International Journal of Animal Research (ISSN:2575-7822)

Incidence of *Clostridium perfringens* in Intestinal Contents of Domestic Livestock Detected by PCR

Asghar Arshi1, Sajad Nikkhah2, Hamidreza Kabiri3, Arman Akbarpour2, Rassoul Hashemzehi4, Behnaz Mansouri2, Ayse Kilic5, Esmaeil Mahmoudi2*

1. Young Researchers and Elite Club, Najafabad Branch, Islamic Azad University, Najafabad, Iran
2. Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran
3. Young Researchers and Elite Club, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran
4. Department of Genetics, Fars Science & Research Branch, Islamic Azad University, Shiraz, Iran
5. Sivrice Vocational High School, University of Firat, 23119 Elazig-Turkey

ABSTRACT

Clostridium perfringens (C. perfringens) is a Gram-positive anaerobic spore-forming bacterium that is widespread in environmental soil and sewage, as well as in animal intestines. C. perfringens is an important pathogen in both human and veterinary medicine. The incidence and numbers of C. perfringens in the intestinal contents of 100 cattle, 86 turkey and 177 chickens from September 2015 to April 2016 were determined. C. perfringens was found in 26%, 22% and 40% of intestinal contents of cattle, turkey and chickens, respectively. In this research samples were tested for isolation of C. perfringens by culturing and biochemical method and then they were confirmed by the polymerase chain reaction (PCR) methods. We suggest that PCR assay could be a replacement of the culture method for quantifying C. perfringens in the intestinal tracts. These research helping us to establish the role of each C. perfringens toxin in animal disease, to investigate the in vivo mechanism of action of these toxins, and to develop more effective vaccines against diseases produced by these microorganisms.

Keywords: Clostridium perfringens, Cattle, Turkey, Chickens, PCR.

*Correspondence to Author:
Esmaeil Mahmoudi. Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, Iran
E-mail: bioshk@ yahoo.com
Tel: +98-9139884428

Introduction

C. perfringens is a Gram-positive, spore-forming, rod shaped anaerobic pathogen, non-motile and is frequently found in the intestinal tract of man and animals (1). The virulence of this bacterium largely results from its ability to produce at least 15 different *C. perfringens* toxins (2). *C. perfringens* strains are classified into five groups (types *A*, *B*, *C*, *D* and *E*) on the basis of their production of four major toxins (known as the alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) toxins) (3). Due to their short reproductive cycle and their worldwide popularity as a food, poultry represent the most highly selected livestock. Throughout the world the majority of broilers are reared using very similar, modern, intensive systems of production where birds are confined for their lifetime within high density housing (4). In poultry, it can cause a deadly disease called necrotic enteritis (NE). Since *C. perfringens* was first described as *Bacillus aerogenes capsulatus* in 1892 (5), the bacterium has been identified as an anaerobe responsible for a wide range of diseases in humans and animals (6). The overgrowth of *C. perfringens* caused Necrotic Enteritis (NE) disease in the small intestine of different animals throughout the world. It is commonly found in the environment (in soil, on the skin and sewage) and in the intestines of animals and humans as a member of the normal flora (7). *C. perfringens* is found in undercooked or improperly sterilized canned foods (germination of endospores) and in water (surface water). The natural contamination source is human and animal faeces transmitted into food products primarily by water. *C. perfringens* produces an extensive range of invasions and exotoxins (8). Also, *C. perfringens* normally grows at 44 °C, whereas some other clostridia are inhibited at this temperature. This property is used in ISO methods to give the medium more selectivity (9). *C. perfringens* is usually considered to be an exclusively extracellular pathogen that secretes powerful cytotoxins that lyse cells and break down connective tissue (1). *C. perfringens* was first implicated as a cause of antibiotic-associated diarrhoea (AAD) in 1984 (10). Once initiated, the disease spreads rapidly through healthy tissues, leading to shock and death if not treated. Shock, which is a common and frequent occurrence in gangrene infections, is most likely due to extracellular toxins produced by *C. perfringens* (11). *C. perfringens*, a part of normal gut flora, is commonly involved in diseases in most domestic animals and some wildlife, including horses, poultry, birds, rabbits, sheep, goats, cattle, mink, ostrich, dogs and cats (12). The primary host immune defense against *C. perfringens* in the earliest stages of a post-traumatic infection is likely to be the phagocytic cells of the innate immune system, mainly PMNs and monocytes/macrophages. In later stages of the infection, when the bacteria have multiplied to the extent that a clinical case of gangrene is evident, there is a profound lack of phagocytes in the immediate vicinity of the bacteria (1). The *C. perfringens* infections in poultry may present as acute clinical disease or subclinical disease. The acute form of the disease leads to increased mortality in the broiler flocks (13). Vaccination is a widely used preventive measure against many infectious diseases in mammals and poultry. *C. perfringens* may cause necrotizing enteritis in several mammalian species including humans, and some of these conditions have been successfully prevented by vaccination (14). Despite some success with vaccines in animal experiments (15), there is no effective vaccine against *C. perfringens* infections (1). Owing to its ability to produce spores under adverse environmental conditions, it is one of the most widespread potential bacterial pathogen in nature as well as in the gastrointestinal tract of most animal species (16). Many heat processes are incapable of inactivating the *C. perfringens* endospores. Survival of spores in these products allows the subsequent outgrowth

