

The Relationship between Microscopic Lesions and Different Types of *Clostridium perfringens* and their Related Toxins by Sandwich ELISA in Cattle

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Abstract

Obviously, one of the most important causes of Enterotoxaemia in domestic animals is *Clostridium perfringens*. In present study, *C. perfringens* type A (56.66%), type D (26.66%) and type B (16.66%) were respectively the most abundant bacteria in intestinal samples of 30 cattle and calves with enterotoxaemia. The incidence of α , β and ϵ toxins in extracted intestinal contents, by Sandwich ELISA method, were determined 89/98%, 99/19% and 33.33% respectively. Histopathologic examination of different organs from 12 cattle and calves with enterotoxaemia and *C. perfringens* affection indicated following lesions; Heart: congestion (40%), hemorrhage (30%), mild myolysis (10%), sarcocystosis (30%), lymphocytic endocarditis (10%), pericarditis (10%) and myocardiocyte hypertrophy (20%). Liver: hepatitis (27%), coagulative necrosis (36%), hemorrhage (19%), severe hepatic fibrosis (9%) and hepatic abscess (9%). Spleen: congestion (33%), hemorrhage (33%), lymphoid tissue depletion (16%), capsular fibrinohemorrhagic Inflammation (16%) and hemosiderosis (33%). Intestine: necrohemorrhagic enteritis (40%), lymphoplasmocytic enteritis (50%), necrotic and suppurative subserosal Inflammation (10%), congestion (10%), edema (10%) and shortening and fusion of Villi (10%). Lymph nodes: congestion (25%), thrombosis and hemorrhage (25%), edema (25%), lymphocytolysis (25%) and fibrinous lymphadenitis (25%). Lung: congestion (36%), hemorrhage (9%), edema (27%), atelectasis (9%), emphysema (9%), embolic pneumonia (9%), purulent bronchopneumonia (18%), fibrinous pneumonia (45%), cuffing pneumonia (18%), Interlobular fibrosis (9%) and pulmonary abscess (9%). Kidney: congestion (12.5%), acute tubular necrosis (25%), glomerular atrophy (12.5%), interstitial nephritis (50%) and hyaline droplets (12.5%). The present data demonstrate that in the laboratory setting the reproducibility, sensitivity and linear dose response of ELISA allows quantitative estimation of different types of bacteria and respective toxins activity. This could be useful for monitoring toxin production. Our study suggested that although some of the lesions were triggered by *C. perfringens*, some may be caused by other pathogens which were not investigated in this research.

Keywords: *Clostridium perfringens*; Histopathology; ELISA; Organs; Bacteriology

Introduction

Clostridium perfringens, anaerobic gram positive *bacillus*, is an important factor of clostridial enteritis in domestic animals [1-5]. Furthermore, at least 17 exotoxins are produced by this bacterium among which 4 of them possess essential role in pathogenesis of clostridial diseases. This bacterium is divided into 5 pathogenic types according to the 4 types of exotoxins (Alpha (α), Beta (β), Epsilon (ϵ), Iota (I)) that are different from clinical point of view according to the type of produced toxins, type of bacteria and also relevant factors of host and environment conditions. In addition, the common manifestations are enteritis, diarrhea, neural signs and accidental death. Thus, this organism causes losses, reduction of growth and large economic damages to ranchers and animal industry [1-6].

Apparently, this bacterium is natural flora of human being and animal bowel; hence recognition of bacteria is not only sufficient. Therefore, determination of exotoxin type and its amount is necessary. The aim of this study was to investigate relationship between histopathology of various organs and different types of *C. perfringens* bacteria and recognition of relevant exotoxins by Sandwich ELISA method in tissue samples of affected cattle and sheep in addition to determination amount of *C. perfringens* bacteria in microbiologic bowel samples.

Materials and Methods

In present study, the histopathological examination of 30 diagnostic *C. perfringens* out of referring samples to veterinary laboratory was carried out in addition to the microbiology diagnostic tests including determination of type of bacteria, type of exotoxin by Sandwich ELISA method, samples of different organs of 12 sick calves and cow cattle which were positive from *C. perfringens*. In the Sandwich ELISA method, sensitive 96-cell plate has been used by specialized monoclonal antibody for α , β and ϵ exotoxins using special kits of Bio-X diagnostics company. The tests have been carried out according to the kit instruction [7-9].

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Histopathologic examination

Tissue specimens including heart, liver, spleen, bowel, lymph node, lung and kidney of each animal were taken (1×1×0.5 cm) and located in neutral formalin 10% for a period of 24-48 hours. Following molding, the samples were put in paraffin and prepared of samples with 5 micron thickness by microtome and stained by H & E method.

