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Research Article

Ultrastructural and Immunohistochemical Investigations in Calves with Coronavirus Pneumoenteritis Syndrome

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Abstract

The aim of present studies was the structural and morphogenetic investigation of spontaneous pneumoenteritis syndrome in newborn and growing calves with regard to confirmation of some structural features of disease morphogenesis. The research was done with 370 calves from 6 cattle farms in 4 regions of the country, at the age of 24 h - 25 days. For rapid antigenic and viral detection of pathogens, Multiscreen Ag ELISA, Bovine respiratory, Pulmotest respiratory tetra ELISA kit for antigenic diagnosis of *BoHV-1, BVDV, BRSV,* and *BPI-3* sandwich test for tissue lysates (BIOX Diagnostics, Belgium) and Rainbow calf scour 5 BIO K 306 Detection of Bovine Rotavirus, Coronavirus, *Escherichia coli* F5, *Cryptosporidium parvum* and *Clostridium perfringens* in bovine stool (BIOX Diagnostics, Belgium) were used. In 5% of cases, laboratory antigenic tests of lung tissue lysates from pneumonic calves detected co-infections with *BoHV-1, BVDV, BRSV* and *BPI-3*. The utilised antigenic, ultrastructural and virological diagnostic methods allowed concluding that they could be successfully used in the diagnostics of pulmonary and gastrointestinal viral infections in juvenile calves. Electron microscopy and immunohistochemical methods of lung and intestinal tissue are also important and applicable for diagnostics and in differential diagnostic recognition of the condition from other common diseases as IBR, BVD, BRSV, *Mannheimia haemolytica, Cryptosporidium parvum*, BRV and *E. coli* K99 (F5).

Keywords: *Calves, Pathology, IHC, Ultrastructure, BCoV*

Coronavirus Pnömoenteritis Sendromlu Buzağılarda Ultrastrüktürel ve İmmunohistokimyasal İncelemeler

Öz

Bu çalışmanın amacı spontan pnömoenteritis sendromlu yeni doğan ve büyüme dönemindeki buzağılarda hastalığın morfogenezine yönelik bazı yapısal özellikleri incelemektir. Çalışma ülkenin 4 bölgesinden 6 çiftlikte toplam 370 adet 24 saatlik ile 25 günlük arasındaki buzağı üzerinde gerçekleştirildi. *BoHV-1, BVDV, BRSV* ve *BPI-3*'ün hızlı tespiti amacıyla Multiscreen Ag ELISA Bovine respiratory, Pulmotest respiratory tetra ELISA kit kullanıldı (BIOX Diagnostics, Belçika). Sığır dışkısında Rotavirus, Coronavirus, *Escherichia coli* F5, *Cryptosporidium parvum* ve *Clostridium perfringens* tespiti amacıyla Rainbow calf scour 5 BIO K 306 kullanıldı (BIOX Diagnostics, Belçika). Vakaların %5'inde *BoHV-1, BVDV, BRSV* ve *BPI-3* pnömonili buzağıların akciğer doku lizatlarında birlikte gözlemlendi. Çalışmada kullanılan antijenik, ultrastrüktürel ve virolojik tanı metotlarının başarılı bir şekilde pulmoner ve gastrointestinal viral enfeksiyonların tespitinde kullanılabileceği belirlendi. Akciğer ve barsak dokularında elektron mikroskopi ve immunohistokimyasal metotlar tanıda uygulanabilir olup IBR, BVD, BRSV, *Mannheimia haemolytica, C. parvum,* BRV ve *E. coli* K99 (F5) gibi diğer yaygın hastalıklardan ayırıcı tanıda önemlidirler.

Anahtar sözcükler: *Buzağı, Patoloji, İmmunohistokimya, Ultrastrüktür, BCoV*

INTRODUCTION

Bovine Corona Virus - BoCV is outlined as one of primary etiological agents causing gastrointestinal diseases in newborn calves (calf diarrhoea - CD), winter dysentery (WD) in adult cattle and respiratory infections in calves and

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cattle in fattening farms - pneumoenteritis syndrome [1]. As early as in 1972, Stair et al.[2] reported pneumoenteritis in calves induced by coronaviruses. The first isolation of BoCV from bronchial secretion and nasopharyngeal washings of calves with signs of pneumonia occurred in 1982 and later, it was determined as bovine respiratory coronavirus (BRCoV) [1,2]. Strains isolated from diarrhoeic newborn calves and cattle have been classified as enteric or enteropathogenic coronaviruses (BECoV) [3]. Thomas et al.[4] affirmed that enteric and respiratory BCoV were the same, although detected in different life cycle stages. They have been isolated from the intestines and lung of calves affected with pneumoenteritis syndrome, and were antigenically and genomically similar [3,4].

