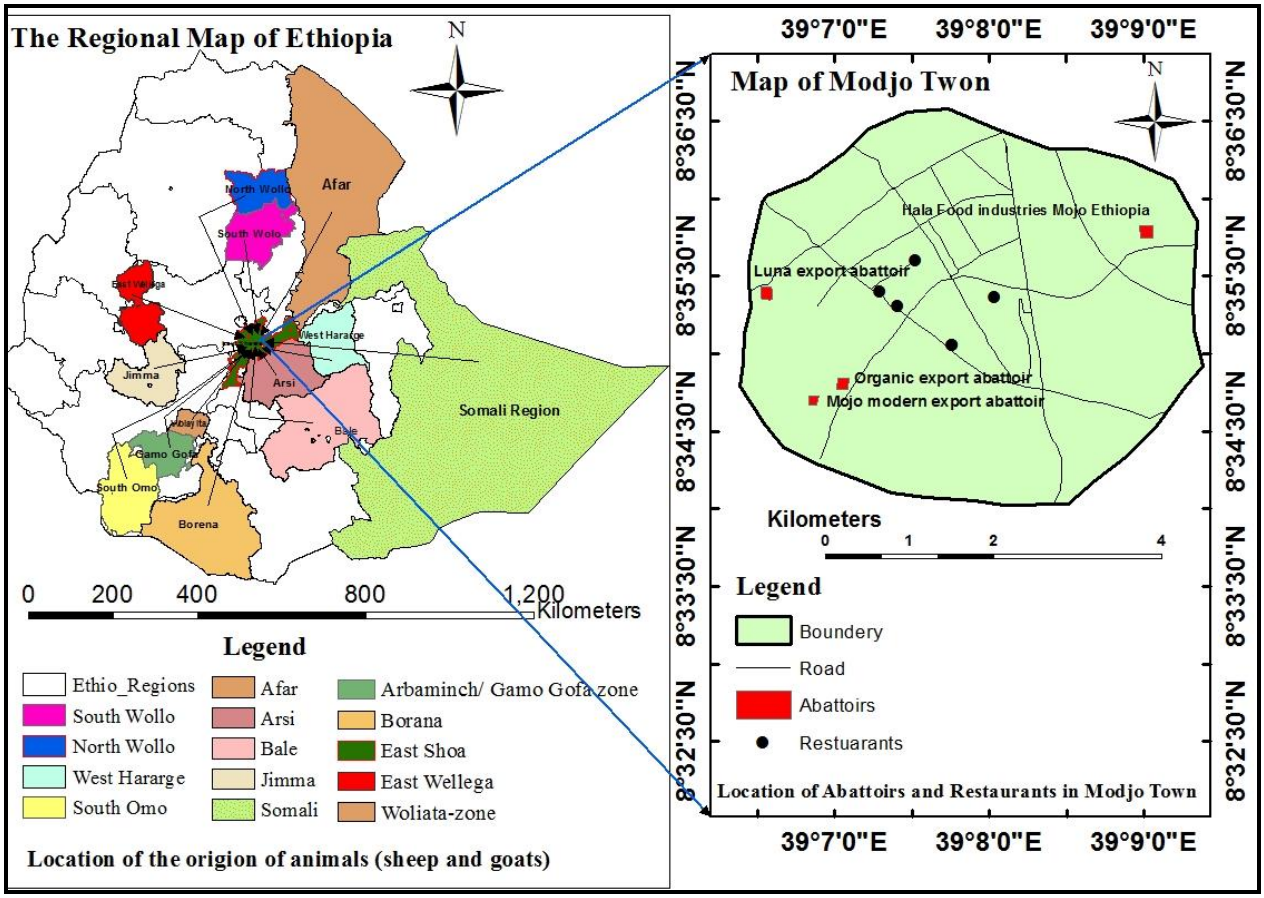


Figure 14: Map of study area showing location of origin of animals and location abattoirs and restaurants in Modjo town

3.2. Study Animals, Study design and Methods of Sampling

The study animals were the small ruminants brought for slaughter as a source of food at abattoirs and different restaurants in Modjo town. In this study a total of 155 lungs (sheep = 86; goats = 69) were examined for pneumonia. A cross-sectional type of study design was conducted for this investigation purposes. The non-probability sampling method was used and lungs with lesions were examined, and sampled purposively from slaughtered animals at abattoirs and restaurants. Samplings from different restaurants found in the Mojdo town was based on delivering cooperation letter, making discussion and accordantly with the manager or representative person.

3.3 Lung collection and Gross examination

The study animal species, and the area of origin from where they delivered, were collected by discussion with the animal care providers or representative person and from the record book of the abattoir.

After slaughter, all suspected lesions of lungs were grossly examined by visualization, palpation and incision. Each lung was inspected and palpated on both lateral and medial sides for the presence of pleural adhesions, nodular lesions and other gross lesions. The texture, consistency, color, adhesion, pattern and distribution of the lesion(s), the nature of exudates if any, and the lobe(s) involved were assessed grossly and recorded. The characteristic gross lesions of pneumonic lungs based on appearance and consistency: cranioventral consolidation with firm/ hard texture for bronchopneumonia, diffuse and elastic/ rubbery with rib imprints for interstitial pneumonia, multifocal and nodular consistency for granulomatous pneumonia were evaluated during routine gross examination and classified as its respective pneumonic lesions and the lesion types with their gross appearance recorded carefully.

3.4. Sample collection and transportation

For histopathological examination the representative sample of lung tissues were collected including active part of the lesions and some of surrounding apparently normal tissue by single cut using a sterile sharp scalpel blade in bottle containing 10% buffered formalin. For bacterial isolation piece of tissues were collected from the pneumonic lung lesions by using a sterile forceps and scalpel blade with handler and then it was immediately put in to sterile screw capped universal bottle. For parasitological examination, lungs suspected with pneumonias were checked for presence of parasites and the whole lungs with a particular parasitic nodule was collected by including the trachea in an icebox for identification of parasites.

The samples were labeled/ coded properly with permanent marker identifying sample source and sample type; including species, and date of sampling. The source of sample was classified as restaurants and abattoirs; while the sample type as the tissue samples. Then the samples were transported on ice box containing ice packs to Addis Ababa University College of Veterinary Medicine and Agriculture (AAU CVMA) Parasitology laboratory for parasitological tests, AAU CVMA Microbiology laboratory and National Veterinary Institute (NVI) laboratory, Bishoftu for bacteriological examination. The tissue samples collected by tubes with 10% buffered formalin were brought to International Clinical Laboratory (ICL), Addis Ababa for histopathology and immunohistochemical processing by using ice box containing ice packs.

3.5 Sample processing

3.5.1. Histopathological examination

The collected lung tissue sample processing and subjecting to histopathological evaluation was done according to the procedure described by Talukder (2007) prior tissue preparation followed by staining. The samples were trimmed, fixed, dehydrated with ascending graded alcohol (70%, 95% and 100%) and cleared with xylene. Then impregnated with molten paraffin wax, sectioned at 5 µm thickness using the microtome, spread on warm water and mounted to microscopic glass

slide. The slides were incubated at 60°C to molten paraffin wax. The sectioned tissue was deparaffinized in three changes of xylene, rehydrated in descending graded alcohol (**Annex I**) and stained with Hematoxylin and Eosin (H and E) (**Annex II**) for routine histopathological examinations. Stained slides were mounted by Distrene plasticizer/ Dibutyl phthalate xylene (DPX) and examined under light microscope using low to high power of magnification (4x - 100x) and photomicrographs were taken for documentation after completing the microscopic assessment of every histopathological lesions.

3.5.2. Immunohistochemistry (IHC) examination

Immunohistochemistry examination was carried out on formalin fixed parafinized tissue sections (5µm) of pneumonic lung lesions by using indirect immunostaining. The biotin-streptavidin methods of detection were used to achieve the staining procedure. The tissue were sectioned by semi-automatic microtome, floated in water bath on 45°C and mounted on the microscopic glass slide charged with 3-aminoethoxypolypropylene. The slide treated at 60°C in the oven for 20 minutes and then slides were deparafinized by xylene and rehydrated by alcohol, and washed with tap water. After these, the sections subjected to epitope retrieval (**Annex IV**), heat induced epitope retrieval (HIER) method was used. The endogenous peroxidase activity is neutralized using peroxidase block which constitutes 3%-4% hydrogen peroxide solution, and kept for 10 minutes at room temperature, then washed 5 minutes in each of with 2 separate PBS solution. This followed by application of protein block sera for 5 minutes to reduce non-specific binding of primary and secondary antibody.

The sections were subsequently incubated with optimally diluted primary antibody at dilution of 1:200 (**Annex XII**) for 60 minutes except for negative control for 3 washes by PBS of 5 minutes each. A biotinylated (biotin-conjugated) secondary antibody incubation applied to detect any tissue-bound primary antibody, and solution of streptavidin-horseradish peroxidase (HRP) complex treatment were performed which binds to the biotin present on the secondary antibody, both for 30 minutes with interposed two time washing 5 minutes in each of with PBS. The sections were further incubated with 3,3'-Di Amino Benzidine (DAB) working solution (prepared from DAB chromogen and DAB substrate buffer) used as a color indicator, for 5

minutes and rinsed in tap water. The slide sections counterstained with Mayer's Hematoxylin, rinsed in water for five minutes, dehydrated, cleared; and then cover slip applied after mounted with Dibutyl phthalate xylene (DPX) mounting medium (**Annex III**). Finally, after this all process the slide examined using light microscope at low to high magnification power for interpretation of the results and the photograph were taken by a digital camera connected to the desktop by software motic image.

3.5.3. Bacteriological examination and isolation of bacteria

The lung tissue samples collected by using a sterile scalpel blade and forceps in to sterile screw capped bottle was transferred to sterile screw capped test tube with 5ml of tryptose broth and incubated at 37°C for 24 hrs aseptically. A loop full of the broth culture was planted on to nutrient agar and blood agar medium and then incubated at 37°C for 24 hours aerobically in an aerophilic incubator. Then, the colonies were sub cultured on to blood agar, Mac Conkey agar, Eosine methylene blue agar, Edwards medium and Mannitol salt agar, and incubated at 37°C for 24 hours to 48 hours and observed for any growth of gram positive and gram negative bacteria.

The pure cultures of single colony type from each agar plate were transferred onto nutrient agar-slants and incubated for 24 hours at 37°C for a series of primary biochemical tests including Grams staining, motility, catalase, oxidase, urease, and secondary biochemical tests such as methyl red, triple sugar iron (TSI), indole test after addition few drops of kovacs reagent and Simmons citrate agar utilization tests (**Annex V**). General procedures for isolation and identification of Gram positive and Gram negative bacteria was done according to the standard procedure (Quinn *et al*., 1994; Quinn *et al*., 2004).

3.5.4. Parasitological examination and parasite identification

All suspected nodules were examined in detail by dissection of lung and lung airways. The trachea and bronchial tree were opened with fine, blunt-pointed scissors, searched for the presence of adult worms. Visible worms were then removed from the opened lungs and transferred to glass beakers containing saline for identification according to morphological and

morphometric characteristics of parasites (Chilton *et al*., 2006; Janquera, 2015a and b). Further, tracheal and bronchial washings were preserved to see smaller parasites if any.

The species of helminths encountered in the lung lesions were determined under the microscope. Lungworms were searched in the small holes and including the hardly visible worms located in the bronchioles and alveoli of the abnormal lung tissues, 1-2 cm^3 parts from the lung lesions were selected and boiled in 40% lactic acid in a water bath for 1.5 hour. After that small (2-3 mm) pieces of those parts were compressed and observed under a light microscope. In this way the lungworms became brighter and sexual structures of the male specimens are clearly visible. The identification of the helminths observed was carried out on the basis of their morphometric characteristics (Boev, 1975).

3.6. Statistical Analysis of Data

The data were entered, coded and scored in Microsoft Excel worksheet (Microsoft Corporation) and STATA version 13 was used for descriptive analysis of frequency and percentages of obtained results.

3.7. Ethical clearance

For this study the ethical clearance was obtained from Animal Research Ethical Review Committee of Addis Ababa University College of Veterinary Medicine and Agriculture for collecting the samples.

4. RESULTS

In the present study an overall of 107 (69.03%) pneumonic lungs were recorded out of a total of 155 lungs (86 sheep and 69 goats) examined, during study period at abattoirs and different restaurants in Modjo town. Out of the total pneumonic lungs, 69.8% (60/86) and 68.12% (47/69) sheep and goats had pneumonic lesions, respectively (**Table 2**). Based on types of exudates, supurative bronchopneumonia (45%) was the most common form of pneumonia followed by 21.5% interstitial pneumonia, 10.3% fibrineous bronchopneumonia, 9.35% granulomatous and 6.54% verminous pneumonia (**Table 3**).

Table 2: Pneumonic lung lesions recorded in sheep and goats slaughtered at export abattoirs/ Restaurants during the study period

Animal species		**Sample source**					**No. of positive**	**Total Percentage**
		Modjo modern	**Organic**	**Luna**	**Halal**	**Restau-rants**		
Caprine	Examined	16	13	18	14	8	47 (68.12%)	107 (69.03%)
	Positive	12	8	14	10	3		
Ovine	Examined	19	16	21	17	13	60 (69.8%)	
	Positive	14	11	16	13	6		

4.1. Pathological changes, gross and microscopic lesions characterization of pneumonia

The different types of pneumonia identified from lesions in affected lungs of caprine and ovine slaughtered in export abattoirs and different restaurants in the study area were summarized in table 3.

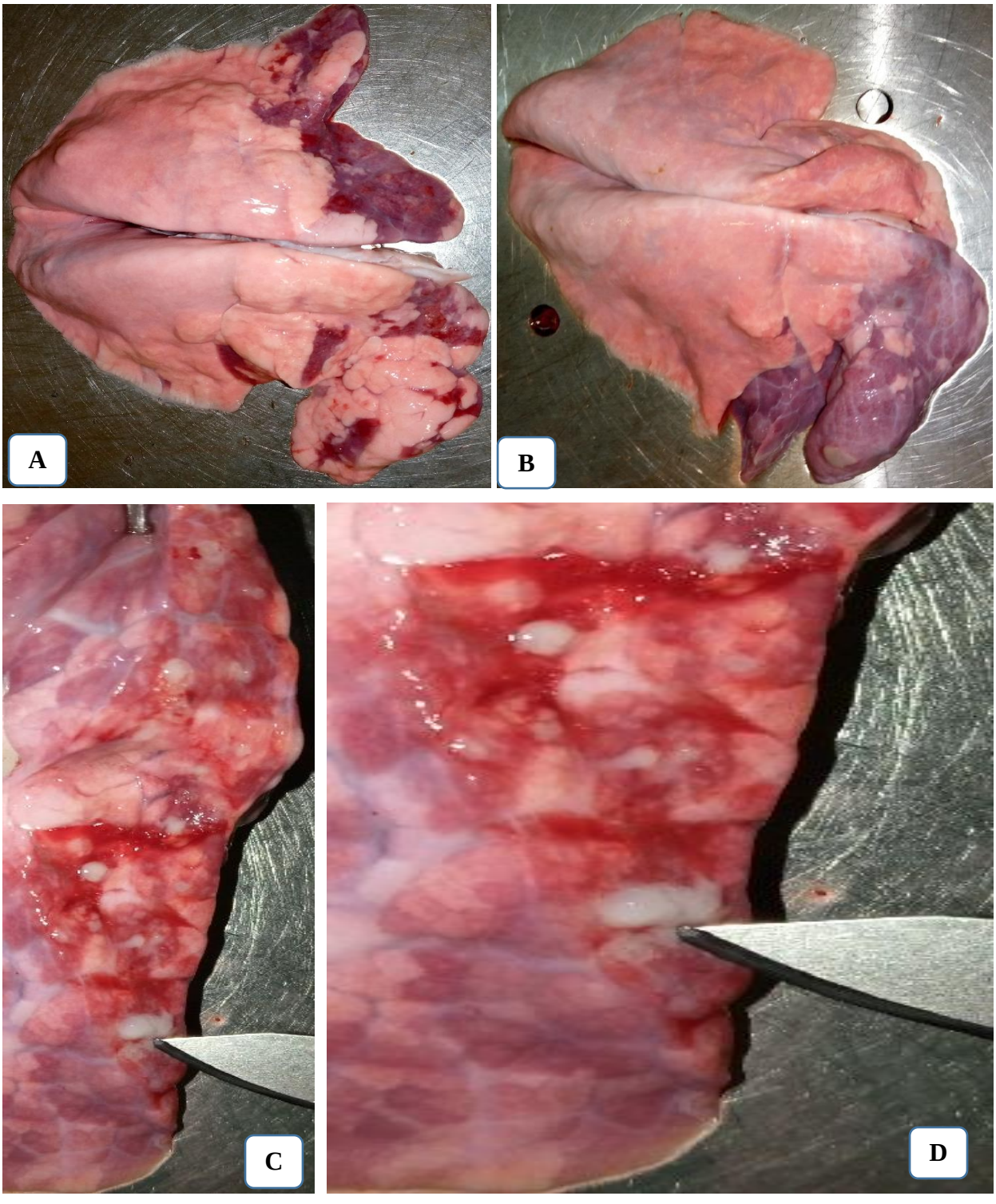
Table 3: Classification of the characterized pneumonia

Pneumonia type	**Caprine** (*N=47*)	**Ovine** (*N=60*)	**Total** (*N=107*)
	Positive (%)	Positive (%)	Positive (%)
Bronchopneumonia (BP)			
Suppurative BP	23 (48.94)	25 (41.7)	48 (45)
Fibrinous BP	4 (8.51)	7 (11.7)	11 (10.3)
Interstitial Pneumonia	8 (17)	15 (25)	23 (21.5)
Granulomatous Pneumonia	6 (12.8)	4 (6.7)	10 (9.35)
Verminous Pneumonia	2 (4.3)	5 (8.3)	7 (6.54)
Aspiration Pneumonia	3 (6.4)	2 (3.3)	5 (4.67)
Embolic Pneumonia	1 (2.13)	2 (3.3)	3 (2.8)

4.1.1. Bronchopneumonia

Suppurative bronchopneumonia was the most frequent (45%) type in current study in sheep and goats of the study area. Grossly, the affected lungs revealed consolidation which was distributed in the cranioventral area of the lung bilaterally (figure 15: **A**) or unilaterally (figure 15: **B**). On palpation the lesion has firm consistency and dark red or pink red to gray and the cut surface of the affected lobes contains visible purulent exudates (indicated in tip of scalpel blade).

