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Research Article

Immunohistochemical and Bacteriological Investigations of *Mannheimia haemolytica* in Sheep Bronchopneumonia

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Abstract

Mannheimia haemolytica infection is one of the most common etiologic agents of sheep pneumonia almost all over the world. Ovine pneumonic Mannheimiosis is characterized by severe fibrinous pleuropneumonia. Subacute to chronic cases progress to purulent bronchopneumonia and its squeals include abscessation and fibrous pleural adhesions. In the present study, lungs of 8986 sheep were inspected grossly in the Ahvaz abattoir and totally 65 lungs with visible signs of bronchopneumonia were selected for pathological and bacteriological examinations. *Mannheimia haemolytica* antigens were detected in 63.07% of immunoperoxidase stained tissue sections while 52.30% of the lungs were positive in bacteriological culture. Suppurative, necrotic and fibrinous types of bronchopneumonia were the most abundant lesions and right cranial lobes, specifically their cranial portions, were the most affected areas. McNemar test showed a significant difference between the diagnostic power of immunohistochemistry (IHC) and bacterial culture in detection of *M. haemolytica* (κ=0.66). Considering IHC as a golden test, sensitivity and specificity of bacterial culture were estimated as 78.05 and 91.67%, respectively. Chi- squared test showed significant correlations between the distribution of the lesions and bacterial isolation (P=0.04), types of lesions and IHC results (P=0.01), and also types of bronchopneumonia and mixed/pure isolation (P=0.008). This study showed the significant role of *Mannheimia haemolytica* in causing pneumonic lesions of studied sheep.

Keywords: *Immunohistochemistry, Mannheimia haemolytica, Sheep, Lung, Bacteriology*

Koyun Bronkopnömonilerinde *Mannheimia haemolytica*'nın İmmunohistokimyasal ve Bakteriyolojik İncelenmesi

Özet

Mannheimia haemolytica enfeksiyonu tüm dünyada koyun pnömonilerinin en yaygın etiyolojik etkenidir. Ovine pnömonik mannheimiozis fibrinli pleuroplnömoni ile karakterizedir. Subakut ve kronik vakalarda purulent bronkopnömoni gelişir ve takibinde abseleşme ve fibrinli plöral yapışma şekillenir. Bu çalışmada, 8986 koyuna ait akciğer Ahvaz kesimevinde makroskopik olarak incelendi ve görsel olarak bronkopnömoni tespit edilen toplam 65 akciğer patolojik ve bakteriyolojik inceleme için seçildi. *Mannheimia haemolytica* antijenleri immunperoksidaz boyalı doku kesitlerinde %63.07 oranında belirlenirken örneklerin %52.30'u bakteriyolojik kültür ile pozitif olarak tespit edildi. Suppuratifi nekrotik ve fibrinli tipteki bronkopnömoniler en yaygın belirlenen lezyonlar iken sağ kranial loblar ve özellikle de onların kranial lopları en çok etkilenen bölgeler olarak belirlendi. McNemar testi immunohistokimya (İHK) ile bakteriyolojik kültür yöntemlerinin *M. haemolytica* (κ=0.66) etkenini tespit etme güçleri arasında anlamlı bir fark olduğunu gösterdi. İHK yöntemi altın test olarak düşünüldüğünde bakteriyolojik kültür yönteminin özgüllüğü ve özgünlüğü sırası ile %78.05 ve %91.67 olarak belirlendi. Ki-kare testi lezyonların yayılımı ile bakteriyal izolasyon arasında (P=0.04), lezyon tipi ile IHK sonuçları arasında (P=0.01) ve bronkopnömoni tipi ile miks/saf izolasyon arasında (P=0.008) ilişki olduğunu gösterdi. Bu çalışma incelenen koyunlarda pnömonik lezyonların şekillenmesinde *Mannheimia haemolytica*'nın önemli rol oynadığını göstermektedir.

Anahtar sözcükler: *İmmunohistokimya, Mannheimia haemolytica, Koyun, Akciğer, Bakteriyoloji*

INTRODUCTION

Bronchopneumonia is the most common type of pneumonia in domestic animals [1], causing great economic losses in lambs [2]. With few exceptions, it is characterized by the cranioventral consolidation of the lungs [1]. Several infectious agents such as ovine respiratory syncytial virus, *Mannheimia haemolytica* and *Pasteurella multocida* have

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been isolated from bronchopneumonia in small ruminants. Generally bronchopneumonia is usually caused by two or more infectious agents working together, but some agents can also cause a significant disease alone [2]. As a rule, the causing agents arrive in the lungs via inhaled air, either from infected aerosols or from the nasal flora [1].

