Kafkas Univ Vet Fak Derg
24 (2): 281-288, 2018
DOI: 10.9775/kvfd.2017.18840

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
Online Submission: http://submit.vetdergikafkas.org

Research Article

Immunohistochemical and Molecular Detection of *Mannheimia* spp. and *Pasteurella* spp. in Sheep with Pneumonia in Kars Province - Turkey [1] [2]

Serpil DAĞ [1] Abdurrahman GÜRBÜZ [2] Hasan ÖZEN [1] Fatih BÜYÜK [3]
Özgür ÇELEBİ [3] Musa KARAMAN [4] Mehmet ÇİTİL [5] Emin KARAKURT [1]

[1] *This work was supported by the Research Fund of Kafkas University (Project Number: 2012-VF-57)*
[2] *This study was presented as poster in 7th National Veterinary Pathology Congress. 8th - 10th September 2014, Kars - TURKEY*

[1] Kafkas University, Faculty of Veterinary Medicine, Department of Pathology, TR-36100 Kars - TURKEY
[2] Kafkas University, Atatürk Health Services Vocational School, TR-36100 Kars - TURKEY
[3] Kafkas University, Faculty of Veterinary Medicine, Department of Microbiology, TR-36100 Kars - TURKEY
[4] Balıkesir University, Faculty of Veterinary Medicine, Department of Pathology, TR-10145 Balıkesir - TURKEY
[5] Erciyes University, Faculty of Veterinary Medicine, Department of Internal Medicine, TR-38039 Kayseri - TURKEY

Article Code: KVFD-2015-18840 Received: 13.10.2017 Accepted: 10.01.2018 Published Online: 10.01.2018

How to Cite This Article

Dağ S, Gürbüz A, Özen H, Büyük F, Çelebi Ö, Karaman M, Çitil M, Karakurt E: Immunohistochemical and molecular detection of *Mannheimia* spp. and *Pasteurella* spp. in sheep with pneumonia in Kars province - Turkey. *Kafkas Univ Vet Fak Derg,* 24 (2): 281-288, 2018. DOI: 10.9775/kvfd.2017.18840

Abstract

In this investigation, presence of *Mannheimia haemolytica* and *Pasteurella multocida* in sheep brought to Kafkas University Faculty of Veterinary Medicine between 2011 and 2013 with a suspicion of pneumonia was studied by immunohistochemical and bacteriological methods. Study materials were 100 sheep lungs. After routine histopathological investigations lungs were immunohistochemically stained with antibodies prepared against both of the agents. In bacteriological investigations, tissue samples were inoculated into 7% sheep blood agar and Mc Conkey agar and incubated for 24-48 h in aerobic conditions, and the suspected colonies were evaluated for *Mannheimia* and *Pasteurella* species. In histological investigations, pneumonias were classified as 28% fibrinous bronchopneumonia, 21% prulent bronchopneumonia, 20% acute-catharal bronchopneumonia, 18% interstitial pneumonia, 11% verminous pneumonia and 2% adenomatous pneumonia. In bacteriological investigations *Mannheimia haemolytica* and *Pasteurella multocida* was detected in 19 and 3 cases, respectively, these numbers were 17 and 2, respectively in immunohistochemical investigations. The results of PCR analysis for both *Mannheimia haemolytica* and *Pasteurella multocida* paralleled with the findings of microbiological culture. The results of the study showed that *Mannheimia haemolytica* is an important cause of pneumonia in sheep in Kars, and *Pasteurella multocida,* although with lesser importance can cause pneumonia in this species.

Keywords: *Mannheimia haemolytica, Pasteurella multocida, Pneumonia, Sheep, Immunohistochemistry*

Kars İlinde Pnömonili Koyunlarda *Mannheimia* spp. ve *Pasteurella* spp.'nin İmmunohistokimyasal ve Moleküler Tanımlanması

Öz

Bu çalışmada Kafkas Üniversitesi Veteriner Fakültesine 2011-2013 yılları arasında pnömoni şüphesi ile getirilen koyunların akciğerlerinde *Mannheimia haemolytica* ve *Pasteurella multocida* etkenlerinin varlığı immunohistokimyasal ve bakteriyolojik yöntemlerle araştırılmıştır. Çalışma materyalini pnömoni şüpheli 100 adet koyun akciğeri oluşturdu. Akciğer doku örnekleri rutin histopatolojik incelemelerin ardından her iki etkene karşı hazırlanan antikorlar kullanılarak immunohistokimyasal olarak boyandı. Bakteriyolojik incelemeler için alınan örneklerin %7 koyun kanlı agar ve Mc Conkey agara ekimleri yapıldı. Aerobik koşullarda 24-48 saat inkübasyonu takiben şüpheli koloniler *Pasteurella* ve *Mannheimia* türleri açısından incelendi. Histopatolojik incelemeler sonucunda pnömonilerin %28'i fibrinli bronkopnömoni, %21'i prulent bronkopnömoni, %20'si akut-kataral bronkopnömoni, %18'i intersitisyel pnömoni, %11'i verminöz pnömoni ve %2'si adenomatöz pnömoni olarak sınıflandırıldı. Bakteriyolojik incelemeler sonucunda 19 koyunda *Mannheimia haemolytica* ile 3 vakada *Pasteurella multocida* tespit edilirken bu oranlar immunohistokimyasal incelemelerde sırasıyla 17 ve 2 olarak belirlendi. PZR analizleri hem *Mannheimia haemolytica* hem de *Pasteurella multocida* için mikrobiyolojik analizler ile paralellik gösterirdi. Çalışmanın sonucunda Kars ilindeki koyun pnömoni vakalarında *Mannheimia haemolytica*'nın önemli derece yer aldığı buna karşın *Pasteurella multocida*'nın da az sayıda olmakla beraber pnömoni etkeni olarak görev yaptığı belirlendi.

