

Detection of bovine respiratory syncytial virus, *Pasteurella multocida*, and *Mannheimia haemolytica* by immunohistochemical method in naturally-infected cattle

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Abstract

Introduction: The aim of this study was to determine the predisposing effect of bovine respiratory syncytial virus (BRSV) on *Pasteurella* spp. infection in naturally-induced pneumonia in cattle by immunohistochemical labelling. **Material and Methods:** Lungs of cattle slaughtered in the slaughterhouse were examined macroscopically, and 100 pneumonic samples were taken. The samples were fixed in 10% neutral formalin and embedded in paraffin by routine methods. Sections 5 µm in thickness were cut. The streptavidin-peroxidase method (ABC) was used to stain the sections for immuno-histochemical examination. **Results:** BRSV antigens were found in the cytoplasm of epithelial cells of bronchi, bronchioles, and alveoles and within inflammatory cell debris and inflammatory exudate in bronchial lumens. *Pasteurella* spp. antigens were detected in the cytoplasm of the epithelial cells of bronchi and bronchioles, and in cells in the lumens of bronchi and bronchioles. Eleven cases were positive for only one pathogen (six for BRSV and five for *Pasteurella* spp.), while 35 cases were positive for 2 pathogens: BRSV plus *P. multocida* (n = 21) or *M. haemolytica* (n = 14). **Conclusion:** The presence of high levels of BRSV in dual infections indicates that BRSV may be the main pneumonia-inducing agent and an important predisposing factor for the formation of *Pasteurella* spp. infections in cattle naturally afflicted with pneumonia.

Keywords: cattle, bovine respiratory syncytial virus, *P. multocida*, *M. haemolytica*, immunohistochemistry.

Introduction

Bovine respiratory disease (BRD) in cattle is a multi-factorial disease complex caused by several viruses (bovine herpesvirus-1, bovine viral diarrhoea virus, infectious bovine rhinotracheitis virus, parainfluenza-3 virus, respiratory syncytial virus, etc.) and bacteria (*Pasteurella* spp., *Corynebacterium* spp., *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, *Mycoplasma* spp., etc.) (31). In domestic ruminants, *P. multocida* and *M. haemolytica* are the most important respiratory pathogens isolated from

respiratory disease (29) that cause pneumonia outbreaks in calves, adult cattle, sheep, and goats (1). These agents are found naturally in the nasopharynx and upper respiratory tract of healthy ruminants (17). When the respiratory defence is damaged as a result of viral infection and stress factors which suppress the local defence mechanisms (37), *Pasteurella* spp. can colonise the lower respiratory tract and cause pneumonic pasteurellosis (9).

Bovine respiratory syncytial virus (BRSV), belonging to the *Pneumovirus* genus of the *Paramyxoviridae* family, leads to large-scale BRD

in cattle and sheep worldwide (7, 8). Although experimental BRSV infections have demonstrated that BRSV alone can induce severe clinical respiratory tract disease and pneumonia as a primary pathogen in calves (11, 14), secondary bacterial pathogens such as *P. multocida*, *M. haemolytica*, and *Histophilus somni* are commonly isolated from BRSV-infected calves (31). In addition, BRSV and *M. haemolytica* seem to have synergistic pathogenic effects in experimentally infected lambs (3).

Immunohistochemistry (IHC) is a safe technique and has been frequently used for diagnostic evaluations of *Pasteurella* spp. (24, 25, 28, 37) and BRSV (14) antigens in formalin-fixed tissues. Experimental studies have investigated the synergistic effect of the antigens in cattle by the IHC method (4, 27, 36). However, to our knowledge, there has been no evaluation by this method of pneumonia originating from BRSV co-existing with *P. multocida* and *M. haemolytica* in naturally infected calves.

This study aimed to determine the predisposing effect of BRSV on *P. multocida* and *M. haemolytica* by macroscopic, histopathological, and immunohistochemical investigations of pneumonic lung lesions occurring in naturally-infected cattle.