In the study of Zhang et al.[5] BCoV was the second most prevalent viral agent isolated from lungs of calves with respiratory infections after bovine herpesviruses BoHV-1.

Bovine coronavirus causes enterocolitis and pneumonia in calves from the 24th h to the 5th month of age. The disease is characterized by a high morbidity rate ranging from 50% to 100%. Mortality rate is usually low, typically less than 2%, with only a few reports describing case fatality associated with this virus [6-10]. The incubation period for BCoV-WD ranges from 2 to 8 days. In small housed herds, the incidence of diarrhea during an outbreak begins with the explosive appearance of signs in 10% to 15% of animals on the first day. The diarrhea may contain a slight to copious amount of mucus. The amount of blood varies from case to case and ranges from just visible flecks or streaks to large clots, or it may be uniformly mixed into the feces. Pyrexia is usually not present during the diarrheal phase of the disease, although it has been reported to precede it by 24 to 48 h or have no consistent relationship. Mild to moderate signs of respiratory disease (eg, cough, nasal discharge) have been inconsistently observed preceding or concurrent with the diarrhea [6,11].

The clinical manifestation of the syndrome depends not only on the BCoV itself, but also on the host, environmental factors, immunological status of the herd, ambient temperature and the occurrence of secondary co-infections with other pathogens [4-7].

According to Craig et al.[7] the most accurate methods of laboratory diagnostics of coronaviruses are immuno-electron, electron microscopy (EM) and enzyme-linked immunosorbent assay (ELISA) as histological changes in the lung and intestines are not always specific for a definite histopathological diagnosis of coronaviral infection. In formalin-fixed specimens from intestinal segment and lungs, an immunohistochemical method has been used [7-9], requiring a specific monoclonal antibody (MAb), bound to the nucleocapsid protein of BCoV. The latter is the most prevalent protein of the virus for its detection in formalin-fixed tissues [8,9]. In non-fixed intestinal and lung specimens, the direct immunofluorescence assay (IFA) was also often used [8]. Daginakatte et al.[9] reported haemagglutination test with mouse red blood cells and polymerase chain reaction as other important techniques for detection of BCoV in tissue samples.

On the basis of literature overview and the relevance of the pathology, we aimed to perform immunohistochemical and ultrastructural investigation of spontaneous cases of pneumoenteritis syndrome in newborn and growing calves with regard to confirmation of some structural features of disease morphogenesis.

MATERIAL and METHODS

Animals and Samplings

The study comprised 370 calves from six dairy cattle farms. The age of animals was from 24 h to 25 days. Samples of 18 dead calves were obtained for antigenic, histopathological, immunohistochemical, lectron microscopy studies. Data presented in *Table 1*.

Antigenic Studies

a) Multiscreen Ag ELISA: For detection of respiratory pathogens were used, Multiscreen Ag ELISA, Bovine respiratory, Pulmotest respiratory tetra ELISA kit for antigenic diagnosis of BoHV-1, BVDV, BRSV, and BPI 3 sandwich test for tissue lysates. (BIOX Diagnostics, Belgium). From all calves, lung samples measuring 0.5 x 0.5 cm were obtained.

b) Rapid Pentavalent Antigenic Strips Test: Rainbow calf scour 5 BIO K 306 Detection of Rotavirus, Coronavirus, *E. coli* F5, Cryptosporidium and *C. perfringes* in bovine stool (BIOX Diagnostics, Belgium) were used. From all calves, faecal samples about 3 g.

