Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Histopathological, Immunohistochemical and Bacteriological Characterization of *Mycoplasma bovis* Pneumonia in Cattle

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ARTICLE HISTORY (16-013)

Received: January 21, 2016
Revised: February 25, 2016
Accepted: March 14, 2016
Published online: March 30, 2016

Key words:
Bacteriology
Cattle
Immunohistochemistry
M. bovis
Pneumonia

A B S T R A C T

The aim of this study was to determine the prevalence of *M. bovis* pneumonia in slaughtered cattle in Bursa region, to perform the histopathological investigation of these cases, to demonstrate the agent with bacteriological and immunohistological examination and to characterize the inflammatory response against the agent. A total of 1413 lungs were examined at slaughter houses and 136 lungs (9.63%) with signs of pneumonia were sampled. Ten pneumonic lungs from the department archive were also included in the study. Bacteriological and immunohistochemical examination revealed *M. bovis* as the cause of pneumonia in 39 animals. In the classification of pneumonia regarding the exudate and the anatomic pattern, the most common pneumonia type was fibrinopurulent bronchopneumonia, non-purulent broncho-interstitial pneumonia and necrotic-fibrinopurulent bronchopneumonia. Necrotic areas were observed in a total of 18 cases. The agent was demonstrated by immunohistochemistry in 24 animals, bronchi and bronchiole epithelium being the most commonly invaded histological structures. Immuno-histochemistry revealed T cell as the most prominent inflammatory cell in *M. bovis* pneumonia, thus supporting the role of cellular defense in the pathogenesis of this pneumonia in cattle.

To Cite This Article: Yilmaz R, Cangul IT, Onat K, Akkoc A, Ozyigit MO and Akdesir E, 2016. Histopathological, immunohistochemical and bacteriological characterization of *Mycoplasma bovis* pneumonia in cattle. Pak Vet J, 36(3): 316-321.

INTRODUCTION

Respiratory system problems, particularly pneumonia, are among the major health problems in livestock production and cause significant economic losses (Ahmad *et al*., 2014). An important proportion of pneumonias in cattle are caused by bacteria, where Mycoplasma play a significant role (Maunsell *et al*., 2011; Kurćubić *et al*., 2014). The prevalence of the disease is high in most parts of the world. In Europe where respiratory problems cause around 576 million Euros of economic losses annually, *M. bovis* is the culprit in one fourth of the cases (Nicholas and Ayling, 2003). *M. bovis* is often present in the upper respiratory tract of cattle without clinical disease. *M. bovis* can be isolated from lungs of cattle without lesions. Macroscopically, affected lung often contains multiple necrotic foci filled with dry yellow to white caseous material (Caswell *et al*., 2010). Interlobular septae can contain linear necrotic lesions. Extensive fibrosis is common, and necrotic sequestra can be present. Acute fibrinous to chronic fibrosing pleuritis occurs in some cases. Histologically, naturally occurring *M. bovis* pneumonia is characterized as subacute to chronic bronchopneumonia that can be suppurative and is usually necrotizing (Gagea *et al.,* 2006; Caswell *et al.,* 2010). Immunohistochemical (IHC) staining reveals large amounts of *M. bovis* antigen, especially at the periphery of lesions (Gagea *et al.,* 2006). Natural infection of *M. bovis* causes cranio-ventral consolidation of the lungs, with caseous or coagulative multifocal lesions, characterized by caseo-necrotic bronchopneumonia with peribronchiolar mononuclear cell cuffing (Khodakaram-Tafti and Lopez, 2004; Gagea *et al.,* 2006). Many naturally occurring cases of *M. bovis* pneumonia are a result of synergistic infections with bovine viral diarrhea virus and the bacterial pathogens *M. haemolytica*, *P. multocida* and *H. somnus* (Haines *et al.,* 2001; Radaelli *et al.,* 2008). The aim of this study was to demonstrate the existence of *M. bovis*, and to define the pathological patterns and the inflammatory response in the lungs of slaughtered cattle.