Microscopic suppurative bronchopneumonia were characterized by inflammatory cell infiltration in to the bronchioles, the lumen fully occupied by large number of neutrophils (center in figure 15: **E**). The peribronchiolar surface engorged in cellular exudate and the bronchiolar membrane was also disrupted.


A
B
C
D

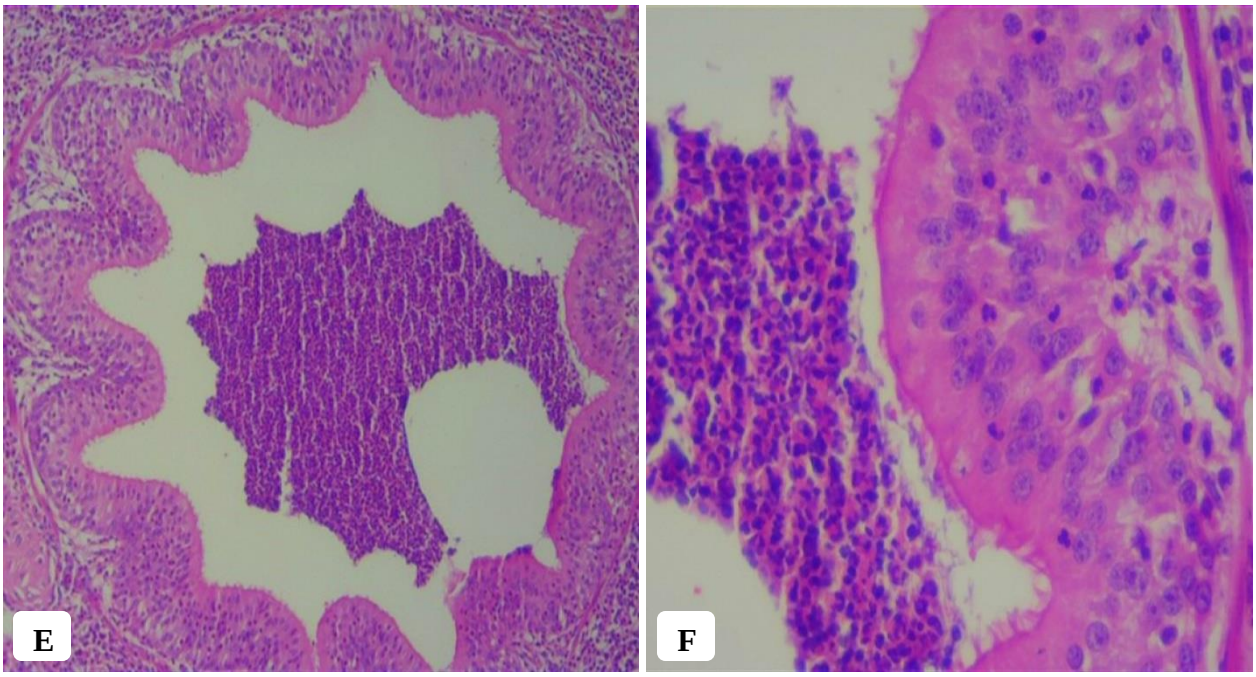
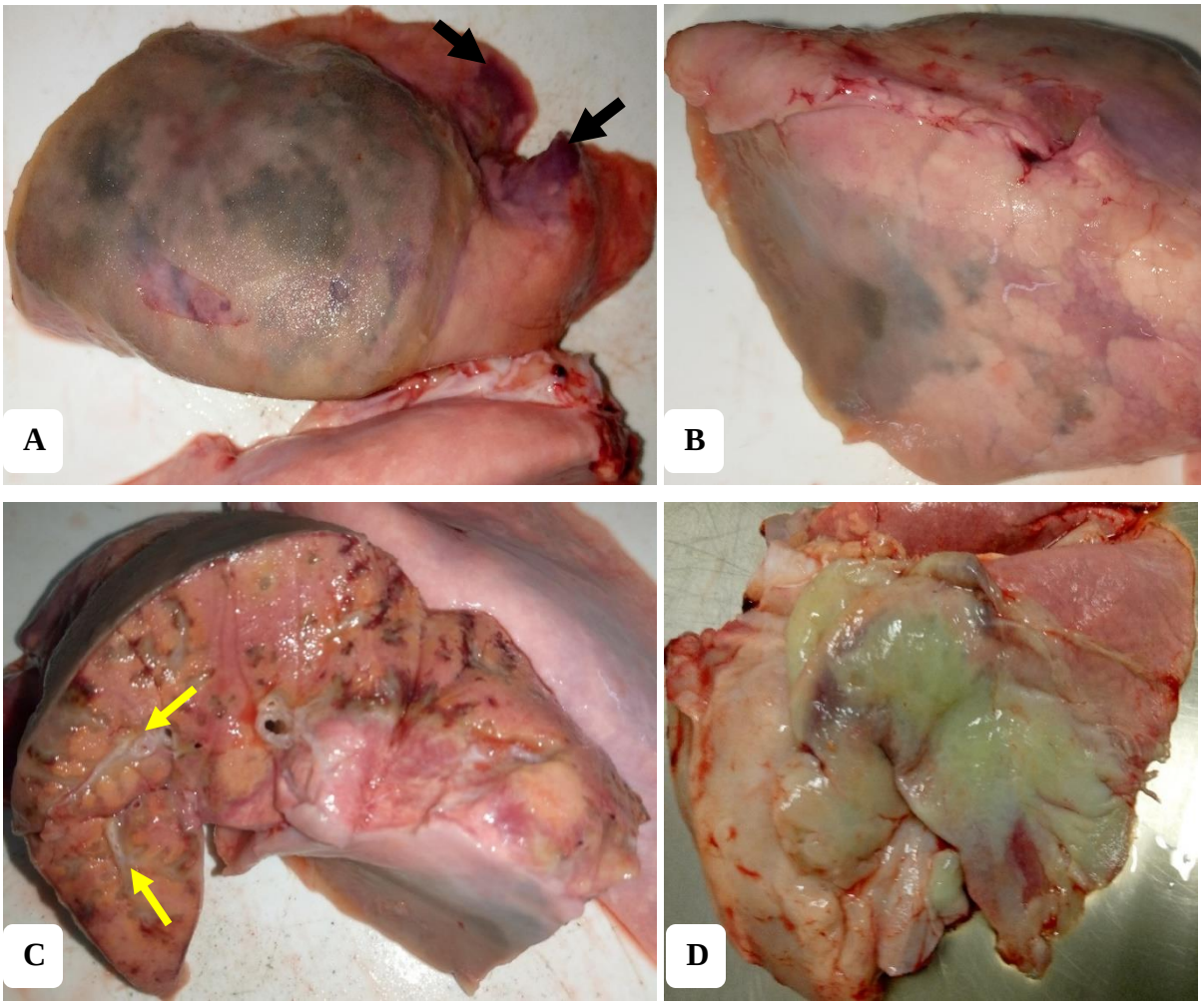


Figure 15: Gross and microscopic lesion of Suppurative bronchopneumonia: cranioventral consolidation (**A** and **B**) with purulent exudate oozes from cut surface (**C** and **D**). Histopathological changes of suppurative bronchopneumonia revealed occlusion of bronchioles lumen by inflammatory cell (neutrophils) (**E**) and large focus of **E** (**F**).

Fibrinous bronchopneumonia: This type of pneumonia was encountered at 10.3% rate which was grossly characterized by deposition of yellowish large fibrin mass coating over the parietal (**A** and **D**) and visceral (**B**) pleural surface of the lung lobe indicated in figure 16. On cut surface the yellow fibrin rich exudate with distended interlobular septa (arrows) accompanied with notable marbled appearance of the affected lung parenchyma (figure 16: **C**). The thick fibrin mass obscure the appearance of the underlying lung and the lesions extending to the parietal fibrinous adhesions in to pericardium of the heart (figure 16: **D**) and other pleural surfaces of lung lobes (caudal lobe) with slight cranioventral consolidation (arrows) in figure 16: **A**.

Histophatologically, the lung tissue sections of fibrinous bronchopneumonia showed presence of variable amounts of fibrin strands in lumens of bronchioles and alveoli. The interlobular septa of lung lobe was widened by inflammatory cell infiltrates and edematous exudates.


A
B
C
D

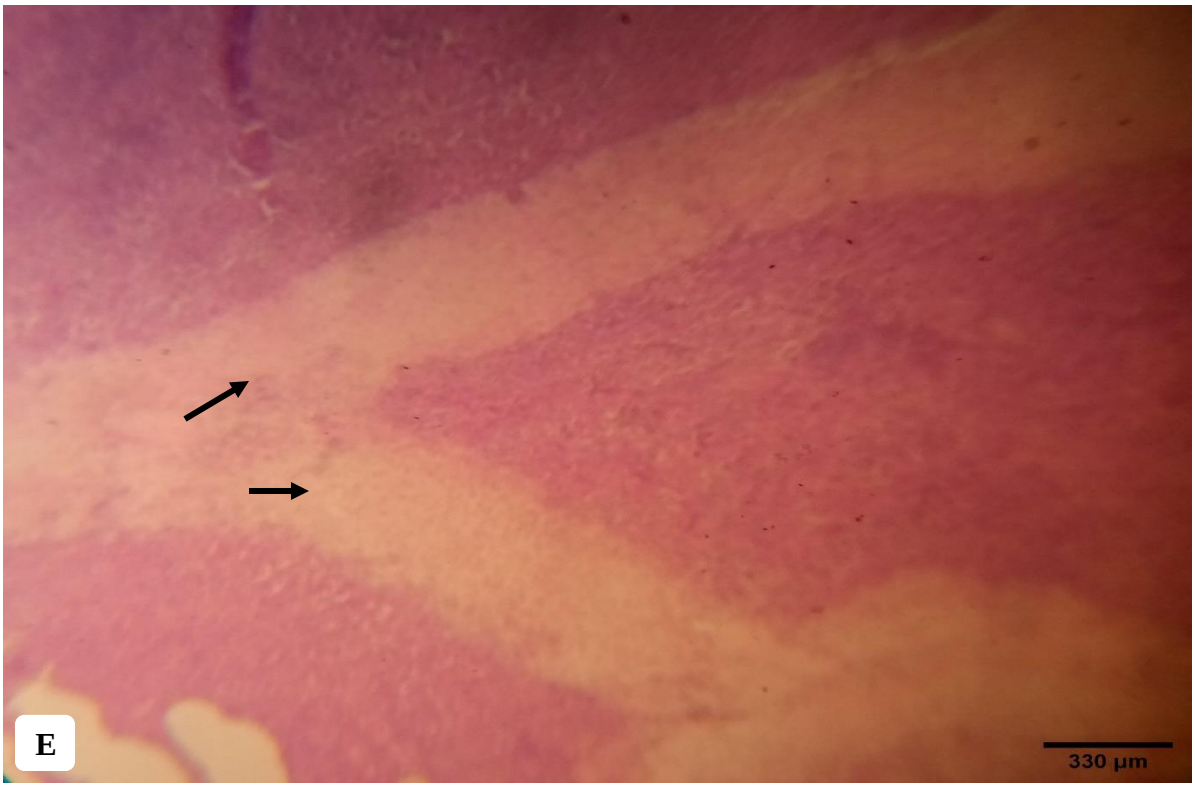
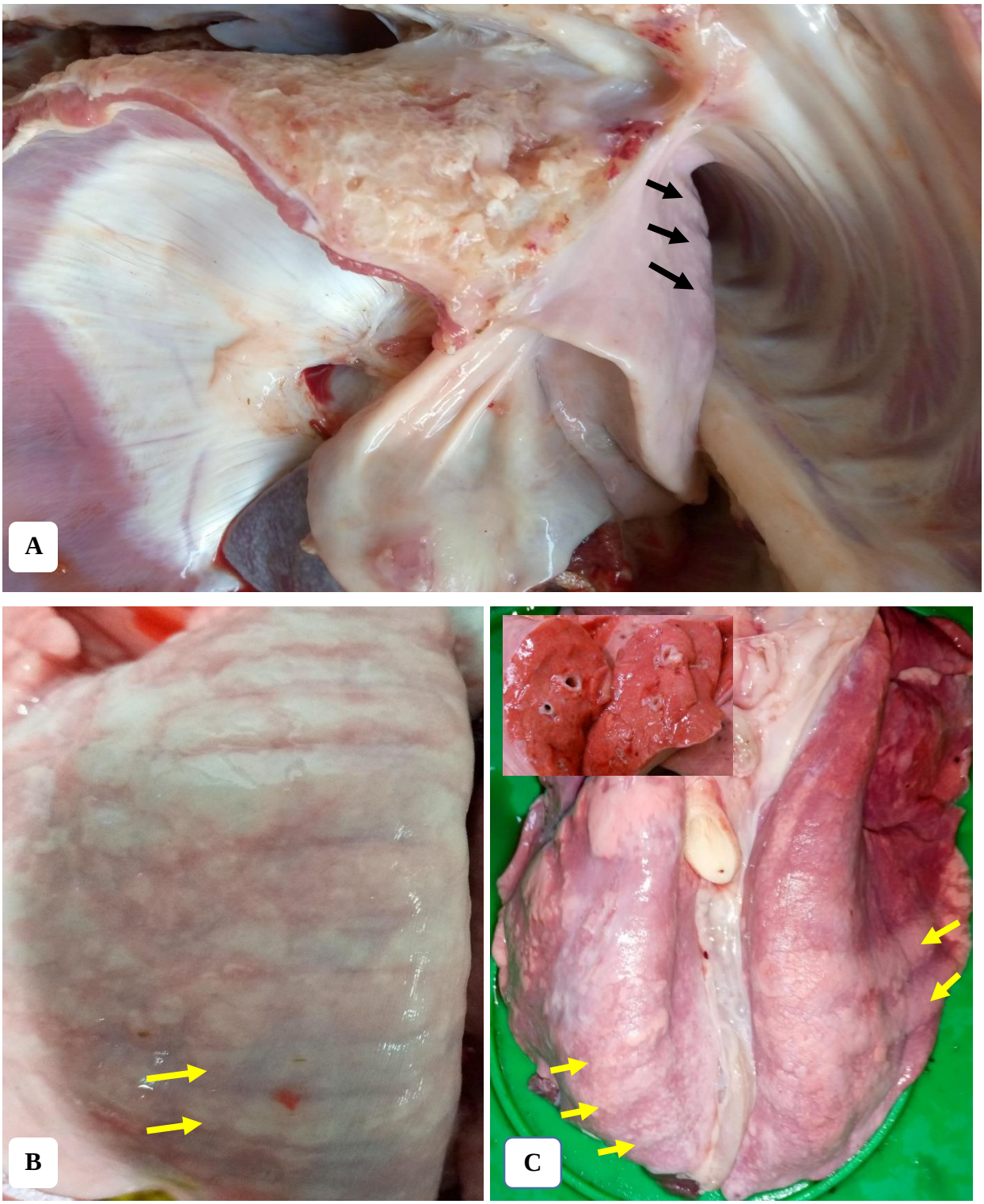


Figure 16: Gross and microscopic lesion of fibrinous bronchopneumonia: fibrin adhesion at parietal (**A** and **D**) and visceral (**B**) surface of lung cranioventrally distributed in to the pericardium of heart (**D**) and caudal lobe of lung (**A**). Cut surface, shows notable interlobular septa dilations and marbled appearances. Sectioned tissue of fibrineous bronchopneumonia showed expansion of interlobular septa (arrows) with fibrin strands (**E**).

4.1.2. Interstitial pneumonia

In the current study interstitial pneumonia was encountered in 21.5% of examined sheep and goats second to the suppurative bronchopneumonia in frequency of occurrence. Out of the total of 23 sheep and goats with interstitial pneumonia, most had the prints of coastal bone at the pleural surface of lung appeared with enlargement in size but in one case the distension and rib impressions were with adhesion at medial surface of ribs and wall of thoracic cavity (figure 17: **A** (in situ) and **B** (out of cavity). Affected parenchyma has meaty or liver like appearances and exhibited a rubbery texture on palpation; clear fluid exudation expressed from cut surface.

Microscopic examination revealed irregular thickening of alveolar septa with infiltration of the leukocytes (lymphocyte, macrophages and a less number of neutrophils) and there was also a proliferation of smooth muscle and slightly presence of edema in the alveolar interistitium.