where spores can germinate and commence growth at temperatures of 43 to 47˚C. In foods such as meats with gravy, heating reduces the oxygen tension (lowered redox) to cause sufficient anaerobiosis in which greater numbers of *C. perfringens* will rapidly divide. Importantly, *C. perfringens* has been documented to have very rapid doubling times, in some cases as low as 7 to 9 minutes in beef broth (17, 18). PCR technique, a molecular biology method, is an interesting alternative for detecting microbiological indicators in water samples based on the detection of genetic material from target bacteria (19). However, understanding of the pathogenicity and physiology of *C. perfringens* is still poor compared with other well studied pathogenic bacteria (3). The study was performed to determine the frequency of *C. perfringens* from cattle, turkey and chickens by culturing and PCR assay.

Materials and methods

Sampling and DNA extraction

In the present study intestinal contents were obtained from 100, 86 and 177 of slaughtered cattle, turkey and chickens, respectively. All animals were healthy and showed no signs of enteritis or other illness. Each sample was placed in a sterile plastic bag, transported to the laboratory and analyzed on the day of slaughter. Initially Cooked meat broth medium was prepared in tubes by mixture of 500 mg of cooked meat with 10 ml distilled water, heated at 80°C for 5 minutes in water bath. This will force Oxygen bubbles leave the medium to give a partial anaerobic medium. 5 gr of Intestine samples were uniformed with 5 ml of PBS (phosphate buffer) in a sterile mortar. The above suspension is added to the cooked meat broth and heated at 65°C for 10 minutes to leave only spore forming microorganisms. Tubes were then incubated for 72 hours at 37°C in an anaerobic CO_2 incubator. In the next step each samples were cultured by streak plate method using a loop on already prepared blood agar plates and incubated for 18 hours at 37°C anaerobic condition. The round, disordered, flat, bright colonies were considered to be clostridium colonies. DNA extraction was performed by boiling method, where few colonies were scratched off the blood agar plates and dissolved in 200µl distilled water in test tubes, and incubated in boiling water bath for 10-15 minutes. The last step was a 12000 rpm centrifugation for 5 minutes, and kept in -20 for further analyses.

Gene amplification

Detection was performed by amplification with the specific primers. The best primer concentrations were identified by performing a series of experiments with varying primer combinations (Table 1). Primers performed at the NCBI with experimental GENINFO BLAST Network Service to assess degree of homology between these primers and other reported sequences.

