Anaerobe 18 (2012) 143–147

Contents lists available at SciVerse ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and toxins

Effects of *Clostridium perfringens* alpha and epsilon toxins in the bovine gut

Winston E. Morris*, Mariana V. Dunleavy, Julián Diodati, Guillermo Berra, Mariano E. Fernandez-Miyakawa

Veterinary and Agronomic Research Centre (CICVyA), National Institute of Agricultural Technology (INTA), Castelar, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 4 February 2011
Received in revised form
10 September 2011
Accepted 6 December 2011
Available online 11 December 2011

Keywords:
Bovine
Calves
Clostridium perfringens
Intestinal loop
Toxin

ABSTRACT

Clostridium perfringens alpha and epsilon toxins produce enterotoxaemia in sheep and goats. However, the information regarding the pathophysiology of alpha and epsilon toxins in the bovine intestine is still scanty. In this study, intestinal loops were performed in the ileum and colon of three one-week-old Holstein and two four-week-old crossbreed calves. Laparotomy was performed in all calves under anaesthesia and four loops -three cm long- were performed in the small and large intestines. For both intestines, loops were inoculated with alpha or epsilon toxins. Tissue samples from all loops were obtained and processed for routine histology and for transmission electron microscopy. Congestion was observed in toxin treated loops. Fluid accumulation in the gut lumen was prominent in all treated loops, but in epsilon treated ones the mucous was also haemorrhagic. The histology revealed large amount of exfoliated epithelial cells in the lumen of alpha toxin treated loops and severe haemorrhage was observed in the lamina propria of epsilon toxin treated colonic loops. Despite some necrotic exfoliated enterocytes, no ultraestructural changes were observed in alpha toxin treated loops, though with epsilon toxin the loops exhibited dilation of the intercellular space in the mucosa of both, small and large intestines. These observations indicate that both, alpha and epsilon toxins can alter the intestinal barrier, in calves and are pathogenic for this species.

1. Introduction

Clostridium perfringens is a sporulating bacteria, often found as a normal inhabitant of the intestine of most animal species and humans [1,2]. *C. perfringens* alpha toxin is produced by all types of *C. perfringens* and it has been associated with a variety of diseases such as gas gangrene in livestock and humans or necrotic enteritis in bovine and poultry. However, necrotic enteritis in poultry has now been related to the effects of NetB toxin, produced by some strains of *C. perfringens* type A [3,4]. Epsilon toxin, on the other hand, is the most potent toxin produced by these bacteria and it is also considered a category B toxin by the Centers for Disease Control and Prevention (CDC) for its potential role in bioterrorism [5]. Epsilon toxin is produced by *C. perfringens* type B and D as a proto-toxin, and it is activated through proteolytic cleavage by the animal's trypsin, chymotrypsin or *C. perfringens* zinc-metalloprotease [2,6] and plays a key role in the pathogenesis of sheep and goat enterotoxaemia [7]. In sheep, this disease is acute and fatal, characterized predominantly by neural signs [8]. In goats, the disease is most commonly an acute or sub-acute enterocolitis, though lung and brain oedema can occur as well [9].

C. perfringens type D alpha and epsilon toxins are also considered as the causative agents for bovine enterotoxaemia, which is known to affect mostly calves [10]. However, this bacteria and/or its toxins have also been detected in the gastrointestinal tract of healthy animals [7,11,12]. Furthermore, it is known that laboratory evidence does not always support the clinical observations of this disease in the bovine, and there are only a few reports about this condition in cattle [2]. Therefore, the information about the clinical and pathologic findings of the disease in calves is scant and contradictory [13,14]. In an attempt to clarify the role of *C. perfringens* type D as a pathogen for cattle, some experimental studies have been performed [16,17]. These studies reported perivascular oedema in the lungs and brain as well as mild acute intestinal haemorrhage after intravenous-toxin or intraduodenal-culture inoculations in calves. However, specific studies on the effects of both, alpha and epsilon toxins in the bovine gut are still scant. In order to provide further knowledge on the effects of *C. perfringens* alpha and epsilon toxins in the bovine gut, the present study, describes the gross, histological and ultrastructural changes of the small and large intestine in one-week-old, and four-week-old calves, after the exposure to alpha or epsilon toxins.