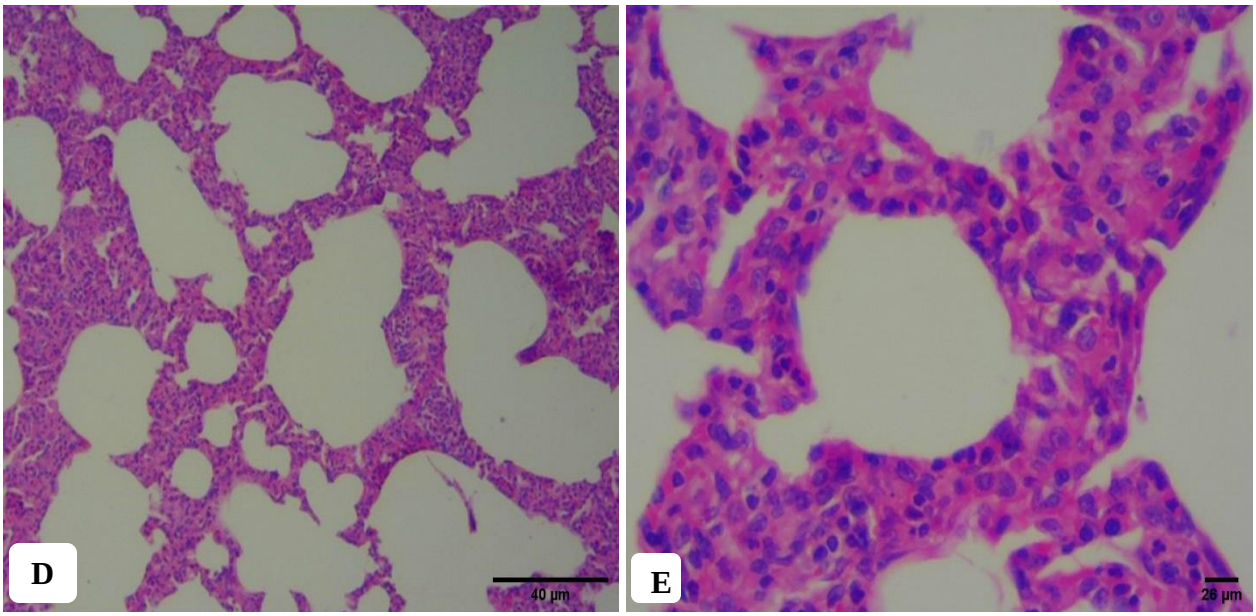


Figure 17: Gross and histopathologic lesion of interstitial pneumonia: inflated lung (**B**) and rib impressions on pleural surface (**A**, **B** and **C**) with meaty appearance of cut surface (incent). Microscopically, the thickened intralveolar septum by inflammatory infiltrates (**D**); high magnification of **D** indicates hyperplasia of smooth muscle cells and dilated alveolar interistitium by neutrophil, macrophages and other mononuclear inflammatory cells infiltration (**E**).

4.1.3. Granulomatous pneumonia

In the present study granulomatous pneumonia was identified in 9.35% (10/107) pneumonic lungs of sheep and goats examined. Grossly, it was characterized by formation of nodules on the lungs. In affected sheep and goat lungs, the granulomatous nodules were diffusely distributed on the pleural surface. These nodules were numerous with varying size and appeared hard in consistency, oval shaped and light or white colored which were observed in the lobes of affected sheep and goat lungs (figure 18: **A**).

On histopathological examination, the granulomatous pneumonia was recognized by the presence of cellular granulomatous rim (a zone of layers consists of various inflammatory cells and few fibrosis in outer layer which were surrounding the central caseous necrotic area) with a

mineralization (figure 18: **C**) and necrotic (figure 18: **B**) area at the center of lesion. The necrosis cover large area and extend in to surrounding tissue and efface the airways.

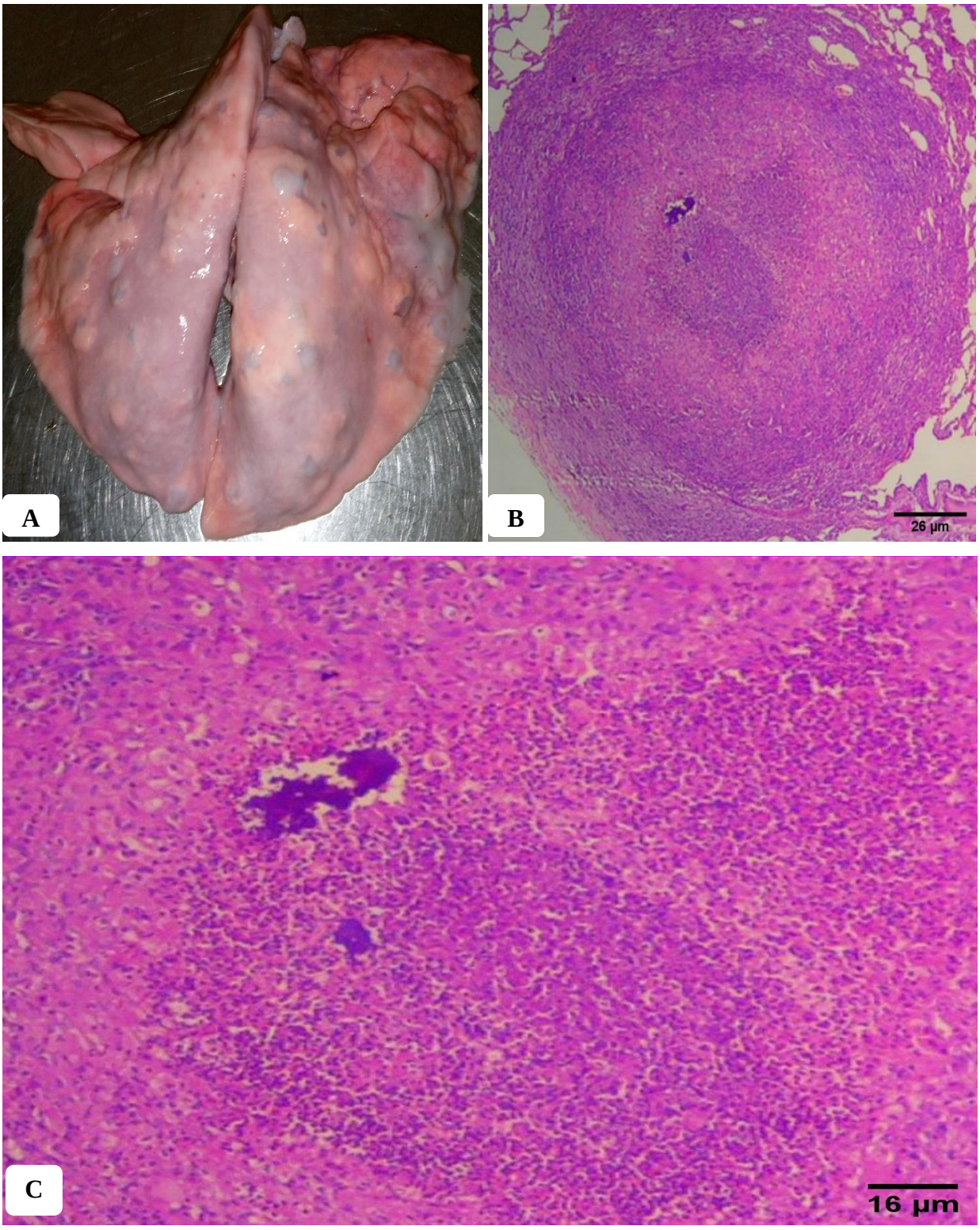
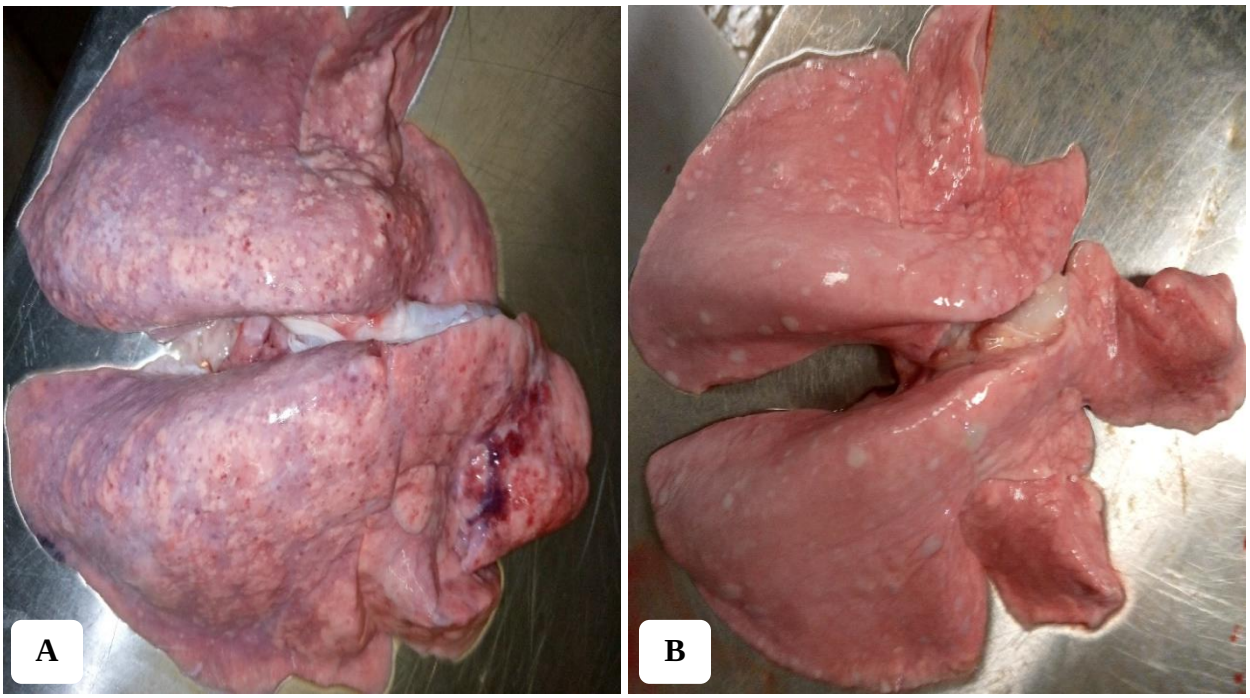


Figure 18: Gross and microscopic lesion of granulomatous pneumonia affected lungs of sheep; gross lesion (**A**) illustrating formation of variable sized nodules diffusely disseminated on the surface of lungs. Histopathological changes (**B** and **C**) indicating caseous necrosis and mineralization at the center of lesion.

4.1.4. Embolic pneumonia

Out of pneumonic lungs, 3 (2.8%) were found to be affected with embolic pneumonia in this study. In gross pathological characteristics, these cases found with the multiple inflammatory foci were reddish and white small nodules in random manner disseminated throughout the entire lobes of lungs, which were looking like hemorrhagic, round, and on palpation small (almost the same sized) nodules can be felt and scattered individual or discrete throughout the lung (figure 19: **A** and **B**).

Histopathologically, in encountered embolic pneumonia cases, the blood vessels of terminal bronchiole were blocked by emboli formed due to infiltration of a few inflammatory cells (figure 19: **C**) and erythrocytes at a places (figure 19: **C** and **D**).



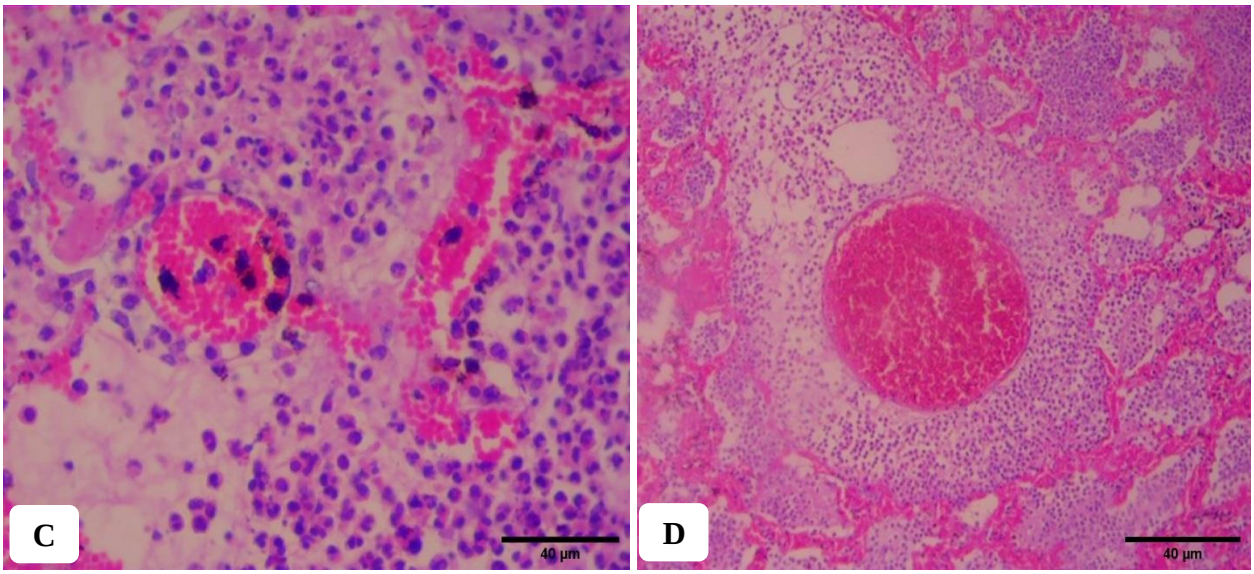


Figure 19: Pathological characteristic lesion of embolic pneumonia: grossly affected lungs of sheep (**A**) and goats (**B**) showed small nodular foci scattered throughout entire lung parenchyma. Photomicrograph of figure **A** and **B** evidenced emboli formation by leukocytic and erythrocytic thrombi of blood vessels (**C** and **D**).

4.1.5. Aspiration pneumonia

In the current study aspiration pneumonia was observed in 5 cases (4.67%) of the examined lungs of sheep and goats. The lungs were enlarged and excess trapped air in the alveoli due to blockage of air way by accumulated foreign objects. On incision the trachea along down to the bronchi and bronchioles showed the presence of light green to brown feed materials in airways and they were trapped in frothy fluids nearly tracheal bifurcation (arrows) in figure 20: **B**.

Histopathological appearance of pneumonia due to aspiration had accumulation of foreign body in the bronchioles and alveolar spaces, and bronchiolar epithelium were desquamated, disrupted and inflammatory cells were observed in the affected area of bronchioles. There was also slimly detectable amount of erupted bronchiolar epithelial revealed amid the foreign body in the lumen of bronchioles (figure 20: **C** and **D**).

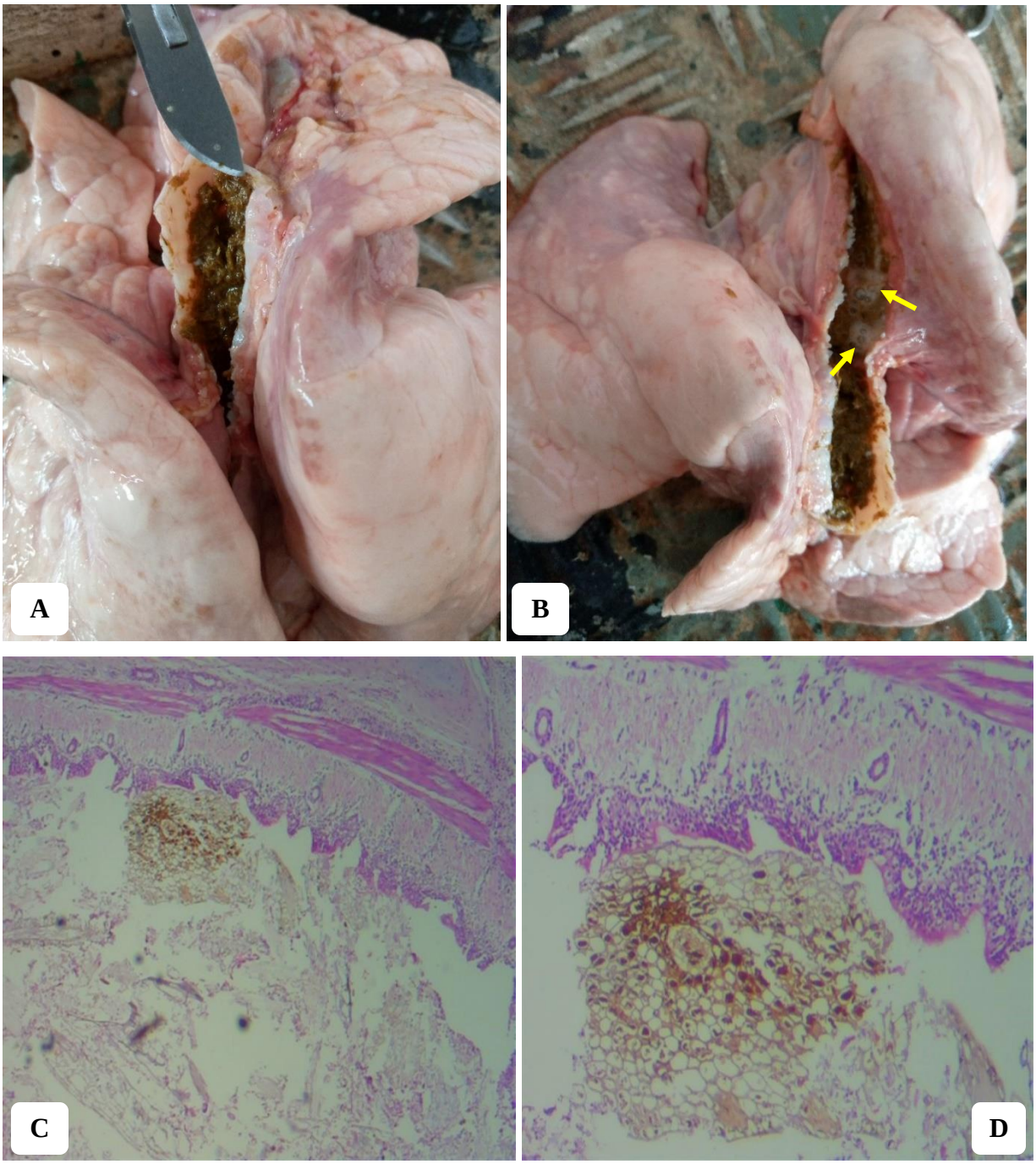


Figure 20: Aspiration pneumonia, gross and microscopic lesion: grossly, inhaled feed ingesta were observed in airway with froths (arrows) and cranial lobe of lung enlarged due to engorgement (figure **A** and **B**). Microscopic picture showed aspirated materials in the bronchioles (**C**); desquamation of bronchiolar epithelium in large focus of **C** (**D**).

