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Characterization of *Clostridium perfringens* Enterotoxin in Food Animals and Human

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Abstract: Enterotoxin (CPE) - producing *C. perfringens* type A is considered one of the most common causes of food poisonings encoded by *C. perfringens* enterotoxin gene (*cpe*). *C. perfringens* is widely dispersed, but enterotoxin gene carrying (*cpe*-positive) isolates are rarely found in nature. So, the aim of this work was to study the occurrence and characterization of *C. perfringens* enterotoxin. A total of 296 rectal swabs collected from different species of food animals and 60 stool samples from human were examined in this study. The results revealed high occurrence of *C. perfringens* in all examined samples. *Cpe-positive C. perfringens* were confirmed in 3.1% of the *C. perfingens* isolates from animals and 3% from human. *Cpe* positive isolates were represented by type A, C and E. Enterotoxin was produced only from *cpe*-positive *C. perfringens* isolates of type A and C.

Key words: *Clostridium perfringens* · Enterotoxin · Food Animals · Man

INTRODUCTION

Clostridium perfringens is the most widely distributed pathogenic microorganism in nature. It is a diverse species, with its strains being divided into five types, A to E; according to the major toxins they produce, include alpha, beta, epsilon and iota toxins, in addition to which, most *C. perfringens* strains produce a range of other toxins or potential virulence factors, such as the *C. perfringens* enterotoxin (CPE) [1]. The ubiquitous distribution of *C. perfringens* has earlier been considered a logical explanation for the common occurrence of *C. perfringens* food poisonings. Therefore, all *C. perfringens* isolates were regarded as potential causative agents for *C. perfringens* type A food poisonings [2]. Currently it is known that only a small minority, less than 5% of global *C. perfringens* isolates produce *C. perfringens* enterotoxin (CPE) and are thus capable of causing food poisonings. The diarrheic and cramping symptoms of *C. perfringens* food poisoning resulted from *C. perfringens* enterotoxin (CPE) encoded by *C. perfringens* enterotoxin gene (*cpe*) [3]. *Cpe* can be detected in *C. perfringens* isolates representing any type (A-E) but not all isolates are, however capable of expressing the gene product, *cpe*-positive *C. perfringens* type E isolates, for example, possess mutations in an open reading frame (ORF), promoter(s) and the ribosome binding site of their *cpe* sequences and therefore do not produce CPE [4]. However, in CPE-associated diseases, the isolate usually represents type A, perhaps because isolates of type A account for more than 95% of global *C. perfringens* isolates [5]. CPE biosynthesis is associated temporally with sporulation and its synthesis begins after the induction of sporulation and increases progressively for at least 6-8 hours. Outbreaks of CPE-mediated food poisoning typically involve a large number of victims and are associated with temperature-abused meat or poultry dishes. So, the this work was planned to study the occurrence and characterization of *C. perfringens* enterotoxin and its role in causing food poisoning.

MATERIALS AND METHODS

Collection of Samples: Nearly 296 rectal swabs were collected from different species of food animals including cattle, buffalo, sheep and goat as shown in Table (1). In addition, stool samples were collected from 60 apparently normal persons in contact with animals.

Isolation of *C. perfringens*: Each sample was inoculated onto a tube of sterile freshly prepared cooked meat medium and then the tube was incubated anaerobically in

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anaerobic jar using anaerobic gas generating kits (Oxoid) at 37°C for 24-48 hours. A loop full from the previously incubated tube was streaked onto the surface of 10% sheep blood agar with neomycin sulphate (200 µg /ml) [6]. The plate was incubated anaerobically at 37°C for 24-48 hours. The plates were examined for the characteristic colonies of *C. perfringens*. Subcultures from the suspected colonies were identified morphologically and biochemically according to Koneman *et al.* [7].

DNA Extraction: To prepare template DNA for the multiplex PCR toxin genotyping assay, pure colonies that showed double zone of haemolysis on blood and confirmed as *C. perfringens* by biochemical tests were grown over night in 5 ml brain heart infusion at 37°C for 24 hour under anaerobic condition.

