Identification of enterototxin harboring gene among *Clostridium perfringens* isolates with different toxin types in Iran

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Abstract:

BACKGROUND: *Clostridium perfringens* is known as the most widely distributed pathogenic microorganism in nature. It is an extremely important pathogen of human and domestic animals. In a commonly used classification scheme, *C. perfringens* is divided into five toxinotypes (A to E) based on the production of four major toxins (alpha, beta, epsilon, and iota). Enterotoxin is not usually used for *C. perfringens* typing but it is a fatal toxin with necrotic activity. Based on our knowledge there is no published scientific report regarding identification of enterotoxin positive *C. perfringens* isolates from animals in Iran. **OBJECTIVES:** To study the presence and frequency of enterotoxin gene among *C. perfringence* isolates with different types. **METHODS:** A specific single PCR assay was developed and used for detection of cpe gene to identify the entrerotoxin harboring isolates among different types of *C. perfringens* isolated from animal enteric diseases in Iran. **RESULTS:** It was found that cpe gene presents among *C.perfringens* isolates types A, B, C and D with 63.6% (7/11), 25% (5/20), 21.4% (3/14), 53.3% (8/15), respectively. Totally 23 of 60 (38.3%) isolates screened by PCR were cpe-positive. **CONCLUSIONS:** This is the first report of cpe- positive isolates of *C.perfringens* causing enterototoxemia in animals in Iran. Further studies to investigate the synergistic effect of CPE toxin in pathogenesis of enteric diseases in animals is suggested.

Introduction

Clostridium perfringens, an anaerobic Gram-positive bacterium, is ubiquitous in the intestinal flora of human and animals, and is also commonly isolated from environmental materials such as soil and water. Moreover, *C. perfringens* is an extremely important pathogen of human and domestic animals. In a commonly used classification scheme, *C. perfringens* is divided into five toxinotypes (A to E) based on the production of four major toxins alpha, beta, epsilon, and iota (Deguchi et al., 2009).

Two other toxins, enterotoxin (CPE) and b2 can be produced by all types of *C. perfringens* (type E only carry silent *cpe* sequences), although they are not used for the conventional typing of this microorganism (Waters et al., 2003; Miyakawa et al., 2007).

CPE- producing *C. perfringens* is also involved in antibiotic-associated diarrhea and sporadic diarrhea in humans (McClane and Chakrabarti, 2004) as well as in animals. Enterotoxigenic strains of *C. perfringens* type A have previously been related to enteritis in several animal species, including dogs, pigs, horses, cattle, sheep, chickens and goats (Miyakawa et al., 2007). *C. perfringens* is widely dispersed, but enterotoxin gene-carrying (*cpe*-positive) isolates are rarely found in nature. Thus, not much is known about reservoirs or transmission routes of this pathogen (McClane, 2005).

Not only humans, but also various experimental animals have been shown to be sensitive to CPE, suggesting that the sensitivity is not restricted to a particular species. Although

the natural target of CPE is intestine, CPE has also been detected in other tissues and organs, including liver and kidneys, after intravenous injection into rats and mice (McClane and Rood, 2001; Bos et al., 2005).

In addition, cultured cells of the intestine, liver, and kidneys from various species have been shown to be sensitive to CPE (Katahira et al., 1997; Sayeed et al., 2008).

Postmortem findings in CPE-positive *C. perfringens* naturally occurring disease of animals and humans have included degeneration and necrosis of enterocytes and villus atrophy (Barker et al., 1997; McClane and Chakrabarti, 2004; Bos et al., 2005).

The enterotoxin produced by *Clostridium perfringens* (CPE) is a simple protein with a molecular weight of 35 kDa with an isoelectric point of 4.3. Known as a causative agent of diarrhea, this organism elicits fluid accumulation in the intestinal tract by altering the membrane permeability of intestinal epithelial cells (Katahira et al., 1997; Bos et al., 2005).

It has heat-and pH-labile biological activity. CPE is made up of two functionally distinct domains: an approximately 22-kDa N-terminal domain that mediates cytotoxicity and an approximately 13-kDa C-terminal domain (C-CPE) that mediates binding (McClane and Chakrabarti, 2004; Taherian Fard et al., 2010; Veshnyakova et al., 2010).