Anahtar sözcükler: *Mannheimia haemolytica, Pasteurella multocida, Pnömoni, Koyun, İmmunohistokimya*

İletişim (Correspondence)
+90 474 2426807/5086
serpildag06@hotmail.com

INTRODUCTION

Respiratory system diseases are common in sheep, and cause important economic losses due to excessive treatment costs, drop in yield, and death [1-4]. Besides various bacterial and viral infectious agents, various stress factors causing hygiene and sanitation problems such as insufficient forage and housing conditions, changes in climate, early weaning, and improper transportation play roles in development of pneumonia [5-8]. Respiratoric mannheimiosis, also known as pneumonic pasteurellosis, is caused primarily by *Mannheimia haemolytica* and *Pasteurella multocida,* and commonly seen in ruminants [9]. *M. haemolytica* is also known as the causative agent of enzootic pneumonia and septicemia in lambs and kids [10,11]. These infectious agents are within the normal flora of ora-nasopharynx, and with the help of predisposing factors and some viral agents can cause pneumonia [12-14]. It is referred that deadly fibrinous lobar or bronchopneumonia is caused chiefly by *M. haemolytica* while *P. multocida* is responsible from the less severe fibrinopurulent bronchopneumonia, though this is not always true [12].

In epidemiologic studies conducted on sheep in Turkey, *M. haemolytica* was detected at 37.3% in Kars [15], 12.5% in Şanlıurfa [16], 11.3-12.7% in Van [17,18], 56.1% in Konya [19], and 2.3% in Elazığ [8]. *P. multocida* was also detected in sheep that showed pneumonia symptoms at 11.2% in Kars [20], 31.6% in Şanlıurfa [16], 10.52% in Konya [19], and 4.3% in Elazığ [8].

The results of the studies in worldwide have been reported different infection rate for *M. haemolytica* as 13% in Norway [21], 21.9-98.7% in Ethiopia [22,23], 4% in Western United States [24] and 25-66.9% in Ethiopia for *P. multocida* [22,23].

In diagnosis of pneumonic pasteurellosis, microbiological (culture and serology) and immunohistochemical methods are commonly used. PCR, which can be applicable on both isolates and tissue samples, is also now often used to detect bacteriological agents. Microbiologic culture technique is quite time consuming and bears the risk of sample contamination [25] while serological tests shows high cross-reaction. On the other hand PCR was suggested to be more sensitive, specific and faster method compared to the other conventional techniques [8,24]. Immunohistochemical detection of the bacterial agents in sections prepared from paraffin tissue blocks was also suggested to be quite successful [25-29].

The purpose of this study is to detect the bacterial agents of *M. haemolytica* and *P. multocida* by the methods of PCR and immunohistochemistry in sheep that show signs of pneumonia. It is also aimed to investigate the extent of pathological changes takes place, the localization of the bacterial agents in tissues, and the correlation between the bacterial presence and the lesion severity.

MATERIAL and METHODS

The study material was 100 lung samples collected from sheep that showed signs of pneumonia at gross examination. All tissue samples were collected at Kafkas University Faculty of Veterinary Medicine. Ethics of the study was confirmed by Kafkas University Laboratory Animals Local Ethics Committee (KAÜ-HADYEK 2011-43).

Polyclonal Antibody Production

For antibody production 10 week old female New Zealand rabbits weighing approximately 2.5 kg were used. Pure colonies of *P. multocida* and *M. haemolytica* were grown in nutrient broth. Pure cells of both bacteria provided by Kafkas University Department of Microbiology were washed several times with sterile water (4500 rpm for 10 min) and suspended in phosphate saline buffer (PBS, pH 7.2). The inoculum containing 10^9 cfu/mL was treated for 30 min at 90°C in a water bath. The immunization was performed subcutaneously (200 µL for per injection site) using 1.0 mL of fresh bacterial cells introduced at five different locations of the rabbit's body. The immunization scheme was shown in *Table 1*. The sera were centrifuged to eliminate the red blood cells and heated at 56°C for 1 h in a water bath. The sera were stored frozen at -20°C in 1.5 mL Eppendorf tubes. The obtained antiserums were purified using a protein-A column (ABICAP, Merck, Germany) to extract the IgG fraction. After purification, the antibodies were neutralized using NaOH (0.5 M) to achieve pH 7.0.

Bacteriological Investigations

Lung tissue samples collected from sheep with pneumonia were inoculated onto sheep blood agar (Oxoid, CM0271) and Mc Conkey agar (Merck, 105465). Samples were incubated at aerobic environment for 24-48 h, and the suspected samples were further investigated for *Pasteurella* and *Mannheimia* species based on the characteristics of colony morphology, hemolysis, oxidase, catalase and indole activities and growth capabilities on Mc Conkey agar plates [30,31].

Pathological Investigations

Lung tissue samples collected at systemic necropsy were fixed in 10% neutral buffered formaldehyde solution and

Table 1. *Immunization and application scheme of rabbits for polyclonal antibody production*

Day Intervals	Applications
Day 0	Pre-immunisation bleed and initial antigen injection
Day 14-21	First antigen booster
Day 28-35	Second antigen booster
Day 35-42	Test bleed
Day 42-56	Third antigen booster
Day 49-66	Blood collection

embedded in paraffin. Following routine procedures, tissue sections at 5 µm were stained with hematoxylin and eosin and investigated under light microscope.

