Immunohistochemical and Molecular Detection of Mannheimia spp. and Pasteurella spp. in Sheep with Pneumonia in Kars Province - Turkey

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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% venous 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, and in Kars, Pasteurella multocida, although with lesser importance can cause pneumonia in this species.

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

Kars İlinde Pnömonil Koyunlarda Mannheimia spp. ve Pasteurella spp.’nin Immunohipstokimyasal ve Moleküler Tanılanması

Öz

Anahtar sözcükler: Mannheimia haemolytica, Pasteurella multocida, Pnömoni, Koyun, Immunohistokimyasal
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 [5-6]. 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 oronasopharynx, 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 fibrinouspulmonary 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 as 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 [8,26]. 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 antisera 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>
<thead>
<tr>
<th>Day Intervals</th>
<th>Applications</th>
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<tbody>
<tr>
<td>Day 0</td>
<td>Pre-immunisation bleed and initial antigen injection</td>
</tr>
<tr>
<td>Day 14-21</td>
<td>First antigen booster</td>
</tr>
<tr>
<td>Day 28-35</td>
<td>Second antigen booster</td>
</tr>
<tr>
<td>Day 35-42</td>
<td>Test bleed</td>
</tr>
<tr>
<td>Day 42-56</td>
<td>Third antigen booster</td>
</tr>
<tr>
<td>Day 49-66</td>
<td>Blood collection</td>
</tr>
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</table>
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 MgCl₂, 0.2 mM dNTPs, 0.3 M forward (5'-GGTACATGATGCTAGATCC-3') and reverse (5'-CCACAGGGTATATTCTGGAC-3') primers, and 10 U/mL Taq DNA polymerase. PCR cycling procedure were as follow; initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and elongation at 72°C for 2 min. A final elongation step was performed at 72°C 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 pheno- typic 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 pneumatic 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 broncho- pneumonia, fibrinous pneumonia, interstitial pneumonia, verminous pneumonia, and adenomatous pneumonia. Numbers of cases according to the histopathological evaluation were shown in Table 2.
In fibrinous pneumonia cases, classical red and gray hepaatisation 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

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**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**

<table>
<thead>
<tr>
<th>Pneumonia</th>
<th>Total Number of Cases</th>
<th>Bacteriology</th>
<th>IHC</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. multocida</em></td>
<td><em>M. haemolytica</em></td>
<td><em>P. multocida</em></td>
<td><em>M. haemolytica</em></td>
</tr>
<tr>
<td>Acute Catarrhal Bp</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Purulent Bp</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Fibrinous Bp</td>
<td>28</td>
<td>2</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Interstitial Pneumonia</td>
<td>18</td>
<td>-</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Verminous Pneumonia</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenomatous Pneumonia</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

*Bp: Bronchopneumonia, IHC: immunohistochemistry*
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 pneumatic 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 [21,28,30].

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 [1,23]. 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 Haziroğlu 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 investigations. It was concluded that \textit{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.

In this study \textit{M. haemolytica} and \textit{P. multocida} were detected in sheep showing signs of pneumonia, and the findings were compared to the previous investigations. It was concluded that \textit{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.

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