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Radiographic, Microbiologic, and Pathologic Study of Pneumonia in Imported Sheep and Goats

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ABSTRACT

This study aimed to determine the main pathogens involved in the pulmonary disorders, clinical signs, radiography, and pathological findings of imported sheep and goats. In the summer of 2019 during repeated visits to the livestock farms, digital radiographs were taken from the thoracic region of the animals that were clinically suspected of pneumonia complications. Afterward, samples were taken following necropsy from 40 cases that died owing to pneumonia, and eventually, lung samples were examined for microbiology and histopathology to isolate causative pathogens and diagnose the type of complication. Radiographically, increased thoracic opacity was evident in all studied animals. The visible pulmonary patterns were mainly the interstitial pattern and to a lesser extent the alveolar and bronchial patterns. The isolated bacteria in sheep were *Pasteurella multocida* (28.57%), *Mannheimia haemolytica* (21.43%), Mycoplasma genus (17.86%), *Escherichia coli* (14.29%), *Corynebacterium ovis* (7.14%), Arcanobacterium (7.14%), and B*acillus licheniformis* (3.57%), and in goats were Mycoplasma genus (42.31%), P*asteurella multocida* (19.23%), *Mannheimia haemolytica* (11.54%), *Corynebacterium ovis* (11.54%), *Escherichia coli* (7.70%), Arcanobacterium (3.84%), and *Bacillus licheniformis* (3.84%), respectively. Based on the histopathologic findings, pulmonary lesions of sheep and goats consisted of 37.5% fibrinosuppurative bronchopneumonia, 27.5% fibrinous bronchopneumonia, 25.0% purulent bronchopneumonia, and 10.0% interstitial pneumonia. Based on the evaluated parameters, in thoracic radiographs, the interstitial pattern was the dominant pulmonary pattern. Also, *Pasteurella multocida*, and Mycoplasma genus were the most common pathogens isolated in the sheep and goats respectively and fibrinosuppurative bronchopneumonia was the most prevalent pulmonary complication.

Introduction

Respiratory involvement is known to be a common disease of small ruminants in rearing countries throughout the world,[1] and indicate a serious problem for the profitability and the welfare of farm animals.[2] In small ruminants, pneumonia is most commonly observed in lambs and goats at an early age when the effect of maternal antibodies declines. The disease can affect animals individually or in groups, often due to a combination of pathogenic organisms, environmental and management factors.[3,4] The clinical signs of pneumonia vary depending on the severity of the disease, including reduced appetite or anorexia, weakness, depression, tachypnea, coughing, nasal discharge, and fever. Pneumonia causes weight loss, decreased production, and reduced wool quality. Its importance is also because

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of sales delays, slaughterhouse waste, mortality and low carcass value.[5]

Mannheimia haemolytica and *Pasteurella multocida*, are found as normal flora particularly in the nasopharynx and tonsils of the healthy domestic ruminant.[6] Mycoplasmas are also recognized as one of the important groups of bacteria responsible for causing respiratory diseases, which can be inapparent or can cause a mild, acute, or chronic disease.[7] Infections induced by these pathogens typically occur because of a poor management system resulting from strict stressful conditions or subsequent secondary infection. Poor farming conditions, overcrowded, sudden environmental changes, transportation stress, and other stressful conditions increase domestic ruminants' susceptibility to pneumonia.[8]

Diagnostic imaging techniques can be used to diagnose respiratory diseases, which are usually non-invasive methods. Ultrasound, radiology, and computed tomographic (CT) scans are some of the most effective diagnostic imaging modalities for diagnosing respiratory problems. Some of these modalities however could not be applied in the field conditions.[9] Determining the etiology of a complication in the lungs is not possible with radiography alone, because different diseases may cause similar radiographic changes.[10]

Hence, in the current study, more diagnostic techniques such as radiography, microbiology, and histopathology were applied to make the precise diagnosis and identify pathogens and to be able to perform effective treatment for herd animals according to the results.

Materials and Methods

Animals

This study was conducted in the summer of 2019 on industrial farms located around the city of Tehran, Iran. The animals of these farms were imported from France to Iran, which included sheep of Romane, Ile De France, Charollais breeds, and goats of Alpine and Saanen breeds.