Material and Methods

An abattoir survey for the prevalence of pneumonia in cattle was carried out on the lungs of approximately 1,400 cattle brought for slaughter, which were predominantly male and originated from dairy and beef herds in Diyarbakir, Turkey. The gross appearance of lesions was recorded, and 100 (14%) pneumonia-suspected lung tissues were sampled. Tissue samples were taken in all four seasons. The effect of seasonal factors on the development of the disease was examined, thus avoiding evaluation of the BRSV and/or *Pasteurella* spp. infections of a specific region or flock at a specific time.

Histopathology. Lung tissue samples taken from sacrificed cattle were fixed in 10% neutral formalin and embedded in paraffin by routine methods. Sections 5 µm in thickness were cut for histological examination. The slides were examined and photographed using an E-400 light microscope (Nikon, Japan) equipped with a DS-Ri2 video camera and DS-U3 microscope video camera controller (Nikon, Japan).

Immunohistochemical staining. The streptavidin-peroxidase method (ABC) was used to stain the sections. After deparaffinising and rehydrating, the sections were rinsed in distilled water. After quenching endogenous peroxidase activity with 3% H₂O₂ (v/v) for 20 min, the slides were washed twice in 0.01 M PBS for 5 min. Heat-induced antigen retrieval was performed with citrate buffer. Before adding the primary antibodies, the slides were incubated with

blocking serum (Histostain Plus Bulk Kit, Zymed, USA) for 15 min to block nonspecific binding. The sections were incubated with polyclonal anti-*P. multocida* and anti-*M. haemolytica* hyper-immune sera in 1:1,000 dilutions and RSV antibody in 1:100 dilution (Thermo Fisher Scientific Inc, USA) and kept at 4°C overnight in a humidified chamber. After incubation, the slides were washed four times in 0.01 M PBS for 5 min, incubated with biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) for 20 min at room temperature (20–25°C), then washed four times in 0.01 M PBS for 5 min. After incubation with the secondary antibody, the sections were incubated with streptavidin-peroxidase (HRP) conjugate (Histostain Plus Bulk Kit, Zymed) for 20 min, then washed four times in 0.01 M PBS for 5 min following the enzymatic incubation. To visualise the reactions, the sections were reacted for 5–15 min with diaminobenzidine (DAB). After the development of the DAB reactions, the sections were counterstained with Gill's haematoxylin. Then, the sections were passed through alcohol and xylene and mounted directly with Entellan mounting medium (Merck, USA). For the positive control, previously *Pasteurella* spp. positive lung tissues (confirmed by the Department of Microbiology and Pathology, Faculty of Veterinary Medicine, Uludag University, Bursa, Turkey) were retained. Lung tissue sections were used as a negative control to verify staining results. The slides were reacted with PBS instead of primer antibodies as negative controls and were examined and photographed.

Statistical analysis. Descriptive statistics for the categorical variables were presented as count and percentages. A chi-squared test was performed to determine the relationship between categorical variables. In addition, multiple correspondence analysis was also carried out to determine relationships among the variables. The statistical significance level was considered to be 5%. The SPSS version 13 statistical programme (IBM, USA) was used for all statistical analysis.

Results

Gross examination. In the macroscopic examination of the lungs with pneumonia, purple-red or grey consolidation areas were found including small haemorrhagic foci in the lobes or lobular extent. The areas were mostly located at the ventrocranial and accessory lobes with gelatinous interlobular septal expansion. Some consolidated areas were dark red. The consolidated lobular areas were patch-like, distributed multifocally and surrounded by pink, emphysematous lobules (Figs 1A, B). Fibrinous pleuritis and coagulative necrosis areas were present in 11 cases. Fibrin filaments were present in seven lungs, with the

pleura clearly thickened and the lungs adhering to certain places on the chest wall. Mucopurulent exudate was widespread in the small bronchi and bronchioles of 13 lungs.



Fig. 1. A – lobular pneumonia in the right cranial and medial lobes and lobular pneumonia in the left cranial lobe. Consolidated areas in red (stars) with small haemorrhagic foci (arrow head). B – cut surface of the lung: normal lobules (stars), purple-red consolidation areas (x), and haemorrhagic areas (arrow heads)

Immunohistochemical findings. In IHC labelling, BRSV, *M. haemolytica*, and *P. multocida* antigens were detected in 41 (41%), 17 (17%), and 23 (23%) out of 100 pneumonic lungs, respectively.

