Investigation of Respiratory Syncytial Virus (RSV) and Parainfluenza-3 (PI-3) virus by histopathological and immunohistochemical Methods in sheep and goats

ABSTRACT

Respiratory Syncytial Virus (RSV) and Parainfluenza type 3 (PI3) virus cause serious respiratory system infections in sheep and goats. It was aimed to investigate the localization and distribution of sheep and goat RSV and PI-3 in lung tissue by histopathological and immunohistochemical methods. The study material consisted of 30 sheep and 24 goat lung paraffin blocks from Samsun and its surrounding regions, which came to Samsun Control and Veterinary Research Institute between 2016-2019. Histopathological changes characterized by bronchial and bronchiolar epithelial degeneration and desquamation, interalveolar septal thickness, epithelialization of the alveolar epithelial surface and inflammatory cells in the alveolar lumen. In addition, hyperplasia of peribronchial lymphoid tissue, hyaline membrane formation and syncytial cells in alveoli were observed less frequently. Althought, bronchiolitis obliterans were not detected in any of the cases. In immunohistochemical staining, RSV antigen was detected in 50% of sheep and 54% of goats, and PI-3 antigen was detected in 40% of sheep and 50% of goats. RSV and PI-3 antigenic distribution of sheep and goats in bronchial and bronchiolar epithelium and cells debris and interalveolar septum were statistically similar ($p>0.05$). PI-3 antigen in goats was detected more intensely than sheep ($p<0.05$) in alveolar macrophages statistically. It was concluded that the localization of RSV and PI-3 antigens in sheep and goat lung tissue was detected similar by this study. In addition, it was determined that RSV and PI-3 antigens were common in sheep and goats and it was thought that vaccination should be done for protection.

Keywords: RSV, PI-3, immunohistochemistry, sheep, goat

INTRODUCTION

Parainfluenza type-3 (PI-3) and Respiratory syncytial virus (RSV) are in the *Paramyxoviridae* family and many hosts also cause respiratory tract infections (Maidana et al., 2012). Most interesting in the biology of paramyxovirus infections is their potential for interspecies infection (Ellis, 2010). RSV is an enveloped, single-stranded, negative-susceptible RNA virus (Zaher et al., 2014) within the genus Pneumovirus (Van der Poel et al., 1995). RSVs have been identified as bovine, sheep, and goat RSV (Eleraky et al., 2001), and all RSV's have been reported to cause cross-species infections (Van der Poel et al., 1995). PI-3 is an enveloped, non-segmented, negative-sensitive RNA virus within the genus Respirovirus (Ellis, 2010; Newcomer et al., 2017). Bovine PI-3 has been reported to cause sheep and goat respiratory infections (Saeed et al., 2016; Stevenson and Hore, 1970).

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Research Article

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Additionally, viral agents such as bovine herpesvirus 1 (BHV-1), bovine viral diarrhea virus (BVDV), and bovine adenoviruses (BAV) cause respiratory tract infection in ruminant species (Al-Hammadi, 2016; Borujeni et al., 2020; Tamer et al., 2018) and cross-species infection between both small and large ruminant species (Yeşilbağ and Güngör, 2009).

RSV and PI-3 infection cause severe bronchopneumonia along with secondary bacterial infections in high-stress situations such as transport, air exchange, and nutrition (Haanes et al., 1997). The agent is transmitted by droplet infection and contaminated animal feed and water (Stott and Taylor, 1985). RSV targets both ciliary and non-ciliary bronchiolar epithelial cells and types II pneumocytes (Viuff et al., 1996), PI-3 virus, epithelial cells of the respiratory tract, and alveolar macrophages (Bryson et al., 1983). There are clinical signs in animals such as fever, weakness, nasal dishcarge, continuous and deep breathing, respiratory distress, cough, and loss of appetite. Histopathological findings are seen in the respiratory tract epithelium such as necrotic bronchiolitis, alveolitis, bronchi, bronchiole and type II pneumocyte hyperplasia, interalveolar septum thickening, atelectasis and lymphoid hyperplasia (Narita et al., 2002; Sacco et al., 2014). RSV, syncytial giant cells formed by non-ciliated bronchiolar epithelial cells with acidophilic cytoplasmic inclusion bodies, are the characteristic findings of the disease (Belknap et al., 1995).

