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Research article

EVALUATION OF RUMINANT ENTEROTOXAEMIA: INSIGHTS FROM A COMPARATIVE STUDY USING ELISA, IMMUNOHISTOCHEMICAL, AND PATHOLOGICAL INVESTIGATIONS

Osman DOGAN[1*], Mustafa ORTATATLI[2]

[1]Konya Veterinary Control Institute, Department of Pathology, Konya, Türkiye; [2]Selçuk University, Faculty of Veterinary Medicine, Department of Pathology, Konya, Türkiye.

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Enterotoxaemia, caused by *Clostridium perfringens* toxins, is one of the most prevalent clostridial diseases in ruminants. The diagnosis is typically based on the detection of *C. perfringens* toxins in the intestinal content; however, challenges remain in achieving a practical, reliable, and definitive diagnosis. We present comparative findings of the enzyme-linked immunosorbent assay (ELISA), immunohistochemical (IHC), and pathological in 150 ruminants (sheep, goats, and cattle) suspected of enterotoxaemia. The present results revealed that 19 out of 150 ruminants were positive for at least one *C. perfringens* toxin (α, β, and ε) by ELISA. Moreover, our findings indicated that macroscopic and histopathological observations were congruent with clostridial enterotoxaemia. IHC for *C. perfringens* stained numerous long, rod-shaped bacteria present in the intestinal lesions in all toxin-positive cases (19/19). Additionally, positive immunohistochemical staining was observed in 115 of 131 toxin-negative ruminants. Our present findings suggest that elevated rates of positive immunohistochemical staining, particularly in histopathological intestinal lesions, may significantly contribute to the pathological diagnosis of enterotoxaemia. It also suggests that when toxin detection is unfeasible, enterotoxaemia can be diagnosed more effectively and securely by concurrently assessing IHC and histopathological findings, hence allowing for the identification of potential positive cases.

Keywords: *C. perfringens*, ELISA, enterotoxaemia, immunohistochemistry, pathology

INTRODUCTION

Enterotoxaemia is a fatal disease in ruminants that leads to toxaemia. Enterotoxaemia caused by *C. perfringens* toxins affects the ruminant industry worldwide [1,2]. *C. perfringens* is found in the intestinal flora of humans, animals, and environmental sources. However, their presence in the host does not lead to disease. Bacterial colonisation and the toxin production following many different predisposing factors are critical in

*Corresponding author: e-mail: osmandogan@tarimorman.gov.tr

the pathogenesis of this disease. *C. perfringens* is divided into seven species based on their ability to produce different toxins, including alpha (α), beta (β), epsilon (ε), iota (ι), enterotoxin, and necrotic enteritis B–like toxins. Especially *C. perfringens* types A, B, C, D, and E cause diseases in ruminants. Additionally, types F and G can cause various diseases in humans and poultry, respectively [3,4].

The diagnosis of enterotoxaemia is based on the detection of *C. perfringens* toxins in intestinal contents. For this purpose, mouse toxin neutralisation test (MNT), enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) methods are used [5-7]. *C. perfringens* toxins degrade quickly when a sample with suspected enterotoxaemia is sent to the laboratory in inadequate circumstances for diagnosis or when time is wasted at this point. In such cases, false-negative results can occur during diagnosis [8,9]. Furthermore, a similar diagnostic mistake may result from the sensitivity of beta toxin to trypsin and its rapid degradation in the intestines [10]. Despite the existing diagnostic instruments, there are problems with practical, reliable, and definitive diagnosis of enterotoxaemia. Therefore, the evaluation of pathological findings is crucial for a final diagnosis, in addition to clinical symptoms, bacteriological identification, and toxin testing. Immunohistochemical (IHC) staining methods detected positive reactions for *C. perfringens* and its toxins in the lesioned intestinal tissues of animals with enterotoxaemia. IHC may be useful for the accurate and complementary diagnosis of enterotoxaemia [8,11,12].

Despite protective measures, enterotoxaemia has become a serious fatal disease that affects ruminant farming recently. Owing to the difficulties in detecting toxins, reliable and rapid diagnostic methods are needed for diagnosis. This study aimed to detect *C. perfringens* toxins (α, β, and ε) by ELISA and to investigate pathological and IHC findings in ruminants with suspected enterotoxaemia.

MATERIALS AND METHODS

Case selection, post-mortem examination, and tissue collection

In this study, one hundred and fifty dead ruminants sent from farms in the Marmara Region of Türkiye to the Pendik Veterinary Control Institute for post-mortem examination were used. The selection criteria for the study were grounded on previously established animals with suspected enterotoxaemia, and samples were collected between January and December 2020 [8,13,14]. These were clinical symptoms such as sudden death, diarrhoea, enteritis, and macroscopic findings such as hydropericardium, haemorrhagic bowel disorders, and pulmonary oedema. All the animals met at least one of the inclusion criteria. Necropsy was performed in all cases, and macroscopic changes were recorded. The intestinal contents were taken into sterile containers for toxin detection. The descriptive details of the study's samples are shown in Table 1. The conducted research is not related to experimental animal use. This study was

granted exemption from requiring ethics approval by the Local Ethics Committee on Animal Experiments of the Pendik Veterinary Control Institute (Decision no.: 03/2020).

Table 1. Distribution of ruminants suspected of dying from enterotoxaemia by season, species and age groups

Year	Seasons	Sheep		Goat		Cattle		Total
		0–6 months	6 > months	0–6 months	6 > months	0–6 months	6 > months	
2020	Spring	21	9	6	1	3	2	42
	Summer	14	8	3	2	2	0	29
	Autumn	7	2	1	0	4	1	15
	Winter	39	8	5	5	5	2	64
Number of case		81	27	15	8	14	5	150

The detection of *C. perfringens* toxins (α, β, and ε) by ELISA

Samples of intestinal contents from all cases were also tested for α, β, and ε using a commercial capture ELISA kit (BIO K 270, www.biox.com) following the manufacturer's instructions. The enzymatic reaction was stopped by the addition of a stopping solution. Optical densities were recorded using an ELISA reader with a 450 nm filter. The results were calculated according to the manufacturer's instructions. *C. perfringens* strains were determined according to toxin types as previously reported by Theoret and McClane [15].

