

Histopathologic, immunoperoxidase and immunofluorescent examinations on natural cattle pneumonia originated from Parainfluenza type 3, Respiratory Syncytial virus, Adenovirus type 3 and Herpesvirus type 1

A. O. CERIBASI^{1*}, M. OZKARACA², S. CERIBASI¹, H. OZER¹

¹Department of Pathology, Faculty of Veterinary Medicine, Firat University, 23119 Elazığ - TURKEY

²Department of Pathology, Faculty of Veterinary Medicine, Atatürk University, 25240 Erzurum - TURKEY

Corresponding author: aoceribasi@firat.edu.tr; aceribasi@hotmail.com

SUMMARY

The aim of this study was to determine Parainfluenza type 3 (PI3), Respiratory Syncytial virus (BRSV), Bovine Adenovirus type 3 (BAV3) and Bovine Herpesvirus type 1 (BHV1) antigens using direct fluorescent antibody technique (DFAT) and immunoperoxidase (IP) staining of frozen and formalin-fixed paraffin-embedded tissues in pneumonic lungs of cattle. For this purpose, the lungs of 2742 male cattle, which were raised in the farms in Elazığ and surrounding areas and brought to the abattoir for slaughtering between January and December 2011, were examined. The macroscopic findings in 263 (9.59%) lungs, which pneumonia was detected only in apical and cardiac lobes, were recorded. Mild (65.02%), moderate (28.14%) and severe (6.84%) consolidations were detected in the pneumonic cases. Pneumonia were microscopically classified as catarrhal-purulent (32.32%), interstitial (45.25%), fibrinous (16.35%) and granulomatous (6.08%). A total of 247 lungs with pneumonia excluding those with granulomatous pneumonia were examined for PI3, BRSV, BAV3 and BHV1 viral antigens by IP and DFAT. PI3, BRSV, BAV3 and BHV1 antigens determined in natural pneumonia cases of cattle were found to be 6.88%, 3.64%, 5.26% and 2.43% by IP method and 10.53%, 6.07%, 6.88% and 4.45% by DFAT, respectively. It can be concluded that these viruses might have important role in the pathogenesis of pneumonia in cattle. In addition, DFAT and IP technique may be used for definitive diagnosis as valuable methods when the difficulties in the determination of specific histologic lesions in natural cattle pneumonia originated from PI3, BRSV, BAV3 and BHV1 antigens were considered. When all the data were considered overall, it can be suggested that there is an urgent need to take necessary measures in order to control and prevent these infections in the country.

Keywords: Parainfluenza type 3, Respiratory Syncytial virus, Bovine Adenovirus type 3, Bovine Herpesvirus type 1, immunoperoxidase, immunofluorescent, histopathology, pneumonie, cattle.

RESUME

Examens histopathologique et immunohistochimique de poumons de bovins atteints d'infection par parainfluenza de type 3, virus respiratoire syncytial, adénovirus de type 3 et type de virus de l'herpès 1

Le but de cette étude était de déterminer dans des poumons de bovins congelés et ou fixés au formolaldehyde la présence d'antigènes de parainfluenza de type 3 (PI3), virus respiratoire syncytial bovin (BRSV), adénovirus bovin de type 3 (BAV3) et l'herpèsvirus bovin de type 1 (BHV-1) révélés par immunofluorescence directe (DFAT) et coloration à l'immunoperoxydase (IP). A cet effet des poumons de 2742 bovins mâles, élevés dans les fermes de la région d'Elazığ abattus entre Janvier et Décembre 2011 ont été examinés. L'examen macroscopique a révélé des lésions dans 263 (9,59%) poumons dans les lobes apicaux et cardiaques. Des lésions de consolidations faibles (65,02%), modérées (28,14%) et graves (6,84%) ont été détectés dans les cas de pneumonie. Les lésions ont été classées après examen microscopique comme catarrhale-purulente (32,32%), interstitielle (45,25%), fibrineuse (16,35%) et granulomateuse (6,08%). Un total de 247 poumons atteints de pneumonie, à l'exclusion de ceux présentant des lésions granulomateuses, a été examiné pour détection des antigènes viraux PI3, BRSV, BAV3 et BHV1 par IP et DFAT. Des antigènes PI3, BRSV, BAV3 et BHV1 ont été retrouvés dans 6,88%, 3,64%, 5,26% et 2,43% des cas par la méthode IP et 10,53%, 6,07%, 6,88% et 4,45% des cas par DFAT, respectivement. Il peut être conclu que ces virus peuvent avoir un rôle important dans la pathogénie des pneumonies chez les bovins. En outre, les techniques DFAT et IP peuvent être utilisées pour le diagnostic lorsque des difficultés sont rencontrées dans l'analyse histologique des pneumonies bovines avec virus PI3, BRSV, BAV3 et BHV-1. Par ailleurs, ces résultats démontrent un besoin urgent de contrôle et prévention de ces infections dans le pays.

Mots-clés: parainfluenza virus de type 3, virus respiratoire syncytial, adénovirus bovin de type 3, herpès virus bovin type 1, immunoperoxidase, immunofluorescence, histopathologie, pneumonie, bovins.

Introduction

Pneumonia is one of the most important diseases that cause economic losses in cattle industry [6, 10, 51]. It has been explained that pneumonia can be arisen from many factors like bacteria, viruses and stress factors which suppress the local defense mechanisms [11]. Parainfluenza Type 3 virus (PI3), Bovine Respiratory Syncytial virus (BRSV), Bovine Adenovirus 3 (BAV3) and Bovine Herpesvirus 1 (BHV1)

are important viral pathogens that cause respiratory tract infections in cattle [11, 16, 21, 29, 34, 47]. Macroscopically, especially consolidated areas with gray-red discoloration and atelectasis were determined in lungs in natural and experimental infections with these viruses [4, 26, 33, 41, 47]. It has been reported that microscopically; necrotic bronchiolitis, alveolitis, hyperplasia in bronchi, bronchioles and type II pneumocytes, interalveolar septums thickening, atelectasis, lymphoid hyperplasia, eosinophilic inclusions

in respiratory tract epithelium characterized by pulmonary lesions observed in these viral infections [6, 26, 30-33, 40, 46].

Only histologic examination of sections stained with hematoxylin eosine (HE) has been reported to be insufficient for the diagnosis because pathologic changes observed in lungs infected with PI3 and BRSV or BAV3 and BHV1 are similar to each other [11]. These viruses may be detected by the methods such as direct virus isolation, cell culture, polymerase chain reaction, immunoperoxidase (IP), indirect fluorescent antibody technique (IFAT), direct fluorescent antibody technique (DFAT) and electron microscopy [2, 18, 19, 22, 27, 30, 33, 45, 46, 51]. Many serological methods are used for the indirect diagnosis [1, 16, 17, 28, 48]. It has been reported that isolation of PI3 were achieved in cell cultures with bovine origin, but isolation of BRSV, which is a labile virus, was difficult due to slow development of the virus in cell culture [12]. Immunofluorescence staining is used for the determination of PI3 and BRSV antigens in the frozen lung samples though its cellular and structural differentiation is difficult [41, 45]. It has been described that IP method provides an opportunity to clearly be showed viral antigens in pulmonary lesions [7].

There are no detailed studies which employed immunoperoxidase and immunofluorescence techniques to investigate the role of these viruses in pneumonia cases of cattle in Turkey. Therefore, the aim of this study was to determine PI3, BRSV, BAV3 and BHV1 antigens using by direct fluorescent antibody technique (DFAT) and immunoperoxidase (IP) staining of frozen and formalin-fixed paraffin-embedded tissues in pneumonic lungs of cattle in Elazig region of Turkey.