During repeated visits to the livestock farms, radiographs in a standing lateral view were taken from the thoracic region of animals that were clinically suspected of pneumonia, by using a flat panel (Konica Minolta AeroDR 1012 digital mobile detector, Japan) digital radiography system (Ecotron 1600 portable x-ray generator, South Korea).

Radiography was not possible in the ventrodorsal or dorsoventral projections because the cases had not received sedation and binding them was not possible due to the large number of cases studied. In some cases, multiple radiographs were taken from the thorax to evaluate all of the lung lobes.

Collection of Samples

The animals were isolated from the herd with respiratory symptoms such as cough, fever, rhinorrhea, and abnormal sounds during the physical examination and monitored them daily. If the livestock showed respiratory symptoms, they were separated from the herd and quarantined at the veterinary hospital for treatment; so, the disease did not spread to the rest of the herd. If the patients recovered during this time, they would return the herd. A total of 40 animals (23 sheep and 17 goats; 9 male and 31 female between 6 months to 6 years old), which showed clinical signs of pneumonia and died during this period, were examined and multiple pieces of lung tissue were collected from each case after necropsy. Some lung samples of each animal in the vicinity of ice were shipped to the microbiology laboratory for isolation of causative organisms and the other samples were placed in 10% buffered formalin solution for routine histopathologic examination. Data regarding each case was recorded on the sample containers.

Microbiology

The lung samples were initially streaked on general microbiological media including blood agar, MacConkey agar and Eosin Methylene Blue (Merck Co., Germany) and then, were incubated at 37 °C for 24 hours. If no growth was observed during this period, the culture media were incubated for another day. If no bacterial growth was visible during 48 hours, results were reported as negative. Before the bacteria could be identified and a pure culture prepared, each was transferred to a new culture medium. Each culture medium contained only one bacterium, which was purified in this manner. The colonies which were grown in the media were morphologically examined and stained by gram staining method. According to the bacteria observed under light microscope, the colonies were purified and assessed by differential media and biochemical tests. In order to examine the infection of the lung with bacteria, suspected colonies were used for complimentary tests including catalase, gelatinase, nitrate reduction, litmus milk, CAMP (Christie-Atkins-Munch-Peterson), coagulase and IMVIC (Indole, Methyl red, Voges-Proskauer, Citrate). Meanwhile, the colonies were inoculated into complimentary media including urea, loeffler serum, TSI (Triple Sugar Iron) and SIM (Sulfide-Indole-Motility). The colonies were also inoculated into sugar liquid media to identify their ability of fermentation of glucose, lactose, mannitol, and sucrose.[11,12]

***Pasteurella multocida* identification.** *P. multocida* are identified on the basis of biochemical tests including: catalase-positive, Indole-positive, no-hemolysis on sheep blood agar, no growth on MacConkey agar, and acid

production from lactose (-).

***Mannheimia haemolytica* identification.** The presumptive *M. haemolytica* colonies were identified using flowing biochemical characteristics: Indole-negative, hemolysis on sheep blood agar, growth on MacConkey agar, and acid production from lactose.

***Escherichia coli* identification.** Suspected bacterial colonies were identified via subculture on Eosin Methylene Blue (EMB) agar (Merck, Germany), and additional identification was made using biochemical tests: TSI, IMViC and growth on MacConkey agar.

***Corynebacterium ovis* identification.** Identification criteria for *C. ovis* isolates include: Gram-positive, pleomorphism in a Gram-stained smear, catalase-positive, oxidase-negative, enhancement of hemolysis test (CAMP), and nitrate reduction.

***Arcanobacterium* identification.** Gram-positive bacteria which grow in Loeffler serum, and pin-point colonies with hemolysis on sheep blood agar were confirmed as *Arcanobacterium*.

***Bacillus licheniformis* identification.** Large, Gram-positive rods, Endospores producer, Citrate-positive, and VP test positive were identified as *B. licheniformis*.

Mycoplasma isolation. All samples were analyzed by polymerase chain reaction (PCR) to identify the *Mycoplasma* genus. Van Kuppeveld *et al*.'s method[13] was employed to extract Mycoplasma DNA by Qiagen Inc., Valencia kit. The sequence of primers with 277 bp-position used to search for *Mycoplasma* genus are as follows:

GPO3F (Forward): 5′-TGGGGAGCAAACAGGATTAGATACC-3′.