Mixed infections of BRSV and *M. haemolytica* (n = 14) or *P. multocida* (n = 21) were observed in 35 cases. Eleven cases were positive for only one pathogen (six for BRSV and five for *Pasteurella* spp.). The distribution of the antigens based on ages and breeds is presented in Table 1. Additionally, the relationship between the antigens and breeds and ages of the animals are shown on two-dimensional charts (Fig. 2).

The IHC labelling of BRSV antigens was observed in the mucosa propria and epithelial cells of bronchi and bronchioles and in alveolar epithelium (Fig. 3A). Immuno-reactivity was also detected in cell debris within the lumen of bronchi, bronchioles, and alveoles. In addition, positive immunostaining was noticed in the cytoplasm of macrophages and syncytial cell formations located in cellular exudate, interstitial areas, and lumen of airways (Fig. 3B). In some bronchiolar and alveolar lumen, immunopositive areas were detected as a thin layer. BRSV positivity had a granular appearance and was located intracytoplasmically. No immune reaction was observed in bronchial glands or peribronchial cartilage tissue.

Positive immunoperoxidase staining associated with *Pasteurella* spp. was detected within the epithelial cells of bronchi, bronchioles, and alveoles (Fig. 3C). Bacterial antigens were also observed in the degenerating neutrophils, macrophages, and desquamated cellular debris in the lumen of bronchi, bronchioles, and alveoli (Fig. 4D), and in the cytoplasm of inflammatory cells located in interlobular and interalveolar septa. The localisation of IHC staining of examined antigens is presented in Table 2.

Table 1. Distribution of the antigens in relation to age and breed in pneumonic cattle lungs

	BRSV	<i>P. multocida</i>	<i>M. haemolytica</i>	BRSV + <i>P. multocida</i>	BRSV + <i>M. haemolytica</i>	Total
Total	6 (13%)	3 (6.5%)	2 (4.3%)	21 (45.7%)	14 (30.5%)	46
One year	3 (11.1%)	2 (7.4%)	0 (0.0%)	14 (51.9%)	8 (29.6%)	27
Two years	3 (15.8%)	1 (5.3%)	2 (10.5%)	7 (36.8%)	6 (31.6%)	19
Montofon	2 (20%)	0 (0.0%)	0 (0.0%)	5 (50%)	3 (30.0%)	10
SAR	2 (8.3%)	2 (8.3%)	1 (4.2%)	11 (45.8%)	8 (33.3%)	24
EAR	2 (16.7%)	1 (8.3%)	1 (8.3%)	5 (41.7%)	3 (25.0%)	12

SAR – South Anatolian Red; EAR – Eastern Anatolian Red

Table 2. Distribution of IHC stained antigens in lung tissue

Antigen	Alveolar epithelium	Alveolar lumen	Bronchi, bronchiole lumen	Bronchi, bronchiole epithelium	Interstitium	Vein lumen	Peribronchial glands
<i>P. multocida</i>	10	17	9	20	5	0	3
<i>M. haemolytica</i>	14	5	7	11	8	0	5
BRSV	16	12	27	29	27	0	0