Immunohistochemical Investigations

Avidin-biotin-peroxidase method (Histastain® Plus Broad Spectrum, Invitrogen Cat No: 859043) with diaminobenzidine substrate (DAB-Plus Substrate Kit, Invitrogen Cat No: 002020) color development with hematoxylin background staining was used for immunohistochemical staining (IHC) in lung tissue sections. Antigen retrieval was accomplished with microwave treatment in Citrate solution. Primer antibodies were diluted at 1:50 dilution with phosphate buffer saline and the sections were incubated with them at room temperature for 1 h. Other procedures were completed routinely and the sections were observed under a light microscope for presence of positive immunoreactivity and the extent of it.

PCR Analysis

Primers designed for PCR amplification of *P. multocida toxA* and *M. haemolytica sodA* genes were used in molecular detection of the bacteria. Total DNA was isolated from the suspected lung tissue samples as follow: tissue samples were homogenized with a homogenizer and then the homogenates were suspended with Tris EDTA (TE) buffer solution and centrifuged at 9000 g for 10 min. Pellets were suspended with TE buffer, and then incubated with lysis buffer (10 mg/mL Proteinase K, 1 M Tris-HCl, 0.5 M EDTA, 10% SDS) at 55°C overnight. DNA isolation was accomplished with phenol-chloroform-isoamyl alcohol. To precipitate DNA, 3M sodium acetate and cold absolute ethanol was added onto the samples and let overnight at -20°C. Finally the samples were centrifuged at 5400 g for 30 min and the pellets were suspended with distilled water. DNA concentration in samples was measured spectrophotometrically at 260 nm.

PCR amplification of *P. multocida toxA* gene was accomplished according to the method described by Kamp et al.[32] with some minor modifications. A PCR sample size of 50 µL contained 100 ng DNA sample, 10 mM Tris:HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 M forward (5′-GGTCAGATGATGCTAGATACTCC-3′) and reverse (5′-CCAAACAGGGTTATATTCTGGAC-3′) primers, and 10 U/mL Taq DNA polymerase. PCR conditions for *P. multocida toxA* gene was accomplished in PCR conditions of initial denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 95°C for 30 sec, hybridization at 65°C for 1 min, and synthesis at 72°C for 2.5 min. A final was extension at 72°C for 20 min was applied.

M. haemolytica sodA gene amplification was performed according to the method described by Guenther et al.[33] with some modifications. The PCR sample size of 50 µL was composed of 100 ng DNA sample, 10 mM Tris:HCl, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 M forward (5′-AGCAGCGACTACTCGTGTTGGTTCAG-3′) and reverse (5′-AAGACTAAAATCGGATAGCCTGAAACGCCTG-3′) primers, and 10 U/mL Taq DNA polymerase. PCR cycling procedure were as follow; initial denaturation at 94ºC for 5 min, 30 cycles of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min, and elongation at 72ºC for 2 min. A final elongation step was performed at 72ºC for 5 min.

All of the PCR products were run in 2% agarose gels containing ethidium bromide and the PCR product bands of 338 bps for *P. multocida toxA* gene and 327 bps for *M. haemolytica sodA* gene were visualized with ultraviolet transilluminator.

RESULTS

Bacteriological Findings

In bacteriological examination, suspicious 20 colonies on blood agar plates that were small, glistening, mucoid and dew-drop like and appeared as Gram negative cocobacilli when stained with Gram stain were further investigated. Out of these colonies 3 (3%) were identified as *P. multocida* and 19 (19%) were *M. haemolytica,* based on the phenotypic characteristics (hemolysis, oxidase, catalase and indole reactivity and growth capabilities on Mc Conkey agar plates).

Gross Necropsy Findings

Total of 100 lung tissue samples that were recognized as pneumonic were investigated in the study. In most of the cases, dark red to gray consolidated firm areas were recognized in the cranioventral lobes of lungs. In some cases, lobar pneumonia, which included the great portion of the lobes with white-gray necrotic areas, was recognized. In verminous pneumonia cases, lesions were seen in the caudal lobes. In acute catarrhal bronchopneumonia cases the lesions were accompanied by hyperemia and edema in the lungs and exudate in the bronchi.

The lungs were not collapsed and the rib traces were found on the lung surface in the cases of interstitial pneumonia. In adenomatous pneumonia cases, the lungs were observed enlarged several folds normally and became heavier and a white marble appearance was detected in the lung pleura. In the cases of verminous pneumonia, caudal lobes were gray colored and had thick nodules. In addition, red colored, atelectatic areas and interstitial emphysema were also seen.

Histopathological Findings

In microscopic examination, the cases were categorized as acute catarrhal bronchopneumonia, purulent bronchopneumonia, fibrinous pneumonia, interstitial pneumonia, verminous pneumonia, and adenomatous pneumonia. Numbers of cases according to the histopathological evaluation were shown in *Table 2*.

Table 2. *Numbers of cattle according to the type of pneumonia, and the cases with M. haemolytica and P. multocida detected with immunohistochemical staining, bacteriological culture methods and PCR*

Pneumonia	Total Number of Cases	Bacteriology		IHC		PCR	
		P. multocida	*M. haemolytica*	*P. multocida*	*M. haemolytica*	*P. multocida*	*M. haemolytica*
Acute Catarrhal Bp	20	-	-	-	-	-	-
Purulent Bp	21	1	1	-	2	1	1
Fibrinous Bp	28	2	16	2	15	2	16
Interstitial Pneumonia	18	-	2	-	-	-	2
Verminous Pneumonia	11	-	-	-	-	-	-
Adenomatous Pneumonia	2	-	-	-	-	-	-