Table 1. Sequence and concentration of primers for PCR

Primers	Primer Sequences (5′ to 3′)	Primer concentrations (nM)		Tms (̊C)	Product Size (bp)
		Forward	Reverse		
CPE	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	600	300	55	233

The PCR reaction mixture (50µl) contained 5 µl of bacterial lysate as template DNA (100 ng), 0.4 mM dNTPs, 5 µl 10 X PCR buffer, 0.25 µl of 5 U/µl *Taq* DNA polymerase, 0.4 uM of each primers (10 pmol/µl) and 2 mM $MgCl_2$. The PCR reaction mixture were placed in an Eppendorf PCR thermal cycler. Amplification was obtained with 32 cycles following an initial denaturation step at 95°C for 5 minutes. Each cycle comprised denaturation at 94°C for 60

seconds, annealing at 55°C for 60 seconds, and synthesis at 72°C for 60 seconds. The final extension step occurred at 72°C for 5 minutes. Then, 10 μL of the amplified product was electrophoresed at 90 V in a 2% agarose gel and stained with ethidium bromide. Amplified bands were visualized and photographed under UV illumination.

Statistical analyses

The frequency of re-isolation of *C. perfringens* were analyzed by the chi-square test using the SPSS 24 (SPSS Inc, Chicago IL, USA) software. P values <0.05 were considered significant.

Results

The quality of the extracted DNA was examined through 2% agarose gel and confirmed. The results showed a high frequency of *C. perfringens* infection in examined samples.

Incidence and number of enterotoxigenic *C. perfringens*: The frequency distribution of *C. perfringens* present in the intestinal contents of domestic livestock is shown in Table 2.

The microorganism was found in 27 (27%), 23 (26.74%) and 58 (32.76%) intestinal contents of cattle, turkey and chickens, respectively. Of the 27 positive samples from cattle, 10 samples contained fewer than 10^2 MPN/100 g, two samples contained $2.3*10^2$ MPN/100 g and one sample contained $4.3*10^2$ MPN/100 g. Of the 23 positive samples from turkey, 10 samples contained fewer than 10^2 MPN/100 g and one sample contained $2.3*10^3$ MPN/100 g. More than half of the 58 positive samples from chickens were found to contain more than 10^2 MPN/100 g (six samples 10^2 to 10^3 MPN/100 g, five samples 10^3 to 10^4 MPN/100 g and one sample $2.4*10^4$ MPN/100 g).

Table 2. Incidence and numbers of *C. perfringens* in intestinal contents of domestic livestock

Source	Number of Samples	Number of Samples	Number of samples containing *C. perfringens* in MPN/100 g			
	Tested	Positive (%)	$<10^2$	10^2-10^3	10^3-10^4	10^4-10^5
Cattle	100	27 (27)	10	3		
Turkey	86	23 (26.74)	10		1	
Chicken	177	58 (32.76)	8	6	5	1

Discussions

C. perfringens, although it is a member of the normal intestinal flora, is regarded as one of the most important causes of intestinal disease in farm animals and wild animals and, to a lesser extent, in humans (20). *C. perfringens* plays a significant role in food-borne human disease and is among the most common food-borne illnesses in industrialized countries (21, 22). Outbreaks are frequently associated with temperature-abused meat or poultry dishes and typically involve a large number of victims (22). The broiler chicken industry is a main source of

animal protein and body fat of broiler chicks is one of the major concerns in chicken industry (23). If a sufficient number of *C. perfringens* cells are ingested from contaminated food, these cells are capable of passage from the stomach to the intestinal tract where, upon sporulation, CPE is released causing the disease state of *C. perfringens* food poisoning (17, 18). *C. perfringens* is recognized as an enteric pathogen in humans, domestic animals, and livestock. This organism is associated with NE, gangrenous dermatitis, clostridial dermatitis, and gizzard erosions in poultry (24).