MATERIALS AND METHODS

The lung tissues examined in the study were collected from slaughter houses located in and around Bursa, Turkey. A total of 1413 lung tissues were examined and 136 lungs showing lesions of pneumonia at various lobes (totally 271 samples) were sampled. The 10 samples that were referred to our laboratory were also included into the study. After fixation in neutral buffered formalin, samples were processed routinely and sectioned for hematoxylin-eosin and immunohistochemical stains. For micro-biological examination, lung tissues were homogenized with ultra-turrax and incubated on Mycoplasma selective agar (MSA) and Mycoplasma selective broth (MSB). The colonies were also tested for biofilm and spot production. *M. bovis* NCTC 10131, obtained from Pendik Veterinary Control Institute, Istanbul, Turkey, was used as the reference serotype. For immunohistochemistry, polyclonal rabbit anti-*Mycoplasma bovis* antibody (provided by Dr. Enrico Radaelli, 1:10,000 diluted), monoclonal rabbit anti-human CD3 (RM-9107-S0, Lab Vision, 1:150 diluted), monoclonal mouse anti-human CD79αcy (M7051, DAKO, 1:50 diluted), polyclonal rabbit anti-human kappa light chains (A0191, DAKO, 1:10,000 diluted), polyclonal rabbit anti-human lambda light chains (A0193, DAKO, 1:10,000 diluted) primary antibodies, and polyclonal biotinylated goat anti-polyvalent secondary antibody (TP-125-BN, Lab Vision) were used. Streptavidin-biotin-peroxidase method was used and the protocols suggested by the manufacturers were applied.

Evaluation Criteria: For classification of pneumonia, type of the exudate and the distribution of the lesions in lung tissue were considered. Presence of necrosis, calcification, fibrous tissue formation, syncytial cells, emphysema, atelectasis or type II pneumocytes were also reviewed. A scoring method similar to that used in a previous study (Radaelli *et al.,* 2008) was used. According to this scoring, the severity of the lesions was scored according to the proportion of the affected lung tissue (1-25% of the lung affected: 1, 26-50: 2, 51-75: 3, >75: 4). In the evaluation of the total inflammatory cells, five areas were selected randomly under 400x magnification and the number of inflammatory cells was counted. Inflammatory cells up to 1-15 were scored 1, 16-30: 2, 31-45: 3, and >45: 4. For the determination of the total pneumonia severity, the scores from the affected area and the inflammatory cell infiltrate were summed. In cases where more than one sample was taken per lung, the most severely affected lung tissue was examined. In the evaluation of the distribution and intensity of inflammatory cell subtypes, a scoring similar to that described above was used. In anti-*M. bovis* stained slides the distribution of the agent within the lung tissue was examined.

Statistical analysis: The data were analyzed with SPSS 13.0 (Chicago, IL, USA). In comparison of more than two groups, Kruskal-Wallis test was used. Mann-Whitney U test was performed to find the group of difference. To find the group differences for category variables, Pearson's chi-square test was utilized. The significance value was set as $P<0.05$.

RESULTS

A total of 1413 lung samples obtained from various slaughterhouses were examined and lesions of pneumonia were observed in 136 (9.63%). *M. bovis* was ascribed as the agent in 39 animals (26.71%, department samples included). The most commonly affected lung lobes were pars cranialis dextra (56.41%).

