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Full Article

Occurrence of Beta2 toxigenic *Clostridium perfringens* isolates with different toxin types in Iran

Jabbari*[1], A.R., Tekyei[2], F., Esmaelizad[3], M., Pilehchian Langroudi[1], R

1 .Department of Anaerobic Bacterial Vaccines Research and Production, Razi Vaccine & Serum Research Institute, Karaj, Iran
2. Faculty of Basic Science, Payame-Noor University, Tehran, Iran
3. Department of Biotechnology, Razi Vaccine & Serum Research Institute, Karaj, Iran

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ABSTRACT

Clostridium perfringens is an important cause of enteric diseases in both human and animals. The bacteria produce several toxins which play key roles in the pathogenesis of diseases and are classified into five toxin types, on the basis of the differential production of Alpha, Beta, Epsilon and Iota toxins. In this study a single PCR assay was developed and used for detection of cpb2 gene to identify the Beta2 harboring isolates among different types of *C. perfringens* isolated from animal enteric diseases in Iran. It was found that cpb_2 presents among *C. perfringens* isolates types A, B, C and D with 54.5% (6/11), 62% (13/21), 42.8% (6/14), 69.25% (9/13), respectively. Totally 34 of 59 (56.7%) isolates screened by PCR were cpb2-positive. This is the first report of cpb2 positive isolates of *C. perfringens* causing enteric diseases of animals in Iran. Further studies to demonstrate the exact role of Beta2 toxin in pathogenesis of the bacterium is suggested.

Keywords: *Clostridium perfringens*, Beta2 toxin, Enteritis

INTRODUCTION

Clostridium perfringens is a gram-positive, endospor -forming, anaerobic bacterium which is found widely in the environment and also in the intestinal tract of humans and animals. The virulence of this bacterium largely results from its ability to produce at least 15 different *C. perfringens* toxins.The production of four major toxins Alpha(α) , Beta(β) Epsilon(ε) and Iota(i) allows the discrimination into five types from A to E (Songer *et al* 1996, Waters *et al* 2003). The major lethal toxins, however are not the only biomedically important toxins, some *C. perfringens* isolates produce other important toxins such as Enterotoxin (CPE) and Beta2 toxin (Gibert *et al* 1997). The B_2 toxin, another recently described virulence factor, is associated with diseases of the gastro intestinal tract in animals. It was produced by *C. perfringens* isolates from piglets with necrotic enteritis and was also found in horses with enterocolitis (Herholz *et al* 1999, Gibert *et al* 1997). Purified CPB2 was reported to be cytotoxic for Chinese hamster ovary cells and to induce hemorrhagic necrosis

*Author for correspondence.Email: a.Jabbari@rvsri.ir

of the intestinal mucosa in a guinea pig ligated intestinal loop (Schotte *et al* 2004). The structure of the toxin is so far unique, as the deduced aminoacid sequence has no notable similarity with beta or any other known toxin .The cpb_2 gene is plasmid –born, at least in some strains, which suggest the potential for mobility and the subsequent transfer of cpb_2 among strains of *C. perfringens* (Gibert *et al* 1997, Shimizu *et al* 2002). The biological activity of the Beta2 toxin is comparable with that of the Beta toxin .It is lethal to mice (minimal dose 3µg) and also causes hemorrhagic necrosis of the intestinal wall in the guinea pig ligated loop model (Gibert *et al* 1997) in contrast to CPE, no fluid accumulation was observed after application of a Beta2- toxigenic *C.perfringens* type A strain to bovine ileal loops (Manteca *et al* 2002). Several studies have shown a wide distribution of Beta2 toxigenic *C. perfringens* strain in various animal species. *C. perfringens* harbouring *cpb_2* was isolated from various ruminants ,horses, pigs and carnivores (Garmary *et al* 2000, Aschfalk & Muller 2001,Gkiourtzids *et al* 2001, Thiede *et al* 2001, Bacciarini *et al* 2003, Baums *et al* 2004). The *cpb_2* gene was detected in four toxin types (A, C, D, E) in different animal species and food isolates (Gibert *et al* 1997). In lambs with dysentery, cpb2-positive strains have been detected (Gkiourtzidis *et al* 2001), although in a low frequency (5%). The detection of *cpb_2*-positive *C. perfringens* type A from a 5-week-old goat with postmortem signs of caprine enterotoxaemia indicates a possible role of Beta2-toxin in this common disease (Dray 2004). The availability of the *cpb_2* gene sequence has provided a powerful genetic tool for the detection of CPB_2 positive *C. perfringens* isolates. PCR assay has been used to detect *cpb_2* positive *C. perfringens* type A isolates from diarrheic piglets (Waters *et al* 2003, Garmory *et al* 2000, Klaasen *et al* 1999), horses with enterocolitis (Herholz *et al* 1999) diarrheic dogs (Thiede & Amtsberg 2001) and calves with enterotoxaemia (Manteca *et al* 2002). The aim of this study was to determine the *cpb_2* - positive *C. perfringens* isolates of different classical types (A, B, C, D) originated from animals in Iran.