Bp: *Bronchopneumonia,* ***IHC:*** *immunohistochemistry*

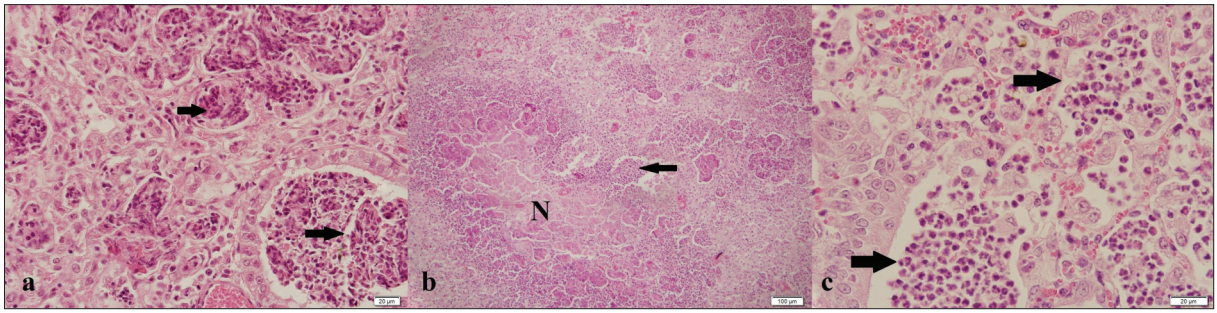


Fig 1. a) Oat cells *(arrows)* in the alveolar and bronchiolar lumens, bar = 50 µm, **b)** Necrosis (N) with numerous mononuclear cellular infiltration *(arrow)* in a case, bar = 100 µm, **c)** A large number of neutrophil granulocytes *(arrows)* in the alveolar and bronchiolar lumen in a case of purulent bronchopneumonia, bar = 20 µm, Hematoxylin-eosin

In fibrinous pneumonia cases, classical red and gray hepatisation phases were observed. Leucocyte infiltration in alveolar and bronchial lumens, hyperemia in alveolar capillaries, fibrin exudation in alveolar lumens and interlobular septa were seen in these cases. In 7 cases of fibrinous bronchopneumonia, spindle shaped leucocytes were also noted in the lumens of alveoli *(Fig. 1a)*. In 10 cases necrotic changes accompanied fibrinous lesions and were named as fibrinonecrotic bronchopneumonia. In these cases, foci of coagulation necrosis that were surrounded by inflammatory cellular infiltration were recognized *(Fig. 1b)*. In some cases, multifocal necrotic bronchitis and bronchiolitis were determined.

Purulent bronchopneumonia cases were characterized by predominant neutrophil infiltration in alveolar lumens *(Fig. 1c)*. In 6 cases, multifocal necrosis accompanied purulent lesions. In interstitial pneumonias, widening in interalveolar septa due to mononuclear cellular infiltration with occasional lymphocyte infiltration around the bronchia and bronchioles were seen.

Capillary hyperemia and neutrophil infiltrations in the alveolar lumen were detected in acute catarrhal bronchopneumonia cases. In the cases of interstitial pneumonia, thickness of the interalveolar septa and hyperplasia of bronchus, bronchiole and lymphoid tissue around the veins were striking. In adenomatous pneumonia cases, alveoli were placed with cubic epithelium and the papillary projections extending to the lumen was determined. In verminous pneumonia cases, hyperplasia in the smooth muscle layer of parenchymal pneumonia and bronchiolitis were seen. Granulomatous structures around the dead larvae and eggs have been identified.

Immunohistochemical Findings

Number of cases showing immunoreactivity for bacterial antigens was given in *Table 2*. Immunoreactivity for *M. haemolytica* antigens was detected in 17 out of 100 cases (17%). 15 animals with fibrinous bronchopneumonia showed positive immunoreactivity including all of the fibrinonecrotic bronchopneumonia cases. The ratio of animals with positive reaction within the total number of 28 fibrinous bronchopneumonia cases was 53.5%. Other 2 cases with positive immunoreactivity were seen in animals having purulent necrotic bronchopneumonia. This yielded a ratio of 9.5% within the purulent bronchopneumonia cases. Immunoreactivity against *M. haemolytica* antigens was observed in the cytoplasm of pneumocytes and the epithelial cells of bronchi and bronchioles, between the oat cells located in the alveoli, cytoplasm of macrophages, and the peribronchiolar glands *(Fig. 2a,b)*. Positive immunoreactivity was also noted around the foci of coagulation necrosis. In cases with necrotic purulent bronchopneumonia, positive immunoreactivity against *M. haemolytica* antigens were observed in the cytoplasm of leucocytes located in the lumens of alveoli, epithelial cells of bronchi and

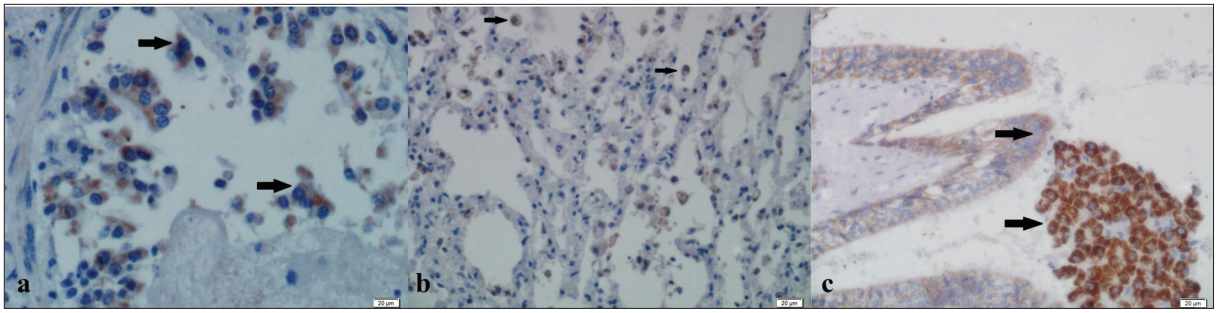


Fig 2. a) Immunopositivity against *M. haemolytica* antigens in spilled bronchiol epithelial cell cytoplasm (arrows), bar = 20 µm, **b)** Immunopositivity against *M. haemolytica* antigens in alveolar macrophage cytoplasm *(arrows),* bar = 20 µm, **c)** Immunopositivity against *P. multocida* antigens *(arrows)* in spilled and intact bronchial epithelial cell cytoplasm, bar = 20 µm, Immunohistochemistry