Gross Pathology Examination

Eighteen carcasses of calves dead after manifestation of

Table 1. *Samples from 18 calves for antigenic, histopathological, immunohistochemical, lectron microscopy studies*

Number of Calves	Samples for Multiscreen Ag ELISA	Samples for Antigenic Strips Test	Samples for Histopathological Examination	Samples for Immunohistochemical Studies	Samples for Electron Microscopy Examination
18	from all calves, lung samples measuring 0.5 x 0.5 sm were obtained.	from all calves, faecal samples about 3 g	from all calves, tissue samples (1 cm x 1 cm) from lungs and bronchial lymph nodes, abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, caecum, colon and rectum	from all calves, tissue samples (1 cm x 1 cm) were obtained from lungs and the ileum	from all calves, tissue samples (1 mm x 1 mm) were obtained from lungs and the ileum

enteritis and respiratory signs were submitted to gross anatomy study.

Histopathological and Immunohistochemical Examination

Tissue samples (1 cm x 1 cm) were collected for histopathological examination from lungs and bronchial lymph nodes as well as from affected proximal and distal gastrointestinal compartments: abomasum, duodenum, jejunum with mesenteric lymph nodes, ileum, caecum, colon and rectum - all intestinal parts with 2.5 cm length. Specimens were fixed in 10% neutral buffered formalin for 48-72 h and embedded in paraffin. Cross sections, 4 µm thick were cut from paraffin blocks on a Leica RM 2235 microtome and stained with haematoxylin-eosin (H/E). Furthermore, tissue samples were obtained from lungs and the ileum for immunohistochemical (IHC) studies. To this end, a monoclonal non-conjugated antibody Monoclonal Antibody anti-mouse unconjugated, Coronavirus pan Monoclonal Antibody (FIPV3-70): sc-65653, 1 mg/mL, (Santa Cruz Biotechnology, Germany) was used.

Protocol for ICH: De-paraffinize sections thoroughly in xylene/synthetic solvent, and hydrate through a graded series of alcohols. Wash twice in water. Outline section with PAP pen. If required, treat with 0.3% (w/v) H_2O_2 in methanol for 15 min to block endogenous peroxidase activity (2% (w/v) H_2O_2/methanol can be used for a shorter time if preferred; Bio-Rad offers peroxide blocking reagent (BUF017B). Wash 3 times in TBS.

If required, include an appropriate antigen retrieval step to enhance the immunostaining (see protocol: Antigen Retrieval Techniques for use with Formalin-Fixed Paraffin-Embedded Sections). Bio-Rad offers a variety of retrieval/antigen unmasking fluids. Wash once in water. Incubate sections for 10 min in 10% normal serum from the species in which the secondary antibody was raised. Tap excess serum off the slides before staining.

Incubate sections with primary antibody for at least 30 min at room temperature in a humid chamber, or overnight at 4°C. Wash 3 times in TBS. Add enzyme-conjugated secondary antibody at the recommended dilution (see specific datasheet for details). Incubate for at least 30 min at room temperature. Wash 3 times in TBS. Incubate with the appropriate substrate solution for the recommended period of time (Bio-Rad recommends the use of DAB substrate with HRP-conjugated antibodies, and Fast Red/Napthol AS-MX for Alkaline Phosphatase-conjugated antibodies). Wash once in water. Counterstain with hematoxylin for 1 min. "Blue" with running water for 5 min. Then wash. Mount in aqueous mounting medium, or alternatively dehydrate through a graded series of alcohols and xylene/solvent. Mount in synthetic mountant.

Electron Microscopy Examination

For detection of ultrastructural lesions, tissue samples were collected from the same sites. They were processed according to the routine electron microscopy technique - fixation for 24 h in 4% glutaraldehyde in 0.1 M phosphate buffer pH 7.3, post fixation for 24 h in 1% OsO_4, dehydration and embedding in Durcupan ACM Fluka. Ultrathin sections were cut on a Reichert ultratome and stained with uranyl acetate and lead citrate. For the TEM study, JEOL 1200 EX transmission electron microscope with accelerating voltage 80 kV was used. All reagents were purchased from Sigma Aldrich - Merck.

RESULTS

The epidemiological survey at the six farms identified that intestinal and respiratory infections as the major health problem in newborn and juvenile calves. In 75% of calves aged between 1 and 15 days, clinical signs comprised digestive disturbances

In the other 25% (mainly between 4 and 10 days of age), respiratory signs were also present along with gastrointestinal ones.