Microscopic pathology and immunohistochemistry

Tissue samples from the lungs, heart, liver, kidney, spleen, brain, abomasum, mesenteric lymph nodes, and intestines were collected and fixed in 10% buffered formalin for 24-72 hours. These samples were processed using standard histological techniques, cut into 5-μm thickness, and stained with haematoxylin and eosin (HE). Sections of the abomasum, mesenteric lymph node, and small and large intestines were processed using an indirect immunoperoxidase technique for *C. perfringens*, as previously described by Diab et al. [16], using the Rabbit Specific HRP/DAB IHC Kit (ab236469, Abcam, www.abcam.com) according to the manufacturer's instructions. Rabbit polyclonal antibody against *C. perfringens* (ab35023, Abcam, www.abcam.com) was diluted 1:200 prior to immunostaining. All sections were incubated with DAB (3,3'-Diaminobenzidine) and Mayer's haematoxylin counterstaining at the end of the process. *A C. perfringens* isolated intestinal section was used as the positive control, as previously described by Navarro et al. [17]. Serial test tissues incubated with an antibody diluent instead of the primary antibody were used as the negative controls. All tissue sections were examined under a light microscope (AXIO Imager.D2, www.zeiss.com).

Statistical analysis

The significance of the relationship between the ELISA and IHC results was investigated using the SPSS software package (IBM Inc. USA). The significance of the differences between the groups was evaluated using Pearson's chi-square test. Spearman's correlation test was used to compare the correlation between IHC findings and ELISA results detected in different layers of intestinal sections. Statistical results with $P<0.05$ were considered significant.

RESULTS

Clostridium perfringens toxins ELISA

At least one *C. perfringens* toxin (α, β, and ε) was detected by ELISA in the intestinal contents of 19/150 (12.67%) ruminants with suspected enterotoxaemia. α-, β-, and ε-toxins were detected in 19/19, 1/19, and 8/19 samples, respectively. The distribution of *C. perfringens* A, C, and D according to the presence of the toxin was 10/19 (52.63%), 1/19 (5.26%), and 8/19 (42.11%), respectively (Table 2). The distribution of enterotoxaemia cases in spring, summer, autumn, and winter seasons was 8, 5, 2, and 4, respectively.

Table 2. Distribution of *C. perfringens* toxin types among animal species and ELISA results

Examined samples			Enterotoxaemia cases	*C. perfringens* type*			
				Type A (α)	Type B (α, β, and ε)	Type C (α, β)	Type D (α, ε)
Sheep	0-6 months	81	8	5	-	-	3
	6> months	27	5	2	-	-	3
Goat	0-6 months	15	3	2	-	-	1
	6> months	8	-	-	-	-	-
Cattle	0-6 months	14	1	-	-	1	-
	6> months	5	2	1	-	-	1
Total (%)		150	19 (12.67%)	10 (52.63%)	-	1 (5.26%)	8 (42.11%)

***C. perfringens* toxin types according to ELISA.**

Gross pathology

Lesions in the intestinal tract of animals with enterotoxaemia were characterised by serosal hyperaemia (Figure 1A), haemorrhagic (Figure 1B) or yellow/greenish fluid content in the intestinal lumen, and mucosal haemorrhage. Haemorrhagic enteritis characterised intestinal lesions in 5 cases of type A, 2 cases of type D, and a calf with type C enterotoxaemia, mainly affecting the small intestine segments (Table 3). These intestinal segments included an abundance of brownish-to-red contents, as well as severe congestion and haemorrhage of the jejunum and ileum mucosa (Figure 1C).

In four cases of type A enterotoxaemia and three cases of type D enterotoxaemia, intestinal sections were enlarged with abundant yellowish content. The main gross alterations outside of the intestinal tract were hydropericardium (Figure 1D) and pulmonary oedema with the accumulation of foamy fluid in the trachea, subepicardial petechiae, diffuse pale colour change in the kidneys and liver, softening of the consistency of the kidneys, and hyperaemia in the meninges. Two cases of type A enterotoxaemia and one case of type D enterotoxaemia showed icteric subcutaneous and peritoneal adipose tissue, congested kidneys (Figure 1E), and a dilated urinary bladder with dark red urine. In four cases of type D enterotoxaemia, narrowing of the sulci in the brain and partial herniation of the cerebellar vermis into the foramen magnum were observed (Figure 1F).