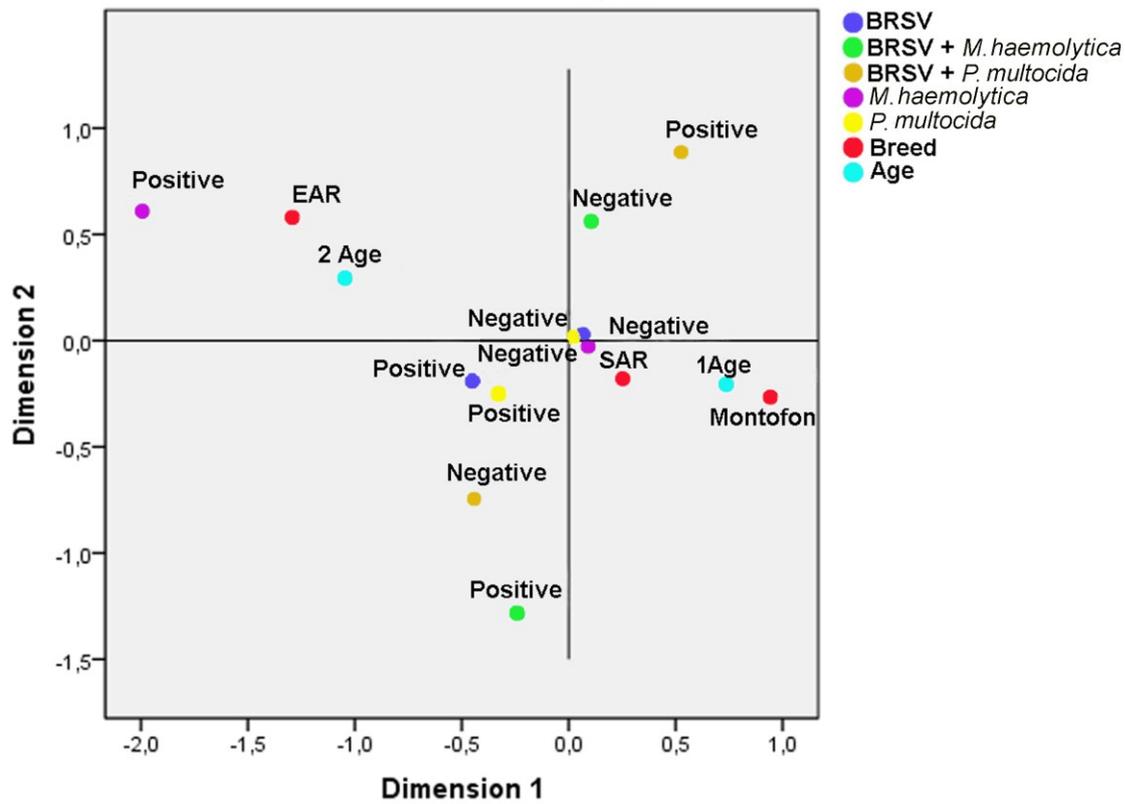


Fig. 2. Relationship between the antigens and breeds and ages of animals

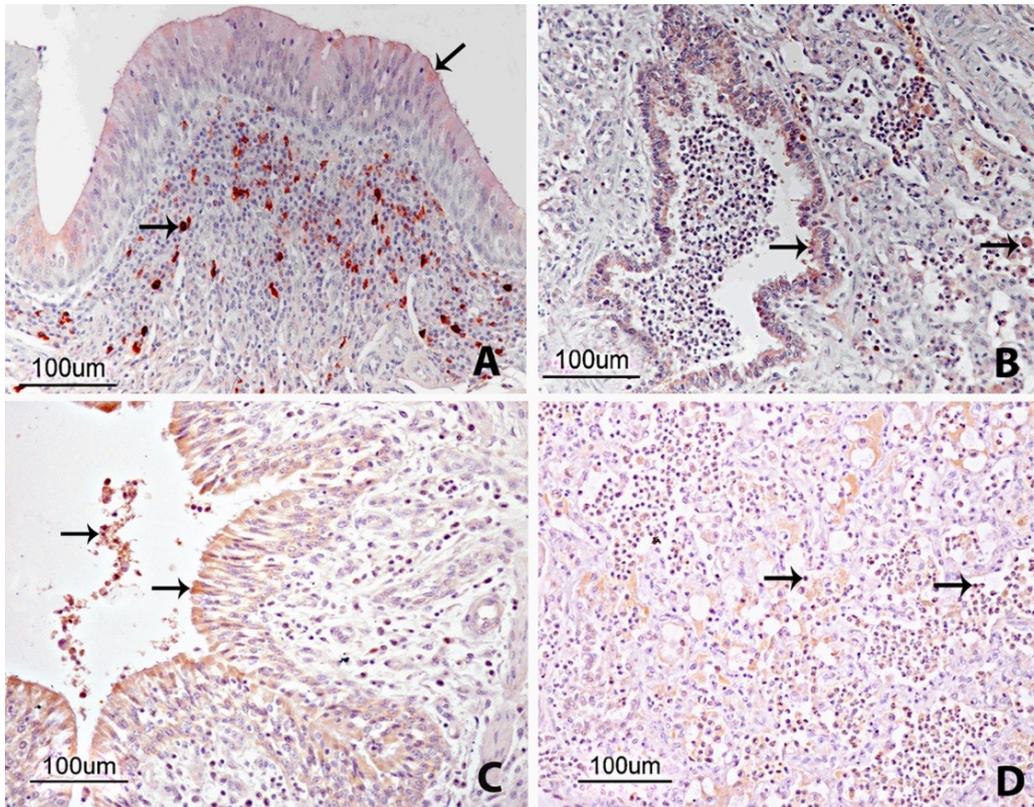


Fig. 3. A – immunopositive staining of BRSV in epithelial cells of bronchi and peribronchial area (arrows), ABC method counterstained with Harris haematoxylin. B – immunopositive staining of BRSV in bronchiolar epithelial cells and inflammatory cells in alveolar lumen (arrows), ABC method counterstained with Harris haematoxylin. C – presence of *M. haemolytica* throughout the bronchi epithelium and in cell debris (arrows), ABC method counterstained with Harris haematoxylin. D – immunopositive staining of *P. multocida* in alveolar content mostly in neutrophils (arrows), ABC method counterstained with Harris haematoxylin

Histopathological findings. Microscopically, broncho-interstitial, catarrhal-purulent, and fibrinous pneumonia was observed in the lung sections. Cases which were immunohistochemically positive for BRSV antigens were examined histopathologically. Broncho-interstitial lesions were generally observed in these cases. Peribronchial and peribronchiolar mono-nuclear cell infiltration with hyperplasia in the bronchi and bronchiolar epithelia was detected. Neutrophils, cellular debris, and an exudate contained in mucus and macrophages were present in the airways. Serous fluid and alveolar macrophages were found in some alveolar lumens (Fig. 4A). The interalveolar septa were expanded because of mono-nuclear