Investigations have been carried out with serological tests such as Virus Neutralization Test (VNT), ELISA, Immunofluorescent Antibody (IFA) and Hemagglutination Inhibition (HI) for the prevalence of ruminant RSV and PI-3 agents (Franco et al., 2020; Tiwari et al., 2016). Immunohistochemical (IHC), Immunofluorescence (IF), Direct Fluorescent Antibody Technique, and Electron Microscope methods are currently used in the lung tissue of ruminant animals to show detailed RSV and PI-3 viruses (Aniță et al., 2015; Jarikre and Emikpe, 2017).

In this study, it was aimed to investigate the localization and distribution of RSV and PI-3 antigen in formalin-fixed, paraffin-embedded lung tissue sections in sheep and goat lung viral pneumonia cases by histopathological and immunohistochemical methods.

MATERIAL and METHOD

The study material consisted of 54 sheep and goat lung paraffin blocks that brought to Samsun Veterinary Control Institute from Samsun and surrounding provinces between 2016 and 2019, showing signs of viral pneumonia.

Histopatological method

Sections 5 µm in thickness were cut for histological examination. The slides were examined and photographed using. Sections 5 µm in thickness were cut and taken normal and poly-L-lysine slides. Normal slides stained with routine Hematoxylin-eosin for the histopathological examination. All slides were examined and photographed using light microscope (Leica DM 400B).

Immunohistochemical Method

Immunohistochemical staining was performed according to the Mouse and Rabbit Specific HRP / DAB IHC Detection Kit-Micro polymer (ab236466) kit procedure. Anti-Mouse RSV monoclonal (Cat NO: ab43812, Abcam, Boston USA) and anti-Goat polyclonal PI-3 (Cat NO: ab 28584, Boston, USA) were used. Sections taken from paraffin blocks to poly-L-lysine slides were washed in distilled water after deparaffinization and dehydration. Firstly, 3% H_2O_2 peroxidase block solution was dropped for 10 min. Proteinase K (ab64220) was

dropped for antigen retrieval and it was waited for 5 min. Then, the protein block solution was poured and kept at room temperature for 10 min. The sections were dropped 1: 100 anti-RSV and 1: 200 anti-PI-3 and left at room temperature for 1 hour. Subsequently, the Mouse Identification Reagent (Complementary) solution was added to the slides and incubated for 30 min and Goat anti-rabbit HRP-conjugate was dropped for 15 min. Slides stained by DAB (3,3'- diaminobenzidine tetrahydrochloride) for 1 min. After counter-staining with Mayer's hematoxylin, slides were closed by coverslips and evaluated under a light microscope (Leica DM 400B). The negative control slides were also stained according to the same procedure and PBS was used instead of the primer antibody. In the immunohistochemical scoring of sections, bronchial and bronchiolar epithelial cells and cells debris, peribronchial glands, interalveolar septum inflammatory cells, alveolar macrophages, and neutrophils were examined semi-quantitatively. Immunohistochemical staining scores were classified as mild (+), moderate (++) and severe (+++) expression according to the criteria determined by Yavuz and Dinçel (2020)

Statistical Analysis

Statistical significance of immunohistochemical scores was evaluated using IBM SPSS Statistics 25.0 software and Mann-Whitney's U test. The criterion for statistical significance was $p<0.05$. All values are presented as mean±standard deviation (median).

RESULTS

Histopatological Results

In this study, degeneration and desquamation of bronchial and bronchiolar epithelium, fibromuscular hypertrophy (Fig 1A), epithelialization, interalveolar septum cell infiltration (Fig 1C) were the most prominent microscopic changes in RSV and PI-3 infections. Lymphoid hyperplasia of the tissue around bronchi and bronchioles was detected in 17 %-25 % of RSV and PI-3 cases (Fig 1B).