Antigenic Findings

The results from laboratory antigenic analyses of lung tissue lysates from pneumonic calves confirmed co infections involving Bo*HV*-1, BVDV, BRSV and BPI-3 in 5% of cases *(Table 2).*

In 75% of gastroenteritis cases, bovine rotaviruses and cryptosporidiae were identified as etiological agents. In 20%

Table 2. *Optical density values higher than 6% were positive for BoHV-1, BVDV, BRSV and BPI-3*

Disease	Positive Control Values	Optical Density Values										
Bo*HV*-1	0.086	0.255	**0.954**	0.051	0.057	0.056	0.097	**0.657**	0.056	0.053	0.053	0.052
BVDV	2.391	0.059	0.056	**0.857**	0.057	0.058	0.061	0.074	0.460	0.060	0.054	0.052
BRSV	0.094	0.067	0.068	0.064	0.066	0.065	0.063	0.066	0.070	0.088	0.071	0.058
BPI-3	2.090	0.055	**0.864**	**0.761**	0.061	0.055	0.056	0.066	0.057	**0.669**	0.073	0.084
Bo*HV*-1	0.062	0.065	0.071	0.070	0.068	0.068	0.073	**0.980**	0.073	0.069	0.077	0.066
BVDV	2.443	0.080	0.070	**0.663**	0.080	0.072	0.071	0.088	0.068	0.107	0.061	0.065
BRSV	0.071	0.090	**0.775**	0.074	0.279	0.070	0.071	**0.666**	0.066	0.073	0.093	0.062
BPI-3	2.354	0.057	0.137	0.090	0.080	0.064	0.069	0.088	0.076	0.072	0.062	0.058

of complicated states with simultaneous occurrence of gastrointestinal and respiratory troubles (pneumoenteritis), bovine coronaviruses were the only pathogens implicated.

Clinical and Macroscopic Findings

In calves with pneumoenteritis, strong dehydration and emaciation were observed. The inspection of the head revealed sunken eyes with bilateral corneal opacity. Visible conjunctival and buccal cavity mucosae were pale and anaemic. The perianal region and tail base were extensively stained with orange-yellow to greenish diarrhoeic faeces with mucous consistency.

Gross changes were identified along the entire length of small and large intestines. The intestinal wall was flaccid and transparent. Intestinal serosa exhibited diffuse hyperaemia with local haemorrhages. The intestinal content was yellow and slimy. Caecal content was pale yellow mixed with blood and a large amount of gas bubbles. Small and large intestinal mucosae were strongly hyperaemic and oedematous, spattered with striated haemorrhages in some areas. The mesenteric lymph nodes of all calves were enlarged and juicy.

The palpation of lungs revealed that they were thickened, with marbled appearance, filled with large amount of cloudy foamy fluid reaching the trachea and primary bronchi. Regional lymph nodes were enlarged and juicy. Areas with interstitial emphysema were observed on the periphery of lung lobes.

The epicardial surface exhibited multiple haemorrhages, most extensive in the coronary margin area. The mucous coats of the trachea, larynx and epiglottis were hyperemic and spattered with single petechiae.

Histopathological Findings

Microscopic lesions were most prominent in the lung and the distal small intestinal compartment. Pulmonary lesions were concentrated in the interalveolar and peribronchial connective tissue. As a result of degeneration, epithelial cells were shed in the alveolar lumen among the serous exudate collection. Interstitial connective tissue was strongly oedematous due both to its impregnation with exudate and infiltration with lymphocytes and macrophages. Part of the small intestine (ileum) villi exhibited strong atrophy and their middle part was infiltrated with numerous lymphocytes. Degenerative and necrobiotic processes predominated in epithelial cells of villi and crypts. The submucosa was oedematous, diffusely hyperaemic and spattered with large haemorrhages, while glands were enlarged secondary to the profuse hypersecretion *(Fig. 1)*. Haemorrhages and hyperaemia were visible in the medullary part of mesenteric lymph nodes.