Materials and Methods

SAMPLE COLLECTION

The lungs of 2742 male cattle, which were raised in Elazig and surrounding areas and were brought to the abattoir for slaughtering between January and December 2011, were examined. The macroscopic findings in 263 (9.59 %) lungs with pneumonia detected only in apical and cardiac lobes were recorded. The tissue samples taken from affected lungs were separated into two parts; one part was fixed in formalin, the other was stored at - 80 C° to obtain frozen sections.

GROSS PATHOLOGICAL EXAMINATION

The severity of pneumonia in apical and cardiac lobes was scored based on the extent of consolidation. Lesions that were determined less than 10%, 10-20% and more than 20% of the apical and cardiac lobe volumes were evaluated as "mild", "moderate" and "severe", respectively. Tissue samples taken from grossly consolidated lungs were fixed in 10% buffered formalin for 48 h and were embedded in paraffin wax before sectioning. The tissues were then stained

with hematoxylin eosin (HE), and finally were examined under light microscopy. IP and DFAT methods were applied to the sections with different types of pneumonia except granulomatous pneumonia.

IP EXAMINATION

IP staining was performed according to the avidin-biotin-peroxidase complex procedure [25]. As the primary antibody; monoclonal mouse anti-PI3 for PI3 (Cat No.: BIO 290, Biox Jemelle, Belgium), monoclonal mouse anti-BRSV for BRSV (Cat No.: BIO 031, Biox Jemelle, Belgium), monoclonal mouse anti-adenovirus type 3 for BAV3 (Cat No.: BIO 292, BIOX Jemelle, Belgium), monoclonal mouse anti-BHV1 for BHV1 (Cat No: BIO 289, BIOX Jemelle, Belgium) were used. IP kits used for other stages were obtained from a commercial company (Ultravision Detection System, Antipolyvalent, HRP/DAB, Thermo Scientific, Cat No: TP-015-HD) and, staining was performed according to the manufacturer's procedure. For IP staining, deparaffinized tissue sections were taken to citrate buffer solution (10 mM citric acid, pH: 6.0) and allowed to stand in the microwave at antigen retrieval step for 20 min. After the sections were allowed to cool and incubated in 70% methanol with 3% H₂O₂ for 10 min. to prevent endogenous peroxidase activity, and were then washed three times with phosphate buffered saline (PBS). The sections were treated with a blocking solution for 10 min. After draining the blocking serum, the sections were incubated with primary antibodies, diluted to 1:50 rate in PBS at 4° C overnight in a humidified chamber. After washing three times with PBS, the sections were treated with biotinylated anti-goat polyvalent secondary antibody for 10 min. Then the sections were washed three times in PBS and treated with the peroxidase-conjugated streptavidin for 10 min. After another PBS bath, the sections were incubated with 3,3 diaminobenzidine (DAB). After color change, the sections were washed tap water and then counterstained with Mayer's hematoxylin (MH). Lung from unaffected cattle was used as a negative controls. For positive controls, primary sera were used instead of non-immun rabbit serum in the pneumonic lung tissues.

DFAT EXAMINATION

DFAT was used for the detection of PI3, BRSV, BAV3 and BHV1 antigens. For this purpose, monoclonal mouse anti-PI3 FITC (Cat No: BIO 030, Biox Jemelle, Belgium) and monoclonal mouse anti-BRSV FITC (Cat No: BIO 032, Biox Jemelle, Belgium), monoclonal mouse anti-BAV3 FITC (Cat No: BIO 039, BIOX Jemelle, Belgium) and monoclonal mouse anti-BHV1 FITC (Cat No: BIO 026, BIOX Jemelle, Belgium) conjugates were provided from a commercial company. Immunofluorescence staining was performed according to the manufacturer's procedure. Lung tissues were stored at -80 °C until frozen sections were taken. Frozen sections cut at 6 microns were placed on 0.01% poly-D-lysine hydrobromide coated slides and were air-dried. Then, the sections were fixed in acetone/PBS mixture for 10 min at room temperature.

	Jan	Feb	Mar	April	May	June	July	Aug	Sep	Oct	Nov	Dec	Total	
The number of cattle lungs examined	276	298	263	287	219	192	178	165	200	222	183	261	2742	
Severity of Lesion														%
Mild	15	19	25	21	15	9	10	7	10	12	13	15	171	65.02
Moderate	7	6	9	10	7	6	2	4	6	5	7	5	74	28.14
Severe	2	2	3	3	2	1	-	1	-	1	2	1	18	6.84
Type of Pneumonia														
Catarrhal-purulent pneumonia	9	7	13	11	8	5	3	3	5	5	8	8	85	32.32
Intertitial pneumonia	11	12	17	15	10	8	6	6	6	10	10	8	119	45.25
Fibrinous pneumonia	3	6	5	7	4	2	1	2	3	2	4	4	43	16.35
Granulomatous pneumonia	1	2	2	1	2	1	2	1	2	1	-	1	16	6.08

TABLE I: Severity, type and distribution of pneumonic lesions by month.

	Jan	Feb	Mar	April	May	June	July	Aug	Sep	Oct	Nov	Dec	Total																											
Number of Cases with P	22/276	24/298	33/263	32/287	23/219	26/192	9/178	9/165	14/200	18/222	16/183	21/261	247/2726																											
	(%7.97)	(%8.05)	(%12.55)	(%11.15)	(10.50)	(%13.54)	(%5.06)	(%5.45)	(%7.00)	(%8.11)	(%8.74)	(%8.05)	(%9.06)																											
Severity of Consolidation																																								
Mild	14	1	2	17	3	4	23	4	8	20	7	9	15	4	5	20	6	8	7	1	2	7	-	1	10	1	2	12	3	4	12	3	4	14	1	2	171	34	51	
Moderate	6	-	-	5	-	1	8	1	2	9	2	3	7	1	1	5	2	3	2	1	1	1	1	-	-	4	1	1	5	1	2	3	-	1	5	1	1	60	10	16
Severe	2	-	-	2	-	-	2	1	1	3	-	1	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1	-	-	2	-	-	16	1	2

P: Pneumonia, IP: Immunoperoxidase, D: Direct Fluoresan Antibody Technique.

TABLE II: Severity of consolidation in 247 cases with pneumonia, and the data related to PI-3, BRSV, BAV3 and BHV1 viral antigens determined by IP and DFAT methods.

After washing with PBS, the sections were treated with the conjugates diluted in a 1:20 ratio with PBS-Evans Blue at room temperature for 1 h. After washing with PBS again, the slides were mounted with glycerol. Finally, the sections were examined under fluorescent microscope, and the results were evaluated. Processes mentioned for the IP method were applied for the negative and conjugate controls.

Results

GROSS PATHOLOGICAL FINDINGS

In this study, 2742 cattle lungs were examined and pneumonia was observed macroscopically in the apical and

cardiac lobes of 263 cases (9.59%). The severity, type and rates of pneumonia are summarized in Table I. Pulmonary lesions were macroscopically characterized with patchy or confluent consolidated purple-red or grey foci and irregular lobular atelectatic foci. In some of the lungs, thickening and cloudy appearance in pleura and adhesions between lobes were detected. In most cases, it was observed that mediastinal lymph nodes were enlarged and the section surface was edematous. The consolidation was detected mild in 65.02%, moderate in 28.14% and severe in 6.84% of cases.