Mannheimia haemolytica is one of the most important bacterial agents of sheep pneumonia [3,4] and is involved in ovine pneumonic Mannheimiosis, septicemia in young lambs, ovine enzootic pneumonia, and sporadic severe gangrenous mastitis in ewes. In pneumonic form, lesions are characterized by severe fibrinous pleuropneumonia. Subacute to chronic cases progress to purulent broncho-pneumonia and its sequels may include abscessation and fibrous pleural adhesions. In contrast to ovine pneumonic Mannheimiosis, chronic enzootic pneumonia, causes only a mild to moderate pneumonia and is rarely fatal [1].

Immunoperoxidase technique (IPT), by clear visualization of antigens, is a preferred method for determining a correlation between histopathological findings, causative organisms, and their location in tissues. Since the identification of *M. haemolytica* with bacterial culture is often difficult in some situations (antibiotic therapy, frozen and autolytic samples, fixed tissue, etc.), immunohistochemical analyses for detecting *M. haemolytica* antigens can be employed to overcome many problems associated with this method [5].

This study was performed to investigate the diagnostic capabilities of IPT and bacterial isolation for the detection of *M. haemolytica* in bronchopneumonic lungs of slaughtered sheep in Ahvaz, southwest of Iran.

MATERIAL and METHODS

Sample Collection and Pathology

Lungs of 8986 sheep were inspected grossly for 4 months (from February to May 2011) in the Ahvaz abattoir and 65 lungs with visible signs of bronchopneumonia were collected for pathologic, bacteriologic, and immuno-histochemical studies. Tissue samples were taken from the affected areas and were fixed in 10% neutral buffered formalin. After routine tissue processing and paraffin embedding, 5 µm thick sections were routinely cut and stained with haematoxylin and eosin for histopathological investigation.

Bacterial Culture and Identification

According to the conventional method, sterile swab samples were taken aseptically from the deep areas of the lesions and cultured on blood and McConkey agar plates. Plates were incubated aerobically at 37°C and examined for growth of bacteria. Characterization of suspected bacterial isolates to *Pasteurella, Mannheimia* and *Bibersteinia* was carried out using classic methods based on bacterial morphology, biochemical tests, and reference tables [6-8]. *M. haemolytica* (local isolate) was used as a positive control for identification procedures.

Preparation of anti-M. haemolytica Polyclonal Antibody

Polyclonal antibody against *M. haemolytica* was prepared with immunization of 2 rabbits by intramuscular administration of 2 ml *M. haemolytica* inoculum prepared with Freund's adjuvant. Immunization was done six times in two weeks intervals [9]. Blood samples were taken two weeks following the last injection. The collected sera samples were evaluated for antibody titer against *M. haemolytica* (local isolate) and cross-reaction with antigenically closest to Pasteurellaceae *(Pasteurella multocida, Bibersteinia trehalosi)* and *E. coli* (representative of gram negative bacteria) by microagglutination, Dot-ELISA and indirect IPT [10,11]. In order to eliminate cross-reactivity of the polyclonal anti-body, it was diluted and adsorb with the above bacteria (whole cell antigens) for 1 h. The mixture was then centrifuged at 4000 rpm for 20 min and the supernatant was evaluated again for probable cross-reactivity.

Indirect Immunoperoxidase Tests

Tissue sections, 3 µm thick, were deparaffinized and rehydrated and antigen demasking was carried out using commercial solution (Target retrieval solution, S1699, DAKO, USA) at 97°C for 20 min [12]. Sections were rinsed in Tris buffer (pH 7.6) before endogenous peroxidase activity was blocked using 3% hydrogen peroxide in absolute methanol for 30 min. A further rinse in tap water for 10 min was followed by application of commercial blocking solution (Protein block, serum free, X0909, DAKO, USA) for 10 min at room temperature. Treatment for 1 h with the primary antibody (rabbit polyclonal anti *M. haemolytica)*, at room temperature and at a 1/200 dilution followed. Sections were then washed three times (5 min each) in Tris buffer before incubation at room temperature for 1 h with secondary antibody (Goat anti-rabbit IgG, HRPO-Conjugated, A9169, Sigma- Aldrich, USA) at 1/800 dilution. Further rinsing for three times (5 min each) in Tris buffer was followed by peroxidase development using DAB (D5905, Sigma-Aldrich, USA) in 1 h and sections were counterstained with Mayer's haematoxylin [10].