cell infiltration, congestion, and type II epithelial cell proliferation. Fibromuscular hyperplasia was observed somewhere in the alveolar walls. Syncytial cell formation was detected in bronchial and alveolar lumens (Fig. 4B).

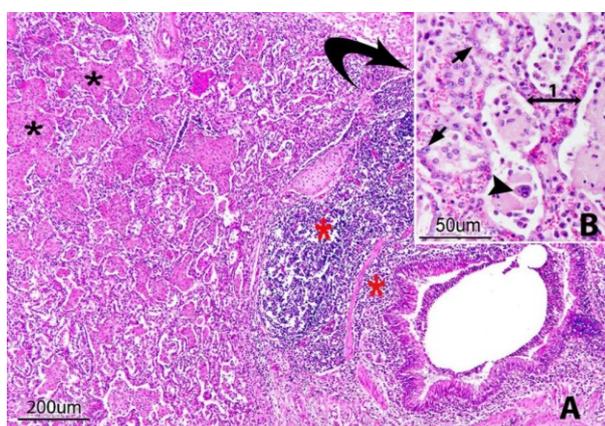


Fig. 4. A – peribronchial mononuclear cell infiltration in case of BRSV infection (red stars). Alveoli filled with serofibrinous exudate and alveolar macrophages (black stars). B – proliferation of type II epithelial cells in the wall of alveoli (arrows), serofibrinous exudate and syncytial cell in the lumen of the alveoli (arrowhead), thickening of the interalveolar septum due to oedema, leukocyte infiltration, and hyperaemia (1) (interstitial pneumonia)

In sections which were immunohistochemically positive for *P. multocida* and *M. haemolytica*, exudative and proliferative lesions were found. Proliferative changes were observed in the lung parenchyma, characterised by epithelialisation (type-II epithelial cell proliferation) in the alveoli. Alveoli and bronchiole were filled with dense infiltration with neutrophils and mononuclear cells mixed with fibrin. Fibrin plugs were also observed in the lymphatic vessels and venules in these regions. Pleura and interlobular septa were enlarged by exudate composed of fibrin and leukocytes (Fig. 5A). Some of these leukocytes were characteristically oat cell-shaped and localised in the alveolar lumens (Fig. 5B). In addition, syncytial cell formation was observed.