MATERIALS AND METHODS

Bacterial strains growth conditions. The *C. perfringens* isolates which were used in this study are listed and described in Table 1. A starter culture (6ml) of each *C. perfringens* isolated were prepared by overnight growth at 37 °C in fluid thioglycolate broth (FTG , Merck) for DNA isolation. An aliquot (0.2ml) of each FTG culture was inoculated into 10 ml of TGY (trypticase3%, glucose 1%, yeast extract 0.5%, cysteine 0.1%) which was then 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 bacterial isolates were cultured in thioglycolate broth and incubated 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. A single colony suspended in 200 µl of HPLC-grade water could be substituted for washed cells obtained from liquid culture and microwaving (10 min at 700 W) could be used in place of boiling. The toxin types of the isolates were determined by a PCR system described previously (Jabbari *et al* 2011, Baums *et al* 2004). For detection of the gene encoding *C. perfringens* Beta2 ـ toxin (cpb_2), PCR primers were designed from the reported nucleotide sequence of the gene. The extracted DNA was then subjected to screening by a cpb_2 – specific PCR. The primer set 5′-AGATTTTAAATATGATCCTTAAC (forward) and 5′- CAATACCCTTCACCAAATACT (reverse) was used to amplify a cpb_2 fragment of 566 bp (Gkiourtzidis *et al* 2001). PCR reaction:

These PCRs used 100 ηg of template DNA, 25 pm each primer, 200µM deoxynucleoside triphosphates, 2.5 mM

$MgCl_2$ and 1U of Taq DNA polymerase in a total volume of 50 µl. The reaction mixture was placed in a thermal cycler (Eppendorf) for an initial period of 5 min at 94 °C (denaturation) and then was subjected to 32 cycles each consisting of 1min at 94 °C, 1min at 58°C (annealing), and 1min at 72 °C (extension) followed by an additional period extension for 10 min at 72 °C. After PCR, the presence of an amplified product was analyzed by subject 1ng an aliquot of each PCR sample to electrophoresis at 100 V in 1.5% agarose gels followed by ethidium bromide staining and visualization under UV illumination.

Sequencing and blasting. Verification of the cpb_2 PCR product was done by DNA sequencing of purified PCR product.The PCR product from amplification of *cpb_2* (566bp) was purified using the PCR product purification kit (Fermentas). DNA sequencing reactions containing the purified PCR product and the PCR primers were performed using an automated tag polymerase cycle sequencing protocol with fluorescently labelled deoxynucleotides. All purified PCR products were sequenced by Applied Bioscience, France.

Analysis of the sequence data. Searches for sequences in GenBank databases were performed by Blast software. The 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 results. *cpb_2* was amplified among *C. perfringens* types A, B, C and D with 54.5, 62, 42.8, 69.2 percent, respectively (Table1).

Table 1. Properties of *C.perfringens* isolates and results of PCR amplification of Beta 2 toxin gene among different toxin types.

Code of study	Animal	Toxin genotype	Beta2 toxin
085	sheep	A	+
072	sheep	A	-
073	sheep	A	-
022	sheep	A	+
094	Lamb	A	+
093	cattle	A	+
095	Calf	A	+
105	Cattle	A	+
122	sheep	A	-
123	sheep	A	-
020	dog	A	-
220	sheep	B	+
213	Lamb	B	+
333	sheep	B	-
227	Lamb	B	+
214	sheep	B	+
221	sheep	B	+
1795	sheep	B	+
236	sheep	B	+
222	sheep	B	+
8071	sheep	B	+
239	sheep	B	-
234	sheep	B	-
237	sheep	B	-
215	sheep	B	+
210	sheep	B	+
228	sheep	B	-
238	sheep	B	-
233	sheep	B	-
231	sheep	B	-
207	sheep	B	+
240	sheep	B	+
316	Lamb	C	-
314	sheep	C	-
312	pig	C	+
305	sheep	C	+
303	sheep	C	-
302	sheep	C	-
301	sheep	C	+
356	sheep	C	-
337	sheep	C	-
327	sheep	C	-
326	goat	C	+
325	sheep	C	+
324	sheep	C	-
321	sheep	C	+
413	sheep	D	-
469	sheep	D	+
450	sheep	D	+
456	sheep	D	+
471	sheep	D	+
447	sheep	D	-
440	sheep	D	+
401	sheep	D	-
431	sheep	D	+
432	sheep	D	+
433	sheep	D	-
453	sheep	D	+
444	sheep	D	+