A rapid boiling procedure was used to prepare template DNA from bacterial strains according to Sheedy *et al.* [8].

Multiplex PCR Assay to Determine the Toxin Genotype of *C. perfringens* Isolates: The multiplex PCR assay of Asten *et al*. [9] was used to detect the presence of genes encoding alpha-toxin (*cpa*), beta-toxin (*cpb*), epsilon-toxin (*etx*), iota-toxin (*iap*) and CPE (*cpe*). Primer sequences were published previously (9). Each PCR had a total volume of 25µl, which contained 5µl of DNA as template, 0.34 mM of each *cpe* oligo, 0.36 mM of each *cpb* oligo, 0.44 mM of each *etx* oligo, 0.5 mM of each *cpa* oligo and 0.52 mM of each *iA* oligo, 1X of PCR master mix and completed to 25 µl by DNase-RNase-free water. Amplification conditions were initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. A final extension step at 72°C for 10 min was followed. Amplification products were electrophorezed in 1.5% agarose gel containing 0.5X TBE at 70 volts for 60 min. and visualized under ultraviolet light. To assure that the amplification products were of the expected size, a 50 bp DNA ladder was run simultaneously as a marker.

Extraction and Purification of *C. perfringens* Enterotoxin According to Waters *et al.* [10]: *Cpe* positive *C. perfringens* isolates that obtained in this study were cultured in CMM then transferred to modified DS sporulation medium and incubated at 37°C for 8 hours under anaerobic condition for enterotoxin production. The sporulated cells were washed once in cold distilled water then suspended in 2 ml of cold saline. The cells were disrupted by sonic treatment (6 Hertz for 20 minutes using sonicator ultra-sonic) and depris was removed by centrifugation at 12000 xg for 20 minutes at 4°C to obtain a clear extract. The supernatant protein was precipitated by addition of ammonium sulfate (4.76g of $(NH4)_2SO_4$ /10 ml supernatant) and incubated overnight at 4°C [10]. The precipitated protein was then collected by centrifugation at 12000 rpm for 30 minutes at 4°C and resuspended in 25µl of sterile phosphate buffer saline. This solution was dialyzed overnight against the same buffer and any precipitate was removed by centrifugation.

Detection of Cytotoxicity of Enterotoxin on Vero Cell: The cytotoxicity of the purified enterotoxin was tested using Vero cell assay [11]. The method involved the rapid killing of Vero cells by enterotoxin produced by *C. perfringens* grown in Duncan and Strong sporulation medium.

RESULTS AND DISCUSSION

Although *C. perfringens* type A forms part of the normal intestinal flora of animals, the enterotoxin producers have been linked to enteritis in animals and food poisoning outbreaks in humans [12]. The role of food animals as the main reservoir and contamination source of *cpe* positive *C. perfringens* needs to be reconsidered [13]. Table (1) shows that the occurrence of *C. perfringens* recovered from rectal swabs of food animals were 58.3% in cattle, 69.6% in buffaloes, 49.4% in sheep and 34% in goat with overall occurrence 54.4%. These results indicated the presence of *C. perfringens* in all animal species and it is referred as the most widely distributed pathogenic microorganism in nature [14]. Therefore, spread of the infection may occur from feces of animals to the surrounding environment. Classically, typing of *C. perfringens* is performed with toxin neutralization test in mice. This procedure consumes a lot of antisera and experimental animals. Additionally it is time consuming. In recent years, molecular techniques such as polymerase chain reaction (PCR) have been used to type *C. perfringens* [15]. Regarding typing of all *C. perfringens* isolates recovered from food animals by using multiplex PCR, Table (1) and photo (1&2) show that the most predominant types of *C. perfringens* isolates were type (A) and then followed by type (C). Two isolates were found to be type D, while only one isolate was found to be type (E). None of the isolates contained type B. In addition to the major lethal toxins, a minority of