In the examination of immunohistochemically positive BRSV + *Pasteurella* spp. cases, interstitial pneumonia and severe exudative pneumonia with focal necrosis and haemorrhage were observed.

Consequently, these lesions were histologically similar to those seen in cases of pneumonic pasteurellosis.

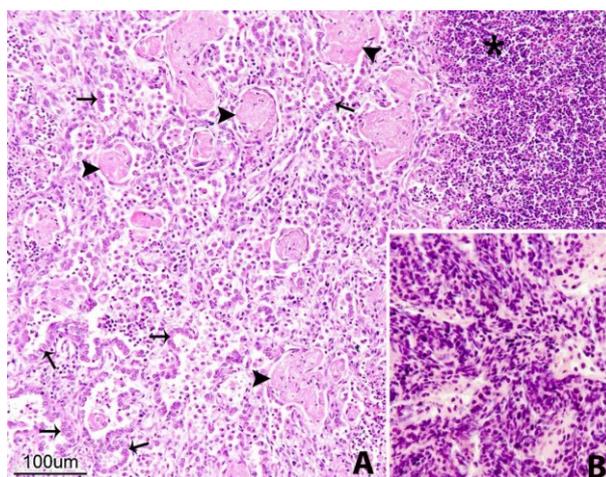


Fig. 5. A – alveoli filled with dense neutrophils in case of pasteurellosis (star). Serofibrinous exudate in the lumen of some alveoli (arrow head) and proliferation of type II epithelial cells in the wall of alveoli (epithelialisation) (arrows). B – oat cells in alveoli

Discussion

Pneumonic pasteurellosis is one of the important infectious diseases caused by *M. haemolytica* and *P. multocida* (1, 9). It occurs in young calves as a component of enzootic pneumonia of beef and dairy calves and following a primary viral infection (6). BRSV is well known as a primary pathogen causing severe BRD, which leads in cattle to secondary infections (31). The predisposing effect of BRSV on *Pasteurella* spp. infection in naturally infected cattle was investigated in this article.

The diagnosis of *Pasteurella* spp. infection by bacteriological methods has been reported to be difficult due to many factors such as antibiotic treatment and frozen or autolytic material (28, 37). Especially when the tissues are contaminated with other microorganisms, a selective medium is required for the isolation of *M. haemolytica* and *P. multocida*. Yener *et al.* (37) reported that IHC using polyclonal antibodies can be a useful tool for the diagnosis of pneumonic pasteurellosis because the positive percentage of *M. haemolytica* antigens detected by the IHC was higher than the results of bacterial isolations in pneumonic lungs of goats slaughtered at the abattoir. Similar results were reported by Ozyildiz *et al.* (28). Detection of *M. haemolytica* antigens in pneumonic lungs by IHC is more reliable than detection by bacterial isolation. It may be explained by the fact that antigens from live and dead bacteria are present in the samples with positive IHC, whereas bacteriological cultures do not detect dead organisms (24). Previous studies reported the detection of *Pasteurella* spp. by PCR (15, 38), ELISA (34), *in situ* hybridisation (2), and

immunoperoxidase technique using polyclonal anti-*M. haemolytica* and anti-*P. multocida* hyperimmune sera on sheep and cattle (1, 9, 20, 21, 28). In this study, the *M. haemolytica* and *P. multocida* were detected by an immunohistochemical method using hyperimmune sera in the cytoplasm of neutrophils and macrophages in the alveolar and bronchial lumens (23), in the cytoplasm of cells located in the bronchial epithelium and peribronchiolar area (28), and in the wall of necrotic alveoli, fibrin, serous exudate, and degenerated leukocytes (22). Histopathologically, neutrophil, macrophage, and fibrin accumulation in the lumens of airways and dilatation of lymphatic vessels were recorded in *Pasteurella* spp. positive sections, as previously reported (22, 29). In addition, the presence of cell clusters with spindle-shaped nuclei was another finding. These cells have oat cell definition and are formed by the action of bacterial toxins on leukocytes accumulated in inflamed alveoli (16, 37).