MGSO (Reverse): 5′-TGCACCATCTGTCACTCTGTTAACCTC-3′.

The compounds required for the polymerase chain reaction in a total volume of 20 µl were prepared as follows: 2 µl template DNA, 0.4 µl of each primer (forward and reverse), 2 µl PCR buffer (10X), 1.6 µl $MgCl_2$ at a concentration of 25 mM, 0.8 µl DNTPs at a concentration of 5 mM, 0.04 µl Taq DNA polymerase and 12.76 µl sterile deionized water. Amplification was performed for 35 cycles. The cycling temperature was denaturation at 94 °C for 45 sec., annealing at 55 °C for 45 sec., and elongation at 72 °C for 90 sec. The final cycle was followed by an extension at 72 °C for 360 sec. The amplified products were run and analyzed on 2% agarose gels and visualized under ultraviolet light after electrophoresis. Distilled water was used for negative control and a previous Mycoplasma positive sample (PTCC ID: 7051) was used for positive control.

Pathology. Multiple samples from the affected area of the lungs were fixed in 10% buffered formalin solution. Specimens were embedded in paraffin and thin sections of 5 µm thickness were obtained. Then, they were stained using hematoxylin and eosin method for routine histopathologic examination.

Results

Clinical Signs

The animals studied in this investigation showed different degrees (moderate to severe) of respiratory involvement. Clinical signs of these cases, mainly included: weakness, coughing, nasal discharge, tachypnea, and abnormal breathing sound in the lung's auscultation. The duration of involvement of the sheep and goats before the radiography was between 2 and 5 days, and the infected animals received non-steroidal anti-inflammatory drugs (NSAID), flunixin meglumine (1.5 mg/kg, IV, Flonex 5%, Razak, Iran) in addition to broad-spectrum antibiotics include oxytetracycline (10 mg/kg, IM, Oxyvet 10%, Razak, Iran) and tylosin (10 mg/kg, IM, Tylomax 20%, Rooyan Darou, Iran) during this period. These two antibiotics were combined because they had a synergistic effect and covered many bacteria, including gram-positive, gram-negative, and mycoplasma.[14]

Radiography Results

Radiographic examinations revealed an increase in lung opacity in all sheep and goats. Pulmonary involvement in radiographs was mainly mild, but in a few cases especially in goats, moderate involvement was reported. Pulmonary patterns were frequently interstitial patterns and to a lesser extent, alveolar and bronchial patterns were observed (Figure 1). Besides, a mixed pattern (bronchointerstitial pattern) was also reported due to bronchopneumonia. The clarity of the pulmonary vessels as well as the aorta was reduced in most animals, but the caudal vena cava was well detectable.

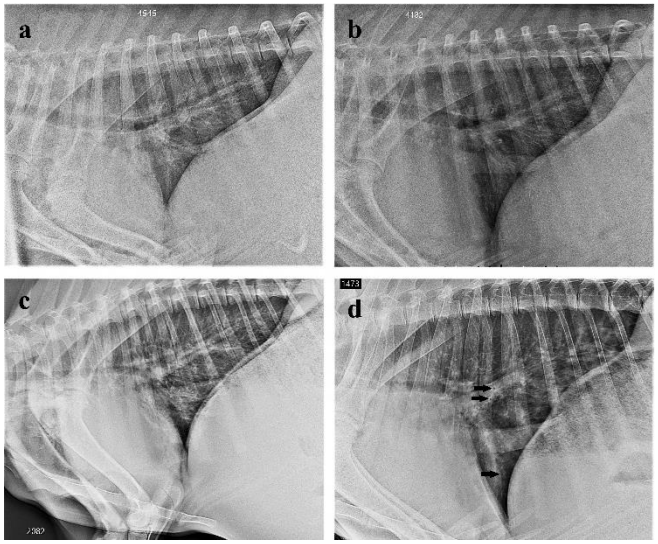

Figure 1. Standing lateral radiographs of caudodorsal thoracic region in two lambs (A and B) and two kids (C and D) with pulmonary involvement. **A.** Mild bronchial pattern, **B.** Mild interstitial pattern, **C.** Mild generalized interstitial pattern, **D.** moderate generalized mixed bronchointerstitial pattern due to bronchopneumonia, the black arrows indicate the bronchial rings.