Histopathological Findings: *M. bovis* pneumonia was classified in seven groups as under:
Fibrinopurulent bronchopneumonia (n=10): Prominent fibrinous and purulent exudate, together with desquamated epithelium and inflammatory cells, was observed within the alveoli. Fibrin plugs were present in lymphatics. **Non-purulent bronchointersititial pneumonia** (n=6): Mononuclear cells were observed around bronchi and bronchioli, as well as the interstitial tissue. **Non-purulent bronchopneumonia** (n=3): Mononuclear cells were observed in the submucosa of bronchi and bronchioles, as well in the alveoli. **Purulent broncho-pneumonia** (n=5): Dense infiltrations of neutrophils were observed in the submucosa of bronchi and bronchioles, as well in the alveoli. **Necrotic-purulent bronchopneumonia** (n=5): Large areas of necrosis, together with dense infiltrations of neutrophils in the submucosa of bronchi and bronchioles, and in the alveoli were observed. **Necrotic-fibrinopurulent bronchopneumonia** (n=6): These cases were similar to the necrotic-purulent bronchopneumonia. However, prominent fibrin exudation was observed within the alveoli and fibrin plugs in some lymph vessels. **Pyogranulomatous pneumonia** (n=4): Pyogranulomatous foci were observed in the lung parenchyma.

Bronchus associated lymphoid tissue (BALT) was hyperplastic in 2, 3 and 4 groups. In addition to the findings described above, areas of emphysema and atelectasis were observed in almost all cases. Caseative necrosis in 11 cases (4 with calcification) and coagulation necrosis in 7 cases were also observed.

Microbiological examination: Of the 146 lung samples examined, *M. bovis* was isolated in 38 cases. In 30 cases *M. bovis* was the only agent isolated, while *P. multocida* (10.52%), *Streptococcus* spp. (5.26%), *A. pyogenes* (5.26%) and *M. haemolytica* (5.26%) were also isolated in other cases. No significant difference could be found (P=0.328) between the severity of pneumonia in only *M. bovis* affected animals (6.16±1.77) and the co-infected animals (5.50±1.85).

Immunohistochemical Staining:
***M. bovis* staining:** In 24 of 39 animals, the agent was observed centrally and peripherally in the necrotic foci (Fig. 1), in the bronchial, bronchiolar, and alveolar epithelium (Fig. 2), within the exudate and within the cytoplasm of neutrophils (Fig. 3), macrophages and alveolar macrophages. The most commonly affected part was the bronchial and bronchiolar epithelium (51.28%), followed by cytoplasm of macrophages (35.89%) and neutrophils (28.20%), and least commonly observed in the alveolar epithelium (7.69%). In microbiological examination the agent was detected in 38 cases and 24 of these cases were also immunohistochemically positive (63.15%). Despite immunopositivity, only in one case, the agent could not be detected in bacteriology.

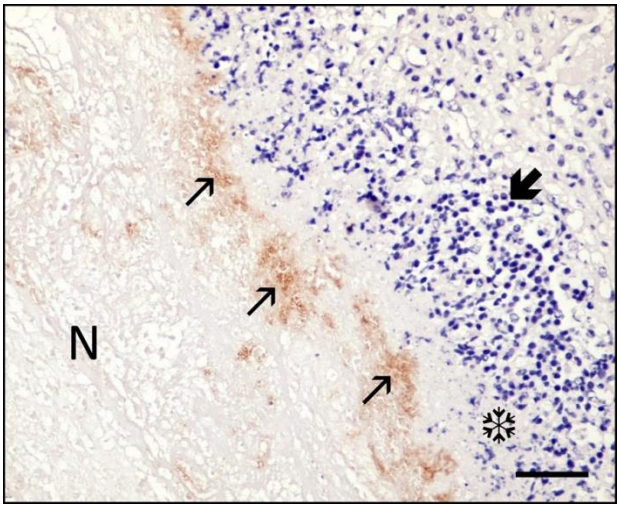

Fig. 1: *M. bovis* agents around coagulation necrosis (N), *M. bovis* (+) agents (arrows), degenerate-necrotic neutrophil leukocytes (snowflake) and other inflammatory cells (thick arrow), streptavidin biotin peroxidase method, DAB chromogen, Bar=50μm.

