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RESEARCH ARTICLE

# Pathological and Microbiological Investigations of Pneumonic Pasteurellosis in Sheep [1]

Zafer OZYILDIZ * Osman Yasar TEL ** Rahsan YILMAZ *
Sule Yurdagul OZSOY *** Oktay KESKIN *

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* Harran University, Faculty of Veterinary Medicine, Department of Pathology, TR-63200 Eyyubiye Campus, Sanliurfa - TURKEY
** Harran University, Faculty of Veterinary Medicine, Department of Microbiology, TR-63200 Eyyubiye Campus, Sanliurfa - TURKEY
*** Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Pathology, TR-31040 Antakya, Hatay - TURKEY

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## Summary

In this study, between March 2010 and March 2011, 110 pneumonia suspected lung tissues were examined histopathologically, immunohistochemically and microbiologically, in Sanliurfa province. After definition of the macroscopic localization of the consolidated areas in the lungs, tissue samples were taken and preserved in cold chain and 10% formalin for microbiological and pathological examinations, respectively. For bacteriological examination of *Pasteurella* spp. lung specimens were inoculated in 7% sheep blood agar and Mc Conkey agar. After routine pathological tissue follow up procedure, tissues were embedded in paraffin and obtained sections stained with Hematoxylin&Eosin (H&E). The cases, having histopathological findings consistent with pneumonia, were forwarded to immunohistochemical (IHC) examinations to know whether lesions related to *Mannheimia haemolytica* and *Pasteurella multocida* using hyperimmune polyclonal rabbit sera in Avidin Biotin Complex Peroxidase (ABC-P). Microbiological, histopathological and immunohistochemical findings were comparatively evaluated in examined animals. *Pasteurella multocida* as a cause of pneumonia were detected in 38 cases of microbiological inoculations. Immunohistochemical staining resulted *Mannheimia haemolytica* (n=35) and *Pasteurella multocida* (n=30) positive. Immunohistochemically both *Mannheimia haemolytica* and *Pasteurella multocida* were positive in 23 cases and 45 animals were negative for both bacteria. The aim of this study is to show importance and role of *Pasteurella* spp, in sheep pneumonia in Sanliurfa region.

***Keywords:*** *Pneumonic Pasteurellosis, Pathology, Microbiology, Immunohistochemistry, Sheep*

# Koyun Pnömonik Pastörelloz'unda Patolojik ve Mikrobiyolojik İncelemeler

## Özet

Bu çalışmada, Şanlıurfa ilinde Mart 2010-Mart 2011 tarihleri arasında, 110 adet pnömoni şüpheli akciğer dokuları histopatolojik immunohistokimyasal ve mikrobiyolojik olarak incelendi. İncelenen akciğerlerde makroskobik olarak konsolide alanların lokalizasyonu belirlendikten sonra bu bölgelerden alınan doku örneklerinin bir kısmı soğuk zincirde muhafaza edilerek mikrobiyolojik inceleme için kullanıldı. Doku örneklerinin bir kısmı %7 lik koyun kanlı agar ve Mc Conkey agara ekildi. Diğer bir kısım ise %10'luk tamponlu formaldehitte tespit edildikten sonra, rutin doku takibinden geçirilip, histopatolojik incelemeler için Hematoksilen&Eozin (H&E) ile boyandı. Histopatolojik olarak pnömoni teşhisi konulan kesitler, poliklonal tavşan *Mannheimia haemolytica* ve *Pasteurella multocida* hiperimmun serumları kullanılarak Avidin Biyotin Kompleks Peroksidaz (ABC-P) Yöntemi ile immunohistokimyasal olarak boyandı. İncelenen hayvanlarda mikrobiyolojik, histopatolojik ve immunohistokimyasal bulgular karşılaştırmalı olarak değerlendirildi. Mikrobiyolojik ekimlerde 38 vakada *Pasteurella multocida* pnömoni etkeni olarak belirlendi. Yapılan immunohistokimyasal boyamalar sonrası toplam 35 hayvanda *Mannheimia haemolytica*, 30 hayvanda *Pasteurella multocida*, 23 hayvanda her ikisinin birden ve 45 hayvanda ise nedeni belirlenmeyen farklı etkenlerin pnömoni sebebi olduğu ortaya konuldu. Bu çalışmanın amacı Şanlıurfa İlindeki koyun pnömonilerinde *Pasteurella* spp.'lerin yeri ve öneminin belirlenmesidir.