Results

Microbiological findings

In this study, bacteriological tests were applied on 30 cattle samples suspected to *C. perfringens* affection and indicated frequency of contamination with *C. perfringens* type A-56.66%, type B-16.66% and type D-26.66%; however, no contamination by type C was observed. By means of Sandwich ELISA method, presence of α , β and ϵ toxins related to this bacterium in the refined intestinal contents was determined equal to 89.98%, 19.99%, 33.33%, respectively (Table 1).

Histopathological findings

In this examination, the tissue samples (heart, liver, intestine, lymph node, lung and kidney) of 12 affected cattle and calves with *C. perfringens* infection have been investigated histopathologically, out of which 10, 1 and 1 cases were related to type A, type B and type D respectively. The results have been depicted respectively in tables 1, 5 and 6.

Heart: Sarcocystosis 30% (3 cases), lymphocytic endocarditis 10% (1 case), pericarditis 10% (1 case) and myocardiocytic hypertrophy have been observed 20% (2 cases) in addition to following lesions (Table 2).

Liver: Severe hepatic fibrosis 9% (1 case), hepatic abscesses 9% (1 case) and fatty liver have been observed 36% (4 cases) in addition to following lesions (Table 3).

Spleen: In addition to the following complications in the histopathological examinations, another complication such as hemosiderosis was observed 33% (2 cases) (Table 4).

Lung: In addition to the following complications, in histopathological examination, symbolic pneumonia 9% (1 case), suppurative bronchopneumonia 9% (1 case), interstitial pneumonia 45% (5 cases), cuffing pneumonia 18% (2 cases), interlobular fibrosis 9% (1 case) and abscess 9% (1 case) have been observed (Table 7).

Kidney: In addition to the following complications, interstitial nephritis 50% (4 cases) and hyaline droplets have been observed around 12.5% (1 case) as well (Table 1).

Discussion

According to the results, the most common type in the reference samples which developed tissue lesions was type A. In references, *C. perfringens* type A is mentioned as the most prevalent type in mammals, avian and environment [5,10]. Many researchers have

Number/type of bacterium	Toxin					
	ϵ		β		α	
	P	N	P	N	P	N
A/17	0	0	0	0	100%	17
B/5	40%	2	100%	5	60%	3
D/8	100%	8	12.5%	1	87.5%	7
Total	33.33%	10	19.99%	6	89.98%	27

Table 1: The rate of different *Clostridium perfringens* toxins by Sandwich ELISA method in the samples referred to veterinary diagnostic labs.

Lesions	N	P	Toxin			Type of bacterium		
			ϵ	β	α	D	B	A
Hyperemia	4	40%	0	25%	100%	0	25% (1case)	75% (3cases)
Hemorrhage	3	30%	33.33%	0	100%	33.33% (1case)	0	66.66% (2cases)
Mild myolysis	1	10%	0	0	100%	0	0	100%

Table 2: The role of different types of *Clostridium perfringens* toxins in each one of cardiac complications.

Lesions	N	P	Toxin			Type of bacterium		
			ϵ	β	α	D	B	A
Hepatitis	3	27%	33.33%	33.33%	100%	33.33% (1 case)	33.33% (1 case)	33.33% (1case)
Coagulative necrosis	4	36%	25%	25%	100%	25% (1 case)	25% (1 case)	50% (2 cases)
Mild myolysis	2	19%	0	0	100%	0	0	100% (2cases)

Table 3: The effect of different types of *Clostridium perfringens* toxins on each one of hepatic complications.

Lesions	N	P	Toxin			Type of bacterium		
			ϵ	β	α	D	B	A
Hyperemia	2	33%	0	0	100%	0	0	100% (2cases)
Hemorrhage	2	33%	0	0	100%	0	0	100% (2cases)
Lymphoid tissue depletion	1	16%	0	0	100%	0	0	100% (1case)
Capsular fibrino-hemorrhagic presplenitis	1	16%	0	0	100%	0	0	100% (1case)

Table 4: The share of different types of *Clostridium perfringens* toxins in producing each one of splenic complications.

recognized *C. perfringens* type A with higher prevalence in comparison with other types of this bacterium in mammals and birds [4,11-14] (Table 8). Diseases resulting from *C. perfringens* in diverse organs indicate several histopathological lesions [5,10]. In current study, 4 cases revealed hyperemia in heart tissues, 3 cases of which were related to *C. perfringens* type A and one case was related to type B. In sample of type A hemorrhage, acute pericarditis, mild myocardiocyte hypertrophy, sarcocystosis, lymphocytic endocarditis and mild myolysis with bacterial colonies were observed. Ghareib and Amer [15] demonstrated histopathological changes in laboratory animals' heart, with type A such as vascular congestion, edema, and degenerative changes in myocardium and congestion only in cases with type D.