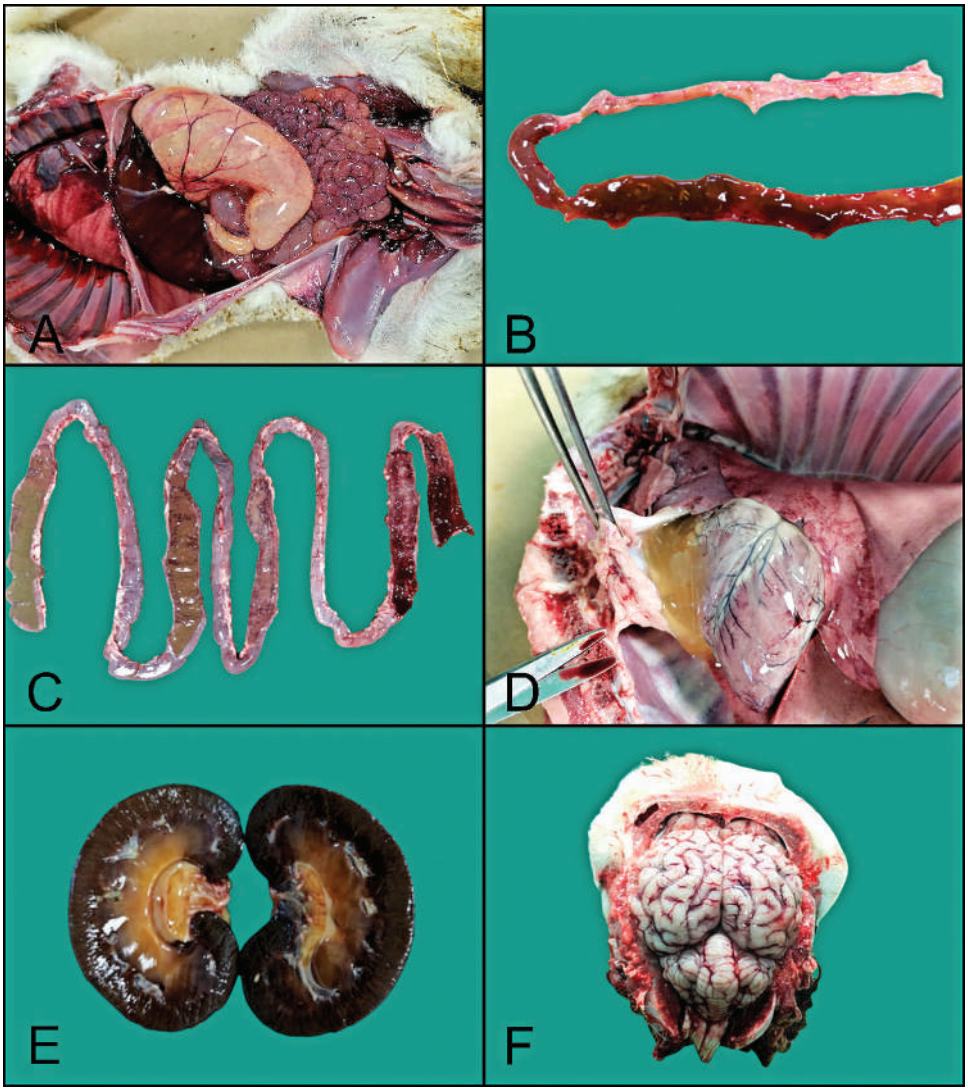

Figure 1. **(A-B)** Lamb with type A enterotoxaemia. **A.** Thoracic and abdominal cavity, dilatation of the abomasum and hyperaemia of the intestinal serosa. **B.** Jejunum, segments of jejunum have haemorrhagic content. **C.** Calf with type C enterotoxaemia. Small intestine, mucosa is covered with yellowish-brown, and haemorrhagic content. Diffuse haemorrhage and congestion on the mucosal surface. **D.** Lamb with type D enterotoxaemia. Thoracic cavity, abundant yellowish-coloured pericardial fluid in the pericardial sac that coagulates on exposure to air. **E.** Lamb with type A enterotoxaemia. Kidney, dark brown-black discolouration of cross-sectional surface of the kidneys and yellowish colouration of the medulla. **F.** Lamb with type D enterotoxaemia. At the opening of the cranial cavity, meningeal hyperaemia, and partial herniation of the caudal portion of the cerebellum into the foramen magnum (cerebellar coning).

The frequency of macroscopic findings in enterotoxaemia and toxin-negative cases is presented in Table 4.

Microscopic pathology and immunohistochemistry

Necrotic and/or haemorrhagic microscopic findings were detected in the gastrointestinal tract of animals with enterotoxaemia (Table 5). Microscopically, the intestine showed desquamation of lining epithelium, inflammatory cell infiltrate, mucosal necrosis (Figure 2A), and haemorrhage in the lamina propria (Figure 2B). A pseudomembrane composed of necrotic desquamated epithelial cells, cell debris, inflammatory cells, fibrin, and bacilli covered the luminal surface of the intestines (Figure 2C). These bacilli were found in groups (or individually) within the intestinal lumen and on the surface of the necrotic mucosa. Also, a few bacilli were encountered in the necrotic lamina propria and the in the crypts (Figure 2D). In cases of enterotoxaemia (7/19), the developmental stages of different parasite species (e.g., intestinal coccidia, nematodes) were observed histopathologically in the intestinal epithelium and mucosa. Pulmonary oedema, renal tubular epithelial degeneration, necrosis, and hyaline casts in tubular lumens; centrilobular necrosis and vacuolar degeneration in hepatocytes; perivascular haemorrhage and perineural oedema in the brain were present outside the intestinal tract. The frequency of the histopathological findings in enterotoxaemia and toxin-negative animals is summarised in Table 6. In all cases with enterotoxaemia, *C. perfringens* antigens were detected by IHC. IHC-positive reactions were also detected in 115/131 (87.79%) of toxin-negative cases. In the intestinal sections of enterotoxaemia cases, IHC-positive reactions occurred in the sloughed epithelium in the lumen (19/19) and necrotic villus tips (19/19), pseudomembranes (10/19), lamina propria (5/19), and crypts (3/19) (Figure 3A-D). IHC-positive reactions were also detected in 115/131 (87.79%) of toxin-negative cases. IHC-positive reactions were detected in the sloughed epithelium in the lumen (112/115), necrotic villus tips (38/115), pseudomembranes (7/115), lamina propria (3/115), and crypt (1/115) localisations in toxin-negative cases. The IHC positivity rates in abomasum sections of enterotoxaemia and toxin-negative cases were 14/19 and 97/131, respectively. Positive reactions were localised in the sloughed epithelium in the abomasal lumen (Figure 3E). In the mesenteric lymph nodes of the studied samples, positive reactions were not detected. Immunoreactivity was absent from the negative control sections (Figure 3F).