As a negative control, a normal sheep lung tissue (based on negative bacteriological and histological findings) was used. For serum control, replicate sections of the pneumonic lungs were processed, substituting the primary antibody with the rabbit pre-immune serum. This serum was free of antibodies against *M. haemolytica, P. multocida, B. trehalosi* and *E. coli* based on the results of Dot ELISA.

Statistical Analysis

McNemar test and kappa coefficient were used to compare the diagnostic power and the agreement of the two diagnostic methods, respectively. Chi square or Fisher

exact tests were also used to evaluate the data distribution. Results are shown using *P*-values with 5% level of significance. All analysis was performed using SAS software (SAS, 9.1).

RESULTS

Pathological Findings

Grossly, the common feature of the selected lungs was the various degrees of consolidation affecting the cranioventral lobes. In 21 cases, consolidation was seen exclusively in the right lung. The lesion in one case was restricted to the left lung and in 42 cases it included both the left and the right lungs. In one case, consolidation was observed in the whole lung. Among the lobes, affected regions were frequently observed in the right cranial lobe, especially in its cranial portion (57 cases). In the cut surfaces, at least one of the following manifestations was noted: multiple irregular pale necrotic foci with 2-7 mm diameter *(Fig. 1)*, grey nodules 1-4 mm in diameter, gelatinous thickening of interlobular septa and suppurative or mucous exudate in the bronchi.

Histologically, suppurative bronchopneumonia was observed in 44 cases, followed by necrotic bronchopneumonia (9 cases), fibrinous bronchopneumonia (8 cases), ovine pulmonary adenocarcinoma (6 cases), bronchointerstitial pneumonia (1 case), and pyogranulomatous pneumonia (1 case).

Suppurative bronchopneumonia was categorized into 3 subtypes; acute, subacute and chronic. Filling of the airspaces (bronchi, bronchioles and alveoli) by infiltrated leukocytes, mainly neutrophils, was a common feature of 3 subtypes, especially the acute ones (10 cases). The microscopic features of OPA (papillary hyperplasia of pneumocytes type II and the Clara cells) was detected in 2 of acute suppurative bronchopneumonic lungs. In 12 subacute suppurative pneumonic cases, there was epithelial hyperplasia of some bronchioles and increasing number of infiltrated mononuclear leukocytes such as macrophages within the airspaces. Chronic suppurative bronchopneumonia (22 cases) was characterized by peri-airways lymphoid hyperplasia, bronchial squamous metaplasia and bronchiolar goblet cell metaplasia.

Necrotic bronchopneumonia was characterized by irregular areas of coagulative necrosis surrounded by a dense zone of necrotic leukocytes, specially neutrophils. Heavy infiltration of neutrophils and/or deposition of fibrin were also seen within the adjacent bronchioles and alveoli. This type of pneumonia was observed in 1 specimen along with OPA.

In fibrinous bronchopneumonia, fibrin was the predominant component of the filling exudates of the airspaces. Hyperemia, dilation of lymphatic vessel, infiltration of neutrophils, and fibrin deposition were common findings in the interlobular septa and the pleura. In one of the affected lungs, the localized chronic suppurative bronchopneumonia was also seen.

Bacteriological Findings

The culture result of the 65 specimens showed bacterial isolation in 62 specimens (95.38%). Because of mixed infections in some specimens, the total number of the isolate increased to 92. The most isolated bacteria were *M. haemolytica* (34; 52.30%) and then *P. multocida* and *B. trehalosi* (12; 18.46%). *M. haemolytica,* that was isolated from 34 lungs, grew in 20 lungs as pure and mixed with other bacteria in 14 specimens *(Table 1)*. *Table 1* shows other bacteria isolated with a low frequency in the lungs.

