Original Paper

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Isolation and genotyping of *Clostridium perfringens* from healthy and diarrheic dogs

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Keywords

isolation; genotyping; Closteridium perfringens; dog

Abstract

Clostridium perfringens has been known as a cause of diarrhoea in dogs. The aim of this research was isolation *C. perfringens* by culturing and toxinotyping by PCR molecular method. In this research 151 dogs' faecal samples were collected from northwest of Iran, 131 of which were apparently healthy, and 20 of which were diarrheic. These faecal samples were cultured on 5% sheep blood agar; the suspected colonies with double homolysis that using multiplex and single PCR assay were admitted to detect toxinotypes of the isolates by specific primers. *C. perfringens* strains were isolated from 5/20 (25%) the diarrheic group and 31/131(23.8%) the non-diarrheic group. All isolates (36/151) were classified as *C. perfringens* type A (cpa+). Fourteen isolates (38.8%) with cpa+cpb2+netB-tpeL- profile and one isolate (2.8%) had cpa+cpe+netB-tpeL- toxin's profile. More studies are needed to elucidate the epidemiology of *C. perfringens* in dogs and its role as a zoonotic agent and public health hazard. Based on author's knowledge, this is the first study performed in order to isolation *C. perfringens* and genotyping from dogs in Iran. The cpa+cpe+ gene was reported from one *C. perfringens* isolated from healthy dogs.

Abbreviations

C. perfringens: *Clostridium perfringens*
PCR: Polymerase Chain Reaction
SBA: Sheep Blood Agar
TSC: Tryptose Sulfite Cycloserine agar
TSN: Tryptose Sulfite Neomycin agar
CPA: *C. perfringens* Alpha toxin
cpb2: *Clostridium perfringens* beta2 toxin
netB: Necrotic Enteritis Toxin B
cpe: *Clostridium perfringens* Entrotoxin
etx: Epsilon Toxin
itx: Iota Toxin
TcdA: *Clostridium difficile* toxin A
TcdB: *Clostridium difficile* toxin B
TcsL: *Clostridium sordellii* lethal toxin
TcnA: *Clostridium novyi* alpha-toxin

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Introduction

Clostridium perfringens is a Gram-positive bacillus, strictly anaerobic bacteria that produce spores, which have a wide host range. In *C. perfringens* diarrheic dogs and cats has been found pathognomonic signs and the spectrum of disease attributed to this organism varies significantly. *C. perfringens* associated diarrhoea can cause clinical signs of small or large intestinal disease, or both in animals. Severity of disease ranges from a mild, self-limiting diarrhoea to a potentially fatal and acute hemorrhagic diarrhoea with severe inflammation of the intestinal mucosa in dogs (Marks et al. 2011).

The outbreaks of acute, often severe diarrhoea in humans, horses, dogs and cats have been related to *C. perfringens*. The existence of four major toxins, alpha (α), beta (β), iota (ι), and epsilon (ε), is the base for typing the microorganism into five toxigenic phenotypes (A, B, C, D and E). Type A produce (α) toxin, type B(α,β,ε), Type c(α,β,), type D(α,ε) , type E(α,i). *C. perfringens* strains in addition to the four major lethal toxins produce several minor toxins and enzymes, which can affect the severity and course of an infection. For example, delta toxin, kappa toxin, the iota toxin, neuraminidases and ureases all have a less important role in the pathogenesis of *C. perfringens* infections (Lepp 2012). Nevertheless, other toxins such as enterotoxin (CPE), possibly the CPB2 toxin, and the recently revealed NetB and perhaps TpeL toxins have major roles in specific diseases. Enterotoxigenic *C. perfringens* type A has been associated with human food poisoning and sporadic diarrhoea, canine acute and chronic large and small bowel diarrhoea and acute hemorrhagic diarrheal syndrome (AHDS).

Clostridium perfringens enterotoxin (CPE) has the most important pathologic effects in human intestines. Unlike the other toxins, CPE is created only via sporulation cells and accumulates in a large inclusion body inside the mother cell, from which it is released after lysis at the end of sporulation(Veshnyakova et al. 2010). Other recently defined virulence factor, the B2 toxin, is associated with diseases of the gastrointestinal tract in animals. It was produced by *C. perfringens* isolates from piglets with necrotic enteritis and was also found in horses with enterocolitis (Garmory et al. 2000). Necrotic enteritis toxin B-like (NetB) is a pore-forming toxin described as a significant virulence factor for necrotic enteritis in broiler chickens (Timbermont et al. 2011). TpeL, a recently-described novel member of the family of large clostridial cytotoxins, is found in *C. perfringens* types. TpeL was reported in *C. perfringens* type A isolates from necrotic enteritis outbreaks (Coursodon et al. 2012).