Table 3. Main gross lesions distribution in enterotoxaemia cases

Case no	Icterus	Lung (Od)	Hd	Heart (Ha)	Kidney (Pk/Co)	Brain (Ed/Hp)	Intestine		Type*
							Mucosal haemorrhage	Fluid content	
Lamb 1	-	+	+	-	+/-	-/+	+	Haemorrhagic	Type A
Lamb 37	-	+	+	-	-/-	+/+	-	Haemorrhagic	Type D
Lamb 43	+	+	+	+	-/+	-/-	+	Haemorrhagic	Type A
Lamb 44	-	+	+	-	+/-	+/+	-	Yellow/greenish	Type D
Lamb 48	+	-	-	+	-/+	-/+	+	Haemorrhagic	Type A
Lamb 50	-	-	-	+	-/+	+/+	-	Yellow/greenish	Type D
Lamb 58	-	+	-	-	-/-	-/-	+	Yellow/greenish	Type A
Lamb 66	-	+	-	+	-/-	-/-	-	Yellow/greenish	Type A
Sheep 9	-	+	-	+	+/-	-/-	+	Haemorrhagic	Type A
Sheep 11	+	+	-	+	-/+	+/+	-	-	Type D
Sheep 12	-	+	-	+	+/-	-/-	-	-	Type D
Sheep 14	-	+	-	-	-/-	-/-	-	-	Type A
Sheep 19	-	+	+	+	-/-	-/-	-	-	Type D
Kid 4	-	+	-	-	+/-	-/-	-	Yellow/greenish	Type A
Kid 12	-	+	-	+	-/-	-/-	-	Yellow/greenish	Type A
Kid 15	-	+	+	-	+/-	-/-	-	Yellow/greenish	Type D
Calf 8	-	+	-	-	+/-	-/-	+	Haemorrhagic	Type C
Cattle 2	-	+	-	+	-/-	-/-	+	Haemorrhagic	Type D
Cattle 3	-	+	-	+	-/-	-/-	+	Haemorrhagic	Type A

Co= Congestion; Od= Oedema; Ha= Haemorrhage; Hd= Hydropericardium; Hp= Hyperemia; Pk= Pulpy kidney.
**C. perfringens* toxin types according to ELISA; + observed; – not observed.

Table 4. Incidence of the gross lesions in enterotoxaemic and toxin-negative animals

Gross lesions	Type A (n=10)			Type C (n=1)			Type D (n=8)			Total incidence (%)	Toxin negative cases (n=131) (%)
	Sheep	Goat	Cattle	Sheep	Goat	Cattle	Sheep	Goat	Cattle		
Icterus	2	-	-	-	-	-	1	-	-	3/19 (15.79%)	2/131 (1.53%)
Hydropericardium	2	-	-	-	-	-	3	1	-	6/19 (31.58%)	9/131 (6.87%)
Pulmonary oedema	6	2	1	-	-	1	5	1	1	17/19 (89.47%)	65/131 (49.62%)
Subepicardial petechial haemorrhage	4	1	1	-	-	-	4	-	1	11/19 (57.89%)	29/131 (22.14%)
Brain hyperaemia	2	-	-	-	-	-	4	-	-	6/19 (31.58%)	18/131 (13.74%)
Cerebellar coning	-	-	-	-	-	-	4	-	-	4/19 (21.05%)	-
Pulpy kidney	2	1	-	-	-	1	2	1	-	7/19 (36.84%)	27/131 (20.61%)
Renal congestion	2	-	-	-	-	-	2	-	-	4/19 (21.05%)	13/131 (9.92%)
Liver pale colour and friable consistency	5	2	-	-	-	-	3	-	-	10/19 (52.63%)	35/131 (26.72%)
Red coloured fluid accumulation in the urinary bladder	2	-	-	-	-	-	1	-	-	3/19 (15.79%)	1/131 (0.76%)
Perineal region soiled with watery diarrheic faeces	2	2	-	-	-	-	1	1	-	6/19 (31.58%)	69/131 (52.67%)
Enlarged mesenteric lymph nodes	5	2	1	-	-	-	3	-	-	11/19 (57.89%)	74/131 (56.49%)
Dilatation of the intestines (gas, liquid content)	3	1	-	-	-	1	1	1	-	7/19 (36.84%)	22/131 (16.79%)
Serosal hyperaemia in the intestines	5	2	1	-	-	1	3	1	-	13/19 (68.42%)	94/131 (71.76%)
Haemorrhagic content in the intestinal lumen	4	-	1	-	-	1	1	-	1	8/19 (42.11%)	19/131 (14.50%)
Yellow/greenish content in the intestinal lumen	2	2	-	-	-	-	2	1	-	7/19 (36.84%)	21/131 (16.03%)
Haemorrhage in the intestinal mucosa	5	-	1	-	-	1	-	-	1	8/19 (42.11%)	40/131 (30.53%)
Parasite in the intestinal lumen	-	-	-	-	-	-	-	-	-	-	6/131 (4.58%)

Table 5. Distribution of microscopic findings in the small and large intestine in enterotoxaemia cases