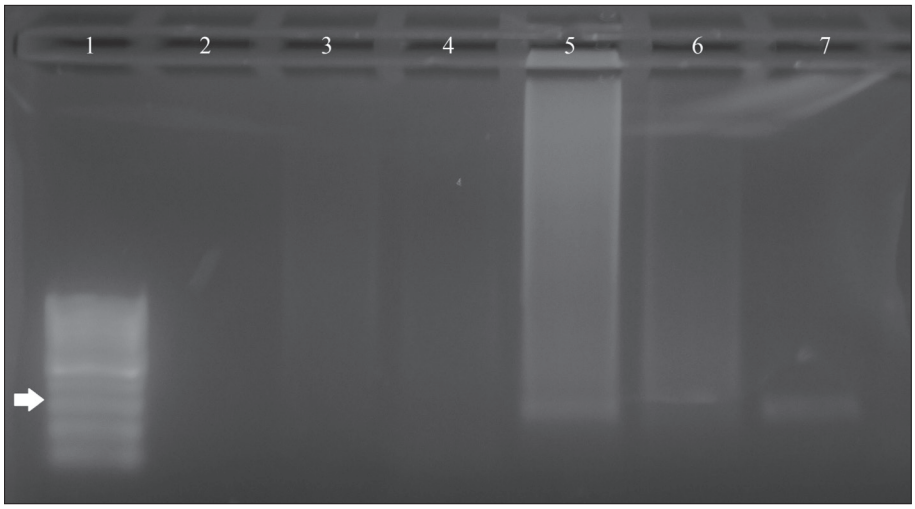
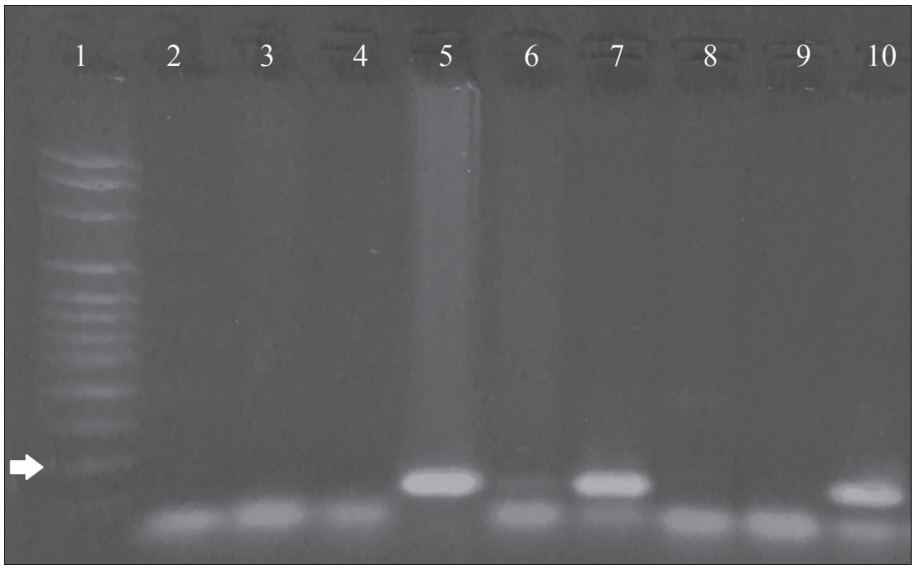


Fig 3. PCR analysis of *P. multocida* isolates from sheep showing 338 bp lanes for *toxA gene*. Lane 1: 100 bp DNA ladder, Lane 2: Negative control, Lane 3-6: Samples, Lane 7: Positive control

Fig 4. PCR analysis of *M. haemolytica* isolates from sheep showing 143 bp lanes for *sodA gene*. Lane 1: 100 bp DNA ladder, Lane 2: Negative control, Lane 3-9: Samples, Lane 10: Positive control



bronchioles, and around the necrotic foci. Positive immunoreactivity against *P. multocida* antigens was detected only in 2 cases with fibrinous bronchopneumonia. The ratio of positive immunoreactivity among the purulent bronchopneumonia cases was detected to be 7.1%. The immunostaining pattern for *P. multocida* antigens *(Fig. 2c),* in general, resembled to that of *M. haemolytica* antigens.

PCR Analysis

PCR amplification of *P. multocida toxA* and *M. haemolytica sodA* genes yielded DNA product bands of 338 bps and 143 bps, respectively. Out of 100 cases, 19 were detected to be positive for *M. haemolytica (Fig. 3)* and 3 for *P. multocida (Fig. 4)* with PCR analysis.