Immunohistochemical Findings

The presence of *M. haemolytica* or its antigens were detected in 41 (63.07%) of 65 lung specimens. IHC positive

Fig 1. Cut surface of left cranial lobe of an affected lung showing sharply demarcated irregular shaped foci of necrosis *(asterisks)* within consolidated region

Şekil 1. Enfekte bir akciğere ait sol kranial lobun kesit yüzeyinde konsalide bölge içerisinde yer alan keskin kenarlı düzensiz şekilli nekroz odakları *(yıldızlar)*

Table 1. *Bacteriological, histopathological and immunohistochemical findings in 65 suspected bronchopneumonic lungs of sheep*
Tablo 1. *Bronkopnömoni şüpheli 65 koyun akciğerine ait bakteriyolojik, histopatolojik ve immunohistokimyasal bulgular*

Lesion	Count	Culture positive		IHC positive		Isolated bacteria of IHC negative lungs										
		Pure	Mixed	Mild	Severe	B. t	P. m	P sp A	M. h	C. s	St. a	St. d	St.	Pr. v	En. a	N. G.
Chronic suppurative bronchopneumonia	22	9	4	15	0	3	1	1	1	0	1	1	0	0	1	1
Subacute suppurative bronchopneumonia	12	6	2	9	0	0	1	2	0	0	0	0	0	0	0	0
Acute suppurative bronchopneumonia	10	4	2	5	0	1	1	1	1	2	0	0	1	0	0	1
Necrotic bronchopneumonia	9	0	4	0	8	1	0	0	0	0	0	0	0	0	0	0
Fibrinous bronchopneumonia	8	0	2	3	0	1	2	0	0	0	0	0	0	1	0	1
Bronchointerstitial pneumonia	1	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Total	62	20	14	33	8	6	5	4	2	2	1	1	1	1	1	3

P. m: *Pasteurella multocida*, ***B.t:*** *Bibersteinia trehalosi*, ***St.:*** *Streptococcus spp.*, ***P. sp A:*** Pasteurella species A, ***St.a:*** *Streptococcus agalactiae*, ***C.s:*** *Corynebacterium pseudotuberculosis*, ***Pr.v:*** *Proteus vulgaris*, ***St.d:*** *Streptococcus dysgalactiae*, ***En.a:*** *Enterobacter aerogenes*, ***N.G.:*** no growth bacteria

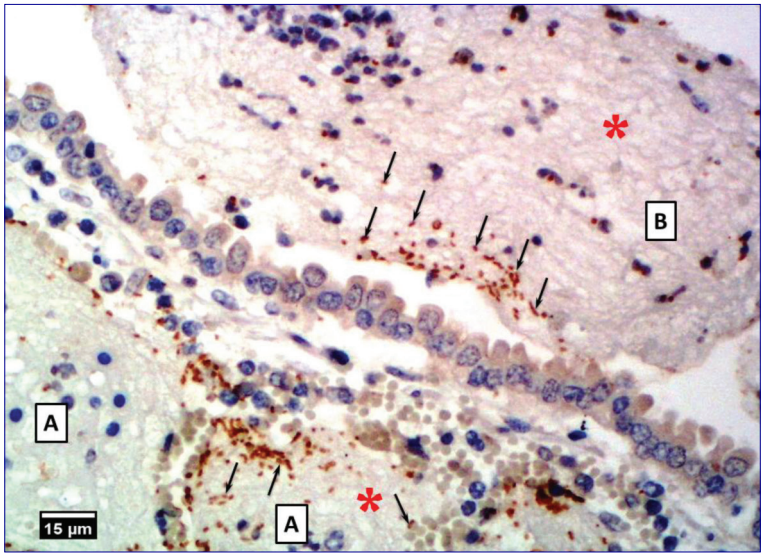


Fig 2. Positive immunostaining of *M. haemolytica (large arrows)* within fibrinous exudate *(asterisks)* in a bronchiole (B) and adjacent alveoli (A), Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 2. Bronşiol (B) ve bitişiğindeki alveollerde (A) fibrinli eksudat içerisinde *(yıldızlar) M. haemolytica (büyük oklar)* pozitif immunboyanma, İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

cases were divided into 2 groups based on the intensity of immunoreactivity.