Case no	Haemorrhage	Necrosis and desquamation	Inflammatory cell infiltration	Pseudomembrane	Parasites	Basophilic rod-shaped bacteria	*C. perfringens*[1]	Type*
Lamb 1	+	+	+	+	-	+	+	Type A
Lamb 37	+	+	+	+	+	+	+	Type D
Lamb 43	+	+	+	+	-	+	+	Type A
Lamb 44	-	+	+	-	+	+	+	Type D
Lamb 48	+	+	+	+	-	+	+	Type A
Lamb 50	-	+	+	-	-	+	+	Type D
Lamb 58	+	+	+	-	-	+	+	Type A
Lamb 66	-	+	+	-	-	+	+	Type A
Sheep 9	+	+	+	+	+	+	+	Type A
Sheep 11	-	+	+	+	+	+	+	Type D
Sheep 12	-	+	+	+	-	+	+	Type D
Sheep 14	-	+	+	-	-	+	+	Type A
Sheep 19	-	+	+	+	+	+	+	Type D
Kid 4	-	+	+	-	-	+	+	Type A
Kid 12	-	+	+	-	-	+	+	Type A
Kid 15	-	+	+	-	+	+	+	Type D
Calf 8	+	+	+	+	+	+	+	Type C
Cattle 2	+	+	+	-	-	+	+	Type D
Cattle 3	+	+	+	+	-	+	+	Type A

**C. perfringens* toxin types according to ELISA; [1]immunohistochemistry; + observed; – not observed.

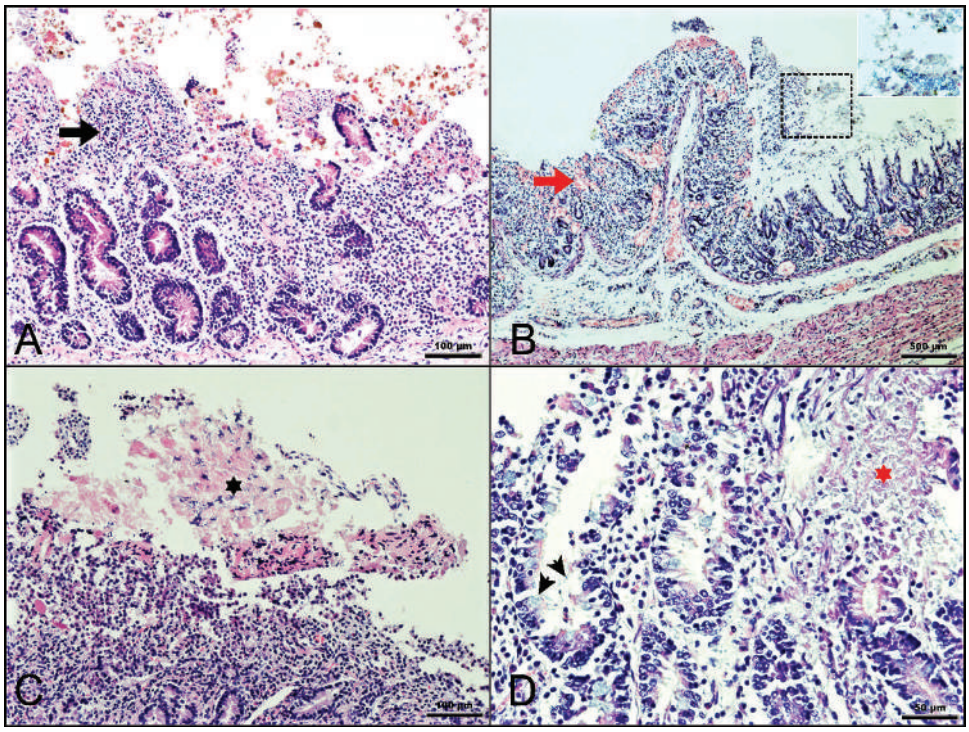

Figure 2. Histopathological findings of enterotoxaemia cases (HE). **A.** Lamb with type D enterotoxaemia. Small intestine, villus epithelium completely sloughed, necrosis of the mucosa and inflammatory cell infiltrations (black arrow) in the lamina propria. **B.** Lamb with type A enterotoxaemia. Small intestine, haemorrhage (red arrow) in the lamina propria, epithelial desquamation and basophilic rod-shaped bacterial clusters in the lumen. Inset: Higher magnification of the square with dashed lines represents necrotic cells and basophilic rod-shaped bacterial clusters in the lumen. **C.** Sheep with type D enterotoxaemia. Small intestine, intestinal mucosa covered with a pseudomembrane (black asterisk) composed of fibrin, cellular debris and rod-shaped bacteria. **D.** Sheep with type D enterotoxaemia. Ileum, mucosal necrosis (red asterisk), eosinophil granulocyte infiltration, basophilic rod-shaped bacteria in the lamina propria and crypts (arrowheads).

Table 6. The incidence of histopathological findings in enterotoxaemic and toxin-negative animals