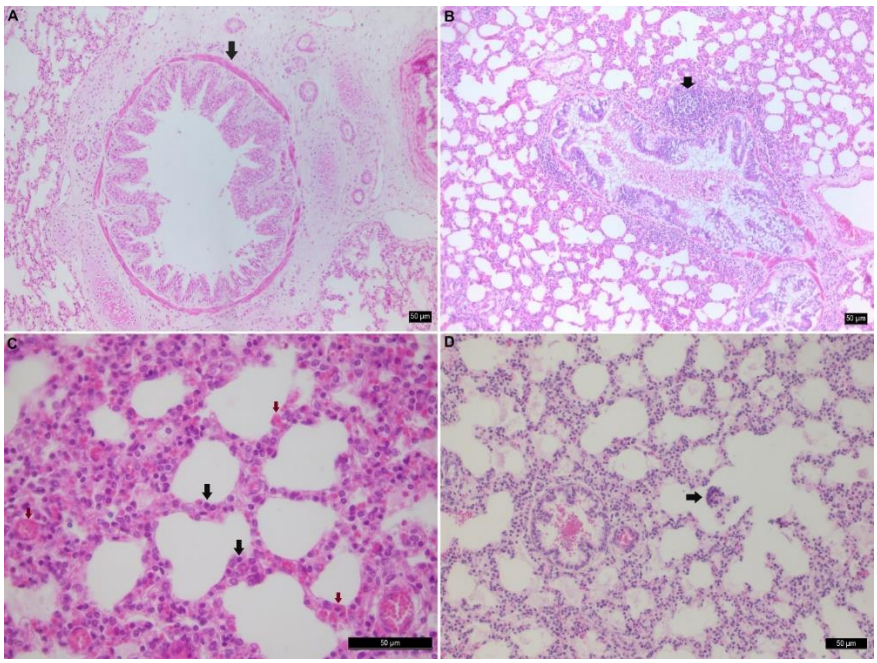

Figure 1. Lung sections, Hematoxylin-Eosin Staining. **A.** Fibromuscular hypertrophy around the bronchial (arrow). Bar: 50 µm **B.** Desquamation of bronchial epithelium and hyperplasia of lymphoid tissue around the bronchial (arrow). Bar: 50 µm. **C.** Inflammatory cells infiltration (black arrows) and hyperemia (red arrow) in the interalveolar septum. Bar: 50 µm. **D.**Syncytial cell (arrow) and inflammatory cells infiltration in the interalveolar septum. Bar:50 µm**.**

Bronchiolitis obliterans, which is characterized by the formation of organized fibrous tissue covered with a single layer of cuboidal epithelium in the bronchiolar lumen, could not be detected in any sheep and goat lung sections. Alveolar epithelization, characterized by the transformation of a monolayer cubic epithelium, was common. In many cases, hyperemia in the interalveolar septum, edema and alveolar macrophages were seen intensely in alveolar lumens. In seven sheep and goats of RSV positive cases, and four sheep and eight goats of PI-3 positive cases, syncytial cells were present in the lumen of the alveoli (Fig 1D). Neutrophilic infiltration in lung tissue was seen in a small number of RSV and PI-3 positive cases.

Immunohistochemical Results

In immunohistochemical staining, RSV antigen was detected in 50% of sheep and 54% of goats, and PI-3 antigen was detected in 40% of sheep and 50% of goats. Statistical evaluation of IHC scoring of sheep and goat RSV and PI-3 antigen in the bronchial and bronchiolar epithelium and cells debris, peribronchial glands, interalveolar septum inflammatory cells, alveolar macrophages are given in Table 1 and 2.

Table 1. Range of the histopathological findings in sheep and goat lung RSV and PI-3 antigen positive cases.