4.1.6. Verminous pneumonia

The overall verminous pneumonia lesions observed in this study were 7 (6.54%) out of 107 pneumonic lesions. The species of parasites identified as well as the rate of percentage were presented in figure 21. As indicated in the figure 21, higher rate 42.9% of infection with *Dictyocaulus filaria* were observed in sheep and goats compared to 28.6% infection with *Muelleries capillaris*. The infection with *Protostrongylus rufescens* and its co-infection with *Muelleries capillaris* were equal value of 14.3% of prevalence.

Grossly, lungs of sheep and goats infected by *D. filaria* appeared hyperemic and slightly enlarged in size. Dissection of the air passages starting from trachea cutting toward to the bronchi and bronchioles revealed the presence of the adult lung worm (*D. filaria*) there (figure 22: **A**); which were thin thread like, white, white to brown in color and have little width in proportion to its length, 40mm to 83mm long (**Annex VII**: A-1 and 2) measured by MacroPATH pro-x digital imaging system. Sterio-microscopic morphology of adult parasite showed posterior end and at anterior end have thick buccal capsule walls (**Annex VII**: A-3 and 4). Gross lesion of *M. capillaris* and *P. rufescens* infected sheep and goat lungs constitutes small nodules in the dorsal surface of caudal lobes (arrows in figure 22: **B** and **C**) and enlarged in size. The nodules had white to grayish color and firm consistency. The parasite recovered from small nodules were larvae of *M. capillaris* with bent tail (**Annex VII**: B-1) and the *P. rufescens* larva which were thin, grey to reddish in color and consisting of a series of wavelike tail (**Annex VII**: B-3).

Histopathologically, adult worms were present in lumen of bronchioles and there was hyperplasia of bronchiolar epithelium with peribronchiolar infiltration of inflammatory cells, edema and disruption of bronchiolar smooth muscle. Similarly, microphotographs of lungs infected with *Muelleries capillaris* and *Protostrongylus rufescens* constitutes the parasite larvae in the alveolar lumen (figure 22: **E** and **F**) and the parasite ova and eggs (figure 22: **E**) efface the alveolar air way. There is infiltration of mononuclear cells with few eosinophil in the alveolar ineristitium (arrows, figure 22: **F**) and lumen. Also thickening of alveolar septum by infiltrated cells (figure 20: **E** and **F**); perialveolar cellular reaction (figure 22: **E**) and a few infiltrates around the worm (figure 22: **F**) were observed.

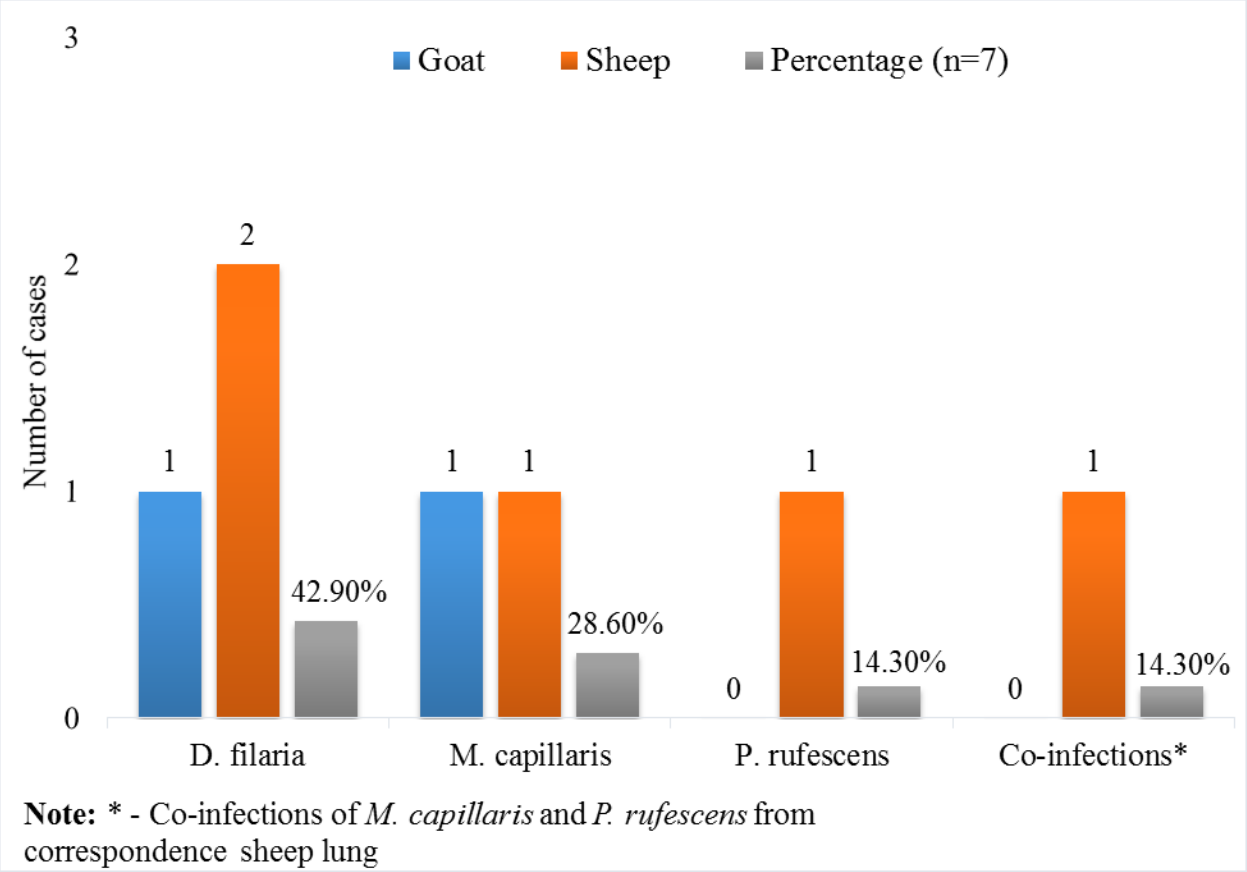
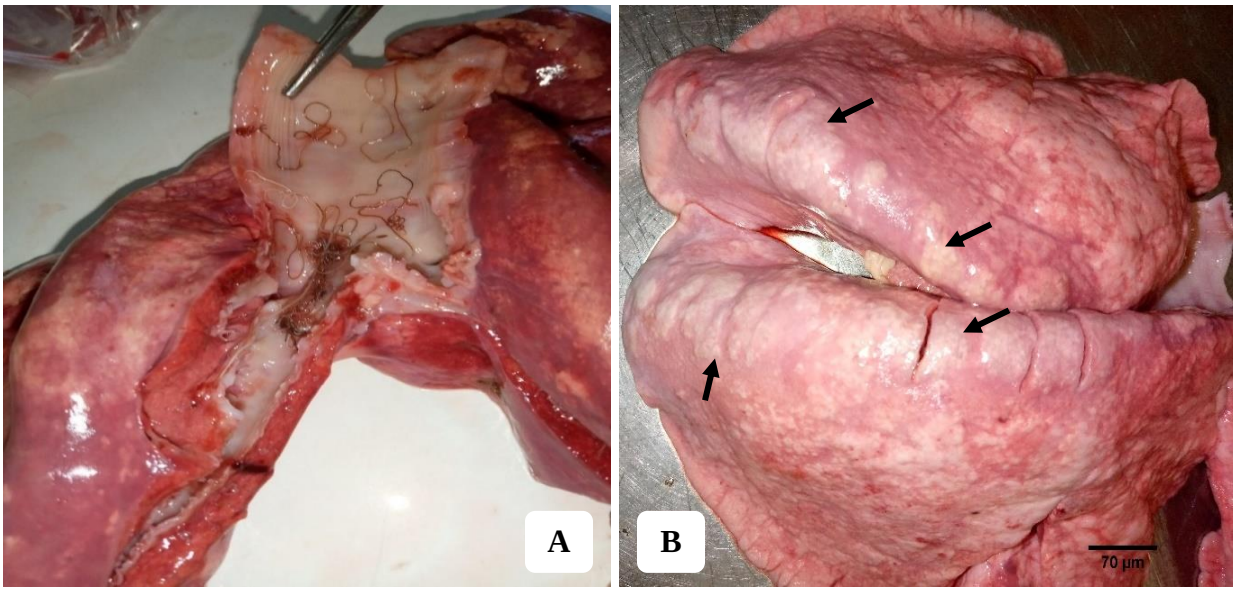
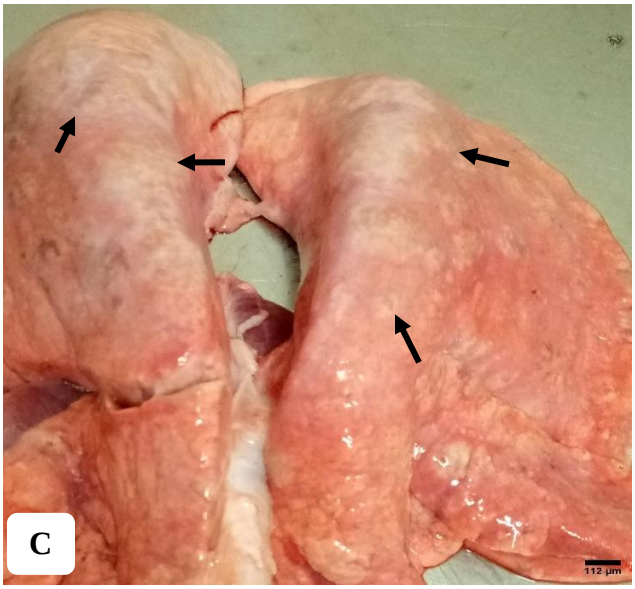
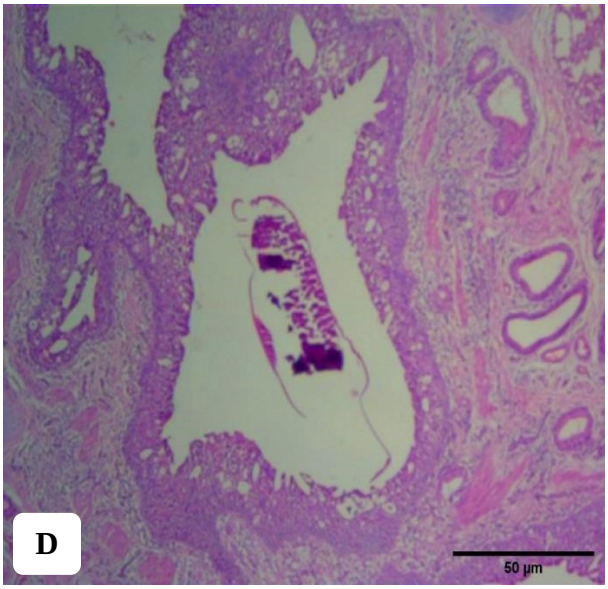
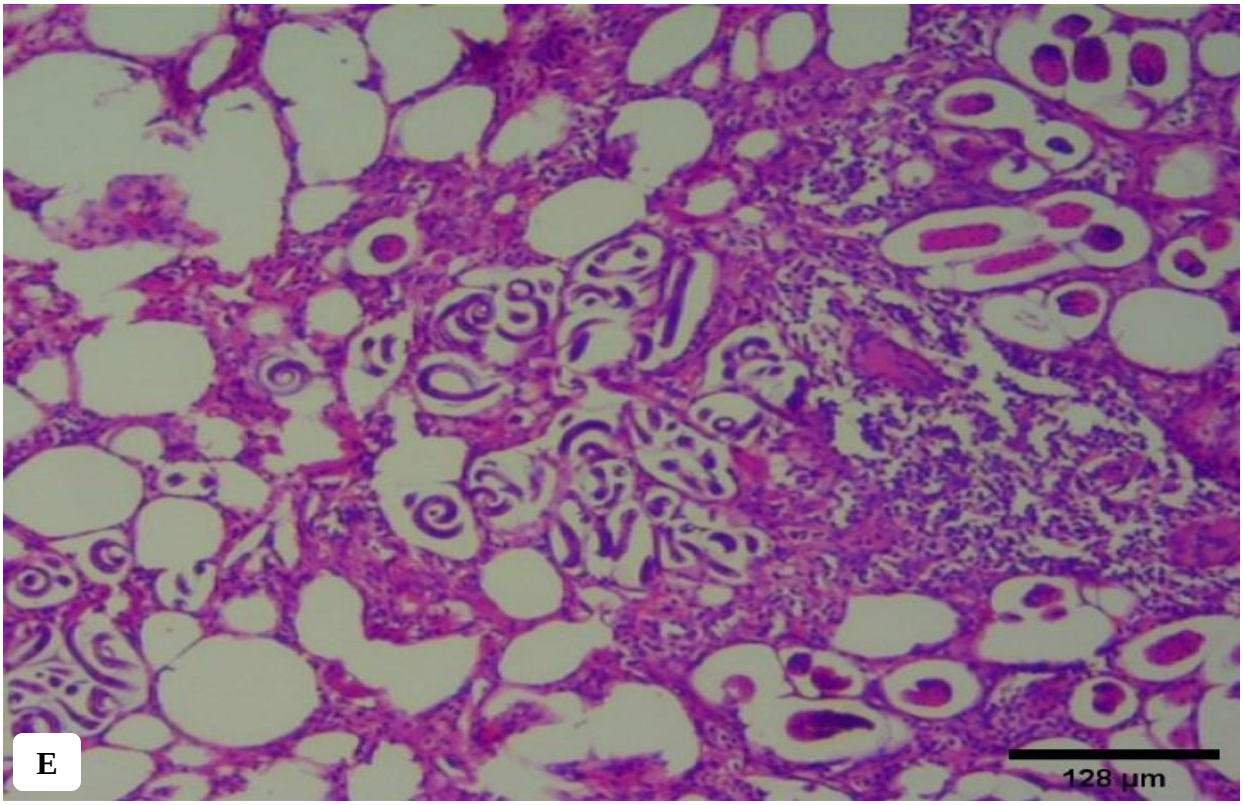


Figure 21: Lungworms identified from pneumonic lungs of sheep and goats




C
112 µm


D
50 µm


E
128 µm

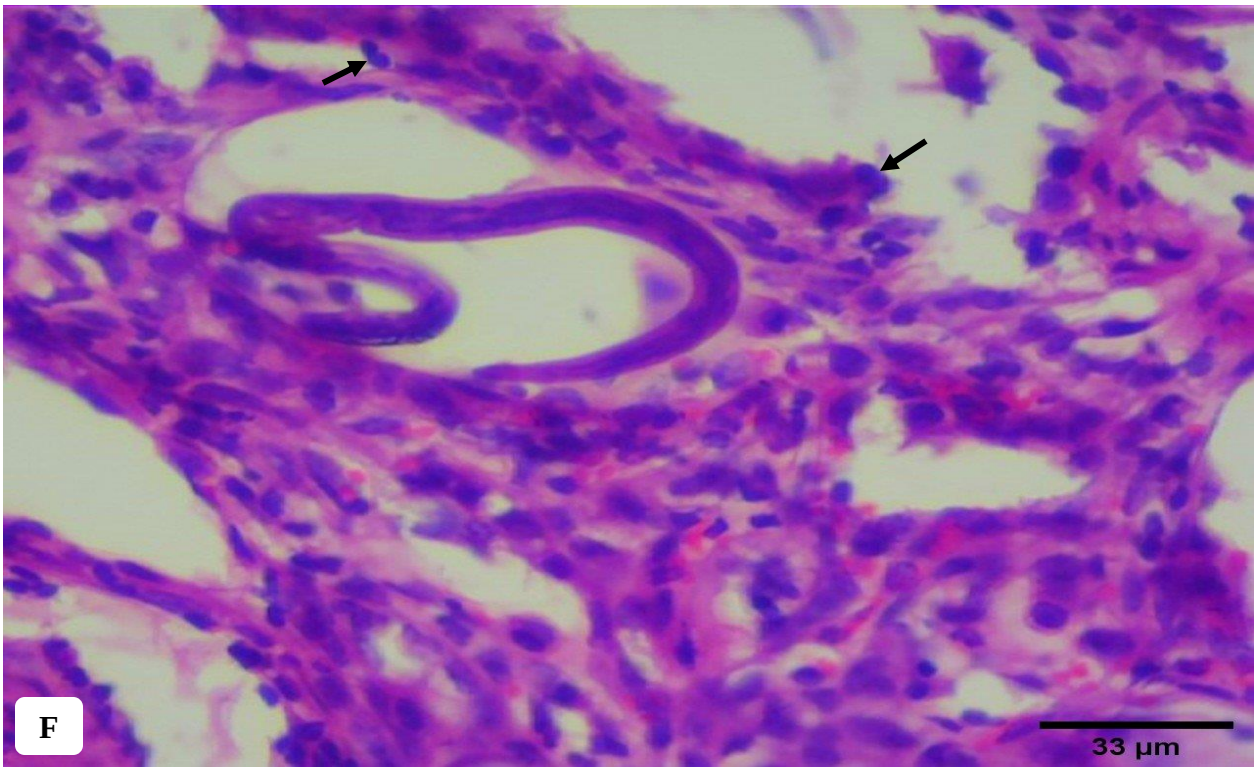


Figure 22: Pathological lesions of verminous pneumonia: affected lung of sheep grossly showing slender thread like worms (*D. filaria*) in the tracheal bifurcation and bronchial tree (**A**). The lungs of sheep (**B**) and goat (**C**) infected with *M. capillaris* and *P. rufescens* were with small firm nodules, respectively. Microscopic picture of affected lung shows cross section of *D. filaria* worm in the lumen of bronchus (**D**). Microscopic picture of *M. capillaris* infected sheep lung showing occlusion of alveolar lumen by parasite larva and eggs (**E**); and goat lung infected with *P. rufescens* showing presence of larvae in alveoli with infiltrated cells around the parasite and in the alveolar septa (**F**).