The enteric pathogenicity of CPE has been evidenced in human volunteer feeding studies, showing that purified toxin causes diarrhea and cramping (Skjelkvale and Uemura, 1977). Animal model studies with isogenic enterotoxin gene mutant have further confirmed Koch's molecular postulates about the requirement of CPE expression to cause gastrointestinal (GI) effects (Sarker et al., 2000; Lahti et al., 2007).

CPE biosynthesis is temporally associated with sporulation. CPE synthesis begins after the induction of sporulation and increases progressively for at least 6-8 hours (Melville et al.,1994; Li McClane, 2006). CPE is not secreted, but accumulates in the cytoplasm, and is localized occasionally in paracrystalline inclusion bodies. CPE may represent up to 15-30% of the total protein present in the cell (Melville et al., 1997; Wen and McClane, 2004). The toxin is released into the intestinal lumen when sporulation is complete and the mother cell lysed, releasing a mature spore (Miki et al., 2008).

The poisoning effect was demonstrated after development of the rabbit ileal loop model in 1968 and the ability to cause diarrhea in humans was shown in 1971. Later on, purified CPE was confirmed to be responsible for diarrhea in animal models and humans (Skjelkvale and Uemura, 1997; Sayeed et al., 2008). In addition, knock-out mutants confirmed CPE to be the toxin responsible for disease caused by CPE-positive strains (Sarker et al., 1999).

The *cpe* gene is chromosomally located in the type A strain that commonly causes food poisoning (Li and McClane, 2006). However, this gene has been documented as being located on a plasmid (Lahti et al., 2007) in type A veterinary isolates as well as in the type A non-food borne strain that causes human gastrointestinal (GI) disease (MacClane et al., 2001).

CPE is being investigated as a candidate marker in cancer diagnostics and as a potential chemotherapeutic agent to treat various cancers (Veshnyakova et al., 2010).

The availability of the *cpe* gene sequence has provided a powerful genetic tool for the detection of CPE positive *C.perfringens* isolates (Miyamoto et al., 2004). PCR assay has been used to detected *cpe* positive *C.perfringens* type A isolates from diarrheic piglets (Waters et al., 2003), goats with enterocolitis (Miyakawa et al., 2007; Dray, 2004) diarrheic dogs (Thiede et al., 2001) and calves with enterotoxaemia (Manteca et al., 2002). However, there is no published scientific report regarding identification of enterotoxin positive *C.perfringens* isolates from animals in Iran. The aim of this study was to determine the *cpe* - positive *C.perfringens* isolates of different classical types (A, B, C, D) originated from ruminants in the country.

Materials and Methods

Bacterial strains growth conditions: The *C.perfringens* isolates used in this study are listed and described in Table 1.

A starter culture (6ml) of each *C.perfringens* isolated was prepared by overnight growth at 37˚C in fluid thioglycolate broth (FTG , Merck) for DNA isolation. An aliquot (0.2 ml) of each FTG culture was inoculated into 10 ml of TGY (tarypticase 3%, glucose 1%, yeast extract 0.5%, cycteine 0.1%) and incubated at 37˚C overnight.

DNA Extraction and specific PCR analysis: Total *C. perfringens* DNA was isolated from the overnight TGY cultures by previously described protocol (Jabbari et al., 2011), briefly, the reference strains and field bacterial strains were cultured in a thioglycolate broth at 37°C for 48 to 72 h in an anaerobic jar. One ml aliquot of each culture was centrifuged (13000 x g 15 min) and the resultant pellets were washed twice and resuspended in 200 µl of HPLC-grade water. After boiling for 20 min and centrifugation approximately 5 µl of lysate was used as template for PCR assay.

The toxin types of the isolates were determined by a PCR system previously (Jabbari et al., 2011; Baums et al., 2003). For detection of the gene encoding *C.perfringens* enterotoxin (*cpe*), PCR primers were designed from the reported nucleotide sequence of the gene.

The isolated DNA was then subjected to screening by a *cpe* - specific PCR.The primer set 5′- GGGGAACCCTCAGTAGTTTCA (forward) and 5′- ACCAGCTGGATTTGAGTTTAATG (reverse) was used to amplify a *cpe* fragment of 500 bp (Baums et al., 2004).