***Anahtar sözcükler:*** *Pnömonik Pastörellozis, Patoloji, Mikrobiyoloji, İmmunohistokimya, Koyun*

**İletişim (Correspondence)**
+90 414 3183909
zaferozyildiz@hotmail.com

## INTRODUCTION

*Pasteurella* spp. bacteria colonize in normal bacterial flora of the nasopharynx, and oral mucosa [1]. Transportation, crowd, climate changes, stress situations such as bad housing conditions are predisposing causes to pasteurellosis. When the respiratory defences of animals are weaken by viral infections *Pasteurella* spp. can colonize the lower respiratory tract in large numbers and induce severe fibropurulent bronchopneumonia. Thus, secondary bacterial infection can be as a major complication of acute respiratory viral diseases, such as those due to: Peste Des Petits Ruminants, Parainfluenza 3, Adenovirus and Respiratory syncytial virus [2-5].

Pneumonic pasteurellosis is one of the most lethal forms of pneumonia with bronchopneumonic and lobar pattern. It is common in many species of pets or wild animals [1,2,10]. Previously *M. haemolytica* and *P. multocida* reported in sheep in different states of disease [6,7]. Pneumonic pasteurellosis is sporadic or enzootic disease, mainly caused by *M. haemolytica* and *P. multocida* and can be seen in all age groups and in every season of the year [3,7-9]. While the acute events tend to form hemorrhagic and fibrino-necrotic bronchopneumonia, subacute to chronic events tend to form fibrinopurulent bronchopneumonia that causes fibrinous pleural adhesion and abscess [6,10]. Macroscopical aspects of lung lesions of pasteurellosis are characterized by red-black to gray-brown in color. There are consolidation areas with significantly gelatinous interlobular septal thickening, fibrinous pleuritis and coagulation necrosis areas in cranioventral lobes [10].

In pneumonic pasteurellosis, responsible agents can be placed within hemorrhagic and fibrinous exudate in bronchus, bronchiole epithelia, alveolar capillary and alveolar lumen sometimes can be placed in the periphery of syncytial cell formations and degenerated spindle-shaped leukocytes (oat cells) and at necrotic areas [6,10].

*Pasteurella* spp. well reproduce at blood agar, is routinely used at isolation [6]. A selective medium is required for the isolation of *M. haemolytica* and *P. multocida* when contaminated with other microorganisms [2]. When samples were taken from slaughterhouse conditions, other saprophytes contamination were often formed [11-13]. Therefore, as well as pneumonic form of the disease, for the detection of acute hemorrhagic-septicemic forms, rapid and sensitive immunoperoxidase technique are important to implement as routine [14]. There are several studies made on sheep and cattle to diagnose the disease with immunoperoxidase technique by using polyclonal *anti-Mannheimia haemolytica* and anti-*Pasteurella multocida* hyperimmune serum [1,3,14,15]. The purpose of this study was to determine the role and incidence of *Pasteurella* spp., in clinical or subclinical pneumonia cases of sheep in Sanliurfa region.

## MATERIAL and METHODS

Between the dates of March 2010 and March 2011, age between 0-2, totally 110 pieces (100 from private DEM-ET Slaughter House, 10 was handed to Harran University, Faculty of Veterinary Medicine, Department of Pathology) pneumonia suspected lung tissues were examined histopathologically, immunohistochemically and microbiologically, in Sanliurfa region.

### *Bacteriology*

For bacteriological examination of *Pasteurella* spp., lung specimens were inoculated in 7% sheep blood agar and in Mc Conkey agar. Petri dishes were incubated at 37°C, in aerobic conditions for 24-48 h. and suspicious colonies were selected and examined for *Pasteurella* spp. Some characteristics of the bacteria such as the colony morphology, hemolytic characteristics, Gram staining, oxidase, catalase, indole and reproduction at Mc Conkey agar, examined according to standard methods [5].