In the microscopical examination, pneumonia were classified as catarrhal-purulent (n=85, 32.32%), interstitial (n=119, 45.25%), fibrinous (n=43, 16.35%) and

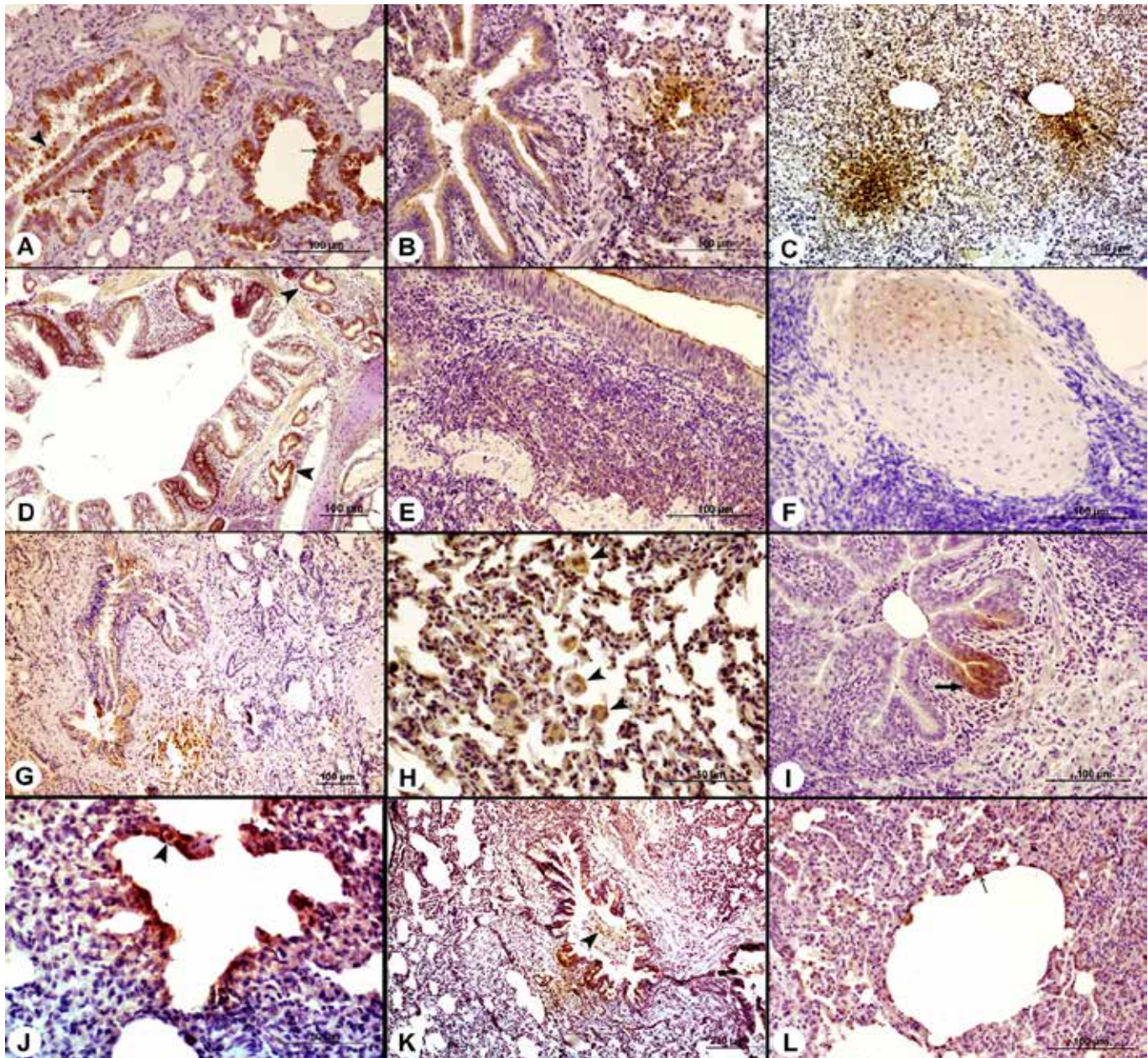


FIGURE 1: A. PI3 viral antigen positivity in bronchioles epithelium (arrows), and desquamative necrotic cells (arrowhead) with immunoperoxidase staining. B. PI3 immunopositivity in alveolar epithelium near the bronchiol. C. PI3 positive IP staining in alveolar macrophages and desquamative necrotic cells in alveol lumen. D. PI3 viral antigen positivity in bronchial glands epithelium (arrowheads). E. PI3 viral antigen positivity in bronchiolar associated lymphoid tissue. F. PI3 immunopositivity in the cartilage tissue of the bronchi. G. BRSV viral antigen positivity in bronchiol and alveol epithelium. H. BRSV immunopositivity in syncytial cells in alveol lumen (arrowheads). I. BAV3 positivity in bronchioles epithelium (big arrow) by IP staining. J. BAV3 antigen positivity in alveolar epithelium nuclei (arrowhead). K. BHV1 antigen positivity in bronchioles epithelium, and desquamative necrotic cells (arrowhead) by IP staining. L. BHV1 immunopositivity in alveolar epithelium (small arrow). Counterstained with MH.

granulomatous (n=16, 6.08%). A total of 247 lungs with pneumonia excluding those with granulomatous pneumonia were examined for PI3, BRSV, BAV3 and BHV1 viral antigens by IP and DFAT.

IP FINDINGS

PI3, BRSV, BAV3 and BHV1 antigens were determined in 17 (6.88%), 9 (3.64%), 13 (5.26%) and 6 (2.43%) of 247 cases by IP staining method, respectively. Mixed infections of PI3+BRSV in 1 case and PI3+BAV3 in 1 case were observed in IP staining. Positive staining was noticed to be generally present in the pneumonic areas.

The immune staining was located as intracytoplasmic in all PI3 positive cases. IP staining for PI3 viral antigens were usually diffuse and less often in granular or linear appearance on the luminal surfaces of bronchioles and alveolar epithelium. Specific IP staining for PI3 viral antigens was detected mostly in bronchial epithelium and less in alveolar epithelium (Fig. 1A). PI3 positivity was more pronounced in alveoli adjacent to bronchioles (Fig. 1B). Also, immunopositivity was observed in the plasma and lymphocytes surrounding bronchi, bronchioles and the blood vessels, and in cellular exudate in the airways. Mild alveolar IP staining was observed in lymphocytes and macrophages and in interalveolar septa, type II pneumocytes, alveolar macrophages, cellular exudate in alveolar lumen and less often in alveolar epithelium (Fig. 1C). Mild positive staining in syncytial cells of bronchioles and alveolar lumen was observed in PI3 positive cases. Furthermore, PI3 viral antigen positivity was observed in the epithelium of bronchial glands (Fig. 1D). In addition, PI3 viral antigens were observed in lymphoid tissue associated with bronchiole (Fig. 1E). PI3 immunopositivity was also prominent in the cartilage tissue cells of the bronchi (Fig. 1F).

Although IP staining for BRSV viral antigens, which was similar to PI3 positive cases, was observed in bronchi, bronchioles and alveolar epithelium, antigenic distribution had a more localized view. It was observed that BRSV positivity had granular appearance in bronchi, bronchioles and alveolar epithelium and, was settled intracytoplasmically (Fig. 1G). In addition, positive immunostaining was noticed in alveolar macrophages in interalveolar septum, cellular exudate, which was poured into bronchioles, and alveolar lumen, and also in peribronchiolar lymphoid tissue. In BRSV positive cases, positive staining in syncytial cells in bronchioles and alveolar lumen was also noted (Fig. 1H). No immune reaction was observed in bronchial glands and peribronchial cartilage tissue.

BAV3 antigen was identified in bronchiolar and alveolar epithelial cells. In addition the positive staining was detected in infiltrating peribronchiolar mononuclear cells. The IP staining for BAV3 antigens was located intracytoplasmically in bronchiolar epithelium (Fig. 1I) but intranuclear staining was noticed in some alveolar epithelial cells (Fig. 1J).