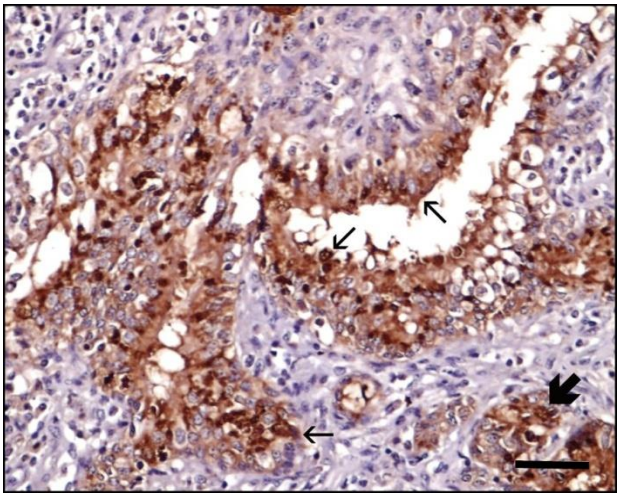

Fig. 2: *M. bovis* agents in bronchioles epithelium (thin arrows) and the bronchioles glandular epithelium (thick arrow), streptavidin biotin peroxidase method, DAB chromogen, Bar=50μm.

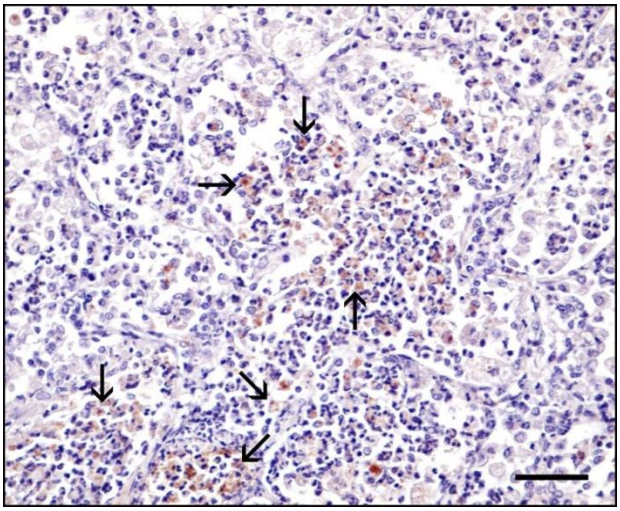

Fig. 3: *M. bovis* agents within neutrophil in the alveolar lumen (arrows), streptavidin biotin peroxidase method, DAB chromogen, Bar=50μm.

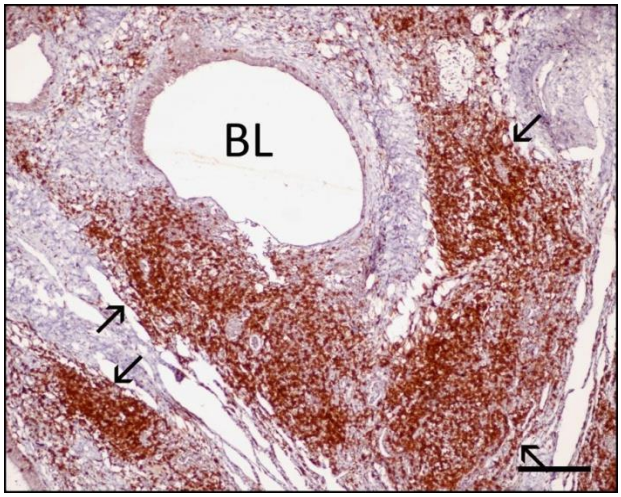

Fig. 4: CD3 (+) T cells in the BALT (arrows), streptavidin biotin peroxidase method, DAB chromogen, Bar=100 μm.