* Corresponding author. Instituto de Patobiología, CICVyA – INTA Castelar, PO Box 25, 1712 Castelar, Buenos Aires, Argentina. Tel./fax: +54 11 46211712.
E-mail address: wmorris@cnia.inta.gov.ar (W.E. Morris).

1075-9964/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.anaerobe.2011.12.003

2. Material and methods

2.1. Animal welfare and care

Three one-week-old Holstein and two four-week-old crossbreed calves were used in this study. The animals were kept with their mothers until the day prior to the surgery.

The animal experiments were carried out according to the recommendations and approval of the Veterinary and Agricultural Research Centre (CICVyA) Animal Experimentation Ethics Committee, at INTA Castelar.

2.2. Toxins

Semi-purified (ultrafiltered) alpha toxin, obtained from a batch for vaccine production (CSL Ltd. Melborune, Australia) was used, as described by Fernandez Miyakawa and Uzal [18]. The crude toxin had been produced using an ovine isolate of *C. perfringens* type A. To avoid loss of toxicity, a freeze dried vial was reconstituted in 25 mM sodium phosphate buffer (pH 7.4) on each occasion. The alpha toxin preparation was tested in a mouse neutralization test with *C perfringens* type A and D antisera, and a lethal dose 50% (LD_{50}) per milliliter was calculated [19]. The purity of alpha toxin is >80%, mouse neutralization test revealed that perfringolysin-O was not present in any of the inocula used [20].

Epsilon proto-toxin was purified as previously described [14] from an overnight growth of a *C. perfringens* type-D culture (strain NCTC 8346). The purity of epsilon proto-toxin was >95% when SDS-PAGE gel was stained with Coomasie blue. Prior to its use, epsilon proto-toxin was reconstituted and activated by incubation at 37° for 30 min with 0.1% trypsin (Sigma).The LD_{50} was estimated as previously described [21].

2.3. Challenging inoculum protocol

Twenty 4 h previous to the surgery, all calves were housed and fasted, with water provided *ad libitum*. Tranquilization was obtained by intravenous injection of ketamine (10 mg/kg, Richmond, Argentina) and acepromazine (1 mg/kg Holliday, Argentina). Anaesthesia was induced by peridural injection of 1 ml of lidocaine (Lidocaina 2%, Invi, Argentina). Laparotomy was performed via the right flank, and the small intestine (ileum) and colon were exposed. Four segments (3 cm long) of ileum and four similar lengths of colon were isolated by a ligature in each animal, avoiding interference with the blood supply and leaving an empty segment of gut (4–5 cm long) between consecutive loops. All loops were flushed and rinsed with saline, to remove any content from the lumen of the bowel. One ml of inoculum containing either alpha (300 LD_{50}) or epsilon (4000 LD_{50}) toxins were injected, separately, into either of two small intestinal and either of two colonic loops, in each animal. The remaining four loops (two small and two large intestine loops) received a similar volume of 25 mM sodium phosphate buffer (pH 7.4) and were used as controls.

The abdominal incision was then closed by separate muscle and skin sutures and the animals were kept deeply anaesthetized with ketamine until euthanasia.

After 4 h, all calves were euthanized and the abdominal cavity was reopened. Both, ileum and colon loops of each animal were removed and weight before and after emptying its content.

The volumes of fluid accumulation in the toxin treated loops were compared with the control loops Wilcoxon signed-rank test (STATISTIX 8.0, Analytical Software).

The tissues were then fixed in 10% buffered formalin, embedded in paraffin wax, sectioned at 4 μm and stained with hematoxylin and eosin. A 1 mm^3 section of each loop, together with a similar section of non-ligated small and large intestine were collected and fixed in 2% buffered glutaraldehyde solution and post-fixed in 1% osmium tetroxide. These tissues were then processed for routine transmission electron microscopical examination. The electron microscopy was performed using a Jeol 1010 transmission electron microscope.

3. Results

All control and toxin treated loops exhibited fluid accumulation (Table 1). The largest amount of fluid accumulated, was observed in the small intestine of an alpha toxin treated loop (3.25 ml).