Table 1: Incidence and typing of *C. perfringens* in the examined rectal swabs from food animals

				Typing of *C. perfringens* isolated from food animals by using multiplex PCR									
		Samples positive to *C. perfringens*		A		C		D		E		cpe	
Animal species	No. of examined samples	No	%	No	%*	No	%*	No	%*	No	%*	No	%*
Cattle	115	67	58.3	62	92.5	2	3	2	3	1	1.5	4	6
Buffaloes	56	39	69.6	34	87.2	5	12.8	0	0	0	0	1	2.6
Sheep	81	40	49.4	40	100	0	0	0	0	0	0	0	0
Goat	44	15	34	14	93.3	1	6.7	0	0	0	0	0	0
Total	296	161	54.4	150	93.2	8	5	2	1.2	1	0.6	5	3.1

Calculated according to the number of positive samples %*

Table 2: Occurrence of *C. perfringens* in human stool

				Typing of positive isolates by using multiplex PCR			
		Samples positive to *Clostridium perfringens*		Type A		Enterotoxin	
Source of samples	No. of examined samples	No.	%	No.	%	No.	%
Stool	60	33	55	33	100	1	3

Table 3: Characterization of *cpe* positive *Clostridium perfringens* isolates.

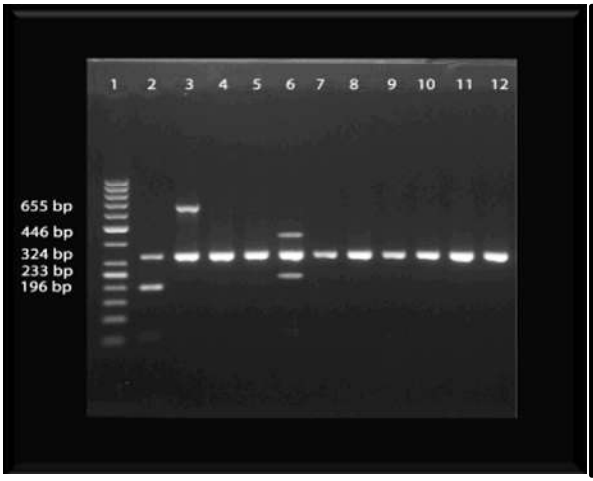
Cpe- positive isolates	Source of isolates	Typing	Spore formation	Enterotoxin Production
Field isolate 1	Feces of cattle	A	Positive	Positive
Field isolate 2	Feces of cattle	E	Positive	Negative
Field isolate 3	Feces of cattle	A	Positive	Positive
Field isolate 4	Feces of cattle	A	Positive	Positive
Field isolate 5	Feces of buffalo	C	Positive	Positive
Field isolate 6	Human	A	Positive	Positive

C. perfringens strains produced an enterotoxin (CPE), which is responsible for the symptoms of common *C. perfringens* type (A) food poisoning [5]. Among the typed *C. perfringens* isolates recovered from food animals, *C. perfringens* carrying enterotoxin genes were isolated only from 5 animals (4 from cattle and one from buffalo, but not isolated from sheep and goat) with overall percentage of 3.1% and regarding to characterization of *cpe* positive isolates recovered from food animal, Table (3) shows that *cpe* positive isolates represent type A (3 strain), type C (1 strain) and type E (1 strain). Because the most prevalent type of *C. perfringens* in food animals were type (A) and *C. perfringens* type C isolates, so the zoonotic characteristic of *C. perfringens* type A and C should be taken in account since some strains have the ability to synthesized enterotoxins.