	RSV (+)		PI-3(+)	
Lesions	**Sheep**	**Goat**	**Sheep**	**Goat**
Degeneration and desquamation of the bronchial and bronchiolar epithelium	87%	69%	83%	75%
Fibromuscular hypertrophy	100%	100%	100%	100%
Lymphoid hyperplasia	20%	23%	17 %	25%
Thickening of the interalveolar septum	100%	100%	100%	100%
Epithelialization	80%	80%	83%	83%
Hyalin membrane	14%	8%	8%	17%
Bronchiolitis obliterans	0%	0%	0%	0%
Syncytial giant cell	47%	54%	33%	67%
Intraalveolar edema	40%	31%	17%	42%
Intracytoplasmic inclusion body	7%	8%	25%	8%
Alveolar macrophage	93%	92%	100%	100%
Neutrophil Infiltration	20%	31%	33%	33%
Hyperemia	87%	100%	100%	100%

Table 2. Statistical results of RSV and PI-3 antigen in sheep and goat lung tissue by IHC method.

	RSV(+) Sheep	RSV(+) Goat		PI-3(+) Sheep	PI-3(+) Goat	
Bronchial epithelial cells and cells in the lumen	2.25±0.21 (2.50)	1.53±0.35 (1.00)	0.114	1.75±0.30(1.50)	1.66±0.35(1.00)	0.843
Bronchial glands	1.93±0.30 (2.50)	1.46±0.36(1.00)	0.398	1.41±0.22(1.00)	1.33±0.15(1.00)	0.713
Bronchiole epithelial cells and cells in the lumen	1.62±0.25 (1.50)	1.69±0.32 (1.00)	0.914	1.58±0.37 (1.00)	1.58±0.15 (1.00)	0.932
In inflammatory cells in the interalveolar septal tissue.	1.43±0.32 (1.50)	1.84±0.33(2.00)	0.374	0.91±0.28 (1.00)	1.41±0.16 (1.00)	0.319
Alveolar macrophages	1.37±0.32(1.00)	2.15±0.29 (3.00)	0.121	0.75±0.25 (1.00)*	1.58±0.25 (1.00)*	0.028*

*Data presented mean value ± standard deviation (median), significant differences (P<0.05) marked with different superscripts.

RSV and PI-3 antigen in lung tissue of sheep and goats, bronchial and bronchiolar epithelial cells and cell debris, in peribronchial glands and cell infiltration interalveolar septum were not statistically ($p>0.05$) significant (Fig. 2 B-C-D). In alveolar macrophages, PI-3 antigen in goats was statistically stained more intensely in IHC staining than in sheep ($p<0.05$). RSV

antigen was found to be positive in the cytoplasm of neutrophils (Fig. 2A) in one sheep and two goats. RSV antigen was not identified in the syncytial cell cytoplasm. Immunopositive staining was detected in bronchial cartilage tissue in PI-3 positive cases.