### *Histopathology*

The lung tissues were fixed in 10% neutral buffered formalin and embedded in paraffin by routine methods. Sections were cut 5 µm in thickness and they were stained with H&E [16].

### *Immunohistochemistry*

***Hyperimmun sera:*** Polyclonal anti-*Pasteurella multocida* and anti-*Manheimia haemolytica* hyperimmune sera were obtained from Adnan Menderes University, Faculty of Veterinary Medicine, Department of Microbiology, Aydin.

***Immunohistochemical staining:*** The selected tissue sections for immunohistochemical staining were dewaxed and rehydrated by routine methods and stained by avidin-biotin-peroxidase complex (ABC-P) technique (Labvision, USA). Antigen retrieval was facilitated by heating the specimens in a citrate buffer (pH 6.0) for 15 min in a microwave owen at a power of 360 W and 600 W respectively. Then the slides were dipped in freshly prepared 0.3% hydrogen peroxide in methanol for 15 min, to block endogenous peroxide activity and followed by incubation with normal goat serum for 20 min at 40°C. The sections were incubated for 60 min at room temperature with one of the following primary antibodies: Polyclonal anti-*Pasteurella multocida* and anti-*Manheimia haemolytica* hyperimmune sera (in 1:1000 dilutions). Colour labeling was developed by a final incubation step using 3.3' Diaminobenzidin (DAB, Labvision, USA). Finally, sections were counterstained with Harris' haematoxylin and mounted. For positive control, previously *Pasteurella* spp. positive lung tissues (confirmed by Uludag University, Faculty of Veterinary Medicine, Department of Microbiology and Pathology) were restained. For the negative control, same sections were incubated with normal goat sera instead of primary antibody.

## RESULTS

Determination of localization of consolidated areas in the sheep lungs, the each lobe of the lungs labeled by given characters A, B, C, D, E, and F *(Fig. 1)*. The localization of consolidated sites in the lungs was shown in *Table 1*. Lung samples were evaluated due to form of pneumonia such as lobar and/or lobular. According to this, the percentage of pneumonia localization was found as 66% (73/110) lobular, 16% (18/110) lobar and 17% (19/110) both of two.

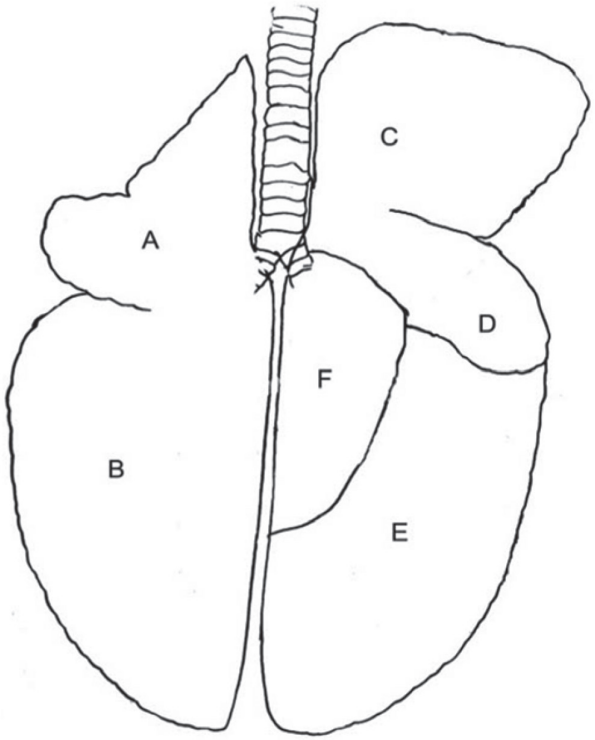


**Fig 1.** Denotation of lobes

(A- Left cranial lobe, B- Left caudal lobe, C- Right cranial lobe, D- Medial lobe, E- Right caudal lobe, F- Accessor lobe)

**Şekil 1.** Lobların isimlendirilmesi

(A- Sol cranial lob, B- Sol caudal lob, C- Sağ cranial lob, D- Medial lob, E- Sağ caudal lob, F- Accessor lob)