DISCUSSION

Pneumonia in sheep and lamb is one of the most important problems in breeding, and pneumonic pasteurellosis is the commonly recognized cause of pneumonia in small ruminants. *P. multocida* and *M. haemolytica* are the bacterial agents of the disease, and cause drop in yield and death, resulting significant economic losses [25,27]. These bacterial agents are also often reported in pneumonia cases in Turkey [8,15,18,19]. *P. multocida* and *M. haemolytica* are normally found in the nasopharynx and the upper respiratory system, and as the immune barrier of the respiratory system fails become active causing pneumonia. *M. haemolytica* induced pulmonary infections are mostly recognized with peracute-acute fibrinosuppurative and necrotic inflammatory response [1].

In studies performed in Turkey aiming the detection of pasteurella species in sheep pneumonia, *M. haemolytica* was recorded between 2.3% and 51.6% [7,17,34,35] while *P. multocida* was seen between 2.9% and 31.6% [7,16,35]. These ratios were seen to change greatly with the ratios of these bacteria detected around the world. Using different detection techniques, *M. haemolytica* was recorded to be between 4% and 98.7% [21-24], and *P. multocida* between 25% and 66.9% [22,23]. A variety of different detection tools, sample collection methods, geographical locations etc. might be in effect for different results besides the actual presence in a location. In the present study, *M. haemolytica* was detected in 19% while *P. multocida* was seen in only 3% of the cases. As compared to the previous investigations, isolation rate of the bacterial agents were in the low end of the scale, probably reasoning to the differences in housing conditions, climate, transportation factors, age, breed and the season studied.

Bacterial culture technique is still the golden standard for detection of *Pasteurella* species though some problems in the process of the technique such as long time requirement in isolation and identification and the need for some special transport media [36] are present. In the current investigation, out of 100 cases 19 were detected to have *M. haemolytica*, and 3 to *P. multocida*, by bacteriological culture technique. In comparison with the results of immunohistochemical investigation, in 1 case no bacterial agents were detected where immunopositive immunoreactivity was seen against *M. haemolytica* antigens in 2 cases of purulent necrotic bronchopneumonia. Sampling from the different areas of the lung samples might be the cause of this difference in the results.

PCR is a commonly used technique in molecular detection of infectious agents. It has high specificity and sensitivity in general compared to the other detection techniques. It has also been reported that PCR is more rapid and specific in determination of *P. multocida* and *M. haemolytica* directly from specimens compared to the standard technique of bacteriological culture [37]. In the current investigation, the bacterial agents were detected in total of 22 cases, from which the bacterial agents were also detected to be positive by bacteriological culture technique. Therefore the results of two detection methods were in complete agreement with each other.

In the present investigation, lung lesions in *M. haemolytica* caused pneumonia mostly showed cranioventral localization. Less frequently, both cranial and middle localization presenting lobar or lobular distribution was seen. In few cases, whole cranial and/or middle lobe or 1/3 of the caudal lobe was affected. This pattern of distribution in *M. haemolytica* caused pneumonia was in accordance with the previous reports [25,28,35].

The results of the immunohistochemical staining and the bacteriological culture technique were in great agreement, having 17 immunopositivity as compared to the 19 with bacteriological culture. The remaining two culture positive samples that were found IHC negative might be explained by the different sampling area and the focal accumulation of bacteria in the lungs. In the present study, *M. haemolytica* positive immunoreactivity was detected in 15 cases with fibrinous bronchopneumonia out of 28 cases. This result paralleled the results of previous investigations, indicating that *M. haemolytica* causes mostly fibrinous or fibrinonecrotic types of pneumonia [1,25]. It has also been noted that the localization of the bacterial antigens correlated the histopathological changes. In tissue sections, positive immunoreactivity against bacterial antigens was observed in the cytoplasm of leucocytes, pneumocytes, bronchial and bronchiolar epithelial cells, epithelial cells fallen off into the lumens of bronchia and bronchioles, around the necrotic areas, and among the oat cells. This immunolocalization pattern was in accordance with the previous investigations [25,27-29].

Immunohistochemical detection technique was reported to be quite useful in showing the bacterial agents of *M. haemolytica* and *P. multocida* [25-28]. In a study conducted by Hazıroğlu et al.[27], *M. haemolytica* was detected by immunohistochemistry in 68.3% of the lambs that showed signs of pneumonia. In a similar study performed by Bemani et al.[29] in Iran, 63.7% positivity was determined in sheep with bronchopneumonia. In this study, positive immunoreactivity against the bacterial antigens was reported in the surface and cytoplasm of epithelial cells, and the cytoplasm of macrophages, leucocytes, and the bronchial exudate. Özyıldız et al.[28] was also reported 31.8% *M. haemolytica* and 27.2% *P. multocida* immunopositivity in 110 sheep with pneumonia. They have indicated that the immunolocalization of both agents were similar; the lumen of alveoli, bronchia, and bronchioles, cytoplasm of leucocytes and the epithelial cells of bronchia and bronchioles, interstitium, lumen of vena, and peribronchial glands. In the current investigation lower rates of immuno-

positivity was detected as compared to the previous studies. On the other hand immunolocalization of the antigens were noted to be similar to those investigations.

Oat cells are commonly described in pneumonias induced by *M. haemolytica*, and was also seen in 7 cases showing fibrinous bronchopneumonia in the current investigation. These cells are known to be due to leucotoxins produced by the bacteria. Widespread foci of necrosis were also seen in 10 cases in the present study. These necrotic changes and the associated exudate are known to be caused by leucotoxins, lipopolysaccharides, and polysaccharides produced by the bacteria as well as the inflammatory substances produced by neutrophils and the other inflammatory cells [1,11,38].