4.2. Immunohistochemical reaction on pneumonic lesions

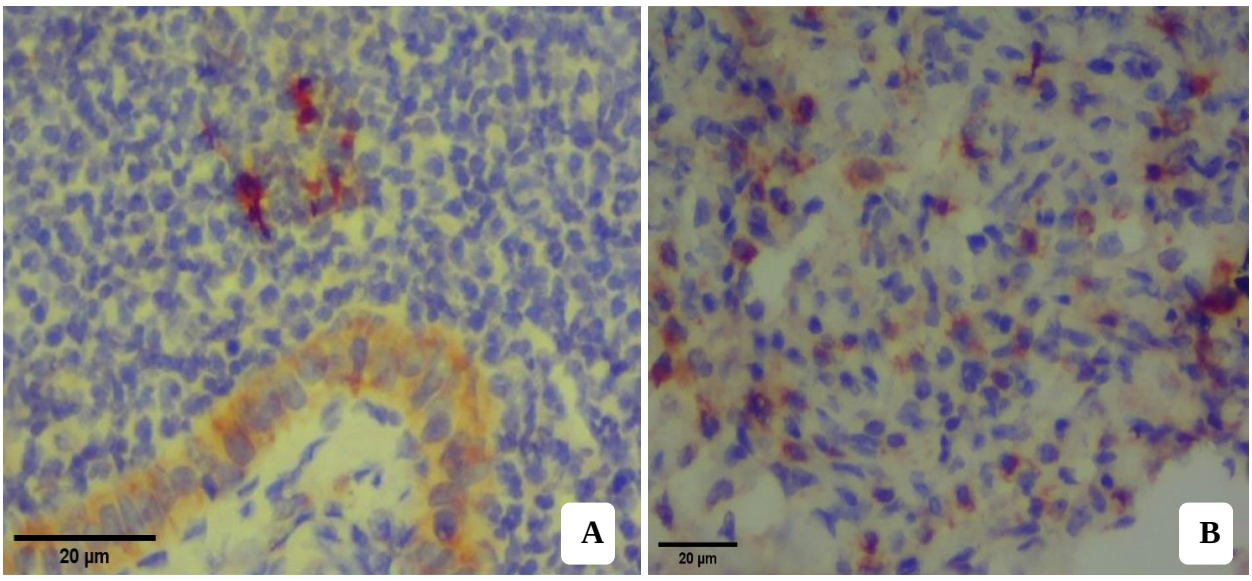
Formalin fixed paraffin embedded pneumonic lung tissue sections (5µm) were stained immunohistochemically by using primary antibody and secondary antibody. The tissue section diagnosed by indirect method of peroxidase detection system. The immunohistochemically detected result expressed on cluster of differentiations 45 (CD45), CD68 and CD20 monoclonal

antibody, and polyclonal antibodies on pneumonic lung tissue sections. The staining intensity was seen more intensely on CD45 incubated lesions than moderate and light expression shown on CD68 and CD20 antibody incubated pneumonic tissue sections, respectively.

Immunohistochemical stained pneumonic lung tissue with common leukocyte antigen (CLA) or cluster of differentiation 45 (CD45) and CD68 primary antibodies, positive reaction revealed within cytoplasm of macrophages and neutrophils on the bronchopneumonia (figure 23: **A** and **B**) and interstitial pneumonia (figure 23: **C**-**E**). These suggests that the existence of infiltration of macrophages and neutrophils in the bronchioles and alveolar septa. In addition, the epithelium of bronchioles also seen with slight immunopositive stain in CD45.

The CD68 immunopositive staining observed in alveolar septa, in the areas of inflammatory cell accumulations. Moreover, the interalveolar septa was thickened because of the infiltration of inflammatory cells. The positive CD68 reaction expressed in the nucleus and intra-cytoplasm of alveolar macrophages but the majority was seen within cytoplasm.

The CD20 labeling was noted in low expression in the majority of immuniostained area but frequently slight positive immunoreactions observed in some polymorphoneuclar cells predominantly macrophages and neutrophils infiltrates constituted in and/or around the necrotic area of granulomatous pneumonia.



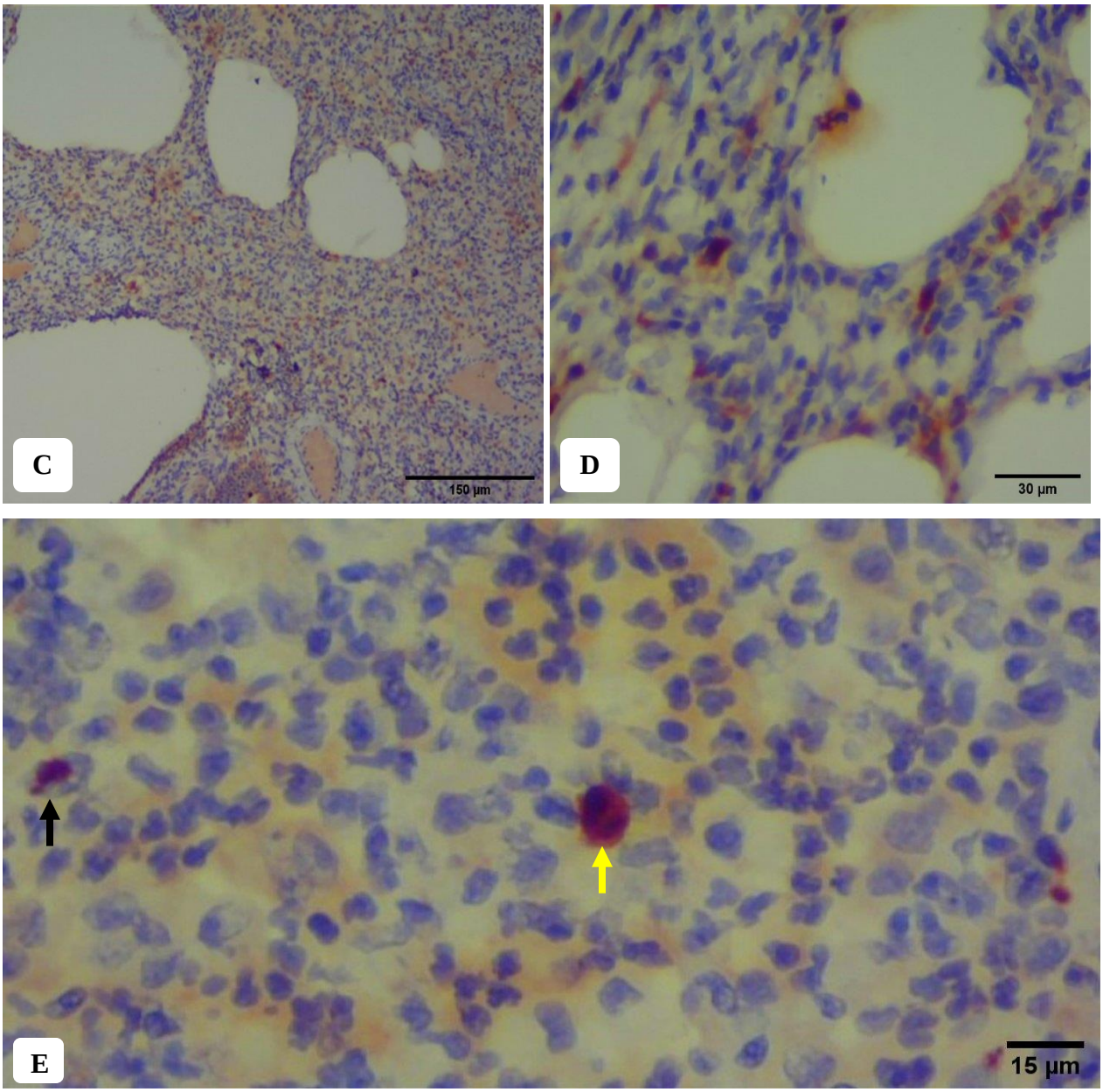


Figure 23: Immuno stained suppurative bronchopneumonia and interstitial pneumonia: CD45 immunopositive cytoplasm stains of macrophages and neutrophils in the bronchioles (**A**) and peribronchiolar surface (**B**) of the suppurative bronchopneumonia. Immunohistochemically stained interstitial pneumonia showed the CD68 positive immunoreaction of alveolar septa infiltrated macrophages (**C** and **D** (high magnification of **C**)) revealed nuclear (black arrow) and cytoplasmic stains (yellow arrow) (**E**).

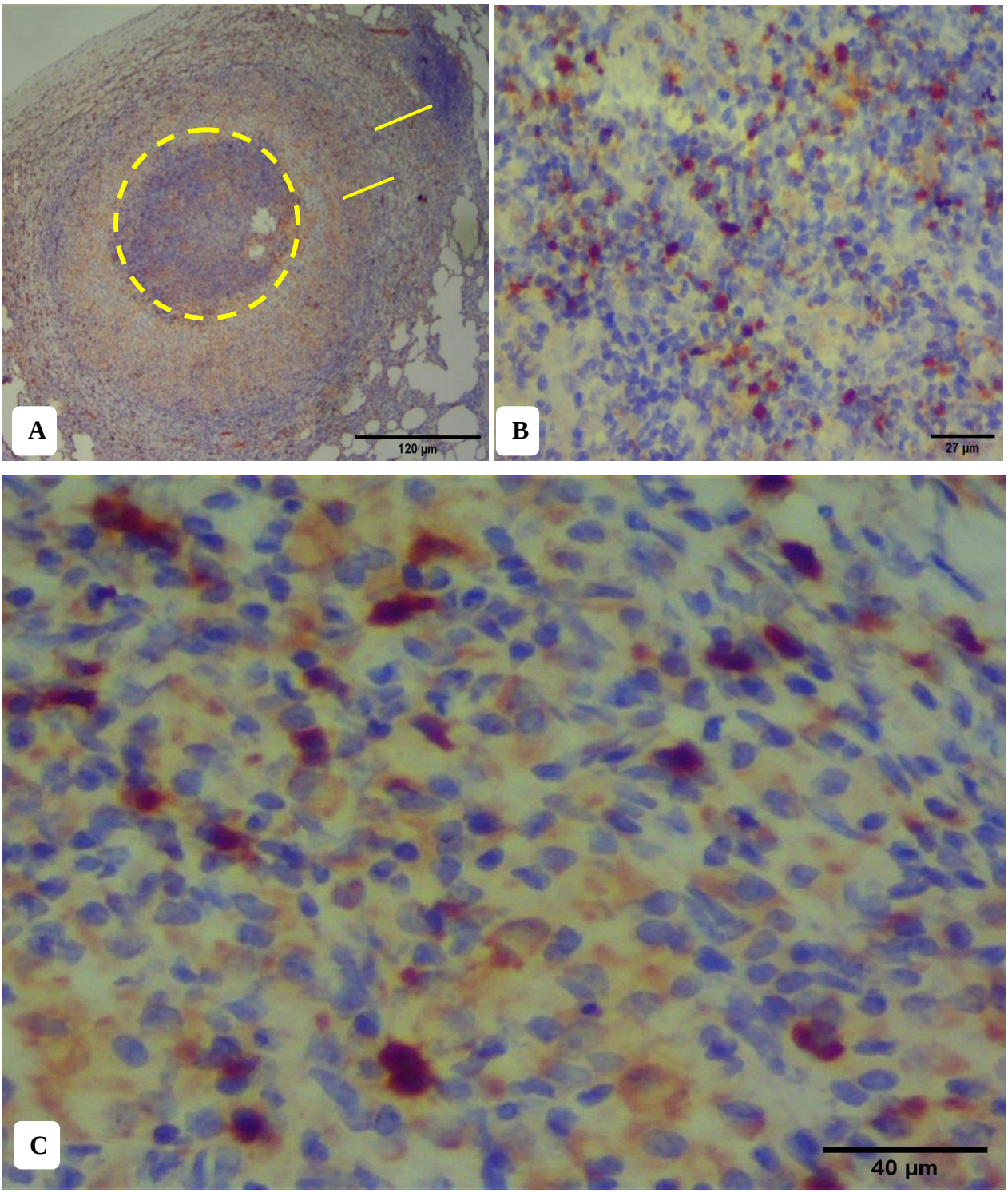


Figure 24: Granulomatous pneumonia immunopositive reaction of CD68 antibody showed cytoplasmic stain of macrophages at the center of the lesion (circled) and granulomatous zone of

layers surrounding the necrotic area (lined) in figure **A**. High focus taken from center of lesion (**B**) and granulomatous layer (**C**).

4.3. Bacterial isolates from pneumonic lungs

Out of 21 lung tissue samples (three from each pneumonia type) were examined bacteriologically, the predominant isolate was *Staphylococcus aureus* with proportion of 42.86% (9/21) followed by *Streptococcus* spp., *Escherichia coli* and *Pasteurella* spp. which were accounted for 28.6%, 23.8% and 19%, respectively (**Table 4**). Among these isolates *Staphylococcus aureus* and *Escherichia coli* were isolated from bronchopneumonia, interstitial, granulomatous and verminous pneumonia. *Streptococcus* and *Escherichia coli* were isolated from bronchopneumonia and interstitial pneumonia. *Staphylococcus aureus* and single case of *Pasteurella multocida* alone isolated from embolic pneumonia and suppurative bronchopneumonia, respectively. The remained cases, out of 19% of *Pasteurella* species, *Mannhemia hemolytica* was detected from fibrineuos bronchopneumonia and verminous pneumonia. Isolates of *Staphylococcus aureus*, *Escherichia coli* and *Mannhemia hemolytica* have shown coinfections on the correspondence lungs with parasitic pneumonias.

Table 4: Bacterial pathogens isolated from the pneumonic lung lesions of sheep and goats of present study

Bacteria isolates	BP		IP	GP	EP	VP	AP	Total isolates	Percentage (*N=21*)
	SBP	FBP							
Gram positive bacteria									
Staphylococcus aureus	3	-	1	1	2	2	-	9	42.86%
Streptococcus	3	2	1	-	-	-	-	6	28.6%
Gram negative bacteria									
Escherichia coli	1	-	2	1	-	1	-	5	23.8%
Mannhemia hemolytica	-	2	-	-	-	1	-	4	19%
Pasteurella multocida	1	-	-	-	-	-			

Note: BP = bronchopneumonia; SBP = suppurative bronchopneumonia; FBP = fibrineous bronchopneumonia; IP = interstitial pneumonia; GP = granulomatous pneumonia; EP = embolic pneumonia; VP = verminous pneumonia.

5. DISCUSSION

The lung is an important organ of respiratory system, however vulnerable to many infectious and non-infectious agents causing various pathological conditions in small ruminants (Ferdausi *et al.*, 2008). Pneumonia is one of the major disease condition causes pathological changes in lungs hence leads to decreased productivity and growth performances, serious financial losses, mortality of sheep and goats. This study was aimed to attain the objectives; gross and histopathological characterization of pneumonic lung lesions in small ruminants, as well detection of inflammatory infiltrates using immunohistochemical methods, and isolation and identification of common involved bacterial and parasitic pathogens and assessed the concurrent occurrence of these pathogens.

The overall pneumonia 69.03% (n=107) (69.85% (60/86) in sheep and 68.12% (47/69) in goats) recorded in examined 155 lungs (86 sheep and 69 goats), of sheep and goats slaughtered in the present study is in agreement with the overall outcome of 69.8% previous report (Emikpe *et al*., 2013). On the other hand, the finding of the present study is higher than the previous prevalence of 30% reported by Chukwuebuka *et al*. (2017). The highest occurrences in this study may be attributed to factors like stress condition from transportation and overcrowding, management system and environmental factors which providing suitable situation for pathogens affect respiratory systems and result small ruminants predisposed to pneumonic lung disease.

In the present study different types of pneumonia were identified based on pulmonary anatomical site and pathological changes observed on gross and microscopic appearances of pneumonic lungs of sheep and goats were bronchopneumonia, which classified in to suppurative and fibrinous bronchopneumonia, interstitial pneumonia, granulomatous pneumonia and embolic pneumonia. In addition, verminous pneumonia and aspiration pneumonia were also encountered in this study.

The observation of suppurative bronchopneumonia (45%) as the most common and most prevalent type of pneumonia as compared to other types of pneumonia in the current study is in

consistent with the previous observation (45.24%) of Azizi *et al*. (2013). In gross appearance, the suppurative bronchopneumonia characterized by cranioventral consolidation with the firm consistency, which were seen in most of examined cases. The affected sheep and goat lungs were dark red or pink red to gray in color and the cut surface of the consolidated lobes contains visible purulent exudate. Histopathologicaly, it was characterized by leucocytic infiltration in to the bronchioles, the lumen fully blocked by large number of neutrophils. The peribronchiolar surface engorged in cellular exudate and the bronchiolar membrane also disrupted. Similarly, this pathological changes were seen associated with the previous work of (kumar *et al*., 2014; Hashemnia *et al*., 2019). The cranioventral consolidation in bronchopneumonia were due to the topographic location which gives tendency to the gravitational sedimentation and deposition of the exudates and infectious pathogens. It is registered that the lesion on bronchopneumonia starts at the bronchiolar-alveolar junction and then the inflammatory lesions can spread downward to the lower portion of the alveoli and upward to the bronchi (Zachary, 2017).