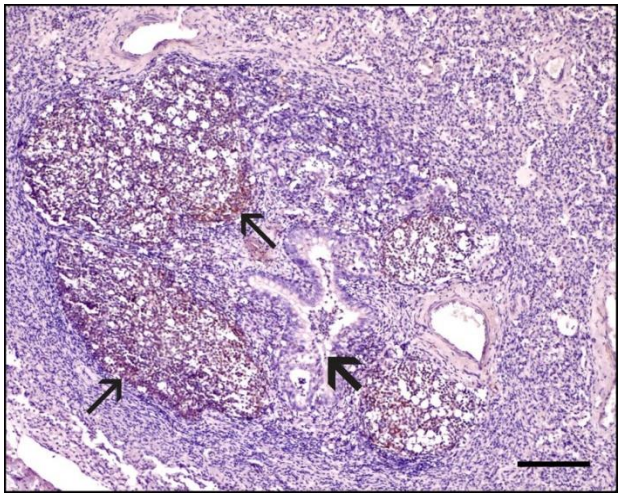

Fig. 5: CD79 (+) B cells in the BALT (thin arrows), streptavidin biotin peroxidase method, DAB chromogen, Bar=200μm.

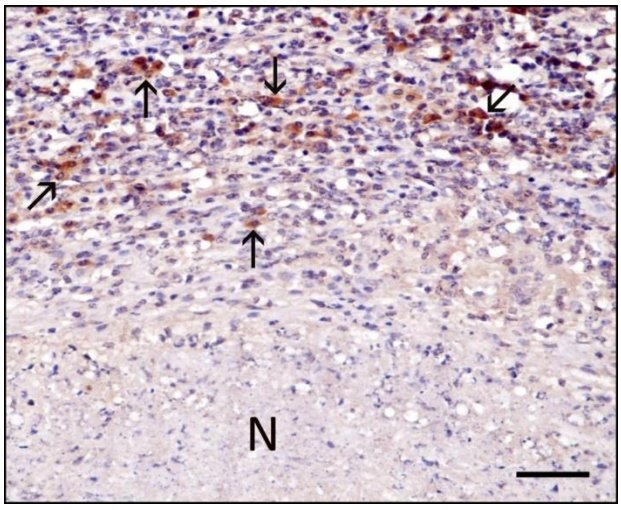

Fig. 6: Kappa light chain (+) plasma cells (arrows), necrotic area (N), streptavidin biotin peroxidase method, DAB chromogen, Bar=50μm.

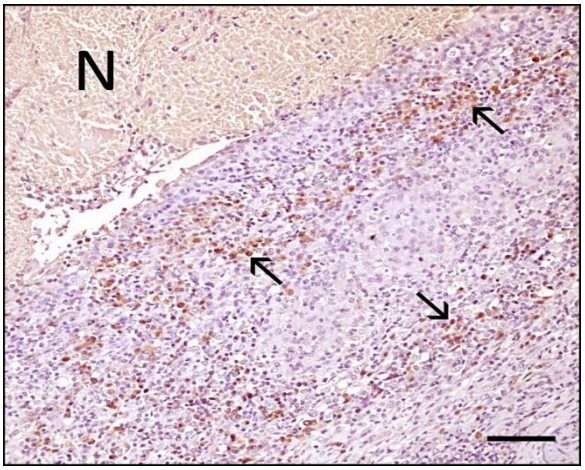

Fig. 7: Lambda light chain (+) plasma cells (arrows), necrotic area (N), streptavidin biotin peroxidase method, DAB chromogen, Bar=100μm.

Table 1: Mean values of the inflammatory cells within variable pneumonia severity of immunohistochemically *M. bovis* (+) animals (mean±SD).