This study was aimed to investigate the prevalence and distribution of the genes encoding the *C. perfringens* toxins in dogs by using multiplex PCR assay, to study possible existence of novel toxins (TpeL and NetB) in the isolates, and to explore the relationship between bacteria and diarrhoea .

Materials and methods

Animals and Sampling

A Total of 151 fresh faecal samples were collected from referred dogs (59 male and 92 female) to a teaching veterinary hospital in northwest of Iran, of which 131 were without diarrhoea, and 20 (10 female and 10 male) were diarrheic. The samples from non-diarrheic and diarrheic dogs were obtained directly from the rectum, at the time of consultation.

One hundred thirty one dogs were clinically healthy in the physical examination and had no previous history (at least for the last month) of antibiotic therapy. However, twenty diarrheic dogs had acute diarrhoea for 4-5 days.

Cultivation and Isolation

Faecal samples were collected every week and at the same week were cultured directly onto 5% SBA using a sterile cotton-tip applicator which lightly coating with a small amount faeces and then streaked for isolation. The SBA was incubated in an anaerobic chamber at 37 °C for 24 h. Colonies with characteristic double haemolytic zones were selected and sub-cultured in TSC and TSN for purification.

The identity of the isolates were confirmed by their colonial and microscopical morphology, haemolytic pattern and Gram staining as previously described (Razmyar et al. 2014). All culture media and additives used in this study were from Merck (Germany). Reference strains of Clostridium perfringens ATCC 13124 (cpa); CIP 106157 (cpa, cpe); CIP 60.61 (cpa, cpb, etx, cpb2); AMJRT003IR (Net b+, TpeL+) were used as positive controls.

DNA Extraction

A single colony of each strain was suspended in 100 µL distilled water, boiled for 10 min and then centrifuged at 10,000 × g for 10 min. The supernatants were collected carefully and used as template DNA for PCR (Aldous et al. 2005).

Multiplex PCR for toxin profiling

Six pairs of primers were used to determine the presence of cpa, cpb, iA, etx, cpe, and cpb2 (Bueschel et al. 2003; Meer and Songer 1997) genes using a multiplex PCR technique for all isolates. The primers used in PCR reaction were provided by (Dena Zist Company, Iran). Amplification reactions were carried out in a 50 µL reaction volume containing 5 µL 10 x PCR buffer, 5 mM dNTPs, 25 mM

Table 1
List of primers used in multiplex and single PCR

Target gene	Primer Sequence (5′–3′)	Amplicon size (bp)	Method of PCR
cpa	5'-GCTAATGTTACTGCCGTTGA-3' 5'-CCTCTGATACATCGTGTAAG-3'	324	Multiplex PCR
cpb	5'-GCGAATATGCTGAATCATCTA-3' 5'-GCAGGAACATTAGTATATCTTC-3'	196	Multiplex PCR
etx	5'-GCGGTGATATCCATCTATTC-3' 5'-CCACTTACTTGTCCTACTAAC-3'	655	Multiplex PCR
itxA	5'-ACTACTCTCAGACAAGACAG-3' 5'-CTTTCCTTCTATTACTATACG-3'	446	Multiplex PCR
cpe	5'-GGAGATGGTTGGATATTAGG-3' 5'-GGACCAGCAGTTGTAGATA-3'	233	Multiplex PCR
cpb2	5'-AGATTTTAAATATGATCCTAACC-3' 5'-CAATACCCTTCACCAAATACTC-3'	567	Multiplex PCR
netB	5'-GCTGGTGCTGGAATAAATGC-3' 5'-TCGCCATTGAGTAGTTTCCC-3'	384	Single PCR
tpeL	5'-ATATAGAGTCAAGCAGTGGAG-3' 5'-GGAATACCACTTGATATACCTG-3'	466	Single PCR

MgCl2, 5U of Taq DNA polymerase, 0.5 mM of each cpa oligo, 0.36 mM of each cpb oligo, 0.36 mM of each cpb2 oligo, 0.52 mM of each iA oligo, 0.44 mM of each etx oligo, 0.34 mM of each cpe oligo, and dH2O. A 10 µL of template DNA was added to the mixture. Reference strains of *C. perfringens* ATCC 13124 (cpa); CIP 106157 (cpa, cpe); CIP 60.61 (cpa, cpb, etx, cpb2) were used as positive controls. Amplification was programmed in a thermocycler (Techne TC-3000, England) as follows: 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min (Bueschel et al. 2003).