Microscopic findings	Type A (n=10)			Type C (n=1)			Type D (n=8)			Total incidence (%)	Toxin negative cases (n=131) (%)
	Sheep	Goat	Cattle	Sheep	Goat	Cattle	Sheep	Goat	Cattle		
Necrosis and desquamation of intestinal villi	7	2	1	-	-	1	6	1	1	19/19 (100%)	131/131 (100%)
Villus blunting	4	2	-	-	-	1	4	1	-	12/19 (63.16%)	16/131 (12.21%)
Haemorrhage in the lamina propria	5	-	1	-	-	1	1	-	1	9/19 (47.37%)	28/131 (21.37%)
Inflammatory cell infiltration in the lamina propria	7	2	1	-	-	1	6	1	1	19/19 (100%)	94/131 (71.76%)
Bacterial clusters at necrotic villus tips and in the lumen	7	2	1	-	-	1	6	1	1	19/19 (100%)	73/131 (55.73%)
Pseudomembrane	4	-	1	-	-	1	4	-	-	10/19 (52.63%)	19/131 (14.50%)
Degeneration and desquamation in crypts	7	1	1	-	-	1	5	1	-	16/19 (84.21%)	94/131 (71.76%)
Stages of parasite development	1	-	-	-	-	1	4	1	-	7/19 (36.84%)	29/131 (22.14%)
Myocardial haemorrhage	4	1	1	-	-	1	3	-	-	10/19 (52.63%)	23/131 (17.56%)
Alveolar oedema	5	2	1	-	-	1	6	1	1	17/19 (89.47%)	82/131 (62.60%)
Vacuolar degeneration in hepatocytes	4	2	-	-	-	1	4	-	-	11/19 (57.89%)	16/131 (12.21%)
Necrosis in hepatocytes	2	1	-	-	-	1	5	-	-	9/19 (47.37%)	29/131 (22.14%)
Renal tubular degeneration and necrosis	6	2	-	-	-	1	5	1	1	16/19 (84.21%)	69/131 (52.67%)
Hyaline cast in the lumen of renal tubule	1	-	-	-	-	-	5	-	-	6/19 (31.58%)	16/131 (12.21%)
Haemorrhage in the renal interstitium	4	2	-	-	-	-	3	1	-	10/19 (52.63%)	26/131 (19.85%)
Eosinophilic fluid exudation in Bowman's cavity	2	2	-	-	-	-	2	-	-	6/19 (31.58%)	7/131 (5.34%)
Capillary hyperaemia in the brain	3	-	-	-	-	-	4	1	-	8/19 (42.11%)	18/131 (13.74%)
Perineuronal oedema	2	-	-	-	-	-	5	1	-	8/19 (42.11%)	19/131 (14.50%)

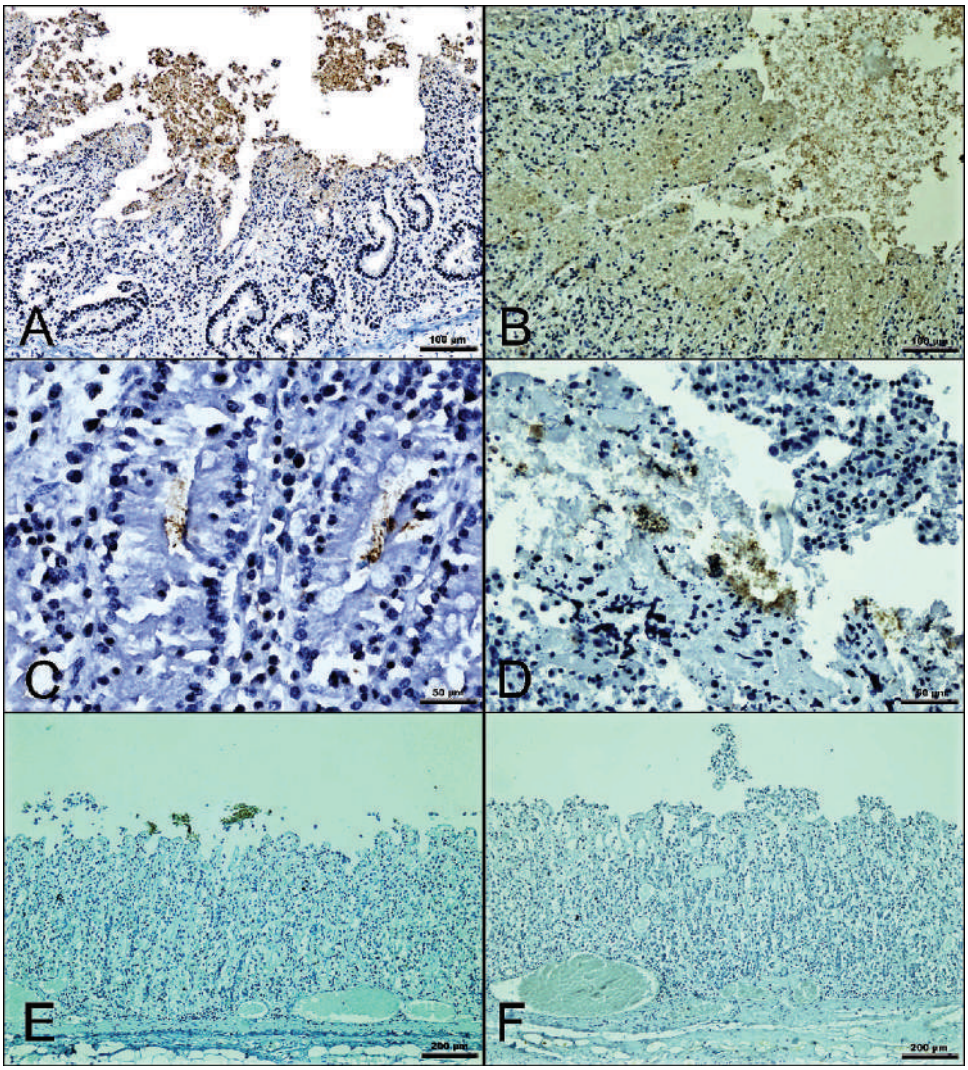

Figure 3. Immunohistochemical findings of enterotoxaemia cases (DAB was used as chromogen). **A.** Lamb with type D enterotoxaemia. Small intestine, *C. perfringens* antibody-positive bacteria in necrotic villus tips and desquamated epithelial cells in the lumen. **B.** Lamb with type A enterotoxaemia. Small intestine, *C. perfringens* antibody-positive bacteria in the necrotic villus tips and desquamated epithelial cells in the lumen. **C.** Sheep with type A enterotoxaemia. Small intestine, *C. perfringens* antibody-positive bacteria in crypts. **D.** Sheep with type D enterotoxaemia. Small intestine, *C. perfringens* antibody-positive bacteria in the pseudomembrane. **E.** Lamb with type A enterotoxaemia. Abomasum, *C. perfringens* antibody-positive bacteria in the desquamated epithelial cells in the lumen. **F.** Lamb with type A enterotoxaemia. Abomasum, absence of positive immunohistochemical staining in the negative control section.