C. perfringens type D produce toxin known as an angiotoxin that causes pericardial effusions in heart [5]. Investigations on type D in calves ascertained heart lesions including hydropericardium, protein-rich fluid, myocardial hemorrhage, endocardial petechiae and subendocardial hemorrhage around mitral valves [3,16-18]. In present

study, one case of infection resulting from type D and one case related to type B, focal coagulation necrosis and hepatitis were observed as well as bacterial colonies in infection arising from type D in some parts of liver. Ghareib and Amer [15] in guinea pigs demonstrated vacuoles degeneration, granular hepatocytes and leukocyte infiltration in portal areas with type D and in that group with type A, they observed hepatitis and congestion in central and portal veins, intravascular hemolysis, necrosis, vacuoles degeneration of hepatocytes and proliferation of kupffer cells. In this study, the lesions caused by type A including fatty liver, hemorrhage, acute hepatic necrosis and fibrosis and in one case, hepatic abscess and bacterial colonies were observed. Also four cases of fatty liver may have been resulted from hunger due to infections or anorexia. *C. perfringens* in spleen developed congestive splenomegaly [5]. In this study, we only separated *C. perfringens* type A from spleen and observed histopathological lesions as hyperemia, hemorrhage, hemosiderosis, lymphatic depletion and capsular fibrinohemorrhagic inflammation. In enterotoxaemia caused by type D in calf also, spleen

Lesions	N	P	Toxin			Type of bacterium		
			ε	β	α	D	B	A
Necrohemorrhagic enteritis	4	40%	25%	0	100%	25% (1 case)	0	75% (4cases)
Lymphoplasmacytic enteritis	5	50%	0	20%	100%	0	20%	80% (4cases)
Diffuse necrotic & purulent subserosal inflammation	1	10%	0	0	100%	0	0	100% (1 case)
Hyperemia	1	10%	0	0	100%	0	0	100% (1case)
Edema	1	10%	0	0	100%	0	0	100% (1case)
Shortness and fusion of villi	1	10%	0	0	100%	0	0	100% (1case)

Table 5: The role of different types of *Clostridium perfringens* toxins in producing each intestinal complication.

Lesions	N	P	Toxin			Type of bacterium		
			ε	β	α	D	B	A
Hyperemia	1	25%	0	0	100%	0	0	100%
Hemorrhage & thrombosis	1	25%	0	0	100%	0	0	100%
Edema	1	25%	0	0	100%	0	0	100%
Lymphocytolysis	1	25%	0	0	100%	0	0	100%
Fibrinous lymphadenitis	1	25%	0	0	100%	0	0	100%

Table 6: The share of different types of *Clostridium perfringens* toxins in producing each one of lymph node complications.

Lesions	N	P	Toxin			Type of bacterium		
			ε	β	α	D	B	A
Hyperemia	4	36%	25%	25%	100%	25% (1 case)	25% (1 case)	50% (2 cases)
Hemorrhage	1	9%	0	0	100%	0	0	100%
Edema	3	27%	33.33%	0	100%	33.33% (1 case)	0	66.66% (2 cases)
Atelectasis	1	9%	0	0	100%	0	0	100% (1case)
Emphysema	1	9%	0	0	100%	0	0	100% (1case)

Table 7: The share of different types of *Clostridium perfringens* toxins in producing each one of pulmonary complications.

Lesions	N	P	Toxin			Type of bacterium		
			ε	β	α	D	B	A
Hyperemia	1	12.5%	0	0	100%	0	0	100%
Acute tubular necrosis	2	25%	50%	0	100%	50% (1case)	0	50% (1case)
Glomerular atrophy	1	12.5%	0	0	100%	0	0	100%

Table 8: The share of different types of *Clostridium perfringens* toxins in producing each one of renal complications.

lesions were observed as splenitis, sub capsular congestion, hemorrhage and reticular tissue disorder [10].

Ghareib and Amer [15] indicated lymphatic follicles decrease and hemosiderosis of spleen in guinea pigs with type A and in those with type D, the splenic lymphatic follicles decrease was solely found [15].

Lulov et al. [19] reported mild hemosiderosis in enterotoxaemia caused by *C. perfringens* type A in calf. In this study, in cases with *C. perfringens* type A infection, intestinal lesions such as lymphoplasmocytic enteritis with shortening and fusion of villi and hemorrhagic enteritis may be caused by concurrent infections with other agents such as rotavirus or coronavirus [20].