Immunohistochemical Findings

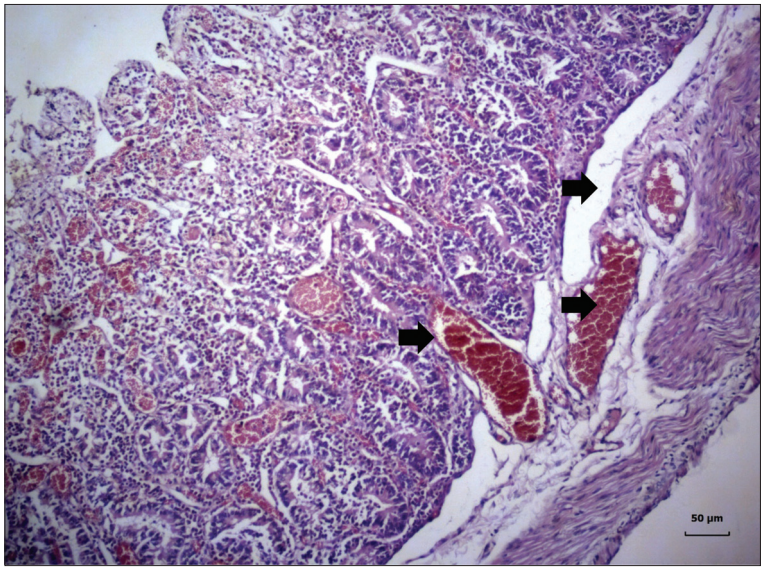
The used immunohistochemical (IHC) technique confirmed the presence of bovine coronavirus antigen in intestinal and pulmonary tissue cross sections *(Fig. 2, Fig. 3)*. This confirmed the field and laboratory antigenic results in calves with pneumoenteritis.

Electron Microscopy Findings

Ultrastructural changes in tissue morphology were detected both in specimens collected from lungs and distal small intestine.

The lung exhibited destruction and desquamation of the respiratory epithelium surrounding the alveoli, type I pneumocytes with enhanced cytoplasmic vacuolation among which a large amount of activated macrophages, lymphocytes and granulocytes had migrated *(Fig. 4-*a,*b,c)*. Coronavirus-like nucleocapsids in double-membrane vesicles were present in the respiratory epithelium of some of tissue samples *(Fig. 4-a)*. Apart the enhanced

Fig 1. Catarrhal haemorrhagic enteritis, submucosal oedema *(arrow)* and vascular hyperaemia in the submucosa *(arrows)* of the ileum of a calf with pneumoenteritis syndrome, H/E, Bar=10 µm



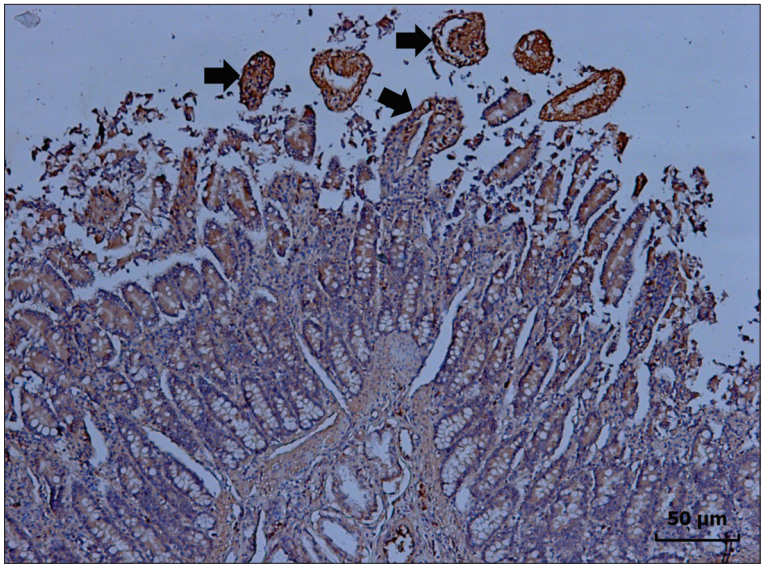
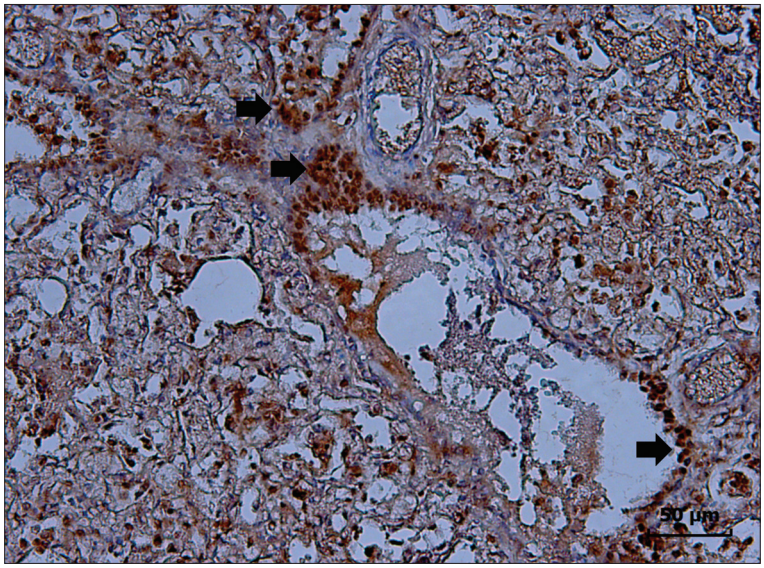