Group 1 which contained 8 specimens (19.51%) was identified by a strong reaction against the antigen in necrotic foci and surrounding areas. The reaction was restricted to fibrinous exudate *(Fig. 2)*, around necrotic cells *(Fig. 3)* and inside surrounding neutrophils and macrophages. In other areas, the presence of the bacteria and antigens were similar to Group 2.

Group 2 (33 specimens; 80.48%) was typified by weak diffuse reaction against the antigen, especially in the exudate or inflammatory cells within the airways or alveoli. The same reaction was seen within or on the luminal surface of the airway's epithelial cells *(Fig. 4)*.

In both groups, the bacterial antigens were seen in the cytoplasm of affected cells in mild, moderate, and severe patterns. In the mild pattern, the antigens were observed in the cytoplasm as multiple large and small inclusions especially in large macrophages *(Fig. 5)*. In the moderate pattern, the cytoplasm had been occupied mainly by the antigens in which nucleus and only the rim of the cytoplasm were observable. In the severe pattern, the cells were completely filled with the antigens *(Fig. 6)*.

Comparison of Bacteriological and IHC Results

McNemar test showed a significant difference between diagnostic power of IHC and bacterial culture in the detection of *M. haemolytica* (The Kappa correlation coefficient was 0.66). *Table 2* demonstrates a correlation between frequency distribution of samples detected by both methods. Considering IHC as a golden test, sensitivity

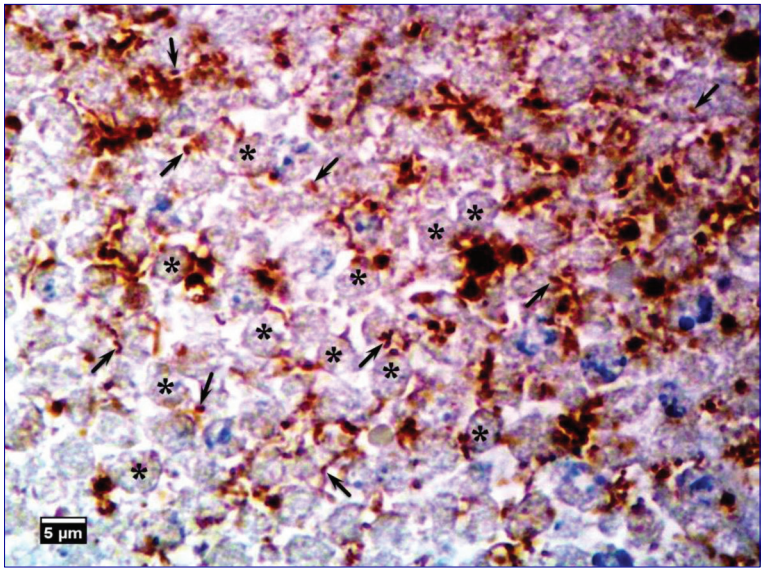
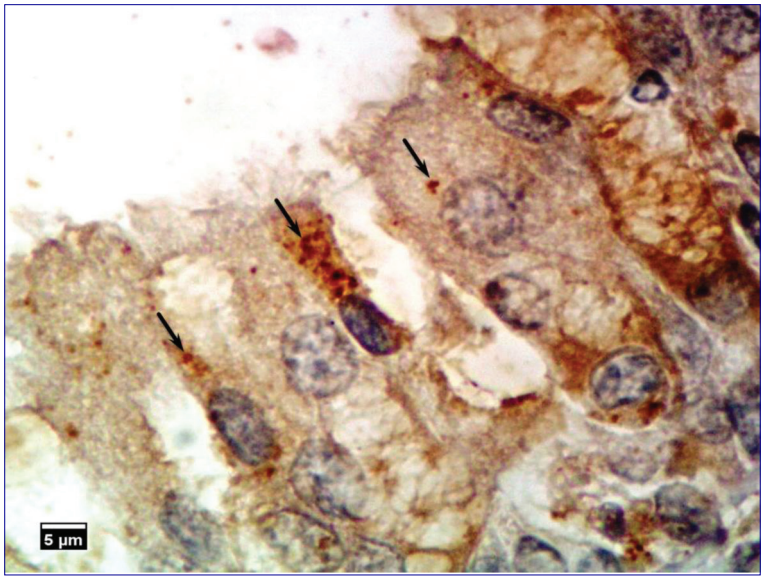