Single PCRs

DNA extracts used as the template for PCR in a 25 µL reaction mixture which contain: 5 µL DNA template, 12.5 µL Taq DNA Polymerase Master Mix RED kit (Ampliqon, Denmark) which contained Tris-HCl 150mM pH8.5, (NH4)2SO4 40mM, MgCl2 1.5mM, 0.2% Tween 20, dNTPs 0.4mM, Ampliqon Taq DNA Polymerase (0.05 unit/µL), 1µL of each forward and reverse of M-primers (table 1) and distilled water to the final volume of 25 µL. The following conditions were used: denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s; annealing at 55°C for 30 s; and extension at 72°C for 1 min; with the final extension step at 72°C for 12 min (Keyburn et al. 2008). To detect TpeL Toxin, PCR condition was the same as described above. AMJRT003IR (netB+, tpeL+) was used as positive control (Coursodon et al. 2012).

PCR products were fractionated by electrophoresis in 1.5% agarose gels and the target was detected by staining with ethidium bromide and examination by UV transillumination. 100 bp DNA molecular weight marker (DenaZist Company, Iran) was used as molecular weight marker.

Statistical Analysis

Data analysis was performed by using SPSS software (SPSS 16). Relationship between Diarrhoea and positive bacteria samples and between bacteria and gender were assessed by Pearson Chi-square. A P value of less than 0.05 was considered significant.

Results

One hundred fifty one faecal samples were collected, 131 from clinically healthy non-diarrheic, and 20 from diarrheic dogs. A total of 36 dogs were positive for *C. perfringens* in culture and multiplex PCR assay (36/151, 23.9%). The median age of non-diarrheic and diarrheic dogs included in this research was not statistically different and was 2.13 years (from 1month to 10 years) and 2.48 years (from 5 months to 7 years). Out of 92 female dogs, 20 (21.8%) and from 59 male dogs, 16 (26.7%) were positive in culture. Seven isolates belonged to dogs with age under 3 years old (23.3%) and 29 isolates to dogs more than 3 years old (24%). Thirty six isolates were used for detection of their toxins by multiplex PCR and single PCR. Twenty-one isolates were only cpa+ (21/36, 58.3%), fourteen isolates were cpa+/cpb2+ (14/36, 38.9%) and one isolate in non-diarrheic dogs was cpa+/cpe+ (2.8%). All of the isolates were classified as *C. perfringens* type A (Table 2). In addition, two separate single PCRs were performed for detection netB and tpeL which were negative.

In respect to being positive for *C. perfringens,* there was significant differences between dogs with and without diarrhoea, while there was not any significant differences regarding the gender of dogs which were positive for *C. perfringens* (Table 3).

Discussion

According to previous studies, direct culture is known as a rapid, accessible with lesser contamination and inexpensive method to detect *C. perfringens*. The solely method to identify *C. perfringens* was based on the presence of characteristic double- haemolytic zones, however, *C. perfringens* phenotypic identification was confirmed by subsequent PCR with specific primers (Goldstein et al. 2012).

C. perfringens strains are currently classified into five toxinotypes (A to E) based on the major toxin production profile (Lebrun et al. 2010). Several studies were performed for typing *C. perfringens* isolated from human, camel, sheep, pig, lamb, dog and wild carnivores. In all of thes studies, *C. perfringens* type A was identified as predominant type of *C. perfringens* (Ahsani et al. 2010; Bueschel et al. 2003; Gibert et al. 1997; Kalender et al. 2005; Mohamed et al. 2010; Silva et al. 2014). From a total of 151 dogs faecal samples, 36 isolates were positive for *C. perfringens* (23.8%) and this rate was lower than previous studies (Goldstein et al. 2012; Marks et al. 2002; Silva et al. 2014; Silva et al. 2013; Weese et al. 2001a). Although, Ossiprandi reported in 2012 a lower isolation rate (14/95: 14.7%). Only in one study, Weese et al. (2001) reported an association between recurrent diarrhoea and *C. perfringens* in 2 dogs. This study confirms for the first time a statistically significant association between *C. perfringens* and diarrhoea in a large population of dogs (p value = 0.017). Molecular detection and profiling by PCR provided further support for diagnosis of *C. perfringens*-associated diarrhoea. Furthermore, high prevalence of cpa+cpb2+ isolates in diarrheic dogs 4/5 (80%) can support the usefulness of PCR assays. *C. perfringens*