Fig 2. Immunohistochemical detection of coronavirus antigen with *MAb - FIPV3* in ileal enterocytes *(arrows)* of a calf with pneumoenteritis syndrome, ICH, Bar=10 µm

Fig 3. Immunohistochemical detection of coronavirus antigen with *MAb - FIPV3* in bronchial epithelium - lung *(arrows)* of a calf with pneumoenteritis syndrome, ICH, Bar=10 µm



destruction of cytoplasmic structures, many pneumocytes had altered nuclei - chromatin margination or chromatin condensation.

Presence of virus-like particles with morphological features and size 80-100 nm similar to that of coronaviruses was detected within the strongly destructed enterocytes and in the interface of adjacent intestinal cells *(Fig. 5-a,b)*. Affected cells had microvilli shed from the apical end, strong vacuolation and lack of clearly defined cell organelles, but with distinct electron-dense intracytoplasmic inclusion bodies.

DISCUSSION

The results of the present study confirmed that bovine coronaviruses (BCoV) were the primary viral agents causing pneumoenteritis syndrome in calves.

Furthermore, we suggest that the occurrence of respiratory and intestinal diseases in calves is associated with the lack of immunoprophylaxis of pregnant cows as well as to faulty elements of the rearing technology of newborn calves [10].

We share the opinion of researchers [5-10] affirming that the clinical manifestation of BCoV-associated pneumoenteritis syndrome is due to the tropism of the virus to the gastrointestinal tract, nasal cavity, proximal trachea and lungs. We agree with the belief [10,11] for a mutual relationship between BCoV and BRDC (bovine respiratory disease complex), causing severe respiratory infections with stunted growth and development of dairy and feedlot calves. Expectedly, this pathology incurs considerable economic losses to farmers [11].

The established macro- and microscopic lesions, as well as ICH findings in the respiratory and digestive tract samples of studied calves due to BCoV replication are large comparable to those reported by others [12]. In this