BHV1 antigen was detected intracytoplasmically in the bronchial, bronchiolar and alveolar epithelial cells and the distribution of this antigen was very restricted. In addition, IP staining was present in the cellular exudate in the lumen of bronchioles (Fig. 1K). Moreover specific immunolabelling of BHV1 antigens was limited in alveolar epithelial cells but more prominent in alveolar macrophages in lumen (Fig. 1L). Intranuclear immunostaining for BHV1 antigens was observed in none of the positive cases.

No immunopositive staining was observed in tissues from healthy lung (negative control) or in the pneumonic lung tissue on which non-immune rabbit serum was added (serum control). With respect to degree of macroscopic lesions; IP positivity determined in pneumonic lungs with PI3, BRSV, BAV3 and BHV1 antigens was mild in 34 cases, moderate in 10 cases and severe in 1 case (Table II).

DFAT FINDINGS

PI3, BRSV, BAV3 and BHV1 viral antigens were detected by DFAT staining in 26 (10.53%), 15 (6.07%), 17 (6.88%) and 11 (4.45%) of 247 lungs with pneumonia, respectively. Mixed infections with PI3+BAV3 in 3 cases, and with PI3+BRSV, PI3+BHV1, BRSV+BHV1 and PI3+BRSV+BAV3 in 1 case each were observed in DFAT staining. All the positive cases with IP staining were also positive for fluorescent staining with varying severity and distribution.

With respect to viral antigens by DFAT staining severity, more intensive fluorescent areas were observed in PI3 positive cases (Fig. 2A) that was followed by BRSV (Fig. 2B), BHV1 (Fig. 2C) and BAV3 positive cases (Fig. 2D), respectively. Specific DFAT staining for each of viral antigens was detected in bronchiolar epithelium intensely and less often in alveolar epithelium as size varying from small to large homogeneous granular masses. Same fluorescent staining was also present in cellular exudate in the airways and interalveolar septum. Fluorescent staining was detected in the cytoplasm though it was difficult to see because frozen sections were thick (Fig. 2E,F,G,H). No fluorescence staining was detected in control sections. In addition, autofluorescence reactions were especially present in capillary vessels. With respect to degree of macroscopic lesions; DFAT positivity determined in pneumonic lungs with PI3, BRSV, BAV3 and BHV1 antigens was mild in 51 cases, moderate in 16 cases and severe in 2 cases (Table II).

HISTOPATHOLOGICAL FINDINGS

Cases which were DFAT positive for only PI3, BRSV, BAV3 and BHV1 antigens were examined histopathologically. Generally, thickening of interalveolar septa and fibrosis, hyperplasia, degeneration or desquamation of bronchiolar epithelium, peribronchiolar and interalveolar mononuclear cell infiltrations and increased numbers of alveolar macrophages were most prominent microscopic changes in all of the cases positive for PI3, BRSV, BAV3 and BHV1

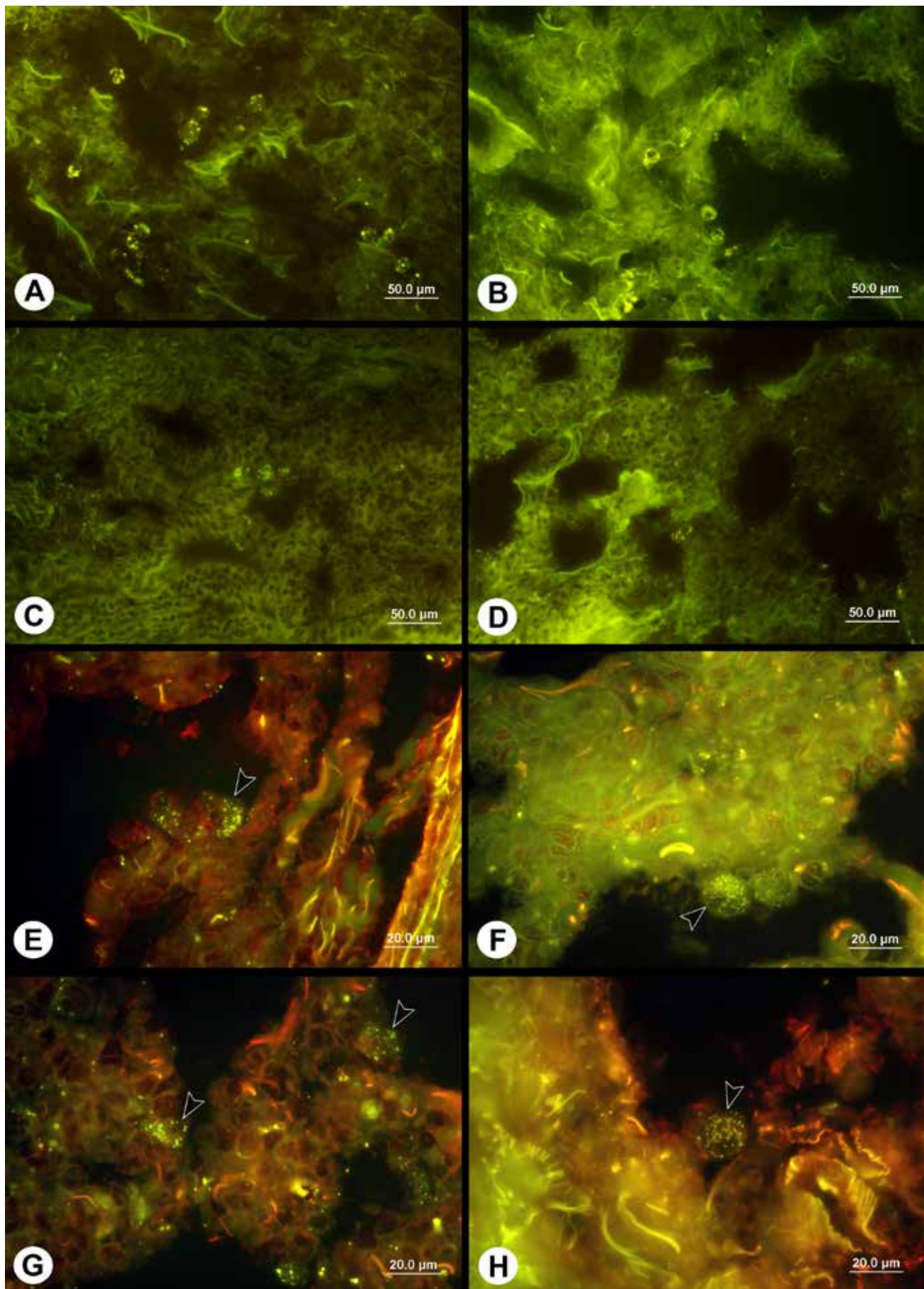


FIGURE 2: A. PI3 fluorescence staining in desquamative necrotic cells, alveolar macrophages and alveolar epithelium in alveoli lumen. B. BRSV positivity in desquamative necrotic cells in bronchioles epithelium by DFAT. C. BHV1 fluorescence staining in alveolar epithelium. D. BAV3 positivity in alveolar epithelium by DFAT. E. Intracytoplasmic PI3 staining in bronchioles epithelium by DFAT (arrowhead). F. Intracytoplasmic BRSV immunofluorescence staining in bronchioles epithelium by DFAT (arrowhead). G. Intracytoplasmic BHV1 fluorescence staining in bronchioles epithelium by DFAT (arrowheads). H. Intracytoplasmic BAV3 antigen immunofluorescence staining in desquamative epithelium in bronchioles lumen by DFAT (arrowhead).