Cellular intensity score*	CD3	CD79	KLC	LLC
2	2.38±0.59^{b}	1.27±0.43^{b}	1.33±0.24^{b}	1.53±0.53^{b}
3	3.37±0.48^{a}	2.34±1.03^{ab}	2.21±0.73^{a}	2.06±1.05^{ab}
4	3.14±0.82^{a}	2.55±1.02^{a}	2.47±0.88^{a}	2.76±0.89^{a}

*Intensity score 1 group was excluded in statistical analyses due to few numbers of animals (n=2); Different letters in the same column refer to significant differences (P<0.05)

Table 2: Mean values of inflammatory cells depending on necrosis of immunohistochemically *M. bovis* (+) animals (mean±SD).

	CD3	CD79	KLC	LLC
No necrosis (n=21)	3.08±0.73	2.10±1.02	1.95±0.94^{a}	1.96±0.99^{b}
Caseous Necrosis (n=11)	3.32±0.40	2.60±1.07	2.68±0.40^{b}	2.77±0.84^{a}
Coagulative Necrosis (n=7)	2.60±1.17	2.07±1.01	1.94±0.85^{a}	2.59±0.95^{ab}

Different letters between the in the same column indicate significant differences (P<0.05).

Inflammatory cells staining:

CD3: Positive reaction was observed as dark brown staining in the cell membranes of the cells located around the bronchi and bronchiole (Fig. 4), in the interalveolar septae, in the interlobular interstitium, pleura and BALT and within the inflammatory cells around the necrotic areas. **CD79:** Positive reaction was observed as dark brown staining in the nuclear membrane and was prominent in cells within the BALT (Fig. 5), and additionally around the bronchi and bronchiole, in the interstitium and periphery of the necrotic areas.

Kappa light chains (KLC): Positive reaction was observed as dark brown staining in the cell membrane of the cells mostly around the necrotic areas (Fig. 6), bronchi and bronchiole and less frequently in the interstitium.

Lambda light chains (LLC): Positive reaction was observed as cytoplasmic dark brown staining of the cells in the interstitium, around the necrotic areas (Fig. 7) and intensely around the bronchi.

In the 39 animals, which are found to be immunohistochemically positive for *M. bovis*, CD3(+) cells are observed to be the most common which were followed by LLC (+) cells, CD79 (+) cells, KLC (+) cells. The cellular intensity scores to estimate pneumonia severity and the immunohistochemistry results were compared to find the distribution of different cell types. As seen in Table 1, immunopositivity for CD79, KLC, LLC antigens tend to increase parallel to the cellular intensity, whereas CD3 positivity is the highest in score-3. As seen in Table 2, KLC positivity was found to be significantly higher in the sections with caseous necrosis compared to those without necrosis (P=0.022) and coagulative necrosis (P=0.027). LLC (+) cells numbers were found to be higher in sections with caseous necrosis in comparison to the ones without necrosis (P=0.022).

DISCUSSION

With this study, the prevalence of *M. bovis* and histopathology of the associated pneumonia has been assessed and bacteriological isolation, identification and immunohistological demonstration of the agent as well as evaluation of the immunological reaction against it has been performed in the bovine lungs which showed pneumonia and were either brought to the Department of Veterinary Pathology or collected from slaughter house. Of 146 lungs showing pneumonia, *M. bovis* was detected in 39 via immunohistochemistry and in 38 via bacteriology. Consequently, the prevalence of *M. bovis* in the region was determined as 26.71 %. Consistent with the previous studies, these results show that *M. bovis* is one of the most common respiratory pathogens in the region (Gagea *et al.*, 2006; Horwood *et al.*, 2014).