It was first recognized as a frequent cause of death among children in New Guinea in the 1960s. It has also been reported to occur among malnourished adults or people with chronic diseases such as diabetes in USA, United Kingdom, Germany and other developed nations (25, 26). Disease outbreaks have been minimized to a significant extent through the use of in-feed antibiotics. However, the incidence of NE in broiler flocks has increased in Western Europe since the implementation of a ban on the growth promotion use of avoparcin in 1997 and of tylosin, spiramycin, virginiamycin, and zinc bacitracin in 1999 (27). Despite the identification of approximately 100 species of *Clostridium,* only a small number have been recognized as relatively common etiologic agents of medical and veterinary importance. Nonetheless, some of these species are associated with very serious diseases (28). The pathogenicity of this bacterium is associated with the production of extracellular toxins produced by some of its strains, such as beta2 toxin (29). The incidence of the disease may have been exacerbated by the use of wheat and barley as basal ingredients in European poultry diets. Dietary grains rich in water-soluble nonstarch polysaccharides (NSP) such as wheat, barley, or rye increase intestinal viscosity through enhanced mucus production (27). *C. perfringens* type *A* food poisoning is one of the most common food borne diseases in the United States. The factor responsible for the characteristic disease symptoms (diarrhea and abdominal cramps) of *C. perfringens* food poisoning has been shown (30). *C. perfringens* types *B* and *C* disease begins in the host intestine (31). Type *B* isolates cause an often fatal hemorrhagic dysentery in sheep, and possibly in other species, while type *C* isolates cause enteritis necroticans (also called pigbel) in humans and necrotic enteritis and/or enterotoxemias in almost all livestock species. Both types *B* and *C* animal disease are often accompanied by sudden death or acute neurological signs (31). Clinical signs and histopathologic findings in type *C* infections are very similar in most livestock animal species. The course of disease can be peracute, acute, or chronic, with signs of the acute and peracute condition including intense abdominal pain, depression, and bloody diarrhea (32). Type *B* and *D* strains are causative agents of fatal enterotoxaemia in domestic animals and occasionally humans (33). In the United States, *C. perfringens* induced gas gangrene affects over 3000 people annually, with a mortality rate of 25%, even when aggressive medical treatment is administered. Gas gangrene caused by *C. perfringens* type *A*, most often occurs when the bacteria enter the host through a deep penetrating wound. If this wound is anaerobic, *C. perfringens* will grow and the clinical signs of gangrene will appear as soon as 6 hours after infection (1). Gas gangrene, or clostridial myonecrosis, is an infection that originates in ischemic tissues in which the blood supply has been cut off due to trauma or circulatory blockages. Once the infection begins, it rapidly spreads to healthy tissues and, if left untreated, the disease is always fatal due to the release of toxins into the bloodstream resulting in severe shock and cardiac stress (34, 35). An importance aspect of pathogenesis of *C. perfringens* induced gas gangrene infections involves the role of the immune system in ischemic tissues. In response to ischemia, connective tissue cells and resident macrophages release monocyte chemoattractant protein-1 (MCP-1) (36, 37, 38). Penicillin is the drug of choice for the prophylaxis and treatment of gas gangrene due to *C. perfringens* (39). *C. perfringens* is a spore forming bacterium and widely occurring pathogen. Vaccines against epsilon and beta toxins is manufactured using secreted toxin by virulent *C. perfringens* types *B* and *D*. Large scale production of vaccines from virulent strains requires stringent safety conditions and costly detoxification and control steps. Therefore it would be beneficial to produce

these toxins in a safe production host and in an immunogenic form (28). The symptoms, predominantly diarrhea and abdominal pain, appear 6 to 24 hour after ingestion of contaminated food. Vomiting and fever are unusual. Death occurs occasionally among debilitated patients, particularly the elderly (40). Death usually is caused by dehydration and occurs among the very young, the very old, and persons debilitated by illness (21). A *C. perfringens* count of >106 cells/g in fecal samples of patients is indicative of *C. perfringens* food poisoning (41). Epidemiological investigations involve enumerating *C. perfringens* in suspected food. Characterization of enterotoxigenic *C. perfringens* strains is not performed routinely, since *C. perfringens* sporulation, which is a prerequisite for CPE production, is limited in the usual culture media (40). The presence of cpb2-positive *C. perfringens* strains in the intestine has been associated with intestinal disease in humans, ruminants, horses, and pigs. However, cpb2-positive *C. perfringens* strains have also been reported in animals and humans without any signs of intestinal disease (20). Miwa et al study the prevalence of *C. perfringens* from chicken intestinal contents was 40% when a direct PCR method from enriched samples (42). The presence of *C. perfringens* in current study was 25% (25 of 100). Many researchers have been used PCR method to detect sequences of interest in *C. perfringens* in purpose of identification and classification (43, 44, 45). According to the high prevalence of *C. perfringens* in Europe For example, the incidence of NE in France increased from 4% in 1995 to 12.4% of reported diseasesin1999 (Drouin, 1999) (7, 46). Bueschel, et al (2003), showed that 85.8% of swine isolates of *C. perfringens* all from type *A* possessed cpb2 gene (47). In a survey of the prevalence of cpb2 in *C. perfringens* field isolates performed in Arizona, USA 197/1537 (12.8%) of bovine isolates were tested positive by PCR assay (Bueschel et al 2003) (47).