Statistical analysis

According to the Pearson's chi-square test, a significant difference was determined between the detection of *C. perfringens* antigens by IHC and ELISA results in the intestinal necrotic villus tips, pseudomembranes, lamina propria, and crypt localisations ($P<0.01$). However, there was no statistical difference between ELISA results and IHC findings in the sloughed epithelium localisation of the intestine and abomasum ($P>0.05$). In addition, Spearman's correlation coefficient analysis showed a positive-moderate correlation in the necrotic villus tips and pseudomembranes and a positive-weak correlation in the lamina propria and crypts ($P<0.05$) (Table 7).

Table 7. Statistical relationship between ELISA toxin detection and anti-*C. perfringens* IHC findings

IHC findings		ELISA		Chi-square	Spearman's correlation	
		Neg	**Pos**	**P-value**	**rs**	**P-value**
Intestinal sections	Neg	16	-	0.107	+0.124	0.161
	Pos	115	19			
Sloughed epitheliums in the intestinal lumen	Neg	19	-	0.076	+0.139	0.116
	Pos	112	19			
Necrotic villus tips	Neg	93	-	P<0.01	+0.485	P<0.05
	Pos	38	19			
Pseudomembrane	Neg	124	9	P<0.01	+0.476	P<0.05
	Pos	7	10			
Lamina propria	Neg	128	14	P<0.01	+0.254	P<0.05
	Pos	3	5			
Crypts	Neg	130	16	P<0.01	+0.255	P<0.05
	Pos	1	3			
Sloughed epitheliums in the lumen of the abomasum	Neg	34	5	0.973	-0.03	0.973
	Pos	97	14			

rs: Spearman's correlation coefficient.
ELISA **Neg** = not toxin detected, **Pos**= toxin detected; **IHC Neg** = not positive IHC staining, **Pos** = positive IHC staining.

DISCUSSION

ELISA, PCR, and MNT analyses showing the presence of *C. perfringens* toxins in the intestinal contents are widely used in the diagnosis of suspected cases of enterotoxaemia. In this study, α, β, and ε toxins were detected by ELISA in ruminants with suspected enterotoxaemia. The toxin positivity rate in this study was 12.67%. In addition, type A and D enterotoxaemia were detected in 52.63% and 42.11%, respectively. However,

type B was not detected, and type C was detected in one calf only. This may be due to the rapid enzymatic degradation of the beta toxin, the main virulence factor of types B and C. Also, consistent with the results of previously reported studies [18,19], types A and D were the most commonly detected enterotoxaemia types. Although types A, B, C, and D enterotoxaemia have been previously detected in calves in Türkiye [20], type D enterotoxaemia in adult cattle was detected for the first time in this study. Enterotoxaemia cases are associated with various predisposing factors such as variable climatic conditions, sudden feed change, and pasture grazing [13,21]. The suspected cases of enterotoxaemia in this study were sampled mostly in the winter (64/150) and spring (42/150) seasons. Enterotoxaemia cases were also most frequently detected in spring (8/19). Moreover, it was speculated that the high rate of cases was associated with inadequate preventive immunisation and a high birth rate.

This study's macroscopic and histopathological changes were consistent with those reported previously [22-24]. Moreover, parasitic enteritis findings were also seen in cases with enterotoxaemia. Parasite invasions in the intestinal mucosa were previously reported as predisposing factors for enterotoxaemia [21,25]. Generally, enterotoxaemia diagnosis based on toxin detection is not possible in the veterinary community due to limited field and laboratory conditions. In such diagnostically limiting conditions, the pathological changes detected in this study may be critical for the preliminary diagnosis of enterotoxaemia [26-28].

Yellow lamb disease caused by *C. perfringens* type A is characterised by icterus, dark red urine, and hemoglobinuric nephrosis [8,28]. In the present study, similar results were observed in lambs with type A enterotoxaemia (2/5). Moreover, pathological findings suggestive of the yellow lamb disease have been previously reported in lambs with type D enterotoxaemia [29]. In this study, unusual findings characterised by icterus, cardiopulmonary lesions, and hemoglobinuric nephrosis were determined in a sheep with type D enterotoxaemia. It was evaluated that these findings may be caused by a *C. perfringens* type D strain that produces high levels of alpha toxin. Thus, type A and D enterotoxaemia should not be ignored in the icterus differential diagnosis in lambs.

In this study, haemorrhagic enteritis was detected in cattle with type A and type C enterotoxaemia. In addition, the intestines of these cases showed coagulation necrosis and haemorrhage of the lamina propria with rod-shaped bacteria around the villus remnants and inside the Lieberkühn crypts. These changes in cattle with type A and C enterotoxaemia were consistent with those previously reported [30,31].

Type D enterotoxaemia is characterised by neuropathological and cardiopulmonary changes [13,32]. In this study, type D enterotoxaemia cases frequently had pulmonary oedema, subepicardial petechiae, hydropericardium, meningeal hyperaemia, and perineural oedema in the brain. Cerebellar coning was also detected in 4 sheep with type D enterotoxaemia. It was speculated that these lesions, which were connected to the circulatory system, were caused by the effect of epsilon toxin on capillary vessel endothelium. It was predicted that these findings could be a marker for type D

enterotoxaemia. These lesions were consistent with the findings of some previously reported experimental studies [11,32-35]. Focal symmetric encephalomalacia and perivascular proteinous oedema are diagnostic findings of subacute and chronic type D enterotoxaemia [13]. However, these findings were not detected in type D enterotoxemic animals. In previous spontaneous and experimental studies, it was reported that epsilon toxin-induced central nervous system lesions were rarely observed for various reasons [13,34-37]. It has been previously reported that the softening of the kidney consistency in animals with type D enterotoxaemia is associated with a postmortem autolytic process [13]. However, in the animals with type A (3/10), C (1/1), and D (3/8) enterotoxaemia in this study, which were those without signs of autolysis in other organs, softening of the renal consistency was also observed in addition to the main macroscopic changes. It was concluded that this lesion, which is associated with autolytic changes, may have developed more rapidly in animals that had died from enterotoxaemia. Therefore, enterotoxaemia may be suspected in cases with softened kidney consistency and no autolytic changes in other organs.