***Table 1.*** *Distribution of pneumonia according to their locations*
***Tablo 1.*** *Lezyonların makroskobik dağılımı*

| Localization | Number | Localization | Number |
|---|---|---|---|
| A | 10 | C+D | 8 |
| B | 1 | C+E | 2 |
| C | 56 | A+B+C | 1 |
| D | 1 | B+C+D | 1 |
| E | 1 | A+C+D | 6 |
| A+C | 10 | C+D+E | 5 |
| D+E | 1 | C+D+F | 2 |
| B+E | 1 | A+C+D+E | 1 |
| A+D | 1 | A+B+C+D+E | 2 |

Macroscopical examinations revealed that the most frequently affected lobe was right cranial lobe (C). Other affected lobes were the left cranial lobe (A), together with left and right cranial lobe (A+C), together with the right cranial and medial lobe (C+D), both two cranial lobe with right medial lobe (A+C+D) respectively. Because of the low-level combinations of the other lobes were excluded from consideration.

Consolidated areas of the lungs were swollen and dark red in color. Affected lung tissues were mostly palped as liver and crusty in consistence *(Fig. 2-A, B, C)*. At cross-section of the lungs; fine foamy fluid or creamy suppuration yellowish, gray in color were detected in some bronchus and bronchiole lumen *(Fig. 2-D)*.

Microscopically, catarrhal-purulent (n=22) and fibrinous (n =24) broncho-pneumonia which has characterized by neutrophil leukocytes and mono-nuclear cell infiltration with fibrin were seen in the bronchi, bronchiole, alveolar lumen and pleura *(Fig. 3-B, C, D)*. Multinucleated syncytial cell formations with presence of spindle-shaped oat cells were observed in the alveolar lumen. In some sections (n =23), widespread neutrophilic infiltration, coagulation necrosis with purulent-necrotic bronchopneumonia were observed in and around of the bronchus and bronchioles *(Fig. 3-A)*. In 21 lung tissue sections, bronchointerstitiel pneumonia was characterized as diffuse mononuclear cell infiltration in and around of bronchus, bronchiole and in 16 lung tissue sections, interstitial pneumonia was characterized as mononuclear cell infiltration in interstitial areas (n =16). In 4 lung tissue sections, pulmonary adenomatosis was seen. The immunohistochemical and microbiological results are shown in *Table 2*. At the end of the immunohistochemical stainings pneumonic pasteurellosis was found in 65 of 110 animals. The immunopositive areas were localized in bronchi, bronchiole *(Fig. 4-B, C)* and alveolar *(Fig. 4-A, D)* lumen and epithelia, interstitium, vein lumen and peribronchial glands. The microscopical distributions of agents are shown in *Table 3*.

## DISCUSSION

Pneumonic pasteurellosis is one of the important infectious diseases of the respiratory system observed in Turkey. Recent studies have shown that the disease is an important health problem at ruminants [10,14,17-19]. In many geographic regions of Turkey, *Pasteurella* spp. responsible for the most pneumonia cases in small ruminants [9,14,15,19]. According to our study *Pasteurella* spp. is holding an important place in sheep pneumonia in Sanliurfa region.

Pasteurella pneumonia is usually indicated lobar distribution and often fibrinous, purulent, necrotic lung infections [10]. In this study pneumonia was lobular rather than lobar (68 animals). This situation may be associated with animals being butchered, thus the lesions are not formed