Songer et al. [14] demonstrated the infections resulting from *C. perfringens* type A in calf, observed intestinal lesions including fatal hemorrhagic enteritis particularly at proximal duodenum with gram-positive bacteria, necrohemorrhagic inflammation of mucous membrane and emphysema.

Denison et al. [17] showed that 17 out of 20 cattle feces samples with hemorrhagic enteritis, *C. perfringens* type A was isolated and in histopathological examinations, they found severe focal submucosal hemorrhage with edema in small intestine. The results raised the relationship between presence of *C. perfringens* type A and hemorrhagic bowel syndrome (HBS).

In current study, 3 cases of necrohemorrhagic lesions were observed and since we have obtained *C. perfringens* type A from these lesions, it is likely that HBS is important in these 3 cases. In Khorasan Razavi province, 2 dairy cattle with this syndrome were diagnosed and a large amount of *C. perfringens* was obtained from the culture of cattle bowel contents [1]. In present study, one case with *C. perfringens* type D manifested intestinal lesions as acute hemorrhagic enteritis with gas bubbles.

Ghareib and Amer [15] in guinea pigs with type A observed intestinal congestion, necrosis and degenerative changes of villi epithelial cells and proliferation of goblet cells and severe leukocyte infiltration and in those with type D, intestinal hemorrhage and Pyre plaques decrease, edema and leukocyte infiltration in thick layer of mucus were observed. Additionally, in this study, intestinal lesions in one case with type B developed severe and diffuse lymphoplasmocytic enteritis. Uzal et al. (2010) [2] demonstrated intestinal lesions of type B mainly in small intestine. However, cecum and large spiral intestine were sometimes engaged. The lesions may be restricted to large intestine. Bloody contents of intestine with fibrin at aerosol surface of the intestine and adhesion are seen. In histopathological investigations, mesenteric hyperemia and focal or diffuse necrotic enteritis and intestinal emphysema can be observed. The luminal surface of intestine is covered by pseudomembrane composed of necrotic and degenerated cells and cell debris, inflammatory cells, fibrin, and large, thick bacilli with terminal spores.

In present study, lesions of lymph nodes included hyperemia, hemorrhage and thrombosis, edema, lymphocytolysis and fibrinous lymphadenitis. However, only *C. perfringens* type A was obtained out of them.

Uzal et al. (2010) [2] and Tajik et al. [21] reported lesions of lymph nodes as hyperemia and enlargement of mesenteric lymph nodes.

In this study, one case of *C. perfringens* type D with acute pulmonary hyperemia and edema with bacterial colonies were observed.

Pulmonary lesions reported in current study are consistent with the findings of other studies [1,21-23]. Furthermore, in pulmonary tissues with *C. perfringens* type A, interstitial pneumonia, purulent fibrinous bronchopneumonia, abscess, interlobular fibrosis, edema, hyperemia, atelectasis, emphysema and bacterial colonies were observed which were correspondent to other researchers' findings [3,11,24,25]. In this study, 5 cases of interstitial pneumonia could be attributed to probable presence of other pathogenic agents like viruses and mycoplasma causing chronic pneumonia.

In case of kidney tissue, in this study one case of severe hyperemia and 4 cases of multifocal lymphoplasmocytic interstitial nephritis was found. Although lymphoplasmocytic interstitial nephritis is not much related to diseases caused by *C. perfringens* bacteria, this lesion may appear in disease such as leptospirosis, theileriosis, and malignant catarrhal fever (MCF).

In current study, acute tubular necrosis in one case of infection caused by *C. perfringens* type D and one case of type A were reported.

Hormitzky and Glastunbury [26] indicated early autolysis of kidneys, hemorrhage, subcapsular and renal cortex hyperemia in cattle following enterotoxaemia.

In a study on 36 cattle kidneys with *C. perfringens*, Ghareib and Amer [15] found hyperemia and vascular hypotrophy, degeneration and necrosis in loop of henle, capsule thickness and hemorrhage and leukocyte infiltration in kidneys with type A and glomerular atrophy, tubular necrosis and degeneration, hyperemia and edema with type D [15].

Conclusion

The present data demonstrate that in the laboratory setting the reproducibility, sensitivity and linear dose response of ELISA allows quantitative estimation of different types of bacteria and respective toxins activity. This could be useful for monitoring toxin production. We hope to take more effective actions to diagnose, control and prevent from these diseases by discovering the relationship between lesions and different types of bacteria and respective toxins. Further work is being undertaken to determine the predictive value of the tests in commercial cattle.

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