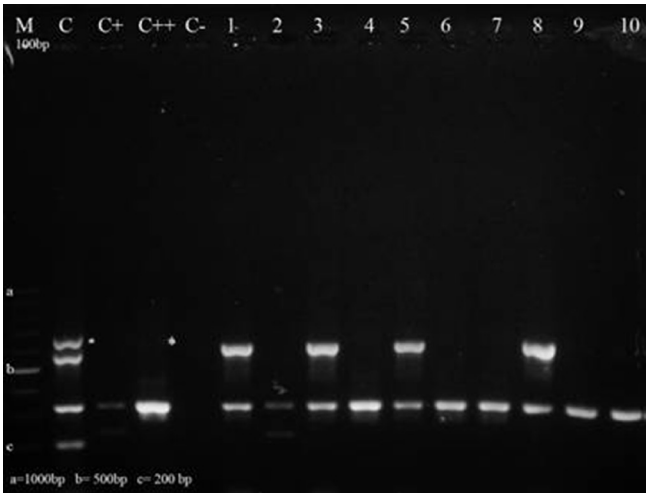

Figure 1
Multiplex PCR typing of *C. perfringens* toxin genes. Lane M 100 bp ladder, lane C reference strain of *C. perfringens* CIP 60.61, lane C+ reference strain *C. perfringens* CIP106157 , lane C++ positive control, lane C- negative control, lane 1-10 *C. perfringens*, lane 1, 3, 5, 8 amplicons for cpa and cpb2 toxin genes, lane 2 amplicons for cpa and cpe genes, other lanes amplicons for cpa gene (Bueschel et al., 2003; Meer and Songer,1997).

Table 2
Identification of *C. perfringens* isolated form canine faecal samples complemented with other data

Dogs	*C. perfringens* type A strains			Total
	$CpA^{+}NetB^{-}TpeL^{-}$	Cpa^{+} $Cpb2^{+}NetB^{-}TpeL^{-}$	$Cpa^{+}Cpe^{+}NetB^{-}TpeL^{-}$	
Diarrheic	1/5(20%) 1/20(5%)	4/5(80%) 4/20(20%)	- -	5/5(100%) 5/20(25%)
Nondiarrheic	20/31(64.5%) 20/131(15.2%)	10/31(32.2%) 10/131(7.7%)	1/31(3.2%) 1/131(0.8%)	31/31(100%) 31/131(23.7%)
Total	21/36(58.3%) 21/151(13.9%)	14/36(38.9%) 14/151(9.3%)	1/36(2.8%) 1/151(0.7%)	36/36(100%) 36/151(23.8%)
Female	12/20 (60%) 12/92(13 %)	8/19(42.1%) 8/92(8.7%)	- -	20/92(21.7%)
Male	9/16(56.2%) 9/59(15.2%)	6/16(37.5%) 6/59(10.1%)	1/16(6.25%) 1/59(1.7%)	16/59(27.1%)
Total	21/36(85.3%) 21/151(13.9%)	14/36(38.9%) 14/151(9.3%)	1/36(2.8%) 1/151(0.7%)	36/151(23.8%)
Age ≤3 years	17/29(58.6%) 17/121(14%)	11/29(37.8%) 9/121(7.4%)	1/29(3.4%) 1/121(0.8%)	29/121(24%)
Age >3 years	4/7(57.1%) 4/30(13.3%)	3/7(42.9%) 3/30(10%)	- -	7/30(23.3%)
Total	21/29(72.4%) 21/151(13.9%)	14/29(48.3%) 14/151(9.3%)	1/29(3.4%) 1/151(0.7%)	36/151(23.8%)

Table 3
Relationship between positive *C. perfringens* dogs with diarrhoea and gender

		Diarrhoea			Gender		
		Negative	Positive	Total	Female	Male	Total
Bacteria	Negative	100	15	115	72	43	115
	Positive	31	5	36	20	16	36
Total		131	20	151	92	59	151
P value				0.017			0.573

Alpha Toxin (CPA) has been shown as the most important major and lethal toxins produced by *C. perfringens* strains in dogs. All types of *C. perfringens* carry the alpha toxin gene cpa, which hydrolyzes phospholipids and sphingomyelin and cause tissue damage, in addition to the lysis of blood cells and epithelial cells (Cavalcanti et al. 2004).