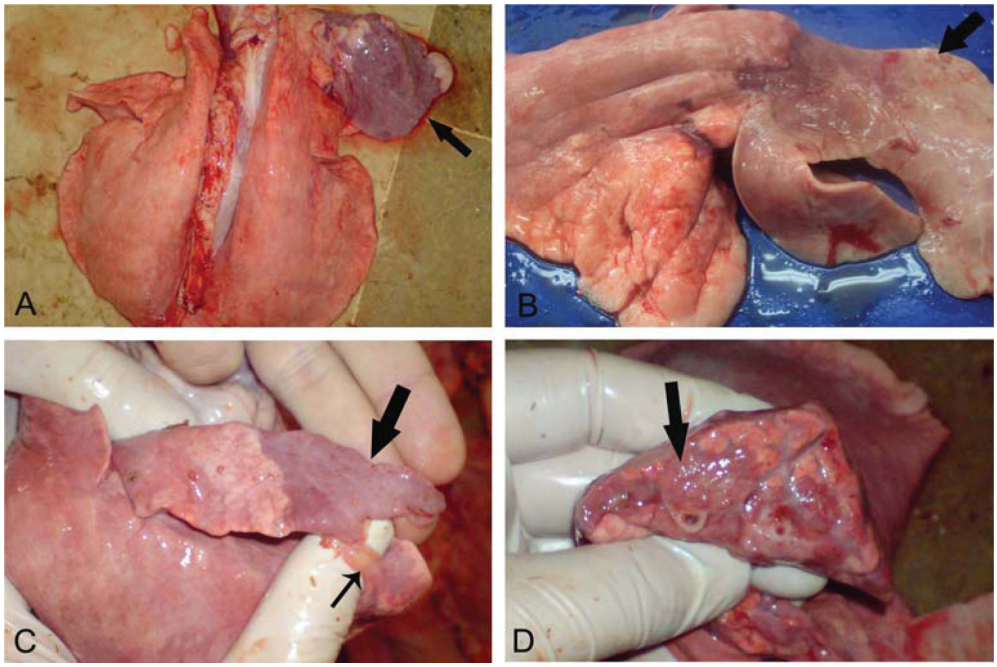


**Fig 2.** A- Right cranial lobe, lobar pneumonia *(arrow)*, dorsal appearance, B- Right cranial lobe with medial lobe, lobar pneumonia *(arrow)*, dorsal appearance, C- Medial lobe lobular pneumonia *(arrow)*, adhesion on visceral pleura *(thin arrow)*, dorsal appearance, D- The cut section of pneumonic areas

**Şekil 2.** A- Sağ kranial lob, lober pnömoni *(ok)*, dorsalden görünüm, B- Sağ kranial lob ile medial lobda lober pnömoni *(ok)*, dorsalden görünüm, C- Medial lobda lobuler pnömoni *(ok)*, viseral plörada adezyon *(ince ok)*, dorsalden görünüm, D- Pnömonik alanların kesit yüzü

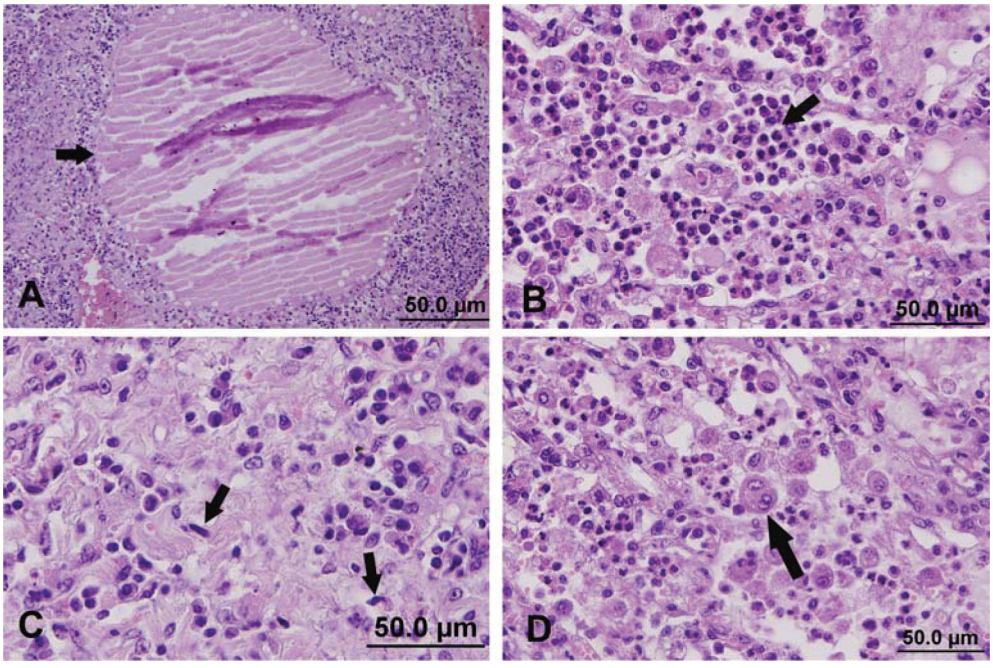


**Fig 3.** A- Coagulation necrosis *(arrow)* surrounded with inflammatory cells, H&E x200, B- Neutrophil leukocytes and mononuclear cell infiltration *(arrow)* in alveoli lumen, H&E x400, C- Oat cells *(arrows)* H&E x400, D- Syncytial cell formation *(arrow)*, H&E x400