PCR reaction: These PCRs used 100 g of template DNA, 25 pm of each primer, 200 µM deoxynucleoside triphosphates, 2.5 mM MgCl2 and 1U of *Taq* DNA polymerase in a total volume of 50 µl. All tubes were placed in a thermal cycler (Eppendorf) for an initial period of 5 min at 94 ˚C (denaturation) and then subjected to 35 cycles, each consisting of 1min at 94˚C, 1min at 52 ˚C (annealing), and 1min at 72˚C, (extension) followed by an additional period extension for 5 min at 72 ˚C.

After PCR, the presence of an amplified product was analyzed by subjecting 1ng of an aliquot of each PCR sample to electrophoresis at 100 V in 1.5% agarose gel followed by ethidium bromide staining and visualization under UV illumination.

Sequencing and blasting: Verification of the *cpe* PCR was done by DNA sequencing of purified PCR product. The PCR product from amplification of *cpe* (500 bp) was purified by Fermentas PCR product purification kit. DNA sequencing reactions containing the purified PCR product and the PCR primers were performed by an automated tag polymerase cycle sequencing protocol with fluorescently labelled deoxynucleotides. All purified PCR products were sequenced by Macrogene, South Korea.

Analysis of the sequence data: Searches for sequences in GenBank databases were

performed by BLAST and comparison of the sequence alignments was done by Megalign software. The alignments of Iranian isolates were compared to each other and the toxin gene sequences of reference strains in the GenBank.

Results

All of the *C.perfringens* isolates were re-identified by biochemical tests as described in Bergey's manual. The characteristics of the isolates were positive in fermentation of glucose, lactose, sucrose, and maltose, hydrolysis of gelatin, production of lecithinase and a positive reverse CAMP test result.

Cpe was amplified among *C. perfringens* types A, B, C and D with 63.6, 25, 21.4, 53.3 percent, respectively (Table 1). Twenty three out of 60 (38.3%) of the *C. perfringens* isolates screened by PCR were *cpe*-positive.

The amplified PCR products were confirmed by analysis of nucleotide sequences and comparison of the sequences with previously reported sequences in GenBank (similarity more than 98%).

Discussion

C. perfringens is referred to as one of the most widely distributed pathogenic microorganisms in nature. Its principal habitats are soil and the intestinal contents of humans and animals (Barker et al., 1993; Bos et al., 2005). *C. perfringens* is also present in marine sediments and sewage (Lisle et al., 2004). It is divided into five toxinotypes (A-E), of which only types A, C and D are associated with disease in man. Type A predominates in the environment and human intestines (McClane and Miyamoto et al., 2004). *C. perfringens* enterotoxin (CPE)- producing *C. perfringens* type A is considered as one of the most common causes of food poisonings in the industrialized world (Wen and McClane (2004).

C. perfringens enterotoxin (CPE) is a single polypeptide of 35 kDa with a unique amino acid sequence (Deguchi et al., 2009; Taherian Fard et al., 2010). *C. perfringens* food-poisoning outbreaks principally involve meat and meat products, although other foods are occasionally implicated (Miki et al., 2008; Wen and McClane, 2004). The symptoms of food-poisoning are caused by CPE, and although other types of *C. perfringens* may also produce CPE, most of these food poisoning cases are caused by *C. perfringens* type A strains (McClane 2001; McClane 2005). *C. perfringens* type A food poisoning outbreaks are usually reported in institutionalized settings and involve large numbers of victims (Bos et al., 2005). Temperature abuse of the food is considered the major contributing factor to this food poisoning, with the most common vehicle being meat or poultry. Optimal conditions for food poisonings arise when contaminated food is held or served at a temperature range of 10-54°C, allowing growth of the organism (Sarker et al., 2000). When large numbers of vegetative cells are subsequently ingested, they sporulate and release CPE into the intestinal lumen (Li and McCane, 2006). As a consequence of this, severe abdominal cramps and diarrhea occur within 8-12 hours, usually with a spontaneous recovery within 24 hours. Fatalities are rare but possible in elderly or debilitated persons (McClane and Chakrabarti, 2004; Bos et al., 2005).