Microbiology Evaluation

In the microbiological study, out of 40 animals (23 sheep and 17 goats) died due to pneumonia, in twenty cases (50%) from the cultures that obtained from lung lesions, only one bacterium was isolated and in seventeen other samples (42.5%) two bacteria were identified from the affected lungs. In three cases (7.5%) of sheep lung tissues, no bacteria were isolated in culture media. Of the total purified bacteria, 81.48% (44 Bacterial colonies) were related to gram-negative bacteria and 18.52% (10 bacterial colonies) were related to gram-positive bacteria.

Isolated bacteria in sheep were in the order of frequency: *P. multocida* (28.57%), *M. haemolytica* (21.43%), *Mycoplasma* genus (17.86%), *E. coli* (14.29%), *C. ovis* (7.14%), Arcanobacterium (7.14%), and *B. licheniformis* (3.57%). Isolated bacteria in goats were *Mycoplasma* genus (42.31%), *P. multocida* (19.23%), *M. haemolytica* (11.54%), *C. ovis* (11.54%), *E. coli* (7.70%), Arcanobacterium (3.84%), and *B. licheniformis* (3.84%), respectively (Figure 2).

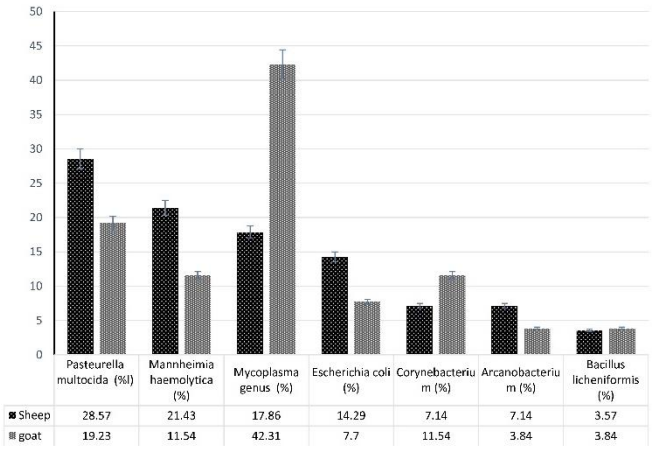

Figure 2. Diagram of bacterial agents isolated from sheep and goats involved in pulmonary disease.

Necropsy Findings

Macroscopically, discoloration, and irregular consolidation were observed in the lung tissue, especially in the cranial lung lobes. Due to the nature of the consolidation, the color of the lungs was dark red in the acute form and gray or pink-gray in the chronic form. In the acute phase, the surface of the lung was wet and, following incision, pus was pulled out. The froth was also observed in the bronchi and ducts. The lung's tissue becomes firm and heavy, and sinks into the stabilizing fluid (formalin).

Histopathology Examination

In the microscopic examination of tissue sections, purulent bronchopneumonia and fibrinous bronchopneumonia with edema, fibrin deposition and inflammatory cells, especially neutrophils, into the alveoli and bronchioles, and areas of coagulation necrosis, interstitial edema between lobules, and hyperemia were observed. In some cases, the purulent or mucopurulent exudate with neutrophil accumulation and cell debris in the alveoli and bronchioles, hyperplasia of bronchial associated lymphoid tissue (BALT) were so severe that the microscopic appearance of the lungs changed. Interstitial pneumonia was seen with thickening of bronchiolar epithelium and accumulation of inflammatory cells around the bronchiole (Figures 3 and 4). Generally, based on histopathologic lesions of sheep and goats' lungs, 37.5% were affected with fibrinosuppurative bronchopneumonia, 27.5% with fibrinous bronchopneumonia, 25.0% with purulent bronchopneumonia, and 10.0% with interstitial pneumonia (Table 1). The relation between histopathological findings and isolated bacteria was shown in Table 2.