Fig 3. Immunopositive staining of *M. haemolytica (arrows)* around necrotic cells *(asterisks)*. Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 3. Nekrotik hücrelerin *(yıldızlar)* çevrelerinde *M. haemolytica* immunpozitif boyanma *(oklar)*. İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

Fig 4. Positive immunostaining of *M. haemolytica* antigens *(arrows)* in some bronchial epithelial cell, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 4. Bazı bronşiol epitel hücrelerinde *M. haemolytica* pozitif immunboyanma *(oklar),* İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası



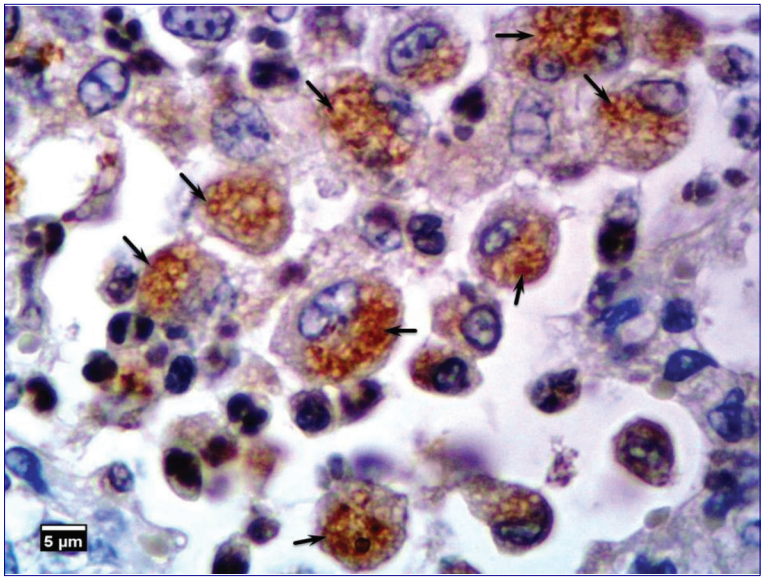


Fig 5. Mild pattern of immunostaining of *M. haemolytica* antigens *(arrows)* in large macrophages within an alveolus, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 5. Bir alveol içerisinde büyük makrofajlarda *M. haemolytica* antijenlerinin orta dereceli immunboyanması *(oklar),* İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

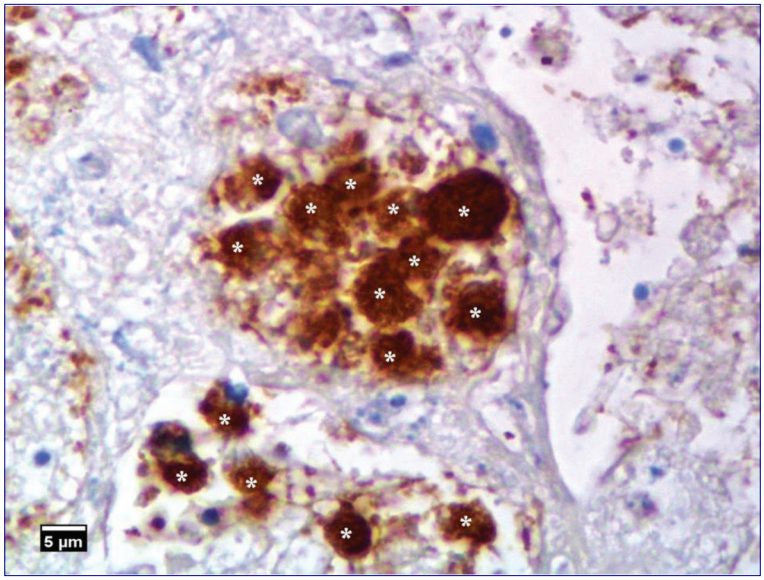


Fig 6. Intense pattern of immunostaining of *M. haemolytica* antigens and filling of macrophages *(asterisks)* within alveoli, Immunoperoxidase test, Mayers haematoxylin counterstain

Şekil 6. Alveol içerisinde makrofajlarda *M. haemolytica* antijenlerinin yoğun dereceli immunboyanması *(yıldızlar)*, İmmunperoksidaz testi, Mayers hematoksilen karşıtlık boyası

Table 2. *Frequency (percentage) of positive and negative samples in IHC or culture methods*
Tablo 2. *İHK veya kültür metodlarında pozitif ve negative örneklerin sıklığı (yüzde olarak)*