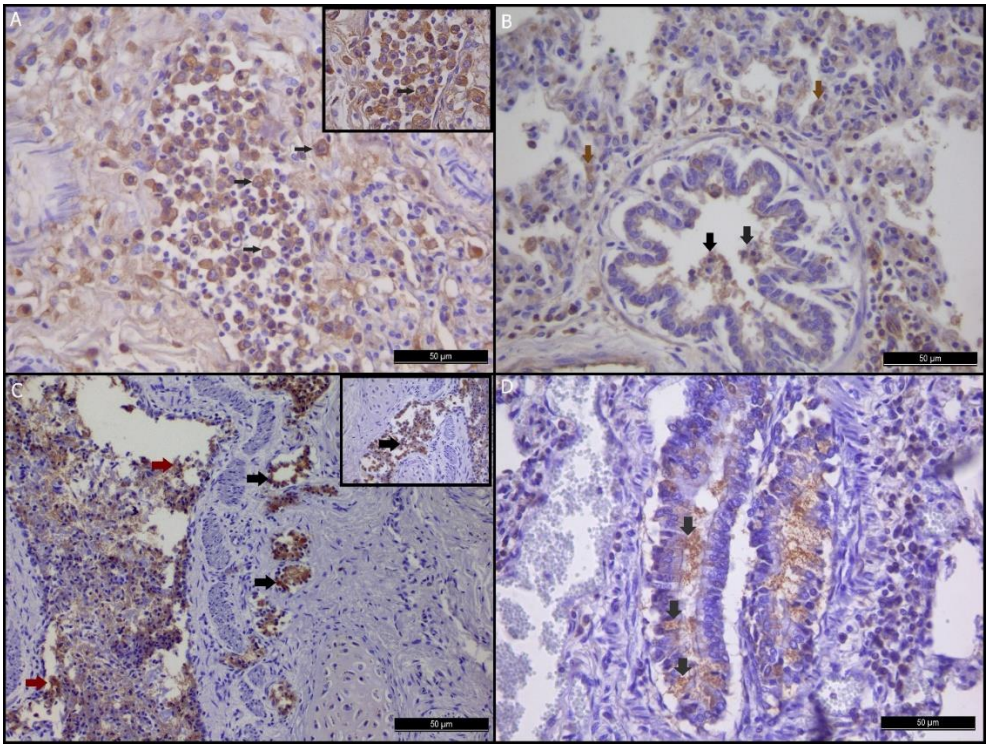

Figure 2. Immunohistochemical staining of sheep and goat lung's. **A.** Anti-RSV positive reactions in alveolar macrophages and neutrophils (arrows). Bar:50 µm. **B.** Anti-RSV positive reactions in the bronchiolar epithelium and cells debris (black arrows) and alveolar macrophages (red arrows). Bar: 50 µm. **C.** Anti-PI-3 positive reactions in the bronchial epithelium and cells debris (red arrows) and peribronchial glands (black arrows). Bar:50 µm. **D.** Anti- PI-3 positive reactions in bronchiolar epithelium and lumen (black arrows). Bar: 50 µm.

DISCUSSION

The anatomical structure of the lung, its mucociliary defense mechanism, lymphoid tissue associated with the mucosa, and phagocytic cells play an important role in defense against microorganisms (Nicod, 2005). Viral agents may suppress the mucociliary system in the airway epithelium and increase susceptibility to bacterial infection (Belknap et al., 1995; Bryson et al., 1991). In the study, lung paraffin blocks infected with natural RSV and PI-3 viruses, in which only viral pneumonia findings were detected, which are not complicated by bacterial agents, were preferred and the distribution of these viruses in the lung tissue was investigated by the IHC method.

BRSV occurs cytopathological changes in both ciliary and non-ciliary bronchiolar epithelial cells and types II pneumocytes (Masot et al., 1995; Viuff et al., 1996). Replication of the PI-3 virus is observed in epithelial cells of the respiratory tract and alveolar macrophages (Bryson et al., 1983). The agents cause viral pneumonia by both damaging the respiratory tract epithelium and inducing cytokines and chemokines in the lung tissue. RSV and PI-3 have histopathological findings such as bronchitis, bronchiolitis, alveolitis, degeneration, desquamation, necrosis or hyperplasia of the bronchiolar epithelium, thickening of the alveolar septum, lymphocyte and neutrophil infiltration, and syncytial cell in the lung tissue (Afshar and Terlecki, 1979; Caswell and Williams, 2007; Sacco et al.,

2014). In addition, it is reported that an increasing number of alveolar macrophages and exudates occur in RSV infections (Ceribasi et al., 2014; Sacco et al., 2014). In this study, histopathological findings caused by RSV and PI-3 factors were determined in sheep and goat lung tissue. As the infection progresses, an attempt to repair necrotic airways results in epithelial hyperplasia and bronchiolitis obliterans, also known as bronchiolitis obliterans (Caswell and Williams, 2007). In the study, bronchiolitis obliterans was not found in paraffin sections of sheep and goat lungs, since acute interstitial pneumonia findings were prominent.