Contamination of the meat by the intestinal contents of slaughtered animals might: Serve as an important source of this pathogen to the food supply (Miki et al., 2008). However, because *C. perfringens* spores are ubiquitously distributed, the contamination might also occur elsewhere in the food chain (McClane and Rood, 2001). Despite wide dispersion of *C. perfringens*, CPE-producing isolates are only occasionally isolated. These have been estimated to represent less than 5% of global *C. perfringens* isolates (Smedley et al., 2004).

Table 1. Properties of *C.perfringens* isolates and results of PCR amplification of cpe gene among different toxin types.

Code of study	Animal	Toxin genotype	*cpe* gene	Code of study	Animal	Toxin genotype	*cpe* gene	Code of study	Animal	Toxin genotype	*cpe* gene
CP01	sheep	A	-	CP21	sheep	B	-	CP41	sheep	C	-
CP02	sheep	A	+	CP22	sheep	B	-	CP42	goat	C	-
CP03	sheep	A	+	CP23	sheep	B	-	CP43	sheep	C	-
CP04	sheep	A	+	CP24	sheep	B	-	CP44	sheep	C	-
CP05	Lamb	A	+	CP25	sheep	B	+	CP45	sheep	C	+
CP06	cattle	A	-	CP26	sheep	B	-	CP46	sheep	D	-
CP07	Calf	A	+	CP27	sheep	B	-	CP47	sheep	D	+
CP08	Cattle	A	-	CP28	sheep	B	+	CP48	sheep	D	+
CP09	sheep	A	+	CP29	sheep	B	+	CP49	sheep	D	+
CP10	sheep	A	+	CP30	sheep	B	-	CP50	sheep	D	+
CP11	calf	A	-	CP31	sheep	B	+	CP51	sheep	D	+
CP12	sheep	B	+	CP32	Lamb	C	-	CP52	sheep	D	+
CP13	Lamb	B	-	CP33	sheep	C	+	CP53	sheep	D	-
CP14	sheep	B	-	CP34	sheep	C	-	CP54	sheep	D	-
CP15	sheep	B	-	CP35	sheep	C	-	CP55	sheep	D	+
CP16	sheep	B	-	CP36	sheep	C	-	CP56	sheep	D	-
CP17	sheep	B	-	CP37	sheep	C	-	CP57	sheep	D	-
CP18	sheep	B	-	CP38	sheep	C	-	CP58	sheep	D	+
CP19	sheep	B	-	CP39	sheep	C	-	CP59	sheep	D	-
CP20	sheep	B	-	CP40	sheep	C	+	CP60	sheep	D	-

Thus, the reservoirs for *cpe*-positive *C. perfringens* type A are not currently understood, and no clear picture has emerged regarding the transmission routes of *cpe*-positive *C. perfringens* type A isolates to the food chain (Wen and McClane, 2004).

Enterotoxigenic *C. perfringens* type A has also been associated with intestinal infections in some domestic animals such as dogs, pigs, horses, and cows (Barker et al., 1993; Marks and Kather 2003, Cave et al., 2002). Moreover, dogs have been demonstrated to possess hospital-acquired diarrhea due to *cpe*-positive *C. perfringens* (Thiede, 2001).

Caprine ulcerative enterocolitis has been associated with enterotoxigenic *C. perfringens* types A (Miyakawa et al., 2007) and D (Uzal et al., 2008). Findings of Baums, et al (2004) showed that 50% of the type D ovine isolates were *cpe*-positive. Enterotoxigenic *C. perfringens* isolates have been identified as the cause of a fatal enterotoxemia in breeding camel (El-Naenaeey, 2000).

The gene (*cpe*) encoding this toxin can be carried on the chromosome or a large plasmid. Interestingly, strains carrying *cpe* on the chromosome and strains carrying *cpe* on a plasmid often exhibit different biological characteristics (Lahati et al., 2007; Deguchi et al., 2009).