**Şekil 3.** A- Etrafı yangı hücreleri ile çevrili koagulasyon nekrozu (ok), H&E x200, B- Alveol lümeninde nötrofil lökosit ve mononükleer hücreler *(ok)*, H&E x400, C- Yulaf hücreleri *(oklar)* H&E x400, D- Sinsityal hücre oluşumu *(ok)*, H&E x400

whole. In addition, animals with the complaint of cough, nasal flow and respiratory distress cannot be completely treated, should be considered.

In the study, consolidated areas were present at the contact surfaces of lobes close to each other, and often detected at the right cranial lobe together with medial lobe.

**Table 2**. *Pasteurella positive animal number according to results of immunohistochemical staining and microbiological isolation*
**Tablo 2.** *IHC boyama ve mikrobiyolojik muayene sonuçlarına göre pastörella pozitif hayvan sayısı*

| Microorganism | IHC (n=110) | Microbiological isolation (n=110) |
|---|---|---|
| *M. Haemolytica* (+) | 35 | 0 |
| *P. Multocida* (+) | 30 | 38 |
| Both of them (+) | 23 | - |
| Both of them (-) | 45 | 72 |

pneumonia (21 cases) was also significant. These findings indicated that important part of pneumonia was also possibly a complication of viral infections. Therefore, this study revealed that in this region viral infections should also be considered.

In this study the microbiological results obtained from a total of 110 animals, were not exactly the same with positive reactions at immunohistochemical stainings. However, immunohistochemically *M. haemolytica*-positive samples were found (n=33), *M. haemolytica* was not isolated