The findings of fibrinous bronchopneumonia 10.3%, recorded in the present study is in line with some previous respective 11.67%; 10.96% and 11.11% reports by different authors (Rather *et al*., 2014; Chukwuebuka *et al*., 2017; Singh *et al*., 2017). This bronchopneumonia grossly characterized by deposition of yellowish fibrin mass on the pleural surface and cut surface showed the distended interlobular septa; however, microscopically, these lesions accompany the presence of fibrin strands in lumens of bronchioles and alveoli. In addition to this, the alveoli also filled with inflammatory cell infiltrates and edematous exudate. Therefore, these the gross and histopathological changes of fibrinous bronchopneumonia were agree well with the description of the aforementioned authors.

The finding of interstitial pneumonia (21.5%), as the most predominant pneumonia in the present study, next to the suppurative bronchopneumonia is in agreement to the earlier report of 23.61% and 21.43% (Yesuf *et al*., 2012; Azizi *et al*., 2013), respectively, but it is higher the previous report of 5.15% (Mugale and Balachandran, 2018) and 8.73% (Hashemnia *et al*., 2019).

The premeditated injury and the inflammatory process in endothelium, alveolar and bronchiolar epithelium may act as a reason for development of interstitial pneumonia. The initial injury

occurred in bronchopneumonia were spread to other portion of lungs and become cause for the happening of this and other pneumonia types. In gross pathological changes, the interstitial pneumonia characterized by presence of prints of coastal bone at the pleural surface and distension of lung come on with adhesion at medial surface of ribs and thoracic cavity. The meaty/ liver like appearances and palpably a rubbery texture exhibited on affected parenchyma of accounted cases were in agreement with the worker mentioned below. Microscopic diagnosis revealed thickening of alveolar septa with infiltration of leucocytic cells (lymphocyte, macrophages and a less number of neutrophils) and there was also a proliferation of smooth muscle and presence of edema. This were accorded with the explanation given by (Jarikre *et al*., 2016; Mugale and Balachandran, 2018).

The finding of granulomatous pneumonia 9.35% (10/107) recorded in this study is in line with the earlier report of 10% prevalence by the work of (Mahdi *et al*., 2015). Grossly, in affected sheep and goat lungs the granulomatous nodules has been seen diffusely distributed in the pleural surface. These nodules were numerous with different size and appeared hard in consistency, oval shaped and light or white colored which were observed in the lobes of affected sheep and goat lungs. Microscopically, there was presence of cellular granulomatous rim with a mineralization and necrotic area at the center of lesion. A zone of granulomatous layers consists of various inflammatory cells and few fibrosis in outer layer which were surrounding the central necrotic area. The necrosis covers large area, which extend in to surrounding tissue and efface the airways. In accordance to this pathological changes, similar work has also been reported by (Yesuf *et al*., 2012; Ahamad *et al*., 2016).

The prevalence of embolic pneumonia 2.8% (3/107) is lower than all the other types of pneumonia in present study is in agreement with the previous report of 2.38% by Azizi *et al*. (2013) but it is higher than the work (0.49%) of Kumar *et al*. (2014). Lungs with embolic pneumonia were grossly characterized by the presence of randomly disseminated multiple inflammatory foci throughout the entire lobes of lungs. Which were appearing like hemorrhagic, round, and on palpation small (almost the same sized) nodules can felt as scattered individual or discrete throughout the lung. Histopathologically, the blood vessels of terminal bronchiole were blocked by emboli formed due to infiltration of a few inflammatory cells and erythrocytes.

The observation of aspiration pneumonia 4.67% (5/107) encountered in the current study coincides with the previous observation of 4.7% prevalence (Mekibib *et al*., 2019). The affected lungs were grossly characterized by presence of ingested feed particles. The lungs were enlarged and incision of trachea along down to the bronchi and bronchioles showed presence of light green to brown feed materials with trapped frothy fluids in airways. Microscopically, accumulation of foreign materials in the bronchioles, and bronchiolar epithelium were desquamated, disrupted and reaction of inflammatory cells observed in the affected area of bronchioles. The pathological lesions description of aspiration pneumonia agreed with observation of Antoniassi *et al*. (2010). The occurrence of aspiration pneumonia associated with various factors, as it is documented (Zachary *et al*., 2017) it considered in animals whose swallowing has been compromised. Moreover, iatrogenic disorder, laryngeal paralysis, drenching or forced application of liquids, inhalation of massive number of microorganisms mingled with materials aspirated were overcome the lung defense and leads to an intense inflammatory response and development of pneumonia (Dusty and Pugh, 2012).

The overall prevalence of 6.54%, verminous pneumonia (*Dictyocaulus filaria*, *Muelleries capillaris* and *Protostrongylus rufescens*) recorded in the present study is in agreement with the earlier overall prevalence 7.63% (Oruc, 2006), on other hand higher prevalence of 22.8% in sheep was obtained by (Baghezza *et al*., 2019), and in sheep (20%) and goats (28.7%) by (Asaye and Alemneh, 2015) were reported.

The prevalence of 42.9% *Dictyocaulus filaria*, 28.6% *Mulleries capillaris*; and the co-infection of *Muelleries capillaris* with *Protostrongylus rufescens* and its 14.3% of prevalence recorded in present study is in agremment with previous respectve prevalence of 44.1%, 23.92% and 14.7% of *D. filaria*, *M. capillaris* and *P. rufescens* by (Fentahun *et al*., 2016; Dutta *et al*., 2017), and the *D. filaria* as the most common prevalent is agreed with the work of Maraqa *et al*. (2005). The reason for variation in prevalence among infection with *D. filaria* and the remaining two lung worm species, *M. capillaris* and *P. rufescens* was related with variation in their life cycle. The direct life cycle accompanied by *D. filaria*, which has short time to reach at infective stage, whereas *M. capillaris* and *P. rufescens* have an indirect life cycle which involve intermediate host of snails and slugs, they takes long period, hence have much lower possibility of infection

and transmission compared to *D. filaria* (Adem, 2016). In addition to this, a change in climate and environment as well as inadequate rearing condition and management system were some of causes influence the transmision of parasites and variation of the occurance.

The gross lesions revealed in lungs of sheep and goats infected by *D. filaria* appeared hyperemic and slightly enlarged in size. Dissection of the air passages starting from trachea cutting toward to the bronchi and bronchioles revealed the presence of the adult lung worm (*D. filaria*) there; which were thin thread like, white, white to brown in color and have little width in proportion to its length. *M. capillaris* and *P. rufescens* infected sheep and goat lungs constitutes small nodular lesion in the dorsal surface of caudal lobes and enlarged in size. The nodules had white to grayish color and firm consistency. Histopathologically, in this verminous pneumonia type, the sectioned layer and structure of parasites which vary in size and shape; larvae and eggs of parasite were observed in Hematoxyline and Eosin (H and E) stained lung tissue. There was hyperplasia of bronchiolar epithelium with peribronchiolar infiltration, edema and disruption of bronchiolar smooth muscle. There was infiltration of mononuclear cells with few eosinophil in the alveolar ineristitium, lumen and around the worm. The similar lesion that observed in interstitial pneumonia, thickening of alveolar septum by infiltrated cells and perialveolar cellular reaction were observed. These gross and microscopic lesions of verminous pneumonia observed in this study were in agreement with the findings reported by different authors (Oruc, 2006; Mishra *et al*., 2018).

For the first time in Ethiopia, the results of this study indicate that immunohistochemical methods using primary antibodies particularly specific cellular markers are useful for detection of their respective infiltrated cells. This method enabled the demonstration of specific cells (leukocyte) and allowing insight in to involved tissue lesions. Thus, the immunohistochemistry allow identification of cell types and colocalization of an antigen with a lesion, thereby dramatically increase diagnostic accuracy (Shi *et al*., 2001). The present study used common leukocyte antigen (CLA) or cluster of differentiation (CD), CD45, CD68 and CD20 to detect the inflammatory cell infiltrates and B-lymphocyte granuloma from formalin fixed paraffin embedded section of lung tissue of sheep and goats with pneumonia. Regarding to the

immunostaing result of this study, most of the immunopositive reactions revealed almost within cytoplasm of the cells.

In consistence with the histopathological examination, immunohistochemcally stained section of lung tissue characterized by leukocyte infiltration mainly macrophages, neutrophil, lymphocyte and a few eosinophil infiltrates. Likewise, thickening of interalveolar septa, epithelial hyperplasia of bronchioles and cellular debris in peribronchiolar sheath were also observed, in coincidence with the earlier published work (Yavuz and Dincel, 2019).

Immuniostaing with CD68 antibody revealed characteristics similar to that obtained with CD45. However, labeling of CD68 was expressed on the macrophages, many of them exhibited intra-cytoplasmic immuniopositive reaction. CD68 is found in lysosomal membranes, cell surface of macrophages in tissue and in the areas of inflammatory cell accumulations. It is membrane protein and closely related to the family of lysosomal related mucin like proteins (Kunisch *et al*., 2004). In this study, in immunohistochemical result, CD68 immunopositive reaction was observed in alveolar septa infiltrated macrophages. This observation was seen related with the description of Yavuz and Dincel, (2019).

In the present study CD20 (B lymphocytes) detected cellular infiltrates were observed in the lungs of goat with granulomatous pneumonia, this show the intense aggregation of inflammatory response. They expressed in and/or around the necrotic area of granuloma. At intense severity of granuloma the majority of B cells found peripherally in cluster by surrounding the caseous necrosis and mineralization (Flynn *et al*., 2011; Aranday *et al*., 2013; Salguero *et al*., 2016). More plasma cells appear in advanced granuloma after activation of B lymphocyte and release of specific antibody for elimination of antigen, this represents that there is high accumulation of antigen in the granuloma (Canfield *et al*., 2002).

The various etiological agents responsible for the respiratory disease occurrences particularly pneumonia. The involvement of bacteria in pneumonia have drawn attention due to severity and variable clinical manifestations (Chakraborty *et al*., 2014). In this study *Staphylococcus aureus* was recorded as the most predominant and with the highest prevalence of 42.86% (9/21) of

bacterial isolate from the pneumonic lungs of sheep and goats during the study period. In line with the present study *Staphylococcus aureus* 40% was reported as the most predominant isolate by Gebremeskel *et al*. (2017) which was attributed to its routine presence on various body parts (Megra *et al*., 2006).

In current study *Streptococcus* spp. 28.8% was recovered from bronchopneumonia and interstitial pneumonia in affected lungs of sheep and goats during the study period. Most of *Streptococcus* and *Staphylococcus aureus* were isolated from the suppurative (purulent) bronchopneumonia. *Streptococcus* spp. is known, as pyogenic bacteria, these two pathogens hypothesized find in association for happening of pneumonic lung syndrome and this suppurative bronchopneumonia were produced by infection of these microorganisms (*Streptococcus* spp. and *Staphylococcus aureus*) (Gebremeskel *et al*., 2017). In addition to this, they were also isolated from fibrinous bronchopneumonia and interistitial pneumonia. The severe localization of these pathogens in the lung may influence in the advancement of pneumonia.

Results of the present study revealed that the *Escherichia coli* was encountered in 23.8% (5/21) of pneumonic lungs of sheep and goats during the study period which agrees with the previous respective prevalence of 24.56%, 25% and 25% by Oruc (2006), Rashid *et al*. (2013) and Chowdhury (2018). *Escherichia coli* can act as the secondary invaders in pneumonic lungs in animals compromised by stress conditions like inadequate hygiene, poor nutrition and adverse ecological conditions. *Escherichia coli* and *Staphylococcus aureus* were isolated from suppurative bronchopneumonia, interstitial pneumonia, granulomatous pneumonia and verminous pneumonia. A single case of *Escherichia coli* isolated from granulomatous pneumonia and suppurative bronchopneumonia.

In the present study *Pasteurella* spp. 19% (4/21) were isolated from pneumonic lungs of sheep and goats. *Pasteurella multocida* was detected from suppurative bronchopneumonia and *Mannhemia hemolytica* was from fibrineuos bronchopneumonia and verminous pneumonia. This result is in line with the previous of Chowdhury, (2018) who reported 20% from pneumonic lungs. In this study *Pasteurella* spp. were the least encountered isolates among the identified bacteria. This is despite the fact that, pneumonia caused by *Pasteurella* spp. bacteria has been

implicated as the most important constraints incurring enormous financial losses to livestock production in Ethiopia (Tibbo *et al*., 2001; Woldemeskel *et al*., 2002). Likewise, in Ethiopia, the respiratory problems due to *Pasteurella* spp. cause high mortality and morbidity and is associated with high therapeutic costs (Ayelet *et al*., 2004).

In general, isolates of *Staphylococcus aureus*, *Escherichia coli* and *Mannhemia hemolytica* showed co-infections with parasitic pneumonia. This may be attributed to herding together of animals from different environments permit spread of infectious agents (bacteria or parasites) between sheep and goats. On certain occasions parasite or bacteria infected sheep and goats were housed and kept together that create a possible condition for occurrence of concurrent infections. Moreover, various conditions including infections as well as co-infections are responsible to induce for the occurrences of pneumonia caused by bacteria and parasites in small ruminants.

6. CONCLUSION AND RECOMMENDATIONS

Pneumonia in small ruminants is regarded as a frequent cause of economic losses in livestock production in the developing country including Ethiopia. Pneumonia is also associated with greater mortality and morbidity, incurred huge costs in treatment and prevention programs and reduced performance in production. The etiology of pneumonia involves many different factors including bacteria and parasites, most of which are known to damage lungs. The results of present study described the major gross characteristics in various types of pneumonia including cranioventral consolidation, purulent exudation, granular nodules, rib impression; whereas granulomatous necrosis, desquamation of epithelium and thickening of interalveolar septa were described as microscopic changes in different types of pneumonia. Results of current study demonstrated that gross and histopathological as well as immunohistochemical method play key roles in characterization of pneumonia in domestic animals. Correct diagnosis and identification of causes of pneumonia requires appropriate characterization of gross and histopathological patterns of pneumonic lesions by using reliable methods.

Based on the above concluding remarks the recommendations are forwarded:

- The impact of pathogens which causes pneumonia in small ruminants including bacteria, parasites and others, requires detail investigation, close monitoring and characterization of pneumonia using highly advanced diagnostic technique like immunohistochemistry (IHC) methods.
- Detailed future research need to be carried out to estimate the economic losses in small ruminants and public health impact of pneumonia and to alleviate the problems.
- Education of animal owners and the whole community to reduce or avoid predisposing factors such as poor managements or husbandry practices, including health disruptions, transportation stress, overcrowding so as to prevent the occurrences and reduce the incidence of pneumonia.

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8. ANNEXES

Annex I: Histopathological Technique Procedures (Takulder, 2007)

1. Fixation of tissue by 10% neutral buffered formaldehyde
2. Trimming part of the tissue in a way that the lesion we require be included or not missed and to fit standard histological processing tissue cassettes (5mm thickness).
3. Tissue specimen processing: fixation of tissue by formalin, dehydrating tissue by increasing alcohols concentration, clearing of tissue by xylene, and impregnation of tissue by paraffin wax.

   Fixation by [Formalin-I 2hr → Formalin-II 2hr] ➔ **dehydrating** by [70% Alcohol 1hr → 95% Alcohol 1hr → 100% Alcohol-I 1hr → 100% Alcohol-II 2hrs →100% Alcohol-III 2hrs] ➔ **clearing** by [Xylene-I 1:30hrs → Xylene-II 1:30hrs → Xylene-III 1:30hrs] ➔ **impregnation** with [Paraffin wax-I 2hrs → Paraffin wax-II 3hrs].
4. Embedding/ Blocking of processed tissue: impregnated tissue is placed in a mould with their labels and then fresh melted wax (54-60^{o}c) is poured and allowed to settle and solidify.
5. Sectioning: sectioning of tissue in 3-5 micron thickness and put on warm water bath to straighten the ribbon, and then adhere on the surface of frost ended and clear slide. Later label and put the slide in incubator at 60^{o}c overnight.

Annex II: Hematoxyline and Eosine stain Procedures (Talukder, 2007)

Staining Procedure:

1. Deparaffinize slides in 3 changes of xylene (xylene-I, xylene-II and xylene-III) for 3 minutes each.
2. Hydrate slides in 100% alcohol and 95% alcohol, 2 changes for 3 minutes each, and rinse in distilled water until ripples disappear from slides.
3. Place in Hematoxylin for 8 - 15 minutes.
4. Rinse in tap water until water runs clear.

5. Decolorize in 1% acid alcohol, 3 - 6 quick dips. Check differentiation microscopically: Nuclei should be distinct; Cytoplasm should be uncolored.
6. Rinse in tap water until ripples disappear from slides.
7. Dip in bluing agent, 3 - 5 long dips.
8. Wash in lake-warm tap water for 5 minutes (37-40°C.)
9. Stain in Eosin for 30 seconds - 2 minutes.
10. Dehydrate in 95% alcohol and 100% alcohol, 3 changes each for 2 minutes.
11. Clear in 3 changes of xylene for 2 minutes each.
12. Mount cover glass with Canada balsam or Deapistix (DPX)
13. Examination of the prepared slides under microscope at low to high magnification power (4x, 10x, 40x and 100x) and finally the photomicrographs taken for documentation of every histopathological lesions.

Annex III: Immunohistochemistry (IHC) Protocols

According to manufactures' instruction for use for the primary antibody detection and detection system all steps involved in IHC test were performed at room temperature (25°C).