Statistical analysis was performed comparing the toxin treated loops with the controls.

Significant differences were observed between small intestine epsilon toxin treated and large intestine alpha toxin treated loops, compared with their respective controls ($p < 0.05$). However, the remaining treatments (small intestine alpha-treated and large intestine epsilon-treated loops) had a higher p-value when compared with their respective controls ($p \leq 0.1$).

The contents of the loops were transparent in the controls and mucous and dense in alpha treated ones. In epsilon treated loops, the mucosa and the mucous content had a haemorrhagic appearance.

Histopathological changes were observed in tissues treated with either alpha or epsilon toxins. In alpha toxin-treated loops, shortening and villus blunting, together with epithelial cell detachment and haemorrhage of the lamina propria was observed in ileal loops (Fig. 1C). Congestion and mild haemorrhage in the lamina propria were observed in the colon loops (Fig. 1D). Abundant exfoliated epithelial cells, together with mucous forming pseudomembrane as well as some leucocytes, were seen in the lumen of both, small and large intestinal alpha toxin treated loops. These changes were more severe in the small rather than the large intestine.

In epsilon toxin treated loops, congestion and erosion of the mucosa with severe haemorrhage, affecting the mucosa, lamina propria and sub-mucosa, with moderate polymorphonuclear infiltrate was observed in either small and large intestinal loops (Fig. 1E and F respectively). In these loops, fibrinonecrotic (diphtheritic) membranes, consisting of mucus, leucocytes exfoliated epithelial cells and erythrocytes were present in the lumen.

The ultrastructure of alpha or epsilon toxin treated loops, revealed epithelial cell detachment and necrosis of detached cells. In epsilon toxin treated loops, haemorrhage of the lamina propria with some polymorphonuclear infiltration was also evident. Although in all loops the microvilli, the cell membranes, nucleus and organelles of the enterocytes appeared intact in the attached enterocytes, in epsilon toxin loops, the intercellular spaces were markedly dilated, predominantly in the large intestine (Fig. 2D) but also in the small intestinal loops as well (Fig. 2B). These separations between adjacent enterocytes were at various sites, between the tight junction and the basal membrane, although no obvious changes were noted in the tight junction or other junction structures. According to their severity, these separations were scored as: 1 (mild), 2 (moderate) or 3 (severe). The average score in small intestine loops was 1.5 ranging from 1 to 2 and in large intestinal loops, the median score was 3, ranging from 2 to 3.

Table 1
Mean and standard error of the fluid accumulation detected in either control or toxin treated small or large intestine (in ml).

	Small intestine	Large intestine
Control	1.44 ± 0.27	1.13 ± 0.42
Alpha toxin	2.30 ± 0.46	2.04 ± 0.50
Epsilon toxin	2.07 ± 0.16	1.58 ± 0.32