Strains carrying *cpe* on the chromosome usually possess higher resistance properties against heat, cold, and nitrates than strains carrying *cpe* on a plasmid (Li and McClane, 2006). In addition, the chromosomal *cpe* strains typically grow faster at optimal temperature, and have a broader growth temperature range, compared to plasmid *cpe* strains or other *C. perfringens* isolates and thus, their presence in retail foods (Wen and McClane 2004) and their predominance in food poisonings (Smedley et al., 2005) are understandable. Chromosomal *cpe* strains may gain access to foods more easily and take advantage of temperature abuse more effectively than strains

with plasmid-borne *cpe* (Sarker et al., 2000).

These complex differences in biological properties, which are likely relevant for foodborne disease, may reflect broad genetic variations between chromosomal *cpe* isolates and other *C. perfringens* isolates (Lahti et al., 2007).

Although the disease-genotype relationship has been described in several studies, no research has been conducted to elucidate the reservoirs for different genotypes. Molecular methods based on specific PCR were used to distinguish *C. perfringens* isolates carrying chromosomal enterotoxin (*cpe*) gene from those carrying plasmid- borne *cpe* genes (Miyamoto and McClane, 2004).

Considering the role of enterotoxin in pathogenesis of *C. perfringens*, this study was conducted to identify whether Iranian animal originated isolates possess this gene.

The frequency of the *cpe* positive isolates was highest among type A with 63.6 %, followed by types D, B and C with 53.3, 25 and 21.4 % respectively.

Interestingly in the present study, in addition to type A, *cpe* gene was identified among types B, C and D of *C. perfringens* isolates from Iran. However, the exact role of enterotoxin in pathogenesis of GI tract and septicemic conditions, especially due to types other than type A isolates in animals and human food-poisoning remains to be understood.

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چکیده

زمینه مطالعه: کلستریدیوم پرفرنجنس به عنوان میکروارگانیسم بیماریزا با انتشار وسیع شناخته شده است. این باکتری در انسان و حیوانات بیماریزایی بسیار مهمی دارد. در یک طبقه بندی کلاسیک کلستریدیوم پرفرنجنس در پنج تیپ توکسینی (از A تا E) بر مبنای ترشح چهار توکسین اصلی (آلفا، بتا، اپسیلون و یوتا) قرار می‌گیرد. آنتروتوکسین به طور معمول در تایپینگ کلستریدیوم پرفرنجنس استفاده نمی‌شود اما یک توکسین کشنده توام با فعالیت نکروتیک محسوب می‌گردد. تاکنون گزارش علمی منتشر شده‌ای در خصوص شناسایی جدایه‌های کلستریدیوم پرفرنجنس از حیوانات در ایران وجود ندارد. **هدف:** در این تحقیق حضور و فراوانی ژن آنتروتوکسین در بین جدایه‌های کلستریدیوم پرفرنجنس تیپ‌های مختلف مورد بررسی قرار گرفت. **روش کار:** آزمایش PCR اختصاصی به منظور شناسایی ژن آنتروتوکسین *cpe* در بین جدایه‌های کلستریدیوم پرفرنجنس جدا شده از مبتلایان به بیماریهای روده‌ای دام‌ها در ایران استفاده گردید. **نتایج:** نتایج نشان داد که ژن *cpe* در جدایه‌های کلستریدیوم پرفرنجنس تیپ‌های A، B، C، D به ترتیب با فراوانی ۶۳٪ (۷/۱۱)، ۲۵٪ (۵/۲۰)، ۲۱/۴٪ (۳/۱۴) و ۵۳/۳ (۸/۱۵) حضور دارد. در مجموع ۲۳ جدایه از ۶۰ جدایه مطالعه شده (۳۱/۳٪) با PCR از نظر حضور ژن *cpe* مثبت بودند. **نتیجه گیری نهایی:** این اولین گزارش از شناسایی موارد *cpe* مثبت در بین جدایه‌ای کلستریدیوم پرفرنجنس عامل آنتروتوکسیمی در دام‌ها در ایران می باشد. مطالعات تکمیلی به منظور بررسی میزان تأثیر افزایشی توکسین *cpe* در پاتوژنز بیماریهای روده‌ای حیوانات پیشنهاد می شود.

واژه‌های کلیدی: کلستریدیوم پرفرنجنس، ژن *cpe*، آنتروتوکسین

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