antigens (Table III). In addition lymphoid hyperplasia was the other significant lesion in these viral infections (Fig. 3A). Also, bronchiolitis obliterans characterized by organized fibrous tissue formation which was covered with a single layer of cuboidal epithelium in the bronchiole lumen was another prominent lesion (Fig. 3B). In some PI3, BRSV, BAV3 and BHV1 positive cases, necrotic bronchiolitis was present (Fig. 3C). In many cases, it was observed that bronchioles adjacent alveolar parahypha was destroyed and atelectatic. These alveoli consistently revealed marked proliferation of type II pneumocytes and increased numbers of alveolar macrophages (Fig. 3D). In some PI3, BRSV and BHV1 positive lungs, alveoli lumina were filled with dark basophilic, oat-shaped clustered leucocytes having elongated or streaming nuclei. In some cases, it was noticed that pleuritis characterized by fibrinous exudate with mononuclear and neutrophil infiltration participated in chronic pneumonia. Intracytoplasmic inclusions were observed in the bronchiolar epithelium of two BRSV positive and one PI3 positive cases (Fig. 3E). In nine PI3 and three BRSV positive cases, syncytial cell formations were present in bronchioles and alveolar lumens (Fig. 3F). Intranuclear inclusion was found in desquamated alveolar epithelium only one BAV3 positive case (Fig. 3G). In some of the PI3, BRSV and BHV1 positive chronic pneumonia cases alveolar epithelialization as

noticed, which was characterized by transformation of single layer cubic epithelium (Fig. 3H).

Discussion

In previous studies, DFAT has successfully been used as a rapid diagnostic method for the diagnosis of PI3, BRSV, BAV3 and BHV1 in respiratory tract infections in cattle [2, 16, 19, 22, 27, 34, 45, 51]. Previous studies have reported that fluorescent antibody technique (FAT) is an advantageous technique in terms of achieving rapid results because preparation and examination of the samples is made within short time (Forghani, Edwards). However, FAT is an inadequate technique to determine appropriate morphological details in tissues [13, 42]. Although processing of formalin-fixed tissues needs more time, histologic results of retrospective studies show that formalin-fixed sections are superior to frozen sections in terms of accurate identification of the cell and tissue types. Moreover, it has been emphasized that IP technique is more sensitive than the mentioned techniques due to virus or antigens in alveoli could not be determined exactly with ultrastructural and immunofluorescence techniques [4]. However, immunogenic epitopes and many antisera used for IP diagnosis are unreactive in fixed specimens due to the damaging effect of fixation [24]. For

Lesions	PI3	BRSV	BAV3	BHV1	Total	%
DFAT positivity	26/247	15/247	17/247	11/247	69/247	27.94
IP positivity	17/247	9/247	13/247	6/247	45/247	18.22
Necrotic bronchiolitis	5/26	4/15	2/17	5/11	16/69	23.19
Bronşiyolitis obliterans	16/26	8/15	10/17	3/11	37/69	53.62
Hyperplasia in bronch and bronchiol epithelium	25/26	12/15	12/17	11/11	60/69	86.96
Desquamation in bronch and bronchiol epithelium	26/26	15/15	17/17	11/11	69/69	100
Degeneration in bronch and bronchiol epithelium	24/26	15/15	17/17	10/11	66/69	95.65
Syncytial cell formation	9/26	3/15	2/17*	0/11	14/69	20.29
Lymphoid hyperplasia	17/26	7/15	9/17	7/11	40/69	57.97
Interalveolar septal fibrosis	22/26	15/15	12/17	6/11	55/69	79.71
Alveolar epithelisation	3/26	3/15	0/17	4/11	10/69	14.49
Oat cell infiltration	9/26	4/15	0/17	2/11	15/69	21.74
Intraalveoli edema	9/26	5/15	4/17	0/11	18/69	26.09
Hyalin membran formation	3/26	4/15	2/17	2/11	11/69	15.94
Pleuritis	8/26	3/15	1/17	3/11	15/69	21.74
Neutrophil infiltration	16/26	5/15	4/17	4/11	29/69	42.03
Mononuclear cell infiltration	26/26	15/15	17/17	11/11	69/69	100
Atelectasia	19/26	12/15	9/17	8/11	48/69	69.57
Intrasitoplasmic inclusion body	1/26	2/15	1/17*	0/11	4/69	5.80
Intranuclear inclusion body	0/26	0/15	1/17	0/11	1/69	1.45
Alveolar macrophage proliferation	24/26	13/15	11/17	9/11	57/69	82.61
Emphysema	6/26	5/15	5/17	1/11	17/69	24.64

*: Mixed infections.

TABLE III: Microscopic findings in PI3, BRSV, BAV3 and BHV1 antigens positive cattle pneumonia.