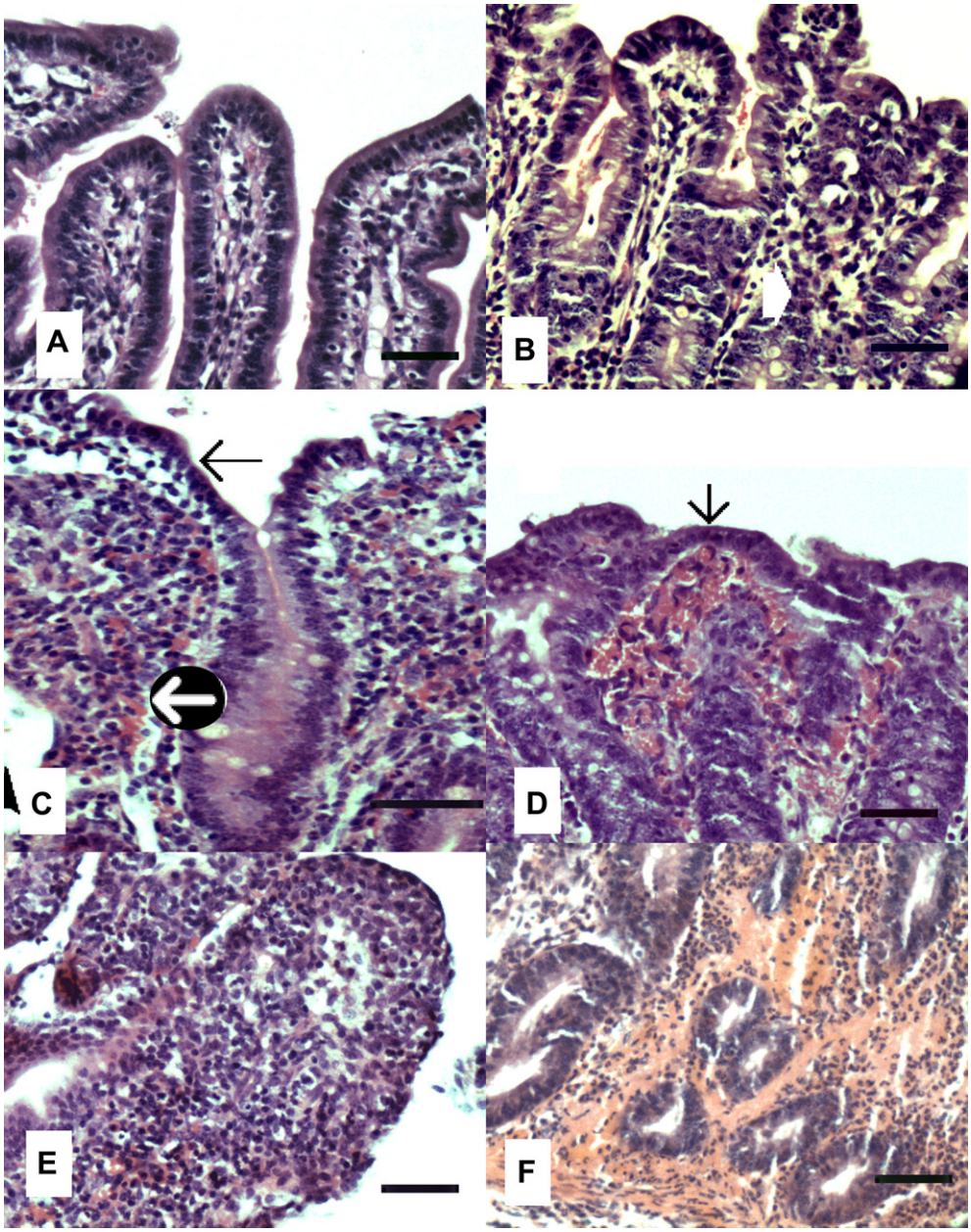

Fig. 1. Histology of bovine small (left column) and large (right column) intestine loops inoculated with vehicle (top), *C. perfringens* alpha (middle) and epsilon toxins (bottom). Control small (A) and large intestinal loops (B), exhibited no evident histological changes. Epithelial cell detachment (arrow) and haemorrhage of the lamina propria (white arrow) can be observed in alpha treated small intestines loops (C), while the large intestine (D) displayed congestion and mild haemorrhage of the lamina propria (arrow). In epsilon treated loops, severe erosion and cell exfoliation can be observed in the small intestine loops (E), while oedema and haemorrhage of the lamina propria, extending from the submucosa, with leukocytic infiltration, can be seen in the large intestine loops (F). Hemoatoxilin and eosin. Bar = 50 μm.

No changes were observed in the endothelial cells of any of these loops, and no histological or ultrastructural changes were observed in any of the control loops (Fig. 1A and B).

4. Discussion

In the present study, some fluid accumulation was noticed in the lumen of all loops, with varying differences; although the largest amount was noted in small intestine alpha toxin treated loops. Significant differences between control and treated loops were also detected.

In all alpha toxin treated loops, the fluid content was mucus but epsilon toxin small and large intestinal loops had a haemorrhagic content. Similar fluid accumulation has been reported in sheep, goats and calves ligated loops treated with *C. perfringens* type A or D culture supernatants, cells or alpha or epsilon toxins [5,17,22–24].