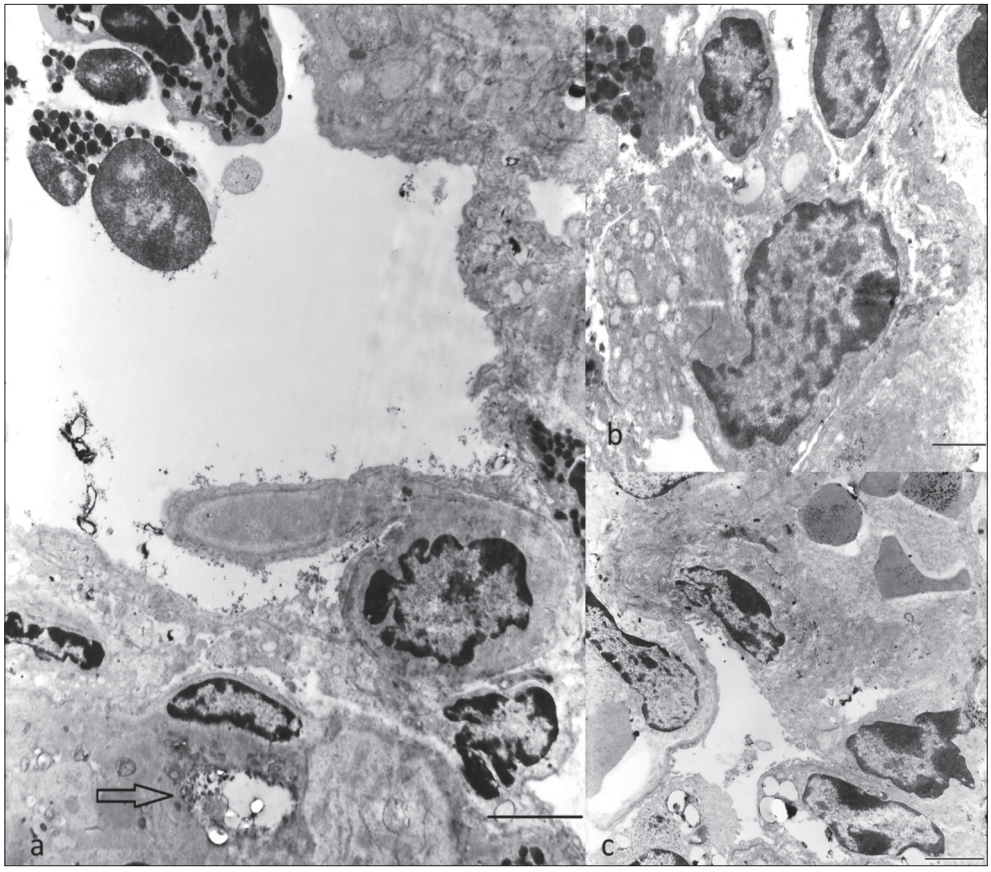


Fig 4. Alveoli with respiratory epithelium and presence of agranulocytes and granulocytes (a, b, c). Pneumocytes with enhanced cytoplasmic vacuolation, nuclear chromatin condensation and margination, cellular detritus to the lumen of the alveolus (a, b, c), activated macrophages (with pseudopods) and granulocytes (a, b); coronavirus-like particles (nucleocapsids) in membrane-confined vesicles *(arrow)* (a). Durcupan, Bar=3 μm (a), Bar=1 μm (b), Bar=2 μm (c)