Zerbini and Ossiprandi (2009) indicated that 23.1% (3/14) of type *A* isolates obtained from diarrheic dogs were cpb2 positive (48). The same method (multiplex PCR) in southeastern Iran showed that The frequency of *C. perfringens* infection among 130 sheep-dung samples were 17.39% type *A*, 21.74% type *B*, 34.78% type *C* and 26.09% type *D* (49). The study by Laura and Ossiprandi (2010) showed that 19 of the 104 (18.3%) faecal and intestine specimens from *C. perfringens* infection (16). Jabbari et al (2011) indicated that frequency of *C. perfringens* were genotype *A* (14.07%), genotype *B* (31.25%), genotype *C* (26.56%) and genotype *D* (28.12%) (33). Jabbari et al (2012) reviewed that *C. perfringens* was 57.6% isolates screened by PCR were cpb2- positive (2). In conclusion the PCR that was developed in this study can be used to type *C. perfringens* isolates in epidemiological studies as an alternative to conventional procedures.

Statement of animal rights

All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Obrien DK, Melville SB. The anaerobic pathogen Clostridium perfringens can escape the phagosome of macrophages under aerobic conditions. Cellular microbiology. 2000; 2: 505-19.

2. Jabbari AR, Tekyei F, Esmaeilizad M, Pilehchian Langroudi R. Occurrence of Beta2 toxigenic Clostridium perfringens isolates with different toxin types in Iran. Archives of Razi. 2012; 67: 133-7.

3. Shimizu T, Ohtani K, Hirakawa H, Ohshima K, Yamashita A, Shiba T, et al. Complete genome sequence of Clostridium perfringens, an anaerobic flesh-eater. Proceedings of the National Academy of Sciences. 2002; 99: 996-1001.

4. Knowles TG, Kestin SC, Haslam SM, Brown SN, Green LE, Butterworth A, et al. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PLoS One. 2008; 3: e1545.

5. Welch WH, Nuttall GHF. A gas producing bacillus (Bacillus aerogenes capsulatus, nov, sepc.) capable of rapid development in the blood vessels after death. Bulletin of the Johns Hopkins Hospital. 1892; 3: 81-91.

6. Yoo HS, Lee SU, Park KY, Park YH. Molecular typing and epidemiological survey of prevalence of Clostridium perfringens types by multiplex PCR. Journal of clinical microbiology. 1997; 35: 228-32.

7. Drew MD, Syed NA, Goldade BG, Laarveld B, Van Kessel AG. Effects of dietary protein source and level on intestinal populations of Clostridium perfringens in broiler chickens. Poultry science. 2004; 83: 414-20.

8. Sartory DP, Field M, Curbishley SM, Pritchard AM. Evaluation of two media for the membrane filtration enumeration of Clostridium perfringens from water. Letters in Applied Microbiology. 1998; 27: 323-27.

9. Wells CL, Wilkins TD. Clostridia (4th edition): sporeforming anaerobic bacilli. Medical microbiology. 1996.

10. Asha NJ, Wilcox MH. Laboratory diagnosis of Clostridium perfringens antibiotic-associated diarrhea. Journal of medical microbiology. 2002; 51: 891-94.

11. Rood JI. Virulence genes of Clostridium perfringens. Annual Reviews in Microbiology. 1998; 52: 333-60.

12. Effat MM, Abdallah YA, Soheir MF, Rady MM. Characterization of Clostridium perfringens field isolates, implicated in necrotic entritis outbreaks on private broiler farms in Cairo, by multiplex PCR. African Journal of Microbiology Research. 2007; 29-32.