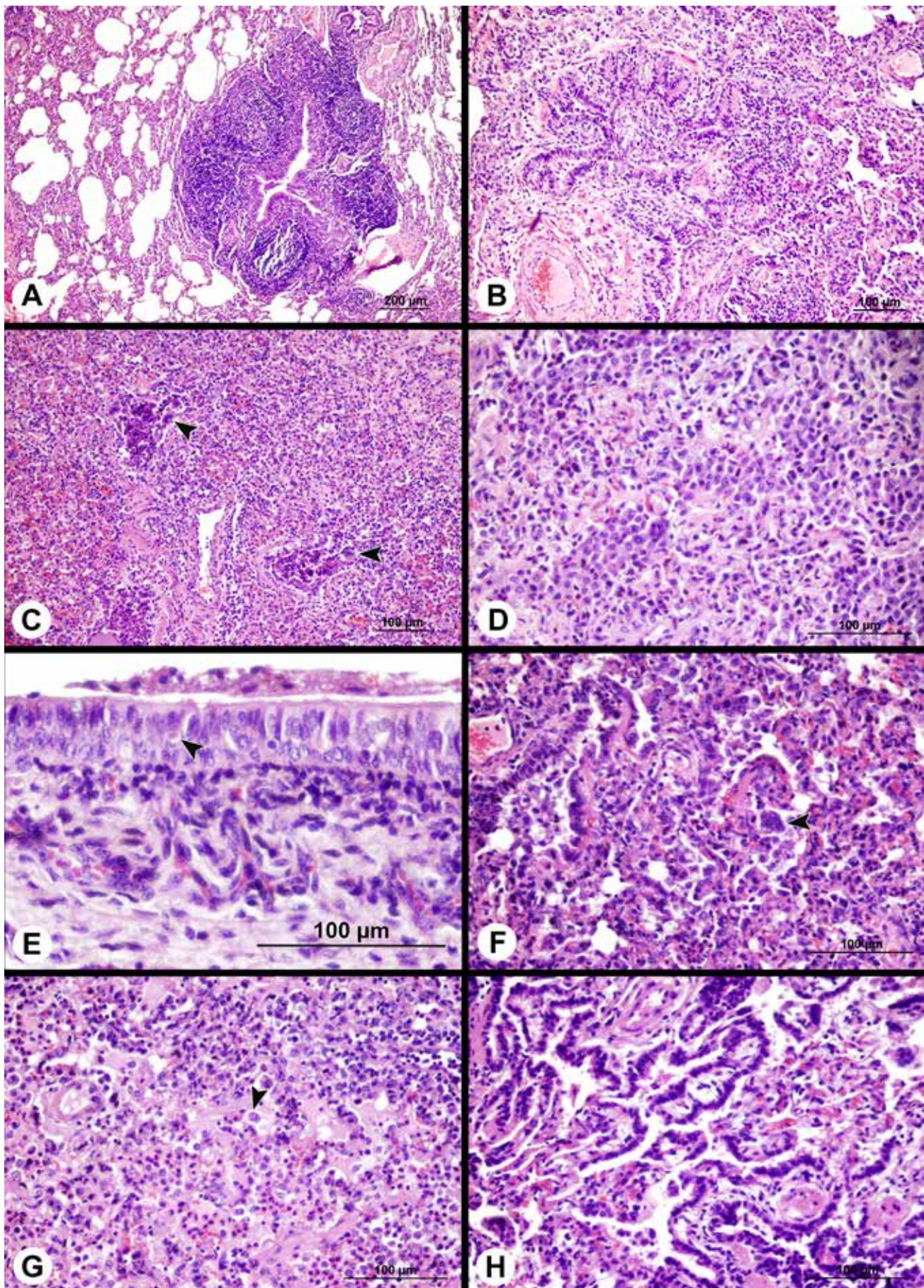


FIGURE 3: A. Lymphoid hyperplasia in peribronchiole. B. Bronchiolitis obliterans characterized by fibrous connective tissue formation and mononuclear cell infiltration in bronchiol lumen. C. Necrotic bronchiolitis and syncytial cell formations (arrowheads). D. Alveolar macrophage proliferations in alveol lumen. E. Intracytoplasmic inclusion in bronchiol epithelium. F. Syncytial cell formation in alveolar lumen. G. Intranuclear inclusion in desquamative alveolar epithelium (arrowhead). H. Alveolar epithelialization characterized by transformation cubic epithelium. HE.

the diagnosis of respiratory diseases in cattle, identification of viral antigens in lesions and association of specific cell types with lesions have been reported to be important due to isolation viruses from healthy animals [25]. In the present study, PI3, BRSV, BAV3 and BHV1 antigens were identified in 69 lungs (27.94%) by DFAT and 45 lungs (18.22%) by IP, of the total 247 pneumonia cases. With respect to distribution of viral antigens, more detailed findings were observed with IP methods than DFAT staining.

It has been reported that PI3, BRSV, BAV3 and BHV1 viral pneumonia infections are microscopically characterized by bronchitis, bronchiolitis, alveolitis, degeneration, desquamation, necrosis or hyperplasia in bronchioles epithelium, thickening of alveolar septa and fibrosis, atelectasis, neutrophil and lymphocytes infiltration, syncytial cell formation and nuclear or cytoplasmic inclusions [11, 26, 28, 30-32, 39]. It has been emphasized that syncytial cells in the lumen of the bronchioles and alveoli seen in PI3 and BRSV infections are originated from combination of the cells due to fusion protein while the viruses spread directly from cell to cell [46]. The histopathological findings of the present study were similar to the results of previous studies, and intracytoplasmic inclusions in two BRSV and one PI3 positive cases, and syncytial cell formations in 9 PI3 and 3 BRSV positive cases were found. However intranuclear inclusion was not observed in any BHV1 positive cases although intranuclear inclusion was seen in only one BAV3 positive case. Earlier studies have suggested that the determination of viral pneumonia-specific lesions like inclusion bodies and syncytial cell formations in PI3, BRSV, BAV3 and BHV1 infected cattle may depend on many factors such as animal species and age, virulence of agent, amount of virus, infection period, and presence of secondary bacterial infections [10, 11, 14, 33, 46]. Thus, definitive diagnosis of virus associated pneumonia has been reported to be made with PCR, culture, virus isolation, electron microscopy, DFAT, IFAT and IP techniques [20, 27, 30, 31, 40, 45].

The mucociliary system has a major role in lung's physical defence against inhaled microorganisms [43]. It has been explained that in some viral respiratory infections, proliferation and penetration of bacteria was caused by the marked decrease in mucociliary cleaning due to destroyed ciliary activity in respiratory tract [10, 14, 30, 44, 47]. It has been described with ultrastructurally studies that BRSV infections damage to the bronchial ciliary apparatus [8]. In addition, it has been suggested that there were multiple mechanisms that contribute to the formation of pneumonia in PI3 virus infections such as prevention of alveolar macrophage functions and protein-rich fluid which helps the development of bacteria in lung and the presence of necrotic cells [15]. In the present study, when the correlation between the presence of viral antigens and the severity of the macroscopic lesions were evaluated, viral antigens have been identified mostly in mild consolidation (DFAT: 51/69, IP:34/45) by both methods. This situation, as described previously, was interpreted to be due to the difficulty in

the determination of the presence of viral antigens with secondary bacterial infection [11].

It has been reported that secondary bacterial infections especially *Mannheimia haemolytica* and *Mycoplasma* spp. occur in viral pneumonia of cattle [10, 11, 30, 46]. In addition, *Mycoplasma* spp. were isolated from natural BRSV lung infections, and it has been interpreted that hyperplasia in the bronchial lymphoid tissue in the lung may be caused by virus or *Mycoplasma* spp. [46]. Additionally, hyperplasia was found in bronchial lymphoid tissue of lungs in experimentally BAV3 infected cattle [26, 32]. Similarly, *Mycoplasma* spp. was isolated from the calves with BRSV infection that cuffing pneumonia was detected in the lung [39]. In the present study, histopathologically oat-shaped inflammatory cells, which are the prominent lesions of *M. haemolytica* or *Pasteurella* spp. infections were identified in 21.74% of DFAT positive lungs, and peribronchial and peribronchiolar lymphoid hyperplasia was observed in 57.97% of DFAT positive cases and it was concluded that these cases could be mixed bacterial infections. Also, it has been reported that multiple viruses could play a role in the formation of respiratory diseases in cattle [3, 35]. In our study, mixed infections were found in 2.83% and 0.81% of cattle with pneumonia by DFAT and by IP, respectively.

It has been identified that BRSV replication occurred in epithelium with and without cilia in respiratory tract and type II pneumocytes [8, 11]. It has been reported that BRSV showed its direct effect due to proliferation in the epithelium of the bronchi and bronchioles as necrosis and desquamation [8]. As well as the direct cytopathic effect of BRSV, it has been reported that it had some indirect effects; alveolar collapse were at the beginning of these effects, which was caused by occlusion of the lumen of small bronchi and bronchioles by cellular exudate, the virus cytokine response and the virus caused surfactant deficiency by proliferating in type II alveolar epithelium [46]. In our study, significant alveolar atelectasis was observed in more than half of the viral positive cases (69.57%), which was concluded to occur due to the reasons mentioned above.

Diffuse cytoplasmic stainings in alveolar and bronchiolar epithelium were observed in PI3 experimental infection of calves with IP method [25]. In natural and experimental BRSV infections in calves, viral antigens in the lung were identified with IP in necrotic cells poured into the lumen of alveoli, bronchioles and bronchi, alveolar and bronchiolar epithelial cells, and syncytial cells in the respiratory tract [6, 46]. Viral antigens were detected in epithelial cells of bronchi, bronchiole and alveoli experimentally BAV3 and BHV1 infected calves by IP method [30, 32]. The findings of the present study are consistent with the results of previous studies with regard to distribution and localization of these viral antigens determined by IP method [6, 25, 30, 32, 46]. In addition, localization of these viral agents throughout the epithelium of the respiratory tract is a epidemiologically important in the spread of infection by nasal secretions and coughing to the susceptible animals.

In experimental PI3 virus infection in calves and lambs; fluorescent foci were determined by DFAT widely in alveoli around the broncho-alveolar epithelium, these areas were referred to as starting points for histopathological changes [42]. Similarly, in cattle infected with BRSV naturally, viral antigens were found in the cytoplasm of bronchioles and alveolar epithelium by FAT [19]. Some studies about cellular localization of PI3, BAV3 and BHV1 antigens show that specific immunofluorescent staining is limited with cytoplasm [2, 5, 42, 51]. In the present study, specific fluorescence staining was prominently detected in bronchioles epithelium and viral antigens were found in cytoplasm of the infected cells by DFAT technique. These results were interpreted as PI3, BRSV, BAV3 and BHV1 related viral pneumonia may be the initial finding of infection in bronchioles epithelium and viral replication occurs in the cytoplasm in cattle.