1. Cut and mount section on slides coated with a suitable tissue adhesive
2. Deparaffinize section in xylene or xylene substitutes.
3. Rehydrate through graded alcohols.
4. Wash slides in running tap water
5. Perform Epitope retrieval as required (see **Annex IV**).
6. Wash slides in de-ionized water.
7. Neutralize endogenous peroxidase using peroxides block for 10 minutes.
8. Wash in PBS for 2x5 minutes.
9. Incubate with protein Block for 5 minutes.
10. Wash in PBS for 2x5 minutes.
11. Incubates with optimally diluted primary antibody for 60 minutes.
12. Wash in PBS for 2x5 minutes.
13. Incubate with Biotiynlated secondary antibody for 30 minutes.
14. Wash in PBS for 2x5 minutes.

15. Incubate with Streptavidin-HRP for 30 minutes.
16. Wash in PBS for 2x5 minutes with gentle rocking.
17. Develop peroxidase activity with DAB working solution (see A**nnex IV**, DAB working solution) for 5 minutes.
18. Rinse slides in water.
19. Counterstain with Hematoxylin.
20. Rinse slides in water for 5 minutes.
21. Dehydrate, clear and mount section.

Annex IV: Antigen Retrieval

1. Prepare a working solution by diluting 1 part Antigen or Epitope Retrieval Solution (ARS) concentrate with 9 parts de-ionized water.
2. Heat 1.5L of the working solution until boiling in pressure cooker. Cover but do not lock lid. Position slide into metal Staining racks (do not place slide close together as uneven staining may occur) and lower into pressure cooker ensuring slide are completely immersed in retrieval solution (the solution should be adequate in amount to immerse the slides completely). Lock lid.
3. Place the pressure cooker (microwave oven resistant plastic holder) with slides in metal Staining racks in to microwave oven.
4. Set the microwave oven for 10 min. time (750-850W)
5. When the pressure cooker reach operating temperature and pressure, remove pressure cooker from heat source (DO NOT OPEN LID THE INDICATORS SHOW THAT PRESSURE HAS BEEN RELEASED).
6. Open lid, remove slides and place immediately in cool tap water for 20 min. in room temperature.
7. The slide placed in disposable immunostaining chamber and arranged in capillary immunostinig slide rack to proceeding IHC steps.

DAB working solution

Add 50µm of DAB chromogen to 1ml of DAB substrate buffer. Use within six (6) hours of preparation.

Annex V: Colony morphology on culture media and biochemical test characteristics of isolated bacteria (Quinn *et al*., 2004).

Features	Gram positive bacteria		Gram negative bacteria		
	S. aureus	**Streptococcus**	**E. coli**	**M. hemolytica**	**P. multocida**
MSA	growth[*1]	—	no growth	—	—
Edward	—	growth[*2]	no growth	—	—
EMB	—	—	growth[*3]	—	—
Hemolysis	+	+	+	+	-
Mac Conkey	—	—	growth[*4]	growth[*5]	no growth
Catalase	+	-	+	+	+
Coagulase	+				
Oxidase	-		-	+	+
Urease			-	-	+
Methyl red			+		
TSI			+ (A/A)	+	+
Indole			+	-	+
Citrate			-		
Motility			+	-	-
VP			-		

[*1]=Small colonies, smooth, cocci, golden color colony, appear as yellow colonies on Mannitol Salt Agar (MSA); [*2] = small, dewdrop like, translucent, smooth and opaque with a raised central portion; [*3] = smooth circular colonies with dark centers and green metallic sheen on Eosin methylene blue (EMB) agar; [*4] = medium size and bright pink colony; [*5] = small pin point, pink colonies about 1-2 mm in diameter; + = positive reaction; - = negative reaction; TSI = Triple sugar iron; VP = Vogueos proskauer

Annex VI: Methods used to identify different bacteria (Quinn *et al*., 2004)

Blood Agar Base

Composition (g/l): Blood agar consists of a base containing a protein source (e.g., Tryptones), infusion from (solid) 2.0; pancreatic digest of casein 13; yeast extract 5.0; sodium chloride 5.0; agar 15.0. Direction: Suspend 40g of powder in 1 liter of distilled water. Mix thoroughly and heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Autoclave at 121°c for 15 minutes and cools the base to 45-50°c and 5-7% sterile sheep blood. Colony growths on blood agar base and haemolysis formation were observed.

Nutrient Agar

Preparation: Suspend 28 grams of the medium in one liter of distilled water. Mix well and leave to stand until the mixture is uniform. Heat with gentle agitation and boil for one or two minutes, or until completely dissolved. Sterilize by autoclaving at 121°C for 15 minutes**.** Dispense in sterile petridishes in 15ml amount. The plates were allowed to solidify at room emprature on flat surface.

Gram Staining

Principle: Gram positive bacteria due to their thick peptidoglycan layer will retain the crystal violet complex even after it is subjected to decolourization with acetone or alcohol. Hence the counter stain Safranin has no action on gram positive cells. But in the case of gram negative, the thin peptidoglycan layer and more lipid contents in the cell wall will easily make them susceptible to the action of decolorizer and hence CVI complex is easily washed out and hence the gram negative cells will the colour of counter stain Safranin.

Procedure:

- Applying a primary stain (crystal violet) for 60 second to a heat-fixed smear of a bacterial culture. Then wash off with tap water.

- ❖ Addition of iodide which remain for 60 second. Then wash off with tape water.
- ❖ Rapid decolourization with ethanol or acetone for only 15-30 second. Then wash off with tape water.
- ❖ Counterstaining with safranin for 60 seconds. Then wash off with tap water and dried with bloating paper.

Oxidase Test

Principle: Anaerobes are oxidase negative and thus can reduce the dye (tetramethyl-p-phenylene diamine dihydrochloride).

Procedure: A piece of filter paper is moistened in a Petridish with 1% aqueous solution of tetramethyl-p-phenylenediamine dihydrochloride. Streak the test bacterium firmly across the filter paper with a glass rod. *Result*: positive → a dark purple colour along the streak line.

Catalase Test

Principle: the breakdown of 3% hydrogen peroxide in to oxygen and water is mediated by the enzyme catalase.

Procedure: a loop of bacterial growth is taken from nutrient agar medium. Then the bacterial cell placed on a clean microscopic slide and a drop of 3% hydrogen peroxide is added. An effectiveness of oxygen gas, within a few seconds, indicates a positive reaction.

Tryptone Soya Broth

Composition (g/l): Pancreatic digest of casein 17.00; enzymatic digest of soya bean 3.00; Sodium Chloride 5.00; Dipotassium hydrogen phosphate 2.50; Glucose 2.50. Final PH: 7.3 ± 0.2 at 25ºC, Distilled water 1 litter.

Preparation: Suspend 30 grams of the medium in one litter of distilled water. Mix well. Heat slightly until complete dissolution of the medium if necessary. Dispense in tubes and sterilize by autoclaving at 121°C for 15 minutes. Larger quantities may require longer sterilization time, but the temperature should not be increased.

Kovac's Reagent

This reagent was composed of 5g para dimethyl amino benzaldyhyde, 75ml amyl alcohol and 25ml concentrated hydrochloric acid. The reagent was protected from light and stored at 4°C for indole test.

Indole Test

Principle: Organisms those possess the enzyme tryptophanase can break down the amino acid tryptophan to indole. When indole reacts with para-dimethylaminobenzaldehye (Kovac's reagent) a pink -colored complex is produced. Tryptophan is plentiful in most media, but growth on blood agar or chocolate agar produces the best effects.

Procedure: Take loopful of inoculum by touching the 3-5 representative colonies with inoculating loop from pure colonies and inoculate Tryptone soya broth tube. Incubate the tube at 37°C for 24 hours and cap left loosen to aerate the tube. After incubation, add 5-10 drops (0.5ml) of Kovac's reagent to the culture broth and agitate gently. Then observe the tube for color change within 5 minutes.

Simmons Citrate Agar

Composition (g/l): Ammonium Dihydrogen Phosphate 1.00; Dipotassium Phosphate 1.00; Sodium Chloride 5.00; Sodium Citrate 2.00; Magnesium Sulphate 0.20; Bacteriological Agar 15.00; Bromthymol Blue 0.08. Final PH: 6.8 ± 0.2 at 25ºC, Distilled water 1 liter.

Preparation: Suspend 24.28 grams of the medium in one liter of distilled water. Heat to boiling to dissolve the medium completely. Dispense in tubes and sterilize in the autoclave at 121ºC for 15 minutes. Cool the tubes in a slanted position so that the base is short (1-1.5 cm. deep).

Test Principle: Citrate contains carbon. If an organism can use citrate as its only source of carbon the citrate in the media will be metabolized. Bromthymol blue is incorporated into the media as an indicator. Under alkaline conditions this indicator turns from green to blue. The utilization of citrate in the media releases alkaline bicarbonate ions that cause the media pH to increase above 7.4 causes the media blue.

Test Procedure**:** Take loopful of inoculum by touching the center of 3-5 representative colonies with inoculating loop and streak it onto the surface of a Citrate slant. Incubate the tube aerobically at 35°C with cap left loosen for 22 hours. After 22 hrs incubation observe the tube for growth and color change.

Triple Sugar Iron Agar

Composition (g/l): Peptone Mixture 20.00; Lactose 10.00; Sucrose 10.00; Sodium Chloride 5.00; Beef Extract 3.00; Yeast Extract 3.00; Glucose 1.00; Ferrous Ammonium Citrate 0.30; Sodium thiosulphate 0.30; Phenol Red 0.024; Bacteriological Agar 12.00. Final pH: 7.4 ± 0.2 at 25ºC, Distilled water 1 litre.
Preparation: Suspend 65 grams of the medium in one liter of distilled water. Bring to the boil to dissolve completely. Mix well and distribute in tubes. Sterilize by autoclaving at 121º C for 15 minutes and cool in a slanted position, as to obtain butts of 1.5 – 2 cm depth.

Test Principle: Bacteria that ferment any of the three sugars in the medium will produce byproducts which will change the color of the red pH-sensitive dye (phenol red). A bacterium that is a non-lactose fermenter and ferments glucose, initially causes a yellow slant/yellow bottom (acid/acid reaction) after 8 hours, but then converts to a red slant/yellow bottom after 24 hours (alkali/acid reaction). Where as if it ferments both lactose and glucose, it results in a

yellow/yellow tube and remains that way due to the large amount of acid produced in the reaction.

Test Procedure*:* By sterile inoculating loop touching the center of colony from isolated pure colony take loop full of inoculum. Streak the inoculum back and forth on TSI agar in tube along the surface of the slant. Incubate the tube with the cap loosened at 35 °C for 22 hours.

Methyl Red Test Solution

Prepared by dissolving 0.04gram of methyl red in 40ml ethanol and volume was made up to 100ml with distilled water.

Test Principle: Some organisms produce acid from the metabolism of glucose in a sufficient quantity to produce a pH of 4.4 in the media. These acids are not further metabolized and are said to be stable acids. At a pH of 4.4 or less the pH indicator methyl red is a bright cherry red. While also some organisms initially produce acid from glucose metabolism but further metabolize the acid produced to neutral end products, such as acetoin, and 2, 3-butanediol. Initially the pH may drop to 4.4 but the neutral end products raise the pH so the methyl red test will be negative. Acetoin and 2, 3-butanediol under alkaline conditions will react with alpha-naphthol (1-naphthol) to produce a mahogany red color.

Test procedure: The tested organism was inoculated into glucose phosphate medium (MR-VP medium) then incubated at 37°C for 48 hours. Two drops of methyl red reagent were added, shaken well and examined. Appearance of red colour indicated a positive reaction, whereas orange or yellow colour indicated a negative reaction.

Motility Medium

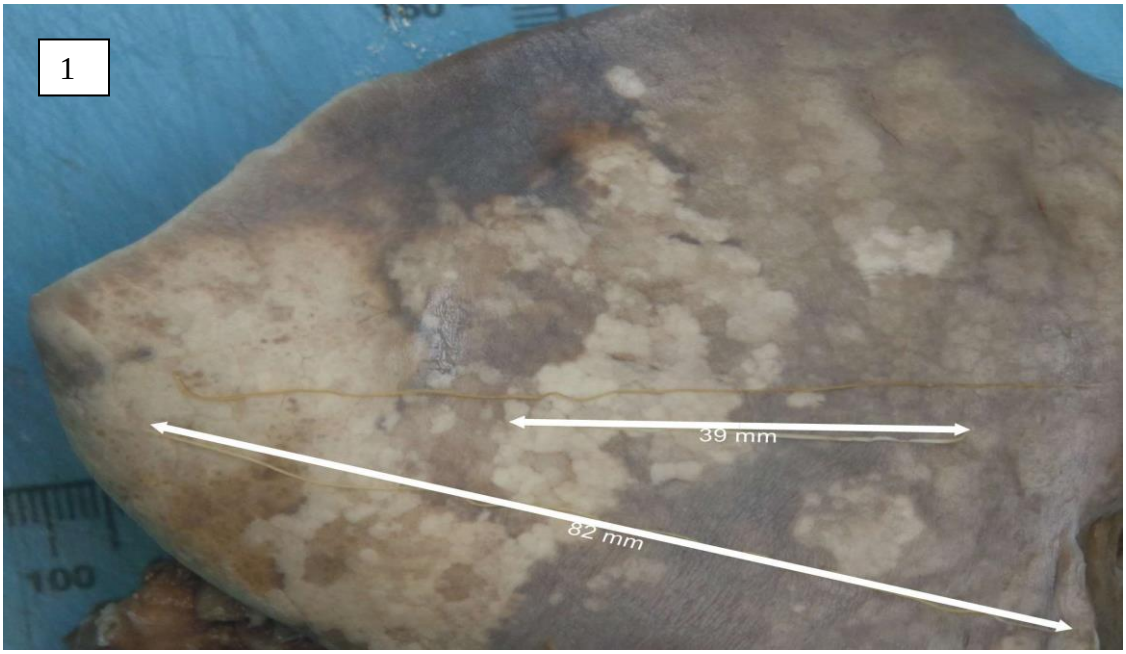
Composition: Peptone (10g), meat extracts (3g), sodium chloride (5g), agar (4g), gelatine (80g) and distilled water (one litre). Preparation: First the gelatine was soaked in water for 30 minutes.

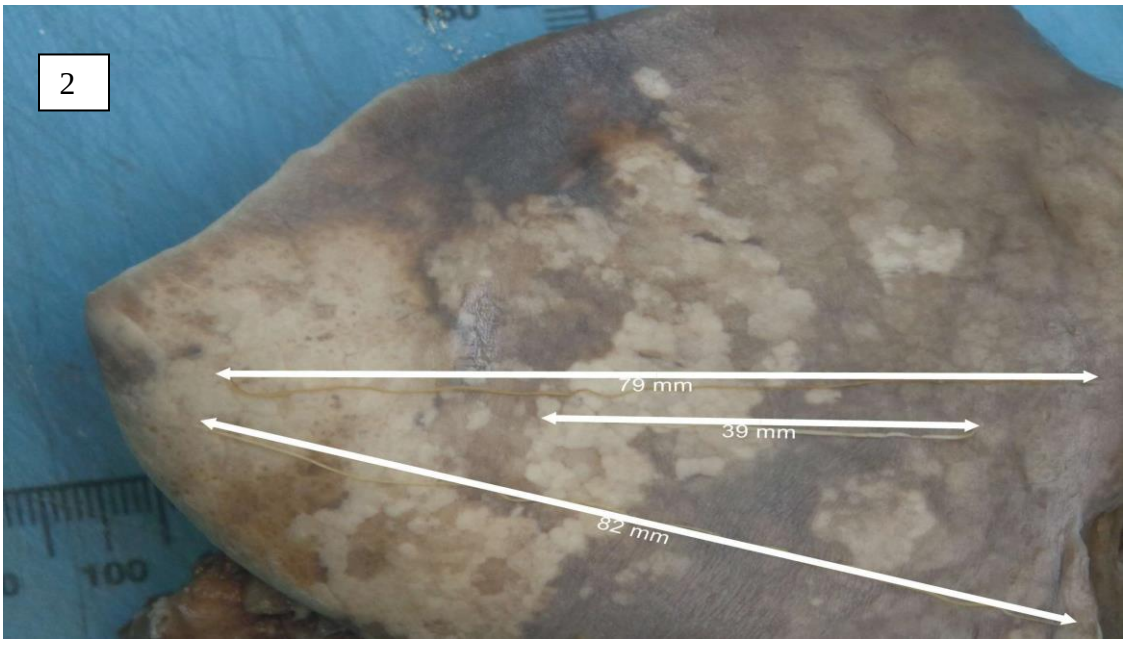
Then other ingredients were added, dissolved by heating, distributed into test tubes and sterilized by autoclaving at 115°C for twenty minutes.

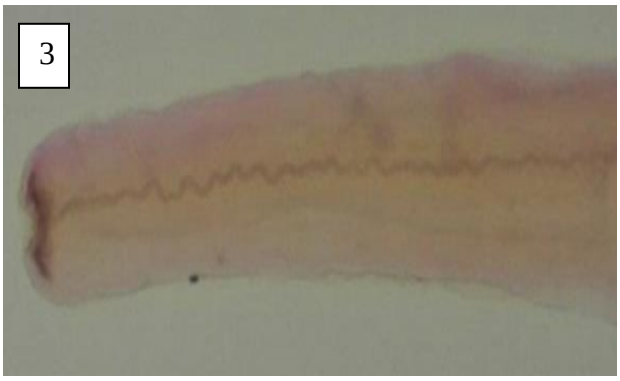
Test procedure and principle: The tube of motility medium was stabbed by inoculums to depth of about five mm and incubated at 37°C. Motile organism migrated through the medium, which become turbid while the growth of non-motile organisms was confined to the stab inoculums.