Alpha toxin induced epithelial cell detachment, tip blunting, erosion and mild inflammation of both the small and large intestine. These changes have been reported in ovine intestinal loops inoculated with *C. perfringens* alpha toxin (300 LD_{50}/ml) [18]. Epsilon toxin, on the other hand, induced more severe changes including severe oedema and haemorrhage of the lamina propria and polymorphonuclear infiltration. The differences in the pathology of either toxin highlight different biological activities that each toxin has in the bowel [5,6,21].

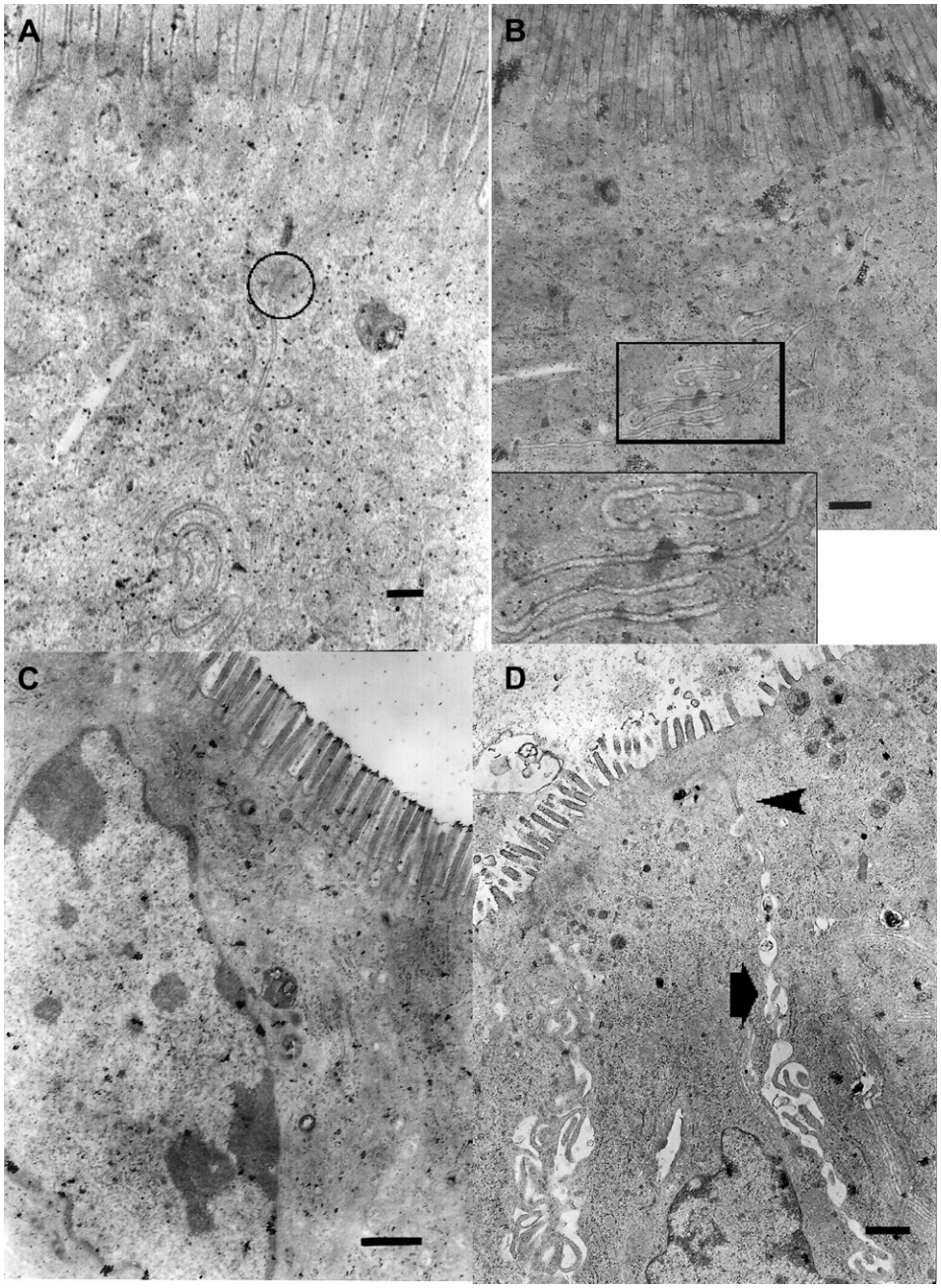

Fig. 2. Transmission electron microscopy of enterocytes of small and large intestinal loops, treated with vehicle (A and C) or epsilon toxin (B and D). Enterocytes from a control small intestinal loop exhibiting well preserved desmosome (circle) and close contact between adjacent cells, in contrast to the epsilon treated loops (B) in which mild (grade 1) separation between adjacent cells (square) can be noted. In epsilon toxin large intestine loops (D), the severe separation (grade 3) is evident (arrow), extending from the apical junction complex (arrowhead) towards the basal membrane. In these cells, the villi seem intact. Bar = 1 μm.

The ultrastructural changes observed in this study, were also more severe in epsilon treated loops, in which dilation of the intercellular spaces was evident, particularly in the colon. Recently, Goldstein and co-workers [25], reported tight junction opening in small intestine ligated loops of rats and mice treated with epsilon toxin, sufficiently enough to enable horseraddish peroxidase to filter through the tight junction [25]. In the present study, the tight junctions were not evidently