The diagnosis of the BRSV is based on clinical signs, gross examination, histopathological findings, and demonstration by immunohistochemical methods of viral antigen in sections of affected tissues (7, 19). It has been reported that isolation of BRSV, a labile virus, was difficult due to slow replication of the virus in cell culture (14). Immunofluorescence and immunohistochemical staining is used for the determination of BRSV antigens in the frozen lung samples, though its cellular and structural differentiation is difficult (30). It has been reported that the immunoperoxidase method provides an opportunity to clearly show viral antigens in pulmonary lesions (10). In this study, replication of BRSV was detected in bronchi, bronchioles, epithelial cells, alveolar cells, and bronchial gland epithelia as intracytoplasmic in naturally infected cattle by IHC methods. In addition, positive immunostaining was noticed in alveolar macrophages, interalveolar septa, cellular exudate, syncytial cells, and bronchiole and alveolar lumens (13, 31). Similar findings associated with BRSV have also been reported in experimentally infected balb/c mice (5).

Histopathological findings of BRSV are characterised by broncho-interstitial pneumonia in cranioventral lobes, bronchitis and bronchiolitis, hyperplasia, degeneration or desquamation of bronchus epithelia, and syncytial cell formations. Expansion of alveolar septa due to congestion, inflammatory cell infiltration (lymphocytes and macrophages), and hyperplasia of type II pneumocytic cells were observed. In addition, an increased number of alveolar macrophages and exudate are reported to be formed in RSV infections (12, 13, 19, 32). Alveolar lumens often contains seroproteinous fluid, cell debris, a few alveolar macrophages, and rarely neutrophils. Neutrophilic exudates may be present in airways (18).

Pneumonic lesions have been reported to be more severe in lambs infected with BRSV and *P. haemolytica*, than in lambs infected with only one pathogen (3, 4). Also necrotic debris and phagocytic

cells were more prominent in the alveoli of lambs infected with BRSV and *P. haemolytica* than in those infected with either of them without the other (4), because the virus leads to lesions that compromise the lungs and thus permit *P. haemolytica* to become established and produce more severe pneumonic lesions than it could produce alone (3). However, evidence of a natural outbreak of respiratory disease in 31 of the 43 calves neither reinforced nor discounted the possibility of interaction between BRSV and *P. haemolytica* infection in the pathogenesis of the disease (35). In this study, we found that combined infections caused by BRSV and *Pasteurella* spp. did not cause more serious lesions in the lung than those produced by the virus or the bacterium alone. This is probably due to the presence of other pulmonary pathogens in cases in which we detected *Pasteurella* spp. alone. The presence of broncho-interstitial pneumonia in *Pasteurella* spp. cases positive for *Pasteurella* spp. alone supports the presence of other predisposed viral infections (26).

In this study, the presence of high levels of BRSV in dual infections with *P. multocida* (n = 14) and *M. haemolytica* (n = 21) indicates that BRSV may be the main pneumonia-inducing agent in naturally-infected calves for *Pasteurella* spp., similar to the results of studies on experimentally-infected calves (3). This result shows that BRSV is a potential cause of epithelial necrosis and hyperplasia, which could easily become predisposed to bacterial colonisation, as mentioned in previous studies (3, 26).

In conclusion, our study is the first work on detection of BRSV, *P. multocida* and *M. haemolytica* in naturally-infected cattle by the immunohistochemical method. This study demonstrates the presence of BRSV as an important predisposing factor and main pneumonia-inducing agent for *P. multocida* and/or *M. haemolytica* infections in naturally-occurring pneumonia in cattle.

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