Discussion

Respiratory diseases are prevalent in all different species of animals, especially herbivores, and are one of the major problems in the livestock industry in the world.[15] Pulmonary involvement in all animal species occurs following a variety of etiologies such as bacteria, viruses, parasites, host immune system insufficiency, environmental factors, and various stressors. Moreover, breed, animal age, nutritional conditions, geographical location, climate change, and low level of health in the flock, are all as factors predisposing pathogens to develop pneumonia.[6] Because pneumonia leads to damages such as mortality, weight loss, reduced production, abattoir elimination, and heavy treatment costs, so far, plenty of studies have been conducted on the histopathology and bacteriology of lung lesions in different geographical conditions

Table 1. Different types of pulmonary complications in sheep and goats based on the histopathologic findings.

Animal		Pulmonary complication			
		Fibrinosuppurative bronchopneumonia	**Fibrinous bronchopneumonia**	**Purulent bronchopneumonia**	**Interstitial bronchopneumonia**
Sheep	Numbers	9	5	6	3
	Percentile (%)	39.13	21.73	26.08	13.04
Goat	Numbers	6	6	4	1
	Percentile (%)	35.29	35.29	23.52	5.88

Table 2. Details of histopathologic features and bacteriological results in 40 cases of sheep and goats pneumonia.

Number of cases (n = 40)	Histopathologic features	Isolated bacteria
15	Fibrinosuppurative bronchopneumonia	Mycoplasma genus + *E. coli* (n = 3) Pasteurella + *Corynebacterium ovis* (n = 3) Pasteurella + Mycoplasma genus (n = 2) Mannheimia + Mycoplasma genus (n = 2) Mycoplasma genus (n = 2) *Mannheimia haemolytica* (n = 1) *Pasteurella multocida + E. coli* (n = 1) Pasteurella + Mannheimia (n = 1)
11	Fibrinous bronchopneumonia	*Pasteurella multocida* (n = 4) Mycoplasma genus (n = 3) *Mannheimia haemolytica* (n = 2) Mannheimia + Mycoplasma genus (n = 1) No bacteria isolated (n = 1)
10	Purulent bronchopneumonia	Arcanobacterium (n = 3) *Mannheimia haemolytica* (n = 2) Mycoplasma + *Corynebacterium ovis* (n = 2) Mycoplasma genus (n = 1) *Pasteurella multocida* (n = 1) *Pasteurella multocida + E. coli* (n = 1)
4	Interstitial pneumonia	*Bacillus licheniformis* (n = 1) *Bacillus licheniformis + E. coli* (n = 1) No bacteria isolated (n = 2)

In the veterinary literature, the use of effective tools such as radiology to examine the thorax has become more widespread than before. The radiography of thorax in large animals has many similarities with small animals in terms of generalities and complications.[10] However, there are not many studies and research in the field of lung tissue radiography in small ruminants.

All of the respiratory diseases, cause marked changes in the radiographic opacity of the lungs, and usually, the pattern of distribution of these lesions is different. In the radiographic examination of the present study, interstitial pattern, and to a lesser extent alveolar and bronchial patterns were observed in the studied sheep and goats, with the severity of involvement varying from mild to moderate.

In a study conducted in 2022, increased opacity of lung field with cotton-wool-like look is detected in chest radiography which is consistent with the results of the present study. [16] The radiographic pattern and distribution of pulmonary lesions was similar to the finding of the early days of pneumonia in another study characterized by bronchointerstitial infiltrates.[17]

Quinn *et al*. (2002), was found a wide variety of colonized bacterial flora in the airways in domestic ruminants with pneumonia. The constant identification and isolation of these organisms from the lungs involved in pneumonia in different species of animals may indicate the role of these organisms in the development of various respiratory syndromes. The population of *M. haemolytica* varied in different species, according to different anatomical regions. For example, the isolation of *M. haemolytica* in the lungs and trachea of goats was lower than in sheep,[18] which was in agreement with the findings of the present study.

In a study conducted in Portugal (2007) on a flock of Saanen goats with severe respiratory involvement with 34% morbidity and more than 20% mortality in kids, after bacteriological studies, species of Mycoplasma such as *M. ovipneumoniae* and *M. arginine* as well as *M. haemolytica* and *P. multocida* were isolated.[19] High prevalence of Mycoplasma has also been reported in association with *M. haemolytica* in lamb lungs in Turkey[20] and in another study in Italy.[21] The results of the present study are consistent with those of previous studies. In our study, Mycoplasma genus, *P. multocida*, and *M. haemolytica* were the dominant organisms isolated from the pneumonic lungs of sheep and goats.