Culture	IHC		
	Positive n (%)	Negative n (%)	Total n (%)
Positive	32 (49.23)	2 (3.08)	34 (52.31)
Negative	9 (13.85)	22 (33.85)	31 (47.69)
Total	41 (63.08)	24 (36.92)	65 (100)

Table 3. *The frequency (percentage) of pulmonary lesions and IHC detection of M. haemolytica*
Tablo 3. *Pulmoner lezyonların ve İHK ile M. haemolytica tespit edilebilme sıklıkları (yüzde olarak)*

Lesion	IHC Results		
	Positive n (%)	Negative n (%)	Total n (%)
Suppurative bronchopneumonia	29 (46.03)	14 (22.22)	43 (68.25)
Necrotic bronchopneumonia	8 (12.70)	1 (1.59)	9 (14.29)
Fibrinous bronchopneumonia	3 (4.76)	5 (7.94)	8 (12.70)
OPA	0	3 (4.76)	3 (4.76)
Total	40 (63.49)	23 (36.51)	63 (100.00)

and specificity of bacterial culture were estimated as 78.05 and 91.67%, respectively.

In the Chi-square test, significant correlations were observed between the distribution of the lesions and the bacterial isolation (P=0.04), types of the lesions and IHC results (P=0.01) as well as the types of bronchopneumonia and mixed/pure isolation (P=0.008) *(Table 3, Table 4)*. In the statistical comparison, the dominant form of bronchopneumonia was compared. The concomitant and low frequent lesions have not been included.

DISCUSSION

In this study, *M. haemolytica* was isolated from 52.3% of samples, while its antigens were detected in 63.7% of lungs by IHC. These results demonstrate a significant difference between bacteriological and immunohistochemical detection of *M. haemolytica*. Such results have been reported in other studies that confirmed preference of immunohistochemistry compared to bacterial culture [5,13,14]. Also, a significant difference was observed between the diagnostic power of IHC and culture in detection of *M. haemolytica* (P=0.03) and an acceptable coefficient of agreement was seen between the results κ) = 0.66), which has not been implied in earlier studies.

Table 4. *The frequency (percentage) of pulmonary lesions and isolation of M. haemolytica*
Tablo 3. *Pulmoner lezyonların ve M. haemolytica izolasyonlarının sıklıkları (yüzde olarak)*

Lesion	Kind of Isolation			
	Negative n (%)	Pure n (%)	Mixed n (%)	Total n (%)
Suppurative bronchopneumonia	17 (27.87)	19 (31.15)	8 (13.11)	44 (72.13)
Necrotic bronchopneumonia	5 (8.20)	0	4 (6.56)	9 (14.75)
Fibrinous bronchopneumonia	6 (6.84)	0	2 (3.28)	8 (13.11)
Total	28 (45.90)	19 (31.15)	14 (22.95)	61 (100.00)

M. haemolytica was not isolated from 9 IHC positive lungs in which 4 samples had necrotic bronchopneumonia (with strong positive reaction) and the others (with weak positive reaction) had chronic suppurative (3 cases), subacute suppurative and fibrinous bronchopneumonia (1 case each). The identification of *B. trehalosi* from 2 lungs with necrotic lesions as a specific sign of Mannheimiosis [4,15], and not the isolation of *M. haemolytica*, may be explainable with the results of Dassanayake et al.[16]. They proved *B. trehalosi*, due to higher growth rate, inhibits the growth of *M. haemolytica in vitro* and concluded that if these patterns occur *in vivo,* they may cause failure to routinely isolate *M. haemolytica* from pneumonic lungs. Negative culture results in the weak IHC lungs may be due to the low concentration of bacteria or dead bacteria in the tissues examined [5]. The negative IHC results of two bacteriologically positive lungs may be explained by different sampling location for both methods as well as the focal accumulation of bacteria in the lungs [5,17].