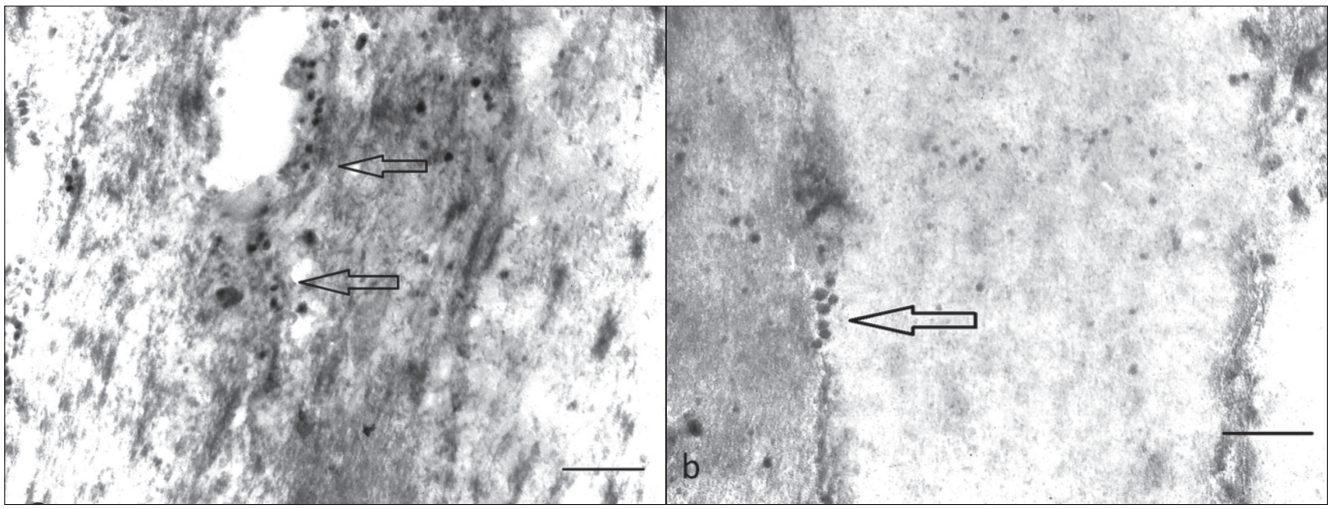


Fig 5. Enterocytes from *lamina epithelialis mucosae*, part of ileal *villi intestinales*. Presence of virus-like particles *(arrow)* in strongly destructed enterocytes (a). No distinct cell organelles are visible (a, b). Presence of virus-like particles at the interface of two adjacent enterocytes (b). Durcupan, Bar=800 nm(a), Bar=600 nm (b)

and other studies [7-12] co-infections with BCoV and other bacterial or viral agents result in higher morbidity and mortality rates in calves up to 14 days of age. BCoV (calf diarrhea, pneumonia in calves and adult cattle, winter dysentery, and combined pneumonia and diarrhea in young and adult cattle) are due to the virus tropism for the intestinal tract, nasal passages, proximal trachea, and lungs [12].

Lung microscopic lesions were indicative for interstitial pneumonia specific for viral respiratory infections [4-12]. In studied samples, there were no intracytoplasmic inclusion bodies in bronchial epithelial cells as reported by others [9-13] in infections with bovine respiratory syncytial virus (BRSV) and bovine parainfluenza 3 virus (BPI3V*)*. We also confirm that interstitial pneumonia is of marked viral etiology unlike bronchopneumonia whose etiology is mainly bacterial *(E. coli, Mannheimia haemolytica, Pasteurella multocida, Mycoplasma bovis, Histophilus somni)* [13].

Microscopic lesions in intestines (atrophy and superficial catarrhal desquamative inflammation) can be associated with viral replication. Unlike these findings, such changes in rotaviral enteritis are observed in the middle part of villi of both small and large intestines [13,14]. The macro- and micro lesions in the lung and the gastrointestinal tract of calves affected by pneumoenteritis are relevant with regard to the differential diagnosis of the syndrome and its differentiation from respiratory (IBR, BVD, BRSV, *M. haemolytica* etc.) and intestinal *(Cryptosporidium parvum*, bovine rotaviruses, bovine coronaviruses and *E. coli* K99 [F5]) diseases in this category of animals.

The present study confirmed that the used antigenic and ICH laboratory tests are of good diagnostic value with respect to viral infections in ruminants [11-14]. IHC can be a very useful test to confirm the etiology. IHC can also be useful in cases with histologic lesions of viral enteritis but for which other microbiologic tests have not confirmed viral infection. Use of IHC for confirming the presence of these enteric pathogens is most successful when tissues are collected and placed in formalin. This sampling method ensures preservation of potentially virus-infected epithelium covering intestinal villi [14,15].

The electron microscopy findings for polymorphonuclear cells and agranulocytes in alveolar spaces and among the respiratory epithelium support the viral etiology of the infection [11-13]. Viral particles whose morphology corresponded to that of the coronaviral agent have been found out both in pneumocytes and enterocytes in some of studied animals. These data evidence that pneumocytes and enterocytes are the primary target cells for coronavirus infection [7-14]. We also affirm that predominating pathological findings were diffuse lesions in alveolar spaces, obliteration and interstitial changes and damage of apical surfaces of intestinal villi resulting from lining epithelium destruction [14,16]. The targets are the conserved nucleocapsid gene for detection of the virus and spike gene for epidemiologic investigation and strain differentiation. At present, there is no commercial test available for BCoV antigen detection in the United States. However, lateral flow immunoassays (LFT) are useful cow and calf-side tests, and are available in European Union for BCoV antigen detection in the feces [11].

The utilised antigenic, ultrastructural and virological diagnostic methods allowed concluding that they could be successfully used in the diagnostics of pulmonary and gastrointestinal viral infections in juvenile calves. Electron microscopy and IHC methods of lung and intestinal tissues are also important and applicable for diagnostics and in differential diagnostic recognition of the condition from other common diseases as IBR, BVD, BRSV, *M. haemolytica, C. parvum*, BRV and *E. coli K99 (F5*).

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