In serological studies carried out in Turkey, the positivity rates ranging between 38.20-91.11%; 46.05-95.55%; 20.07-95.8% and 17.1-61.50% in cattle were reported for PI3, BRSV, BAV3 and BHV1 infections, respectively [9, 17, 18, 23, 35-38, 48-50]. On the other hand, the frequency of PI3, BRSV, BAV3 and BHV1 antigens in pneumonic male cattle in Elazig region were determined as 6.88%, 3.64%, 5.26% and 2.43% by IP, and 10.53%, 6.07%, 6.88% and 4.45% by DFAT, respectively. The positivity rates obtained in this study were low when compared with those reported previously. This might be explained by the use of the different methodologies and sampling procedures which did not include calves and cows. It can be concluded that these viruses might have important role in the pathogenesis of pneumonia in cattle. In addition, DFAT and IP technique may be used for definitive diagnosis as valuable methods when the difficulties in the determination of specific histologic lesions in natural cattle pneumonia originated from PI3, BRSV, BAV3 and BHV1 antigens were considered. When all the data were considered overall, it can be suggested that there is an urgent need to take necessary measures in order to control and prevent these infections in the country.

Acknowledgements

The authors acknowledge to Firat University, Scientific Research Projects Unit (FUBAP; Project number: VF. 11. 11.) for the financial support.

References

1. - AKCA Y., BURGU I., GUR S., BILGE DAGALP S. A study on investigation of occurrence of some virus infection in Buffaloes in Turkey. *Revue. Méd. Vét.*, 2004, **156**, 268-271.
2. - ALKAN F. Rapid diagnosis of bovine anenoviruses subgroup 1 infections in cattle with acute respiratory disease by direct immunofluorescence technique. *Vet. J. Ankara Univ.*, 1998, **45**, 181-184.
3. - ALKAN F., OZKUL A., KARAOGLU M.T., BILGE S., AKCA Y., BURGU I., YESILBAG K., OGUZOGLU T.C. Seroepidemiological studies on respiratory infections of cattle caused by viruses. *Vet. J. Ankara Univ.*, 1997, **44**, 73-80.
4. - BELKNAP E.B., CISZEWSKI D.K., BAKER J.C. (1995). Experimental Respiratory Syncytial Virus Infection in Calves and Lambs. *J. Vet. Diagn. Invest.*, 1995, **7**, 285-298.
5. - BLANC A.M., BEROIS M.B., TOME L.M., EPSTEIN A.L., ARBIZA J.R. Induction of humoral responses to BHV-1 glycoprotein D expressed by HSV-1 amplicon vectors. *J. Vet. Sci.*, 2012, **13**, 59-65.
6. - BRODERSEN B.W., KELLING C.L. Effect of concurrent experimentally induced bovine respiratory syncytial virus and bovine viral diarrhea virus infection on respiratory tract and enteric diseases in calves. *Am. J. Vet. Res.*, 1998, **59**, 1423-1430.
7. - BRYSON D.G., EVERMANN J.F., LIGGITT H.D., FOREYT W.J., BREEZE R.G. An immunoperoxidase method of detecting respiratory syncytial virus antigens in parafin sections of pneumonic bovine lung. *Am. J. Vet. Res.*, 1988, **49**, 1121-1126.
8. - BRYSON D.G., PLATTEN M.F., MCCONNELL S., MCNULTY M.S. Ultrastructural features of lesions in bronchiolar epithelium in induced respiratory syncytial virus pneumonia of calves. *Vet. Pathol.*, 1991, **28**, 293-299.
9. - BURGU I., AKCA Y. Serological investigations on some viral infections in cattle in Gelemen state hatchery. *Vet. J. Ankara Univ.*, 1982, **29**, 506-512.
10. - CARRIERE P.D., MAXIE M.G., WILKIE B.N., SAVAN M., VALLI V.E.O., JOHNSON J.A. Exposure of Calves to Aerosols of Parainfluenza-3 Virus and *Pasteurella haemolytica*. *Can. J. Comp. Med.*, 1983, **47**, 422-432.
11. - CASHWELL J.L., WILLIAMS K. The respiratory system. In: JUBB K.V.F., KENNEDY P.C., PALMER N. (eds.): *Pathology of Domestic Animals*, 5th ed. MAXIE M.G. (eds). Saunders Elsevier, Edinburgh, UK, 2007, 594-622.
12. - CISZEWSKI D.K., BAKER J.C., SLOCOMBE R.F., REINDEL J.F., HAINES D.M., CLARK E.G. Experimental reproduction of respiratory tract disease with bovine respiratory syncytial virus. *Vet. Microbiol.*, 1991, **28**, 39-60.
13. - CUTLIP R.C., LEHMKUHL H.D. Pulmonary lesions in lambs experimentally infected with ovine adenovirus 5 strain RTS-42. *Vet. Pathol.*, 1986, **23**, 589-593.
14. - CUTLIP R.C., LEHMKUHL H.D., BRODGEN K.A., HSU N.J. Lesions in lambs experimentally infected with ovine adenovirus serotype 6 and *Pasteurella hemolytica*. *J. Vet. Diagn. Invest.*, 1996, **8**, 296-303.
15. - DAVIES D.H., LONG D.L., MCCARTHY A.R., HERCEG M. The effect of parainfluenza virus type 3 on the phagocytic cell response of the ovine lung to *Pasteurella haemolytica*. *Vet. Microbiol.*, 1986, **11**, 125-144.
16. - EDWARDS S., WHITE H., NEWMAN R.H., NIXON P. A veterinary services scheme for the rapid