Bacterial culture of one IHC negative lung with necrotic lesions was negative for *M. haemolytica* and positive for *B. trehalosi*. It was proved that necrotic lesions in pneumonic sheep can be induced by other bacteria such as *Histophilus somni*, *Trueperella pyogenes* [18] or *B. trehalosi* [4]. Although *B. trehalosi* is a known cause of septicemia in lambs over 5 months [1], it has been isolated from pneumonic lungs [19]. Sasani et al.[20] isolated the bacterium from fibrinous pneumonic lung of a dead lamb with coagulative necrosis (without signs of septicemia). Also in an experimental study by Onderka and Wishart [21], *B. trehalosi (Pasteurella haemolytica* biotype T) has been isolated from the big horn sheep lungs with necrotizing fibrinopurulent broncho-pneumonia.

In the present study, 0.68% of all examined lungs had bronchopneumonia in which suppurative, necrotic and fibrinous types were recognized in 67.69, 13.85 and 12.31% of the selected lungs, respectively. In a retrospective study by Oruc [3], *M. haemolytica* was isolated from 56.14% of the lungs and the most frequent isolation was reported from fibrinous pneumonia (42.19%) and catarrhal-purulent bronchopneumonia (15.63%). In our study, the broncho-pneumonic IHC positive lungs were mostly suppurative (46.03%) and the necrotic and fibrinous bronchopneumonia were in the next ranking (P=0.01). In addition, most chronic and some subacute suppurative bronchopneumonic lungs had known features of chronic enzootic pneumonia: bronchiolar epithelial metaplasia, bronchial epithelial hyper-plasia and peri-bronchiolar lymphoid hyperplasia [19,22,23]. The etiology of the chronic enzootic pneumonia is complex. The disease is often subclinical [19] and has devastating effects on animal growth rate and food conversion ratio [24]. *Mycoplasma ovipneumoniae*, *M. haemolytica* and some viruses are known to cause the disease [19]. Sheehan et al.[25], identified the sign of the chronic enzootic pneumonia in 60% of suspected pneumonic lambs but in 90% of them, isolation of *Mycoplasma ovipneumoniae* or IHC detection of its antigen was reported. In this study, *M. haemolytica* was isolated from 30% of lungs, especially with purulent lesions. Due to observation of the largely intact infiltrated leukocytes and the lobular pattern of lesions, the authors concluded that the role of *M. haemolytica* in the pathogenesis of chronic enzootic pneumonia appears significantly different from acute pneumonic pasteurellosis. Therefore, because of having no following *Mycoplasma* and the pneumonic viruses in our study, we cannot ignore the probability occurrence of chronic enzootic pneumonia in some animals. In other words, isolation of the bacteria from chronic and/or subacute purulent bronchopneumonic lungs with weak immunoreaction may support a secondary role for *M. haemolytica* in this type of pneumonia. Some authors believe that although mycoplasmas can interfere with host ciliary activity and therefore provide the invasion of other pathogens such as *M. haemolytica* [23], they may also modulate growth or toxin production of *M. haemolytica* [19].

In this study, a significant association was observed between *M. haemolytica* isolation (pure or mixed) and the types of bronchopneumonia. In acute bronchopneumonia, particularly fibrinous and necrotic, *M. haemolytica* was isolated mixed, and in more chronic lesions, it was isolated pure. *M. haemolytica* is a single cause of ovine pneumonic Mannheimiosis [1] and infection with the pneumonic viruses can increase the severity of the disease. However, the role of other ubiquitous opportunistic bacteria in ruminants' population [2] in reducing the resistance of animals to the *M. haemolytica* challenges and facilitating rapid proliferation and descent of *M. haemolytica* into the lower respiratory tract and induction of fatal bronchopneumonia should not be ignored [26,27].

In summary, in the present study, *M. haemolytica* or its antigens were identified by either bacterial culture or IHC in 66.15% of the samples. The sheep lung has anatomical features (minor collateral ventilation and extensive inter-lobular septa) which limits its ability to resolve pneumonic episodes and the capacity to expel alveolar exudate [28]. Therefore, occurrence of chronic pneumonic lesions in ruminant is more frequent in sheep than other animals [4]. However, due to the high storage capacity of the lung, focal pneumonic lesions often remain clinically silent but the presence of inflammatory exudate (even mild) has disruptive effects on respiratory performance. Thus detection of 55.55% and 59.25% of *M. haemolytica* antigens in subacute or chronic lesions (suspected chronic enzootic pneumonia) and acute lesions (suspected respiratory Mannheimiosis), respectively showed the significant role of the bacteria in causing pneumonic lesions in the studied sheep.

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