13. Immerseel FV, Buck JD, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R. Clostridium perfringens in poultry: an emerging threat for animal and public health. Avian Pathology. 2004; 33: 537-49.

14. Lin YT, Labbe R. Enterotoxigenicity and genetic relatedness of Clostridium perfringens isolates from retail foods in the United States. Appl Environ Microbiol. 2003; 69: 1642-6.

15. Williamson ED, Titball RW. A genetically engineered vaccine against the alpha-toxin of Clostridium perfringens protects mice against experimental gas gangrene. Vaccine. 1993; 11: 1253-8.

16. Laura Z, Ossiprandi MC. Molecular toxinotyping of clostridium perfringens cattle isolates by multiplex PCR. Annali Della Facoltà di Medicina Veterinaria di Parma. 2010; 71-80.

17. Smedley JG, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA. The enteric toxins of Clostridium perfringens. Reviews of Physiology, Biochemistry and Pharmacology. 2004; 152: 183-204.

18. Sawires YS, Songer JG. Clostridium perfringens: insight into virulence evolution and population structure. Anaerobe. 2006; 12: 23-43.

19. Tantawiwat S, Tansuphasiri U, Wongwit W, Wongchotigul V, Kitayaporn D. Development of multiplex PCR for the detection of total coliform bacteria for Escherichia coli and Clostridium perfringens in drinking water. The Southeast Asian Journal of Tropical Medicine and Public Health. 2005; 36: 162-9.

20. Allaart JG, van Asten AJAM, Vernooij JCM, Gröne A. Effect of Lactobacillus fermentum on beta2 toxin production by Clostridium perfringens. Applied and environmental microbiology. 2011; 77: 4406-11.

21. Brynestad S, Granum PE. Clostridium perfringens and foodborne infections. International Journal of Food Microbiology. 2002; 74: 195-202.

22. Lindström M, Heikinheimo A, Lahti P, Korkeala H. Novel insights into the epidemiology of Clostridium perfringens type A food poisoning. Food Microbiology. 2011; 28: 192-8.

23. Mersmann HJ. Beta-adrenergic receptor modulation of adipocyte metabolism and growth. Journal of Animal Science. 2002; 80: E24-E29.

24. Rahimi S, Kathariou S, Grimes JL, Siletzky RM. Effect of direct-fed microbials on performance and Clostridium perfringens colonization of turkey poults. Poultry Science. 2011; 90: 2656-62.

25. Songer JG. Clostridia as agents of zoonotic disease. Veterinary Microbiology. 2010; 140: 399-404.

26. Johnson EA, Summanen P, Finegold SM. Clostridium. Manual of Clinical Microbiology. 2007; 9: 889-910.

27. Collier CT, Van Der Klis JD, Deplancke B, Anderson DB, Gaskins HR. Effects of tylosin on bacterial mucolysis, Clostridium perfringens colonization, and intestinal barrier function in a chick model of necrotic enteritis. Antimicrobial agents and chemotherapy. 2003; 47: 3311-7.

28. Langroudi RP, Pour KA, Shamsara M, Ghorashi SA. Synthetic construct of Clostridium perfringens epsilon-beta fusion protein gene. International Conference on Biotechnology and Environment Management. 2011; 18: 126-131.

29. Tolooe A, Shojadoost B, Peighambari SM. Molecular detection and characterization of cpb2 gene in Clostridium perfringens isolates from healthy and diseased chickens. Journal of Venomous Animals and Toxins including Tropical Diseases. 2011; 17: 59-65.

30. Wnek AP, Strouse RJ, McClane BA. Production and characterization of monoclonal antibodies against

Clostridium perfringens type A enterotoxin. Infection and immunity. 1985; 50: 442-8.

31. Uzal FA, Vidal JE, McClane BA, Gurjar AA. Clostridium perfringens toxins involved in mammalian veterinary diseases. Open Toxinology Journal. 2010; 3: 24-42.