- diagnosis of viral infections in ruminants, using immunofluorescence. *State Vet. J.*, 1988, **42**, 41-47.
17. - EROL N., GUR S., YILDIRIM Y., TAN M.T. A serological investigation on Parainfluenza 3 and Bovine Adenovirus infections in dairy cow enterprises in Aydin province. *J. Fac. Vet. Med. Kafkas Univ.*, 2007, **13**, 43-47.
 18. - ERTURK A., CIZMECI S.G., BARUT M.F. Viral Etiology of Respiratory Diseases of Cattle (2002-2005). *J. Etlik Vet. Microbiol.*, 2006, **17**, 29-42.
 19. - FLORES F.E., WEIBLEN R., MEDERIOS M., BOTTON A.S., IRIGOYEN F.L., DRIEMER D., SCHUH F.L., MORAES M.S. A retrospective search for bovine respiratory antigens in histological specimens by immunofluorescence and immunohistochemistry. *Pesq. Vet. Bras.*, 2000, **20**, 139-143.
 20. - FORGHANI B. Diagnosis by viral antigen detection. In: JEROME K.R. (ed.): *Lennette's Laboratory diagnosis of viral infections*. 4th. ed. Informa Healthcare, New York, London, 2010, 113-132.
 21. - FULTON R.W., PURDY C.W., CONFER A.W., SALIKI J.T., LOAN R.W., BRIGGS R.E., BURGE L.J. Bovine viral diarrhoea viral infections in feeder calves with respiratory disease: Interactions with *Pasteurella* spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. *Can. J. Vet. Res.*, 2000, **64**, 151-159.
 22. - GRAHAM D.A., FOSTER J.C., GERMAN A., MCLAREN E., ADAIR B.M., MERZA M. Evaluation of an immunofluorescent antibody test to detect bovine herpesvirus 1-specific IgM. *J. Vet. Diagn. Invest.*, 1999, **11**, 324-329.
 23. - GURSES E. Serological investigation of viral respiratory tract infections in cattle. Selcuk University, Institute of Natural and Applied Sciences, Department of Biology, 2008, Master Thesis.
 24. - HAINES D.M., CHELACK B.J. Technical considerations for developing enzyme immunohistochemical staining procedures on formalin-fixed paraffin-embedded tissues for diagnostic pathology. *J. Vet. Diagn. Invest.*, 1991, **3**, 101-112.
 25. - HAINES D.M., KENDALL J.C., REMENDA B.W., BREKER-KLASSEN M.M., CLARK E.G. Monoclonal and polyclonal antibodies for immunohistochemical detection of bovine parainfluenza type 3 virus in frozen and formalin fixed paraffin-embedded tissues. *J. Vet. Diagn. Invest.*, 1992, **4**, 393-399.
 26. - IDE P.R., THOMSON R.G., DITCHFIELD W.J.B. Experimental Adenovirus Infection in Calves. *Can. J. Comp. Med.*, 1969, **33**, 1-9.
 27. - KEUSER V., SCHYNTS F., DETRY B., COLLARD A., ROBERT B., VANDERPLASSCHEN A., PASTORET P.P., THIRY E. Improved antigenic methods for differential diagnosis of bovine, caprine, and cervine alphaherpesviruses related to bovine herpesvirus 1. *J. Clin. Microbiol.*, 2004, **42**, 1228-1235.
 28. - KIMMAN T.G., STRAVER J., ZIMMER G.M. Pathogenesis of naturally acquired bovine respiratory syncytial virus infection in calves: morphologic and serologic findings. *Am. J. Vet. Res.*, 1989, **50**, 684-693.
 29. - MATTSON D.E. Naturally occurring infection of calves with bovine adenovirus. *Am. J. Vet. Res.*, 1973, **34**, 623-629.
 30. - NARITA M., KIMURA K., TANIMURA N., ARAI S., TSUBOI T., KATSUDA K. Immunohistochemical characterization of calf pneumonia produced by the combined endobronchial administration of bovine herpesvirus 1 and *Pasteurella haemolytica*. *J. Comp. Pathol.*, 2000, **123**, 126-134.
 31. - NARITA M., KIMURA K., TANIMURA N., TSUBOI T. Pneumonia Induced by Endobronchial Inoculation of Calves with Bovine Herpesvirus 1. *J. Comp. Pathol.*, 2000, **122**, 185-192.
 32. - NARITA M., YAMADA M., TSUBOI T., KAWASHIMA K. Bovine Adenovirus Type 3 pneumonia in dexamethasone-treated calves. *Vet. Pathol.*, 2003, **40**, 128-135.
 33. - NARITA M., YAMADA T., TSUBOI T., KAWASHIMA K. Immunohistopathology of calf pneumonia induced by endobronchial inoculation with serum bovine adenovirus 3. *Vet. Pathol.*, 2002, **39**, 565-571.
 34. - NETTLETON F., HERRING J.A., HERRING A.J. Evaluation of an immunofluorescent test for the rapid diagnosis of field infections of infectious bovine rhinotracheitis. *Vet. Rec.*, 1983, **112**, 298-300.
 35. - OKUR GUMUSOVA S., YAZICI Z., ALBAYRAK H., CAKIROGLU D. Seroprevalence of bovine viral respiratory disease. *Acta Vet. (Beograd)*, 2007, **57**, 11-16.
 36. - OZDARENDELI A., KANDIL M. Serological Survey of the Infection of Parainfluenzavirus Type 3 in Cattle in Malatya. *Turk. J. Vet. Anim. Sci.*, 1997, **25**, 223-226.
 37. - OZGUNLUK I., GUR S. The seroprevalence of Bovine Adenovirus (BAV type 1, 2 and 3) Infection in Cattle in Southeastern Anatolia Project (GAP) Area. *Harran Univ. J. Fac. Vet. Med.*, 2012, **1**, 1-5.
 38. - OZTURK F., TOKER A. Serological determination of bovine adenovirus type1, type2 and type3 in cattle in Konya Agricultural Institution. *Eurasian J. Vet. Sci.*, 1988, **4**, 213-218.
 39. - PIRIE H.M., PETRIE L., PRINGLE C.R., ALLAN E.M., KENNEDY G.J. Acute fatal pneumonia in calves due to respiratory syncytial virus. *Vet. Rec.*, 1981, **108**, 411-416.
 40. - SACCO R.E., MCGILL J.L., PILLATZKI A.E., PALMER M.V., ACKERMANN M.R. Respiratory Syncytial Virus Infection in Cattle. *Vet. Pathol.*, 2014, **51**, 427-436.
 41. - SACCO R.E., NONNECKE B.J., PALMER M.V., WATERS W.R., LIPPOLIS J.D., REINHARDT T.A. Differential Expression of Cytokines in Response to Respiratory Syncytial Virus Infection of Calves with High or Low Circulating 25-Hydroxyvitamin D3. *PLoS one*, 2012, **7**, e33074.
 42. - STEVENSON R.G., HORE D.E. Comparative pathology of lambs and calves infected with parainfluenza type 3. *J. Comp. Pathol.*, 1970, **80**, 613-618.
 43. - TRAUB-DARGATZ J.L., MCKINNON A.O., THRALL M.A., JONES R.L., BRUYNINCKX W., BLANCQUAERT

- A.M.B., DARGATZ D.A. Evaluation of clinical signs of disease, bronchioalveolar and tracheal wash analysis, and arterial blood gas tensions in 13 horses with chronic obstructive pulmonary disease treated with prednisone, methyl sulfonmethane, and clenbuterol hydrochloride. *Am. J. Vet. Res.*, 1992, **45**, 1671-1678.
44. - TRIGO E., LIGGITT H.D., BREEZE R.G., LEID R.W., SILFLOW R.M. Bovine pulmonary alveolar macrophages: Antemortem recovery and in vitro evaluation of bacterial phagocytosis and killing. *Am. J. Vet. Res.*, 1984, **45**, 1842-1846.
45. - VAN DER MAATEN M.J. Immunofluorescent studies of bovine parainfluenza 3 virus II. experimentally infected calves. *J. Comp. Path.*, 1969, **33**, 141-147.
46. - VIUFF B., UTTENHAL A., TEGTMEIER C., ALEXANDERSEN S. Sites of Replication of Bovine Respiratory Syncytial Virus in Naturally Infected Calves as Determined by InSitu Hybridization. *Vet. Pathol.*, 1996, **33**, 383-390.
47. - YATES W.D.G. A review of infectious bovine rhinotracheitis, shipping fever pneumonia and viral-bacterial synergism in respiratory disease of cattle. *Can. J. Comp. Med.*, 1982, **46**, 225-263.
48. - YAVRUS., SIMSEK A., YAPKICO., KALEM. Serological evaluation of viral infections in bovine respiratory tract. *Acta Vet. (Beograd)*, 2005, **55**, 219-226.
49. - YESILBAG K., GUNGOR B. Seroprevalance of bovine respiratory viruses in North-Western Turkey. *Trop. Anim. Health. Prod.*, 2008, **40**, 55-60.
50. - YILDIRIM Y., YILMAZ V., FARAJI MAJARASHIN A.R. Seroprevalance of Viral Respiratory System Infections in Cattle in the Border Provinces of North-East Turkey. *J. Fac. Vet. Med. Kafkas Univ.*, 2009, **15**, 601-606.
51. - ZHU Y.M., YU Z., CAI H., GAO Y.R., DONG X.M., LI Z.L., SHI H.F., MENG Q.F., LU C., XUE F. Isolation, identification, and complete genome sequence of a bovine adenovirus type 3 from cattle in China. *Virology*, 2011, **22**, 557-564.