In the present study, both to *M. bovis* (+) and *M. bovis* (-) in animals of the most commonly affected lung lobes were pars cranialis dextra (56.41%). But, according to statistical analysis determined that no significant differences between the lobes in terms of *M. bovis* settlement. In this study, lesions in *M. bovis* related pneumonias were classified in 7 groups depending on the distribution and type of exudate, which were fibrino-suppurative bronchopneumonia, non-suppurative bronchointerstitial pneumonia and necrotizing fibrino-suppurative bronchopneumonia. In comparison to the classification of Radaelli *et al.* (2008), catarrhal bronchopneumonia, eosinophilic bronchointerstitial pneumonia and the pneumonia with necrosis and fibrin were not observed in this study. Also necrotizing and fibrinonecrotizing pneumonia, as mentioned by Khodakaram-Tafti and Lopez (2004) were not observed either. In previous immunohistochemical studies, considering the distribution of lesions, *M. bovis* have been shown to be bronchogenic (Radaelli *et al.*, 2008; Hermeyer *et al.*, 2011; Hermeyer *et al.*, 2012). In this study, bronchopneumonia in 33 and bronchointerstitial pneumonia in 6 animals, concisely affection of airways was observed. Additionally, of 24 animals which revealed positive reaction for the agent in immunohistochemistry, in 20 bronchial and bronchiolar and only in 3 alveolar location of the agent was shown. This finding supports the opinion about the spread of the agent from the airways to the interstitium. The caseative necrosis, coagulation necrosis and pulmonary abscesses have been were defined in *M. bovis* related pneumonias (Adegboye *et al.*, 1995; Hermeyer *et al.*, 2011; Hermeyer *et al.*, 2012).

In this study, of the 39 *M. bovis* positive animals, necrosis has been observed caseifying (Byrne *et al.*, 2001; Hermeyer *et al.*, 2011; Hermeyer *et al.*, 2012) and coagulation necrosis (Haines *et al.*, 2001; Rodriguez *et*

al., 2015). In addition to caseation, there was calcification of the necrotic areas in four lungs. Detection of the agent within and in the margins of the necrotic areas suggests the direct role of the agent in necrosis formation. In previous studies, the role of *M. bovis* in formation of necro-suppurative foci in the lungs could not be revealed (Adegboye *et al.,* 1995), however the interaction between a toxin in complex polysaccharide structure and the immunity in form of over reaction was mentioned to be probable explanation (Rodriguez *et al.,* 1996). Radaelli *et al.* (2008) have mostly reported chronic cases rather than necro-suppurative process, like follicular bronchitis or obliterative bronchiolitis which progress in brocho-interstitial or interstitial pneumonia form. This is consistent with other natural or experimental infection studies, which evaluated the pneumonias caused by *M. bovis* (Adegboye *et al.,* 1995). Immunohistochemistry in these studies revealed few numbers of the agent within the airway epithelium and within histiocystes in the peribronchiolar interstitium. Therefore, few intraepithelial bacteria can result in chronic inflammation by evading from humoral immunity and alveolar macrophages (Thomas *et al.,* 2003).

In this study, positive reaction was observed intracytoplasmically within the airway epithelium in 20 animals out of 24 in which the agent could be demonstrated immunohistochemically. Antigen shift is an effective way of the agent to evade the immunity (Rosengarten *et al.,* 1994; Sibylle *et al.,* 2015). Additionally, *M. bovis* is known to damage neutrophil function (Thomas *et al.,* 1991).