In this study *M. haemolytica* and *P. multocida* were detected in sheep showing signs of pneumonia, and the findings were compared to the previous investigations. It was concluded that *M. haemolytica* is an important infectious agent causing lobar bronchopneumonia and even death in lambs and sheep. The agent is also involved in purulent pneumonia. It was also seen that bacteriological culture, immunohistochemical staining, and PCR techniques could be used in detection of the bacteria almost with same safety. However, immunohistochemistry could give the chance of determining the tissue distribution of the bacterial agents, and hence could be used in routine diagnosis.

REFERENCES

1. Ackerman MR, Brogden KA: Response of ruminant respiratory tract to *Mannheimia (Pasteurella) haemolytica*. *Microbes Infect*, 2 (9): 1079-1088, 2000. DOI: 10.1016/S1286-4579(00)01262-4

2. Barbour EK, Nabbut NH, Hamadeh SK, Al-Nakhli HM: Bacterial identity and characteristics in healthy and unhealthy respiratory tracts of sheep and calves. *Vet Res Commun*, 21 (6): 421-430, 1997. DOI: 10.1023/A:1005855318665

3. Davies DH: Aetiology of pneumonia of young sheep. *Preg Vet Microbiol Immun*, 1, 229-248, 1985.

4. Özen H, Karaman M, Şahin M, Özcan K: Pnömonili Sığırlarda *Mycoplasma bovis*, *M. dispar*, *M. bovirhinis* ve *M. mycoides* subsp. *mycoides* (küçük koloni tipi)'in PZR ile belirlenerek patolojik bulguların incelenmesi. *Kafkas Univ Vet Fak Derg* 15 (1): 125-133, 2009. DOI: 10.9775/kvfd.2008.108-A

5. Binns SH, Cox IJ, Rizvi S, Gren LE: Risk factors for lamb mortality on UK sheep farms. *Prev Vet Med*, 52 (3-4): 287-303, 2002. DOI: 10.1016/S0167-5877(01)00255-0

6. Gökçe E, Erdoğan HM: Neonatal kuzularda pnömoni: Yaygınlığı ve etki eden kimi risk faktörleri. *Kafkas Univ Vet Fak Derg*, 14 (2): 223-228, 2008. DOI: 10.9775/kvfd.2008.58-A

7. Kaya O, Erganiş O: Koyun ve kuzu pnömonileri üzerinde etiyolojik survey. *Veterinarium*, 2 (3-4): 27-29, 1991.

8. Özbey G, Muz A: Pnömonili koyun ve keçilerin akciğerlerinden aerobik bakteri izolasyonları ve izole *Pasteurella multocida* ve *Mannheimia haemolytica*'nın polimeraz zincir reaksiyonu ile saptanması. *Turk J Vet Anim Sci*, 28, 209-261, 2004.

9. Mohamed RA, Abdelsalam EB: A review on pneumonic pasteurellosis (respiratory mannheimiosis) with emphasis on pathogenesis, virulence mechanisms and predisposing factors. *Bulg J Vet Med*, 11 (3): 139-160, 2008.

10. Quinn PJ, Markey B, Carter ME, Donelly WS, Leonard FC: Veterinary Microbiology and Microbial Disease. Blackwell Science, 137-143, 2002.

11. Ewers C, Luebke-Becker A, Wieler: *Mannheimia haemolytica* and the pathogenesis of enzootic bronchopneumonia. *Berl Munch Tieraerztl Wochensch*, 117, 97-115, 2004.

12. Milli UH, Hazıroglu R: Veteriner Patoloji. Cilt 2, s. 80-85. Tamer Matbaacılık, Ankara, 1997.

13. Catry B, Baele M, Opsomer G, de Kruif A, Decostere A, Haesbrouck F: tRNA- intergenic spacer PCR for the identification of *Pasteurella* and *Mannheimia* spp. *Vet Microbiol*, 98, 251-260, 2004. DOI: 10.1016/j.vetmic.2003.10.027

14. Sathiamoorthy S, Shewen PE, Hodgins DC, Lo RYC: *In vivo* gene expression in *Mannheimia haemolytica* A1 during a time-course trial in the ovine host. *Vet Microbiol*, 158, 163-171, 2012. DOI: 10.1016/j.vetmic.2012.01.033

15. Gürbüz A, Şahin M: Sığır ve koyunlara ait pnömonili akciğerlerden *Pasteurella haemolytica*'nın izolasyonu, identifikasyonu, biyotiplendirilmesi ve antibiyotiklere olan duyarlılıklarının belirlenmesi. *Kafkas Univ Vet Fak Derg*, 9 (2): 169-175, 2003.

16. Tel OY, Keskin O: Koyun akciğerlerinden *Pasteurella multocida* ve *Mannheimia haemolytica* izolasyonu ve antibiyotiklere duyarlılığı. *YYU Vete Fak Derg*, 21 (1): 31-34, 2010.

17. İlhan Z, Keleş İ: Biotyping and serotyping of *Mannheimia (Pasteurella) haemolytica* isolated from lung samples of slaughtered sheep in the Van Region. *Turk J Vet Anim Sci*, 31 (2): 137-141, 2007.

18. Solmaz H, İlhan Z: Pnöymonili koyun akciğerlerinden izole edilen *Mannheimia haemolytica* izolatlarının bazı antibiyotiklere *in vitro* duyarlılıklarının belirlenmesi. *Avkae Dergisi*, 1, 15-18, 2011.

19. Oruç E: The pathologic and bacteriologic comparison of pneumonia in lambs. *Turk J Vet Anim Sci*, 30, 593-599, 2006.

20. Beytut E, Otlu S, Sözmen M: Kars Bölgesi koyunlarında gözlenen pnömoniler üzerine patolojik ve etiyolojik incelemeler. *Kafkas Univ Vet Fak Derg*, 8 (1): 31-39, 2002.