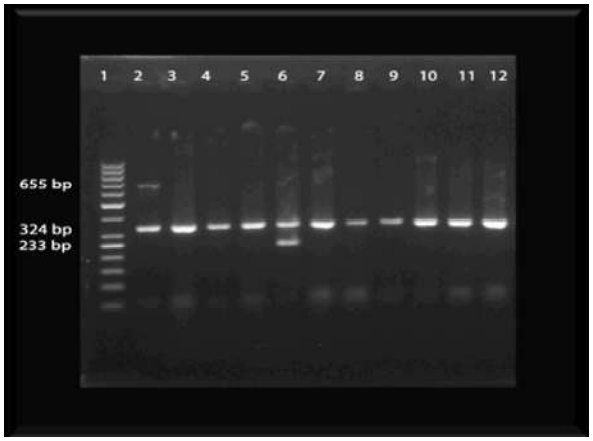
C. perfringens enterotoxin (CPE) is responsible for the diarrhea and cramping symptoms of human [16]. In the present study (Table 2) the occurrence of *C. perfringens* among stool of apparently healthy people who are in contact with animal was 55% and related to typing of isolates by using multiplex PCR, the isolated strains were type A and only one isolate contained enterotoxin gene.

The occurrence of *cpe*-positive *C. perfringens* type A in the feces of healthy persons and the full capacity of these strains to produce CPE regarded as a risk factor for spread of *cpe*-positive *C. perfringens* type A food contamination. From all previous results, it was found that *C. perfringens* occur at high level in all types of examined samples and this is of high significance because some strains have the ability to synthesize enterotoxins which are responsible for causing the symptoms of *C. perfringens* food poisoning. So, the present study focused on study the occurrence and characterization of *C. perfringens* enterotoxin as well the *in vitro* expression of this gene to produce *C. perfringens* enterotoxin. In relation to that, the occurrence of C. *perfringens* enterotoxin genes were detected in five isolates from animals (3.6%) and one isolate from human (3%).

CPE is synthesized during sporulation and thus, sporulation in vitro is essential to measure the production of CPE of an isolate [17]. In the present study, *cpe*-positive *C. perfringens* isolates were sporulated in modified Duncan Strong medium and the resultant purified supernatant was examined for production of enterotoxin. Vero cell assay is rapid assay that measures the biological activity of *C. perfringens* enterotoxin. This method involves the rapid killing of Vero cells by enterotoxin produced by *C. perfringens* grown in Duncan and Strong sporulation medium [11]. The cytotoxicity of Vero cell in the present study, revealed that 5 isolates of 6 show cytotoxicity and only one isolate does not show any cytopathic effect, enterotoxin production was negative, in spite of the presence of the enterotoxin gene. Sporulation is essential prior to detection of CPE from a *cpe*-positive *C. perfringens* isolates, since CPE is formed in the cell during sporulation. Successful sporulation was confirmed by phase-contrast microscopy and the culture was sonicated until more than 95% of the spores were free, determined by phase-contrast microscopy [11]. In the present study, the six *C. perfringens* isolates carrying enterotoxin gene were studied for their ability of sporulation on Duncan and Strong (DS) sporulation medium and enterotoxin production. It was noted from the results that *C. perfringens* enterotoxin is directly related to the ability of the microorganism to sporulate as 5 out of 6 *C. perfringens* strains grown on DS sporulation medium were spore formers and enterotoxin positive. It was noted that one *cpe* positive *C. perfringens* isolates was sporulated well on modified DS sporulation medium while enterotoxin production was negative and this isolate concerning to type E. From these results, it was found that *Clostridium perfringens* plays a significant role in food poisoning since it was isolated at high incidence from all types of examined samples. Only *cpe* positive *C. perfringens* type A and C were able to express enterotoxin which is responsible for causing the symptoms of *C. perfringens* food poisoning.



Lane 1: 50bp DNA ladder.
Lane 2: *C.perfringens* type C
Lane 3: *C.perfringens* type D.
Lane 6: *C.perfringens* type E, enterotoxin.
Lane 4, 5 and 7 to 12: *C. perfringens* type A



Lane 1: 50bp DNA ladder.
Lane 2: *C. perfringens* type D.
Lane 6: *C. perfringens* type A, enterotoxin.
Lane 3 to 5 and 7 to 12: *C. perfringens* type A

Agarose gel electrophoresis of PCR product of some *C. perfringens* isolates

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