Although, the role of other toxins in canine diarrhoea is still unclear but some studies have focused mainly on the cpe gene detection by using PCR or immunoassays (Marks et al. 2002; Thiede et al. 2001; Weese et al. 2001a; Weese et al. 2001b). In the present study, as expected, all of the isolates were type A, the results corroborated the findings of other studies (Marks et al. 2002; Ossiprandi et al. 2012; Silva et al. 2013). However, dissimilar to other studies, our results identified a hypothesis about role of *C. perfringens* Type A in canine diarrhoea and almost entirely with clinical use.

There is inconsiderable published data determining the distribution of the genes encoding the other toxins in a population of healthy and diarrheic dogs (Marks et al. 2002). Beta2 is a novel toxin that has been implicated as a cause of necrotizing enterocolitis in different animal species, including dogs (Gibert et al. 1997; Herholz et al. 1999; Thiede et al. 2001). The prevalence of the cpb2 toxin in healthy dogs has previously been reported in up to1- 15% of healthy dogs and in 5-33% of diarrheic dogs (Ossiprandi et al. 2012; Silva et al. 2014; Silva et al. 2013).

In the current study, fourteen isolates were positive for cpb2 (38%), of which, 4/5(80%) and 10/31 (32.2%) belonged to diarrheic and healthy dogs respectively. Although, the presence of the cpb2 toxin gene in diarrheic faecal samples did not mean that the gene was expressed or whether toxin production was associated with development of diarrhoea.

Thiede et al reported the cpb2 has been detected in conjunction with the cpe in diarrheic samples. The major virulence factor of *C. perfringens* is the CPE enterotoxin, which is secreted upon invasion of the host gut, and contributes to food poisoning and the other gastrointestinal illnesses. It is responsible for the disintegration of tight junctions between endothelial cells in the gut (Katahira et al. 1997). Several studies reported CPE range between 14.4% to 48% (Goldstein et al. 2012; Silva et al. 2014; Silva et al. 2013; Weese et al. 2001b), however, in this study we identified only one CPE isolate 1/31(3.2%). Certainly, the existence of the cpe gene does not suggest its expression; reported incidence may represent the normal carriage of the cpe gene in dogs, and may not essentially imply of its role in canine diarrhoea (Goldstein et al. 2012). Therefore, PCR assay can be as method for detection of cpe gene in healthy dogs' faeces, when the owner has got suspected diarrhoea.

Necrotic Enteritis Toxin B (NetB): NetB is a newly discovered toxin associated with necrotic enteritis of chickens. This toxin has a limited amino acid sequence similar to the beta toxin (38%) of *C. perfringens* and to the alpha toxin of Staph. aureus (Keyburn et al. 2010). The role of NetB in necrotic enteritis of poultry is well established, but full details of the pathogenesis of this disease remains to be understood (Keyburn et al. 2010). Another novel toxin, recently; TpeL is similar to other large clostridial cytotoxins such as *Clostridium difficile* toxin A (TcdA) and toxin B (TcdB), *Clostridium sordellii* lethal toxin (TcsL) and *Clostridium novyi* alpha-toxin (TcnA) (Amimoto et al. 2007). tpeL can be presented on the cpb plasmid in type B and type C isolates (Fernandez-Miyakawa et al. 2007). More recently it was recognized in type A necrotic enteritis strains from chickens. Many studies have been conducted to identify NetB and TpeL in poultry (broilers with necrotic enteritis, ostriches, chicken) (Bailey 2013; Coursodon et al. 2012; Johansson et al. 2010; Keyburn et al. 2010; Razmyar et al. 2014; Timbermont et al. 2014). One research revealed detection of netB and tpeL from horses that all of the isolates were negative for both genes (Mehdizadeh Gohari 2012). Based on the best authors' information, there is no study for detection netB and tpeL in dogs. In this study, investigation of all of *C. perfringens* isolated from dogs for netB and tpeL was done and the findings showed lack of these genes in dog's isolates. There is only one study reporting detection of netB from wild carnivore species in Brazil (Silva et al. 2014).

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