altered although the dilation of the intercellular spaces suggests that subtle changes were present. These observations indicate that epsilon toxin alters the integrity of the intestinal epithelium, affecting the fluid homeostasis, at least through the paracellular pathway as well as the integrity of the intestinal barrier as a whole. This could allow the toxin to cross the epithelial barrier, affecting other structures in the lamina propria, inducing the inflammatory changes and, finally, reaching the blood stream.

Both, one-week-old and four-week-old calves had similar changes, indicating that both categories are probably equally susceptible.

It has been demonstrated —in a mouse model- that alpha and epsilon toxins have a synergic effect when they were inoculated intravenously, but none when they were inoculated intragastrically [20]. The results of the present study indicates that both, alpha and epsilon toxins induce changes in the bovine small and large intestine independently, though through different pathogenic

mechanism. It also stands clear that he small intestine is more susceptible to alpha toxin while the colon appears to be more susceptible to epsilon.

Although there has been some controversy on the role of *C. perfringens* type D as a cause of bovine sudden death [13], the results of this study together with previous ones [15,16] demonstrate that the toxins of this microorganism can induce clinical and pathological changes to the intestine of calves.

Acknowledgments

The authors thank Mrs. Blanca García for her technical help. This study was sponsored by: PICT Jóvenes Investigadores-2005 (N° 21-31684), Ministerio de Ciencia y Técnica (MINCyT), Presidencia de la Nación, Argentina.