Toxin detection is essential for the definitive diagnosis of enterotoxaemia. However, field conditions, disruptions during the sampling process, and the labile nature of toxins frequently pose limitations. Therefore, it has been recommended that toxin detection assays should be supported by clinical and pathological findings [8]. Although *C. perfringens* is a component of the ruminant microbiota, its detection by IHC in intestinal sections may not initially appear diagnostically relevant. Nevertheless, *C. perfringens* antigens were identified by IHC in the lesioned sections of the abomasum, small intestine, and large intestine. The IHC-positive reactions observed in all ruminants diagnosed with enterotoxaemia were consistent with previously reported intralesional IHC findings in *C. perfringens*-induced gastrointestinal diseases [11,12,31,38]. Moreover, a high rate of IHC-positive reactions (87.79%) was identified in toxin-negative cases. Notably, a substantial number of IHC-positive reactions (112/115) were localised in the epithelial cells shed into the intestinal lumen. This was hypothesised to be associated with the endogenous presence of *C. perfringens* as part of the microbiota. No statistically significant association was observed between IHC-positivity and the presence of toxins in the luminal epithelial debris. Disruption of the intestinal epithelial barrier may facilitate the translocation of bacteria and their toxins into deeper layers of the intestinal wall [11,38]. The demonstration of *C. perfringens* antigens by IHC in necrotic villus tips, pseudomembranes, the lamina propria, and crypts was found to be statistically significant. Therefore, IHC findings, in conjunction with histopathological evaluation, were considered critical for the pathological diagnosis of enterotoxaemia, complementing toxin detection assays. The significance of IHC staining in suspected cases of enterotoxaemia was supported by its agreement with macroscopic and histopathological alterations. One of the limitations of this study is the unavailability of specific antibodies against *C. perfringens* toxins for IHC analysis. Furthermore, the study relied solely on ELISA for toxin detection, without incorporating molecular

diagnostic approaches such as PCR or toxin gene sequencing. This methodological constraint may have limited the sensitivity and specificity of toxin identification.

CONCLUSION

In conclusion, macroscopic and histopathological findings were consistent with clostridial enterotoxaemia. Intralesional bacilli observed in the intestines were confirmed to be *C. perfringens* by IHC. It can be said that IHC will make an important contribution to the diagnosis of enterotoxaemia, especially in the presence of histopathological findings. Furthermore, it's possible to identify potential cases of enterotoxaemia that are overlooked because of issues with the detection of extremely sensitive toxins. Detailed immunohistochemical and molecular studies involving this microorganism and its toxins are recommended in the future to determine the exact role of *C. perfringens* in the macroscopic and histopathological changes observed in enterotoxaemia.

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Authors' contributions

OD and MO contributed to the design and conception of the study. OD collected samples and completed the laboratory procedures. Both authors read and approved the final version of the manuscript.

Declaration of conflicting interests

The author(s) reported no potential conflicts of interest concerning the research, authorship, or publication of this article.

ORCID iDs

Osman Dogan https://orcid.org/0000-0001-8579-3203
Mustafa Ortatatli https://orcid.org/0000-0002-3713-813X

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EVALUACIJA ENTEROTOKSEMIJE PREŽIVARA: UVID IZ UPOREDNE STUDIJE PRIMENE ELISA-e, IMUNOHISTOHEMIJSKIH I PATOLOŠKIH ISTRAŽIVANJA

Osman DOGAN, Mustafa ORTATATLI

Enterotoksemija, koju izazivaju toksini koje proizvodi *Clostridium perfringens*, jedna je od najčešćih klostridijalnih bolesti kod preživara. Dijagnoza se obično zasniva na detekciji toksina *C. perfringens* u crevnom sadržaju; međutim, i dalje postoje izazovi u postizanju praktične, pouzdane i definitivne dijagnoze. Predstavljamo uporedne nalaze enzimski povezanog imunosorbentnog testa (ELISA), imunohistohemijskog (IHC) i patološkog testa kod 150 preživara (ovaca, koza i goveda) za koje se sumnja na enterotoksemiju. Sadašnji rezultati su pokazali da je 19 od 150 preživara bilo pozitivno na najmanje jedan toksin *C. perfringens* (α, β i ε) pomoću ELISA testa. Štaviše, naši nalazi su pokazali da su makroskopska i histopatološka zapažanja bila podudarna sa klostridijalnom enterotoksemijom. IHC test za *C. perfringens* je obojio brojne dugačke, štapićaste bakterije prisutne u crevnim lezijama u svim slučajevima pozitivnim na toksin (19/19). Pored toga, pozitivno imunohistohemijsko bojenje je primećeno kod 115 od 131 preživara negativnih na toksin. Naši sadašnji nalazi ukazuju na to da povišene stope pozitivnog imunohistohemijskog bojenja, posebno u histopatološkim crevnim lezijama, mogu značajno doprineti patološkoj dijagnozi enterotoksemije. Takođe sugeriše da kada je detekcija toksina nemoguća, enterotoksemija se može efikasnije i sigurnije dijagnostikovati istovremenom procenom IHC i histopatoloških nalaza, što omogućava identifikaciju potencijalno pozitivnih slučajeva.