32. Songer JG. Clostridial diseases of small ruminants. Veterinary Research. 1998; 29: 219-32.

33. Jabbari AR, Afshari Far AR, Esmaelizad M, Pilehchian Langroudi R, Abdolmohammadi Khiav L. Molecular typing of toxigenic Clostridum perfringens isolated from sheep in Iran. Archives of Razi. 2011; 66: 81-86.

34. Asmuth DM, Olson RD, Hackett SP, Bryant AE, Tweten RK, Tso JY, et al. Effects of Clostridium perfringens recombinant and crude phospholipase C and theta-toxin on rabbit hemodynamic parameters. The Journal of Infectious Diseases. 1995; 172: 1317-23.

35. Stevens DL, Troyer BE, Merrick DT, Mitten JE, Olson RD. Lethal effects and cardiovascular effects of purified alpha- and theta-toxins from Clostridium perfringens. The Journal of Infectious Diseases. 1988; 157: 272-9.

36. Birdsall HH, Green DM, Trial J, Youker KA, Burns AR, MacKay CR, et al. Complement C5a, TGF-beta 1, and MCP-1, in sequence, induce migration of monocytes into ischemic canine myocardium within the first one to five hours after reperfusion, Circulation. 1997; 95: 684-92.

37. Gourmala NG, Buttini M, Limonta S, Sauter A, Boddeke HW. Differential and time-dependent expression of monocyte chemoattractant protein-1 mRNA by astrocytes and macrophages in rat brain: effects of ischemia and peripheral lipopolysaccharide administration. Journal of neuroimmunology. 1997; 74: 35-44.

38. Ivacko J, Szaflarski J, Malinak C, Flory C, Warren JS, Silverstein FS. Hypoxic-ischemic injury induces monocyte chemoattractant protein-1 expression in neonatal rat brain. Journal of Cerebral Blood Flow & Metabolism. 1997; 17: 759-70.

39. Stevens DL, Maier KA, Mitten JE. Effect of antibiotics on toxin production and viability of Clostridium perfringens. Antimicrobial agents and chemotherapy. 1987; 31: 213-18.

40. Fach P, Popoff MR. Detection of enterotoxigenic Clostridium perfringens in food and fecal samples with a duplex PCR and the slide latex agglutination test. Applied and environmental microbiology. 1997; 63: 4232-36.

41. Shandera WX, Tacket CO, Blake PA. Food poisoning due to Clostridium perfringens in the United States. The Journal of Infectious Diseases. 1983; 147: 167-70.

42. Heikinheimo A, Korkeala H. Multiplex PCR assay for toxinotyping Clostridium perfringens isolates obtained from Finnish broiler chickens. Letters in applied microbiology. 2005; 40: 407-11.

43. Baums CG, Schotte U, Amtsberg G, Goethe R. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. Veterinary Microbiology. 2004; 100: 11-6.

44. Park JY, Kim S, Oh JY, Kim HR, Jang I, Lee HS, et al. Characterization of Clostridium perfringens isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poult Sci. 2015; 94: 1158-64.

45. Goncuoglu EM, Ayaz ND, Ormanci FS, Hidebrandt G. Molecular typing of Clostridium perfringens isolated from turkey meat by multiplex PCR. Letters in Applied Microbiology. 2008; 47: 31-4.

46. Drouin P. Retour en force de l'entérite nécrotique. Filieres Avicoles. 1999; 78-9.

47. Bueschel DM, Jost BH, Billington SJ, Trinh HT, Songer JG. Prevalence of cpb2, encoding beta2 toxin, in Clostridium perfringens filed isolates: correlation of genotype with phenotype. Veterinary Microbiology. 2003; 94: 121-9.

48. Zerbini L, Ossipranadi MC. Molecular typing of Clostridium perfringens isolated from dogs by toxin gene amplification. Annual of Veterinary Medicine Faculty Parma. 2009; 29: 115-28.

49. Ahsani MR, Mohammadabadi MR, Shamsaddini MB. Clostridium perfringens isolate typing by multiplex PCR. Journal of Venomous Animals and Toxins including Tropical Diseases. 2010; 16: 573-8.