In previous studies, it was determined that the areas where BRSV was detected in the lung by the immunohistochemical method were virus replication areas (Masot et al., 2000). In studies conducted with natural and experimental RSV infection of ruminants (Ceribasi et al., 2014; Jarikre and Emikpe, 2017; Masot et al., 2000; Viuff et al., 1996; Yener et al., 2005), bronchiolar epithelial cells were determined by the IHC method in type II pneumocytes, epithelial cells of bronchial glands, syncytial cells, alveolar macrophages, and exudate in the bronchial, bronchiolar, and alveoli lumen. In the study, it was determined that the intensity of RSV antigen staining in sheep and goats was statistically similar in bronchial and bronchiolar epithelial cells and cell debris, peribronchial glands and interalveolar inflammatory cells infiltrations. Also, unlike other studies, RSV antigen was determined to be positive in the neutrophil cytoplasm of one sheep and two goats. In this study, RSV antigen was not found in the syncytial cell cytoplasm in both sheep and goats, while the presence of RSV antigen in the syncytial cell cytoplasm of the IHC method was reported in the lung tissue of natural and experimental RSV infection of sheep and goats (Jarikre and Emikpe, 2017; Redondo et al., 2003).

In ruminant naturally infected with PI-3 virus, bronchial, bronchiolar, and alveolar epithelium, exudate in the lumen and syncytial cells were also identified by the IHC method (Çeribasi et al., 2012; Yener et al., 2005). In this study, similar to previous studies, PI-3 antigen was stained on bronchial and bronchiolar epithelial cells, peribronchial glands, interalveolar inflammatory cells in sheep and goats. In addition, PI-3 antigen was statistically significant in sheep and goat alveolar macrophages and stained more intensely in goats. Ceribasi et al. (2014) and Yener et al. (2005) detected PI-3 positivity in bronch cartilage tissue in cattle and goats, and in the study, PI-3 antigen was found in bronch cartilage in both sheep and goats.

PCR, Culture, Virus Isolation, Electron Microscopy, DFAT, IFAT, and IP techniques are used to determine the prevalence of RSV and PI-3 virus in naturally infected flocks in our country and in the World (Emikpe et al., 2019; Jarikre and Emikpe, 2017; Sharma et al., 2017; Tiwari et al., 2016). In previous studies, the immunohistochemical method (IHC) was used to determine the presence of RSV and PI-3 antigen in respiratory tract infections of ruminants and their distribution in the lung (Ceribasi et al., 2013; Haines et al., 1992; Yener et al., 2005). The immunohistochemical method is seen as an advantageous technique in reaching the diagnosis with the histological results of retrospective studies, although more time is needed to process formalin-fixed tissues. For the IHC method to be successful, tissue fixation and the preferred antiserum technique and quality must be good (Haines et al., 1992). In the study, it was determined that the prevalence of natural PI-3 infection with immunoperoxidase (IP) method in goats was lower than the study of Yener et al. (2005) and higher than the study of Çeribasi et al. (2012). In addition, the prevalence of RSV viral antigen was higher than the rate determined by the IHC method Ceribasi et al. (2013). The prevalence

of RSV in sheep herds in our country by the immunohistochemical method and its distribution and localization in lung tissue was presented for the first time with this study. The immunohistochemical method is thought to be an effective method that can be used to identify RSV and PI-3 virus from archive materials.

CONCLUSION

It was concluded that the localization of RSV and PI-3 antigens in sheep and goat lung tissue was detected similar by this study. In addition, it was determined that RSV and PI-3 antigens were common in sheep and goats and it was thought that vaccination should be done for protection.

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Ethical approval: This study was approved by the Animal Experiments Local Ethics Committee of Samsun Veterinary Control Institute Directorate (Approval no: 2019/3-09.05.19)

Conflict of interest: There is no conflict of interest between the authors

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