References

[1] Mantis N. Vaccines against the category B toxins: Staphylococcal enterotoxin B, epsilon toxin and ricin. Advanced Drug Delivery Reviews 2005;57:1424–39.
[2] McClane BA, Uzal FA, Fernández Miyakawa M, Lyerly D, Wilkins T. The Enterotoxic Clostridia. In: Dworkin M, Falkow S, Rosenburg E, Schleifer KH, Stackebrandt E, editors. The Prokaryotes, vol. 4. New York: Springer-Verlag; 2006. p. 698–752.
[3] Ceci L, Paradies P, Sasanelli M, de Caprariis D, Guarda F, Capucchio MT, et al. Haemorrhagic bowel syndrome in dairy cattle: possible role of *Clostridium perfringens* type A in the disease complex. Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine 2006;53:518–23 (Erratum in: Journal of Veterinary Medicine Series A-Physiology Pathology Clinical Medicine. 54: 55, 2007).
[4] Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, et al. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. PLoS Pathology 2008;8(4):e26.
[5] Manteca C, Daube G, Jauniaux T, Linden A, Pirson V, Detilleux J, et al. A role for the Clostridium perfringens beta2 toxin in bovine enterotoxaemia? Veterinary Microbiology 2002;86:191–202.
[6] Minami J, Katayama S, Matsushita O, Matsushita C, Okabe A. Lambda-toxin of *Clostridium perfringens* activates the precursor of epsilon-toxin by releasing its N- and C-terminal peptides. Microbiol Immunology 1997;41:527–35.
[7] Uzal FA, Songer JG. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. Journal Veterinary Diagnostic Investigations 2008;20: 253–65.
[8] Finnie JW. Pathogenesis of brain damage produced in sheep by *Clostridium perfringens* type D epsilon toxin: a review. Australian Veterinary Journal 2003; 81:219–21.
[9] Titball RW. Bacterial Phospholipases *C. Microbiological*. Reviews 1993;57: 347–66.
[10] Brown CC, Baker DC, Barker IK. Alimentary system. In: Grant Maxie M, editor. The Jubb, Kennedy and Palmer's pathology of Domestic animals. 5th ed. Sounders Elsevier; 2007. p. 1–296.
[11] Miyata S, Matsushita O, Minami J, Katayama S, Shimamoto S, Okabe A. Cleavage of a C-terminal peptide is essential for heptamerization of *Clostridium perfringens* epsilon-toxin in the synaptosomal membrane. Journal of Biological Chemistry 2001;276:13778–83.
[12] Losada-Eaton DM, Uzal FA, Fernández Miyakawa ME. *Clostridium perfringens* epsilon toxin is absorbed from different intestinal segments of mice. Toxicon 2008;51:1207–13.
[13] Niilo L, Avery RJ. Bovine "Enterotoxemia" I. *Clostridium perfringens* types isolated from animal Sources in Alberta and Saskatchewan. Canadian Veterinary Journal 1963;4:31–6.
[14] Niilo L. *Clostridium perfringens* in animal disease: a review of current knowledge. Canadian Veterinary Journal 1980;21:141–8.
[15] Uzal FA, Ghoddusi M, Kelly WR, Rozmanec M. Comparison of the effects of *Clostridium perfringens* type D culture supernates in ligated intestinal loops of goats and sheep. Journal Comparative Pathology 1999;121:127–38.
[16] Filho EJ, Carvalho AU, Assis RA, Lobato FF, Rachid MA, Carvalho AA, et al. clinicopathological features of experimental *Clostridium perfringens* type D enterotoxemia in cattle. Veterinary Pathology 2009;46:1213–20.
[17] Uzal FA, Kelly WR, Morris WE, Assis RA. Effects of intravenous injection of *Clostridium perfringens* type D epsilon toxin in calves. Journal Comparative Pathology 2002;126:71–5.
[18] Fernandez Miyakawa ME, Uzal FA. Morphologic and physiologic changes induced by *Clostridium perfringens* type A alpha toxin in the intestine of sheep. American Journal of Veterinary Research 2005;66:251–5.
[19] Stern M, Batty I. Pathogenic Clostridia. London: Butterworths; 1975. pp. 96–101.
[20] Fernandez-Miyakawa ME, Jost BH, Billington SJ, Uzal FA. Lethal effects of *Clostridium perfringens* epsilon toxin are potentiated by alpha and perfringolysin-O toxins in a mouse model. Veterinary Microbiology 2008;127: 379–85.
[21] Sayeed S, Fernandez-Miyakawa ME, Fisherm DJ, Adams V, Poon R, Rood JI, et al. Epsilon-toxin is required for most *Clostridium perfringens* type D vegetative culture supernatants to cause lethality in the mouse intravenous injection model. Infection and Immunity 2005;73:7413–21.
[22] Uzal FA, Kelly WR. Experimental *Clostridium perfringens* type D enterotoxemia in goats. Veterinary Pathology 1998;35:132–40.
[23] Manteca C, Daube G, Pirson V, Limbourg B, Kaeckenbeeck A, Mainil JG. Bacterial intestinal flora associated with enterotoxaemia in Belgian Blue calves. Veterinary Microbiology 2001;81:21–32.
[24] Fernandez Miyakawa ME, Uzal FA. The early effects of Clostridium perfringens type D epsilon toxin in ligated intestinal loops of goats and sheep. Veterinary Research Communications 2003;27:231–41.
[25] Goldstein J, Morris WE, Loidl CF, Tironi-Farinatti C, McClane BA, Uzal FA, et al. *Clostridium perfringens* epsilon toxin increases the small intestinal permeability in mice and rats. PLoS One 2009;4:e7065.