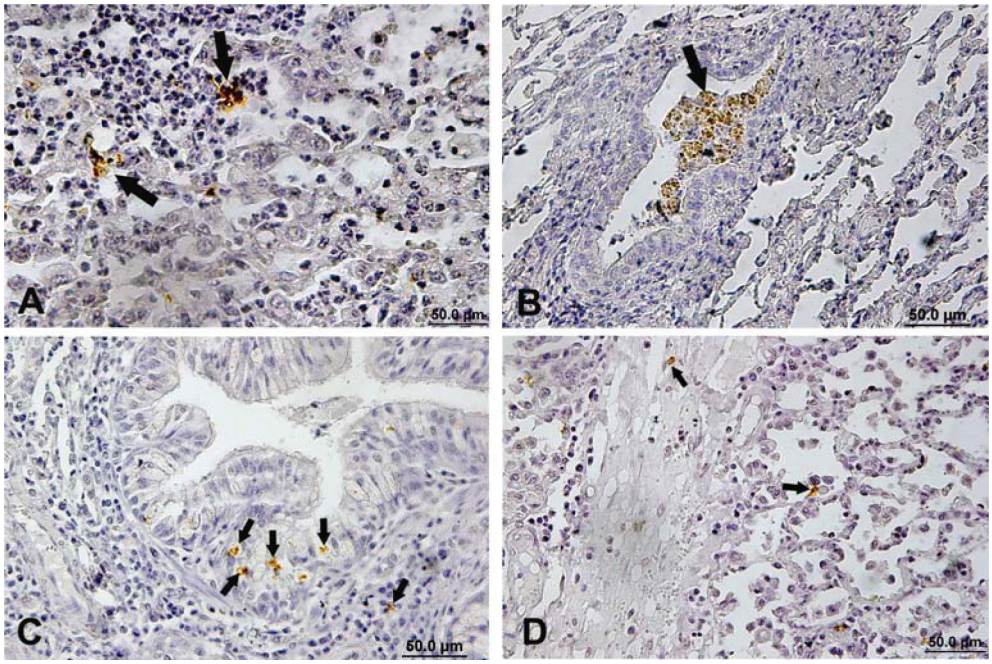


**Fig 4.** A- Alveolar lumen, immunpositive staining in neutrophil leukocytes *(arrows)*, ABC method counterstained with Harris haematoxylin x400, B- Bronchiole lumen, immunpositive staining in exudate *(arrow)*, ABC method counterstained with Harris haematoxylin x400, C- Bronchiole epithelium and peribronchial area, immunpositive staining in epithelium and mononuclear cell *(arrows)*, ABC method counterstained with Harris haematoxylin x400, D- Alveolar lumen and interstitial area, immunopositive agents (arrows), ABC method counterstained with Harris haematoxylin x400

**Şekil 4.** A- Alveol lümeni, nötrofil lökositler içinde pozitif boyanmış etkenler *(oklar)*, ABC, Harris haematoxylin X400, B- Bronşiol lümeni, eksudat içerisinde pozitif boyanmış etkenler *(ok)*, ABC, Harris haematoxylin x400, C- Bronşiol epiteli ve peribronşiol bölge, bronşiol epiteli ve mononükleer hücre içerisinde pozitif boyanmış etkenler *(oklar)*, ABC, Harris haematoxylin x400, D- Alveol lümeni ve intersitisyel alan, serbest haldeki etkenler *(oklar)*, ABC, Harris haematoxylin x400

**Table 3.** *The distribution of agents at lung tissues with IHC staining*
**Tablo 3.** *IHC boyamalarda etkenlerin akciğer dokusundaki dağılımı*

| IHC (+) Agent | Alveolar epithelium | Alveolar lumen | Bronchi, bronchiole epithelium | Bronchi, bronchiole lumen | Interstitium | Vein Lumen | Peribronchial glands |
|---|---|---|---|---|---|---|---|
| *P. Multocida* | 23 | 17 | 25 | 23 | 17 | 2 | 5 |
| *M. Haemolytica* | 27 | 30 | 29 | 24 | 10 | 3 | 4 |

This situation supports that the disease either spread by the endobronchial way or spread by contact to the adjacent lobe pleura like other bacterial agents such as *E. coli*, *Corynebacterium* spp, *Mycoplasma* spp. [11,13]. In histopathological examination, the predominant lesion was bronchopneumonia (catarrhal-purulent: 22, fibrinous: 24, purulent-necrotic: 23). However broncho-interstitiel

in terms of any microbiological cultivation results. Classic inoculation methods give reliable results in the diagnosis of pasteurellosis [20,21]. The histopathologic diagnosis shows the type and the presence of pneumonia, the presence of the agent in the tissue can be detected by immunohistochemical methods [1,14,15]. Barely hygienic conditions in slaughter houses is not satisfactory, the lungs are put in

bulk containers gregariously and hence the inevitability of contamination with other bacteria, because of unconscious use of antibiotics, the reproductive ability of the agent is weak or dead, possible causes of incoordination between the results of microbiological and immunohistochemical examinations. Similar problems were encountered in previous studies [13,14,19]. Immunohistochemical methods are revealing to bacterial agent either free or inside of phagosome. Because of this, immunohistochemical staining method is more sensitive than classic microbiological methods. Present results showed that IHC can be used routinely for the diagnosis of pasteurellosis. Nevertheless for the definitive diagnosis of pneumonic pasteurellosis, combined results of microbiological isolation and immunohistochemical staining will give more reliable results.

Pneumonic pasteurellosis causes serious field losses and animal death [17]. Biggest problems in this subject are; breeders in the region does not take precautions or treat their animals unless there are large number of deaths and incompleted treatments or random medicine use makes disease asymptomatic or chronic thus tricking breeders into thinking that their animals are get well. At the same time because of the animals are housed in closed barns, in inadequate ventilation especially during the winter months and as a result of ammonia irritation *Pasteurella* spp. opportunism is increases. By changing the care and feeding conditions and the vaccination studies in the region, is thought to be important steps in solving the problem.

As a result, by this study we described pneumonic pasteurellosis in sheep by pathologic, immunohistochemical and microbiologic methods in Sanliiurfa region.

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