Bacteriological examinations of the lungs of slaughtered sheep with pneumonic lesions were performed in southwestern Iran and *P. multocida* (24.53%), *Staphylococcus aureus* (20.75%), *Klebsiella pneumoniae* (15.09%), *Corynebacterium pseudotuberculosis* (7.55%), and *Actinomyces pyogenes* (1.89%) were isolated, respectively.[6] In another study of 120 sheep slaughtered at the Garmsar abattoir, *Pseudomonas*, *Proteus*, *Arcanobacterium pyogenes*, Klebsiella, Enterobacter, Streptococcus, *E. coli,* and *Pasteurella trehalosi* were isolated from pulmonary lesions.[22] However, the findings of the current study do not support the previous research. The probable reason for diversity in isolated bacteria from slaughtered samples in previous studies may be due to genetic variation of the breed which influences their immune response.

A common feature of the histopathology of mycoplasma pneumonia is an increase in the number of mononuclear cells in the lung tissue that are found in the areas around the bronchi and vessels.[23]

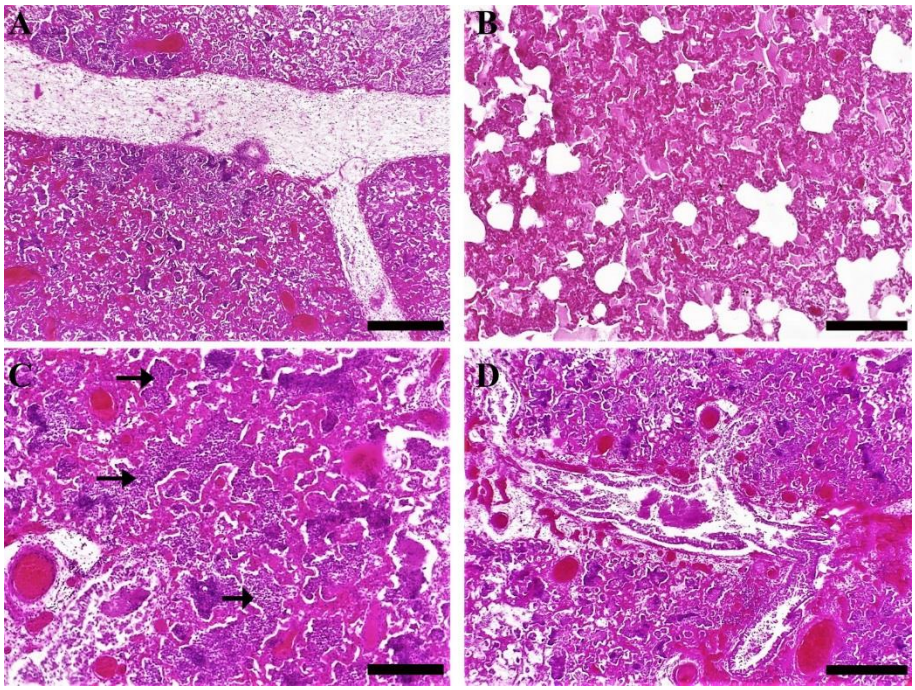

Figure 3. Microscopic appearance of lung lesions (H&E stain: Bar: 50 µm). Fibrinosuppurative bronchopneumonia (A). Edema formation in the alveoli and bronchioles (B). Infiltration of inflammatory cells, especially neutrophils, into bronchioles (black arrows) (C). fibrinosuppurative bronchopneumonia(D).