In additional to *M. bovis* in eight animals, *P. multocida*, *Streptococcus spp*., *A. pyogenes* ve *M. haemolytica* have been isolated in the present study. Possibility for mix infections as a result of immunosuppression caused by *M. bovis* have been previously reported (Vanden Bush and Rosenbusch, 2003; 2004). Consistent with our results, principal pathogens which can be isolated from the lungs in addition to *M. bovis* as previously reported are *P. multocida*, *M. haemolytica*, *H. somni*, *A. pyogenes*, *E. coli*, *P. aeruginosa* ve *Salmonella spp*. (Giovannini *et al.,* 2013). In this study the frequency of co-infections was 20.51% and *P. multocida* was the most common co-pathogen. It was reported that the pulmonary lesions are enhanced in case of co-infection (Haines *et al.,* 2001; Khodakaram-Tafti and Lopez, 2004). In this study, *M. bovis* was isolated and identified in 38 lungs and was demonstrated immunohistochemically in 24 lungs of 148 in total (16.21%). This condition which was considered to be a technical failure initially has been revealed by other researcher as well. Radaelli *et al.* (2008) have isolated *M. bovis* in 16 of the 64 calves (25%) with pneumonia but could immunohistochemically show the agent in only 7 of them (43.75%). They relate this condition of immunohistochemical negativity despite bacteriological isolation, to the catarrhal bronchointerstitial pneumonia and reported that the pneumonia is more bronchogenic necro-suppurative or fibrinonecrotizing in animals which showed positive reaction in immunohistochemistry claiming that immunodecetability of the agent is associated with the severity of the pneumonia. Similar studies report that strong positive immunoreaction is related more likely to chronic-active, suppurative or necrotizing bronchopneumonia (Shahriar *et al.,* 2002). Consistent with this, in our immunohistochemical analysis the agent was detected easily within or around the necrotic foci, whereas careful examination was necessary in animals showing no or minimal necrosis. In addition to necrotic areas, the agent was demonstrated within the airway and airspace epithelium and within the neutrophils and macrophages in intraluminal exudate.

Due to statistical proximity (P=0.053) to the limit levels of the correlation between immunohistochemical detectability and presence of necrosis, it is believed that presence of necrosis is related to higher possibility to detect *M. bovis* immunohistochemically. Radaelli *et al.* (2008) observed positive immunohistochemical reaction for *M. bovis* in all cases of co-infection. In our study positive immunoreaction was seen in 4 animals of the 8 co-infection cases in total which points to the insufficiency of one single method based diagnosis of *M. bovis* pneumonias.

On contrary to the previous studies, in one case, it was possible to demonstrate the agent immunohistochemically whereas bacteriological isolation couldn't be possible. Considering the equality of methods used for culture and immunohistochemistry in all cases, antibiotic application prior to slaughter might be an explanation for this phenomena. Despite its immuno-suppressive abilities, following the infection *M. bovis* causes an immunological reaction, which needs to be evaluated in details on order to understand the disease and improve treatment options. In order to reach the goal about demonstrating the distribution of the inflammatory cells in *M. bovis* pneumonias, immunohistochemistry on CD3 for T cells, CD79 for B cells, KLC and LLC for plasma cells was evaluated. Rodriguez *et al.* (2000) revealing the increase of mainly CD3+ T-lymphocytes and also B-lymphocytes and plasma cells within the BALT in experimental mycoplasmal pneumonias of goats, have pointed to the importance of cellular immunity. In our study, parallel correlation between the number of the inflammatory cells and cellular intensity grade. Considering the mean inflammatory cell numbers, T-lymphocytes were found to be the most common inflammatory cell in *M. bovis* pneumonias, which is consistent with the studies pointing the importance of the cellular immunity in *M. bovis* infection (Rodriguez *et al.,* 2000; Vanden Bush and Rosenbusch, 2003). In this study, the difference in numbers of inflammatory cells based on immunohistochemical reaction or in intensity of inflammation among single and co-infections was not significant. Based on necrosis status the difference in T and B cell populations was not significant, where as in KLC (+) and LLC (+) cell populations was remarkable. Further evaluation of this condition is believed to inputs in understanding the mechanisms of necrosis in *M. bovis* pneumonias. As a conclusion *M. bovis* is shown to be a common pathogen causing bovine pneumonias in the region. Although there can be bacterial co-infections, *M. bovis* was found to be the primary cause of these pneumonias. The severity of the pulmonary lesions suggested the high pathogenicity of the regional agent. It is expected to provide more information for a better understanding of the pathogenesis and improve diagnosis of the disease by immunohistochemical results.

Author's contribution: RY and ITC designed of study. RY, KO, AA, OMO and EA collected samples and processed histopathology and immunohistochemical staining, evaluated of results and writing the manuscript. All authors read, revised intelligently and approved the final version.

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