A representative of cpb2 fragment in agarose gel is shown in Figure 1. Totally 34 of 59(57.6%) of *C. perfringens* isolates screened by PCR were *cpb2*-

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%).

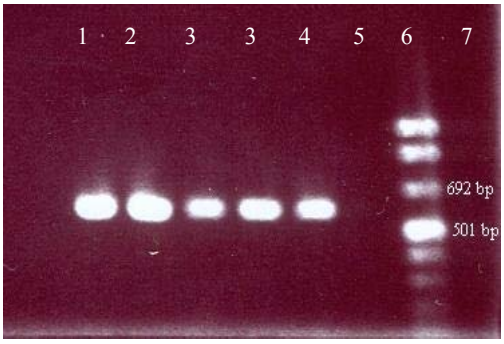

Figure 1. PCR amplification of b2 gene of the *C. perfringens* isolates produced 566 bp fragment. Lanes 1-5: the PCR products with the DNA from *C.perfringens* isolates. Lane 6: control negative. Lane 7: DNA size marker.

DISCUSSION

Regarding the importance of Beta2 toxin in pathogenesis of *C. perfringens*, the present study was conducted to identify if the Iranian *C. perfringens* isolates possess this gene. This is the first report of Beta2 toxin gene among animal isolates of *C. perfringens* in Iran. The finding of the present study showed that 57.6%of the *C. perfringens* isolates tested were *cpb2* positive. The frequency of the *cpb2* positive isolates was highest among type D with 69.2% followed by types B, A, and C with 62, 54.5 and 42.8 % respectively. Our study showed that all major toxin types of A, B, C and D *C. perfringens* isolates in Iran may possess the cpb2 gene. Zerbini and Ossiprandi (2009) showed that 23.1% (3/14) of type A isolates obtained from diarrheic dogs were *cpb2* positive. Bueschel, et al (2003), found that 85.8% of swine isolates of *C. perfringens* all from type A possessed *cpb2* gene. The high rate of occurrence of *cpb2* positively among strains from pigs with enteritis confirmed that Beta 2 plays a role in pathogenesis of these infections in piglets (Bueschel *et al* 2003). Beta 2 toxin, encoded by the cpb_2 gens, has been implicated in the pathogenesis of porcine, equine and bovine enteritis by type A *C. perfringens* (Manteca *et al* 2002, Herholz *et al* 1999, Waters *et al* 2003). Garmory et al (2000) investigated the presence of *cpb2* gene among *C. perfringens* isolated from foal, piglet, lamb and calf enteritis, the gene encoding *C. perfringens* Beta2 toxin (cpb_2) was present in 50% of the isolates genotyped. However, the prevalence of this gene varied between animal isolates.

Van Asten et al (2010) reviewed the occurrence and role of the beta2 toxin positive isolates of *C. perfringens* in endemic diseases of domestic animals, wild animals and human. In lambs with dysentery, cpb_2 harboring strains were detected at a low frequency (7/117)(Gkiourtzidis *et al* 2001). 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 (Bueschel *et al* 2003). Recent epidemiological studies suggested that *C. perfringens* isolates carrying the gene encoding Beta2 (cpb_2) are strongly associated with clostridial GI diseases in domestic animals, including necrotic enteritis in piglets and typhlocolitis in horses (Waters *et al* 2003, Herholz *et al* 1999). As it is shown in Table1, 51.8% (n= 54) of *C. perfringens* isolates originated from sheep were harbouring *cpb2* gene. Our study showed that *cpb2* gene existed among all major toxin types of A, B, C and D *C. perfringens* isolates in Iran. However, the exact role of beta2 toxin in pathogenesis of GI tract of animals and molecular mode of action of the toxin remains to be understood.

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