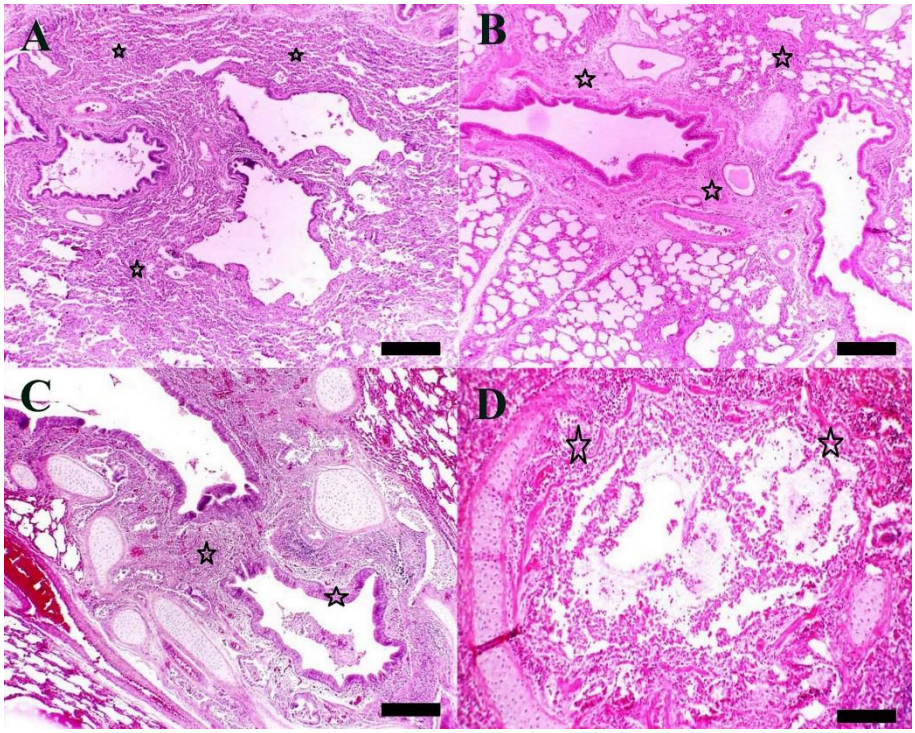

Figure 4. Microscopic appearance of lung lesions (H&E stain; Bar: 100 µm). Interstitial pneumonia with thickening of bronchiolar epithelium (A). Interstitial pneumonia with accumulation of inflammatory cells around the bronchiole (B). Suppurative bronchopneumonia. Hyperplasia of bronchiolar epithelium and hyperplasia of BALT (C). Suppurative bronchiolitis with attenuation of the bronchial epithelium (D).

In our study, histopathologic changes in 16 cases (5 sheep and 11 goat), which Mycoplasma was isolated and confirmed by PCR methods, revealed interstitial pneumonia with tissue thickening due to infiltration of neutrophils, macrophages, lymphocytes, and fibrous tissue proliferation, BALT hyperplasia, and infiltration of mononuclear cells into the airways and blood vessels. These results match those observed about mycoplasma in pulmonary lesions in earlier studies.[24,25]

Azizi *et al.* examined histopathologic lesions of the lungs of sheep pneumonia and reported purulent bronchopneumonia, interstitial pneumonia, bronchointerstitial pneumonia, fibrinous bronchopneumonia, and embolic pneumonia, respectively. In present study, fibrinous-suppurative bronchopneumonia, fibrinous bronchopneumonia, and purulent bronchopneumonia were the main histopathological lesions.[6]

In three cases of sheep lung tissue, no bacteria were isolated in culture media, but histopathology revealed interstitial pneumonia in two lambs and bronchopneumonia in another case. They may have pneumonia due to other pathogens.

Based on the clinical signs and the results of this study, sheep with severe clinical signs of pneumonia, indicated no severe pulmonary involvement in radiographic images. This is probably due to the susceptibility of the sheep to pneumonia, which is lost before the occurrence of visible pulmonary changes on the radiograph. But goats revealed more pronounced radiographic changes.

In conclusion, the microbiological investigations and pathogen isolation conducted in this study have revealed the presence of pathogenic organisms, including *Pasteurella multocida*, *Mannheimia haemolytica*, and Mycoplasma genus, associated with pneumonia in sheep and goats. Given the susceptibility of these animals, the absence of long-term resistance during disease outbreaks, and the potential for pneumonia-induced mortality, it is imperative to promptly administer suitable antibiotics. Timely treatment, coupled with vigilant monitoring until complete recovery, is strongly recommended to safeguard the health and well-being of the affected livestock.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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