21. Poulsen LL, Reiner TM, Rikke LS, Bisgaard M, Christensen H, Olsen JE, Stuen S, Bojesen AM: Occurrence of haemolytic *Mannheimia* spp. in apparently healthy sheep in Norway. *Acta Vet Scand*, 48, 19, 2006. DOI: 10.1186/1751-0147-48-19

22. Marru HD, Anijajo TT, Hassen AA: A study on ovine pneumonic pasteurellosis: Isolation and identification of *Pasteurellae* and their antibiogram susceptibility pattern in Haramaya District, Eastern Hararghe, Ethiopia. *BMC Vet Res*, 9, 23, 2013. DOI: 10.1186/1746-6148-9-239

23. Berhe K, Weldeselassie G, Bettridge J, Christley RM, Abdi RD: Small ruminant pasteurellosis in Tigray region, Ethiopia: Marked serotype diversity may affect vaccine efficacy. *Epidemiol Infect*, 145 (7): 1326-1338, 2017. DOI: 10.1017/S095026881600337X

24. Shanthalingam S, Goldy A, Bavananthasivam J, Subramaniam R, Batra SA, Kugadas A, Raghavan B, Dassanayake RP, Jennings-Gaines JE, Killion HJ, Edwards WH, Ramsey JM, Anderson NJ, Wolff PL, Mansfield K, Bruning D, Srikumaran S: PCR assay detects *Mannheimia haemolytica* in culture-negative pneumonic lung tissues of bighorn sheep *(Ovis canadensis)* from outbreaks in the western USA, 2009-2010. *J Wildl Dis*, 50 (1): 1-10, 2014. DOI: 10.7589/2012-09-225

25. Yener Z, İlhan F, İlhan Z, Sağlam YS: Immunohistochemical detection of *Mannheimia (Pasteurella) haemolytica* antigens in goats with natural pneumonia. *Vet Res Commun*, 33, 305-313, 2009. DOI: 10.1007/s11259-008-9178-z

26. Haritani M, Nakazawa M, Oohashi S, Yamada Y, Hazıroğlu R, Narita M: Immunperoxidase evaluation of pneumonic lesions induced by *P. haemolytica* in calves. *Am J Vet Res*, 48, 1358-1362, 1987.

27. Hazıroğlu R, Diker KS, Turkarslan J, Gülbahar MY: Detection of *Mycoplasma ovipnomoniae* and *Pasteurella haemolytica* antigens by an immunoperoxidase technique in pneumonic ovine lungs. *Vet Pathol*, 33 (1): 74-76, 1996. DOI: 10.1177/030098589603300108

28. Özyıldız Z, Tel OY, Yılmaz R, Yurdagül Özsoy Ş, Keskin O: Pathological and microbiological investigations of pneumonic Pasteurellosis in sheep. *Kafkas Univ Vet Fak Derg*, 19 (1): 103-108, 2013. DOI: 10.9775/kvfd.2012.7281

29. Bemani E, Esmaeilzadeh S, Gharibi D, Ghorbanpoor M: Immuno-

histochemical and bacteriological investigations of *Mannheimia haemolytica* in sheep bronchopneumonia. *Kafkas Univ Vet Fak Derg*, 23 (1): 7-14, 2017. DOI: 10.9775/kvfd.2016.15679

30. Quinn PJ, Carter ME, Markey B, Carter GR: Clinical Veterinary Microbiology, Wolfe Publication, London, UK, 1999.

31. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC: Color Atlas and Textbook of Diagnostic Microbiology, 5th edn., Lippincott Williams & Wilkins, Philadelphia, 1997.

32. Kamp EM, Bokken GCAM, Vermeulen TMM, deJong MF, Buys HECM, Reek FH, Smits MA: A specific and sensitive PCR assay suitable for large-scale detection of toxigenic *Pasteurella multocida* in nasal and tonsillar swabs specimens of pigs. *J Vet Diagn Invest*, 8 (3): 304-309, 1996. DOI: 10.1177/104063879600800305

33. Guenther S, Schierack P, Grobbel M, Lübke-Becker A, Wieler LH, Ewers C: Real-time PCR assay for the detection of species of the genus Mannheimia. *J Clin Microbiol*, 75 (1): 75-80, 2008. DOI: 10.1016/j.mimet.2008.05.008

34. Kırkan Ş: Aydın Yöresinde Koyunların Solunum Sisteminde İnfeksiyon Nedeni *Mannheimia (Pasteurella) haemolytica*'nın Biyotip ve Serotip Tayini, Elektroforez ve PCR İle Tanısı. *Doktora Tezi,* Adnan Menderes Üniv. Sağlık Bil. Enst., 2003.

35. Hazıroğlu R, Diker KS, Gülbahar MY, Akan M, Güvenc T: Studies of the pathology and microbiology of pneumonic lungs of lambs. *DTW*, 101, 441-443, 1994.

36. Townsend KM, Hanh TX, Boyle DO, Wilkie I, Phan TT, Wijewardana TG, Trung NT, Frost AJ: PCR detection and analysis of *Pasteurella multocida* from the tonsils of slaughtered pigs in Vietnam. *Vet Microbiol*, 72, 69-78, 2000. DOI: 10.1016/S0378-1135(99)00188-1

37. Kılıç A, Muz A: Pnömonili sığır akciğerlerinden bakteri izolasyonları ve izole Pasteurella'ların Polimeraz Zincir Reaksiyonu ile saptanması. *Turk J Vet Anim Sci*, 28, 217-223, 2004.

38. Slocombe RF, Malark J, Ingersoll R, Derksen FJ, Robinson NE: Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. *Am J Vet Res*, 46, 2253-2258, 1985.