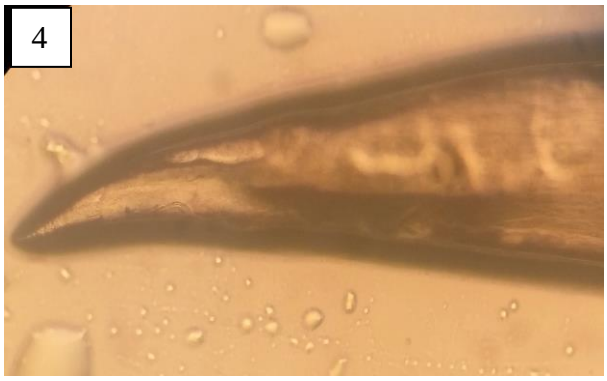
Annex VII: Morphology of parasites identified and recovered from lungs with the parasitic pneumonias.

A. *Dictyocaulus filaria* identified from sheep lung: Adult *D. filaria* 40mm to 83mm (**1**, **2**) long measured by MacroPATH pro-x digital imaging system (formalin fixed lung tissue of verminous pneumonic lung). Anterior (**3**) and posterior (**4**) end of D. filarial shown by stereomicroscopic photograh.

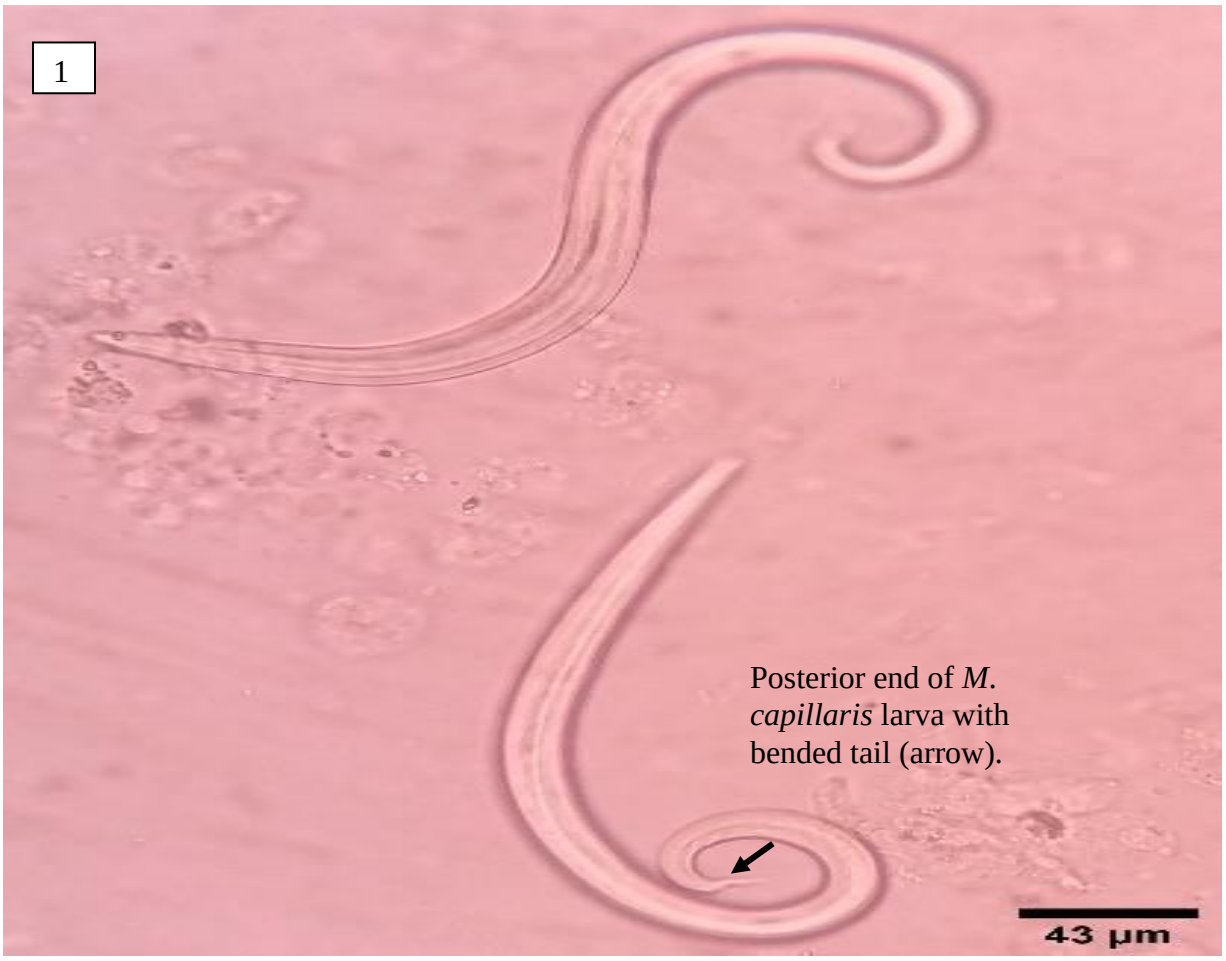


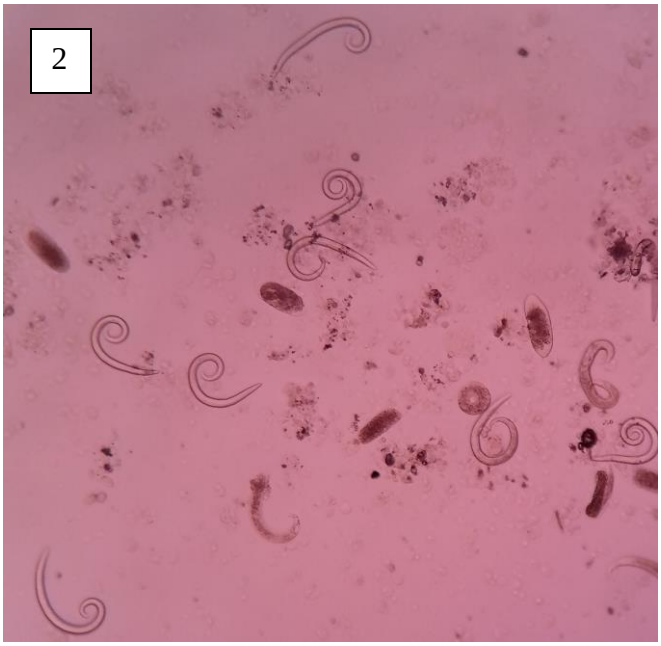


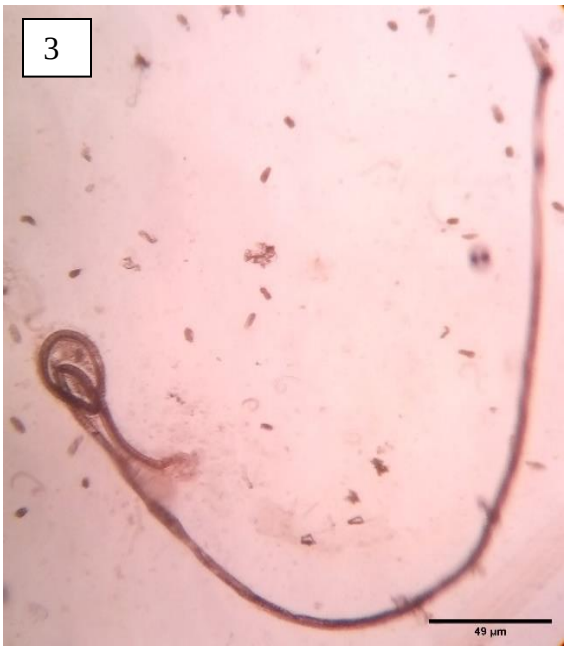




B. Parasitic larvae recovered from nodular lesion of lungs: *Muelleries capillaris* larva with bended tail (1) and larvae with egg (2); *Protostrongylus* larvae (3).

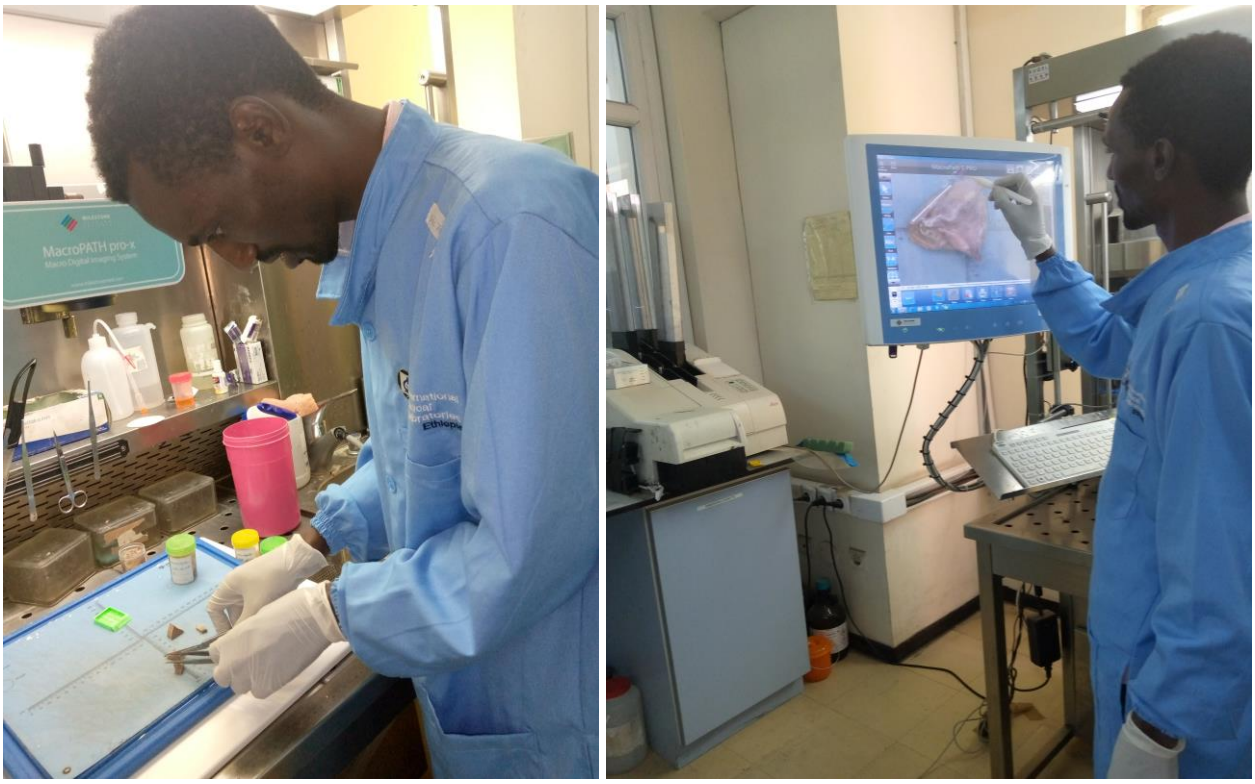






Annex VIII: Photographic demonstration of field and laboratory activity

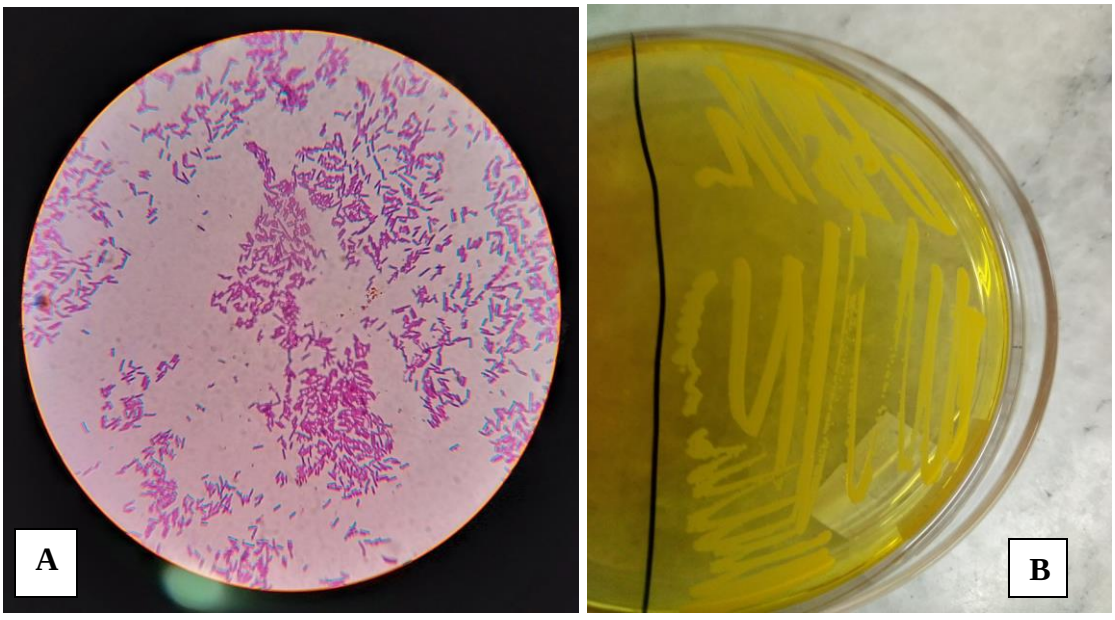
Sheep and goats brought to slaughter were in lairage



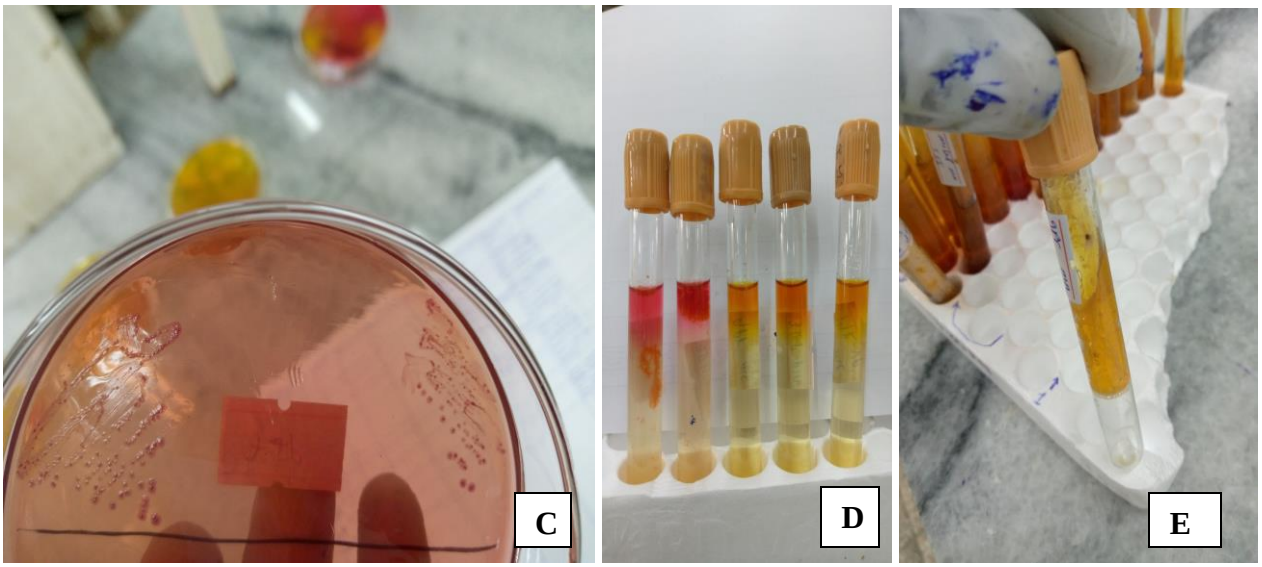
Grossing and morphologinc imaging of lesion by MacroPATH pro-x digital imaging system

Tissue proccessing: embeding, sectioning and staining

When performing IHC activity in histopathology lab of ICL



Short rod microscopic morphology of *Pasteurella* spp. in Gram staining (**A**); the yellow small colonies of *Staphylococcus aureus* on Mannitol Salt Agar (**B**).



The pink small pin point colonies of *P. hemolytica* on Mac conkey agar (**C**); biochemical test result revealing motility positive with red ring and negative in Methyl red broth (**D**) and A/A in TSI agar of *E. coli* (**E**).

Annex IX: Gross pathological lesion recording formats

No.	Date	Sample code	Abattoirs/ Restaurants	Animal Origin	Species	Gross appearance & description of lesions	Pneumonia type	Sample type	Remark

Annex X: Histopathological tissue process recording format

No.	Date	Sample code (code given on gross tissue sample container)	Gross lesions description	Description of histopathological findings	Remark

Annex XI: Immunohistochemistry (IHC) staining format

Rack station number									
Antibody									
Sample identification code									

Annex XII: Recommended information's offered on the antibodies and IHC reagents.

No.	Primary antibodies	Dilution rate	Diluent	Secondary antibody	Epitope retrieval method	Chromogen used	Source
1	CLA (CD45)	1:200	PBS	Biotinylated 2^{ndry} Ab (Horse anti-rabbit IgG)	HIER	DAB	United kingdom (Leica biosystems) supplied by ICL
2	CD68	1:200	PBS	Biotinylated 2^{ndry} Ab	HIER	DAB	
3	CD20	1:200	TBS	Biotinylated 2^{ndry} Ab	HIER	DAB	

Note: CLA-common leucocyte antigen; CD-clusters of differentiation; PBS-phosphate buffered saline; TBS-Tris-buffered saline; Ab-antibody, HIER-heat induced epitope retrieval; DAB-diaminobenzidine