

Full Length Research Paper

Identification of four major toxins of *Clostridium perfringens* recovered from clinical specimens

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A simple polymerase chain reaction (PCR) procedure was used to identify four toxotypes of *Clostridium perfringens* collected from different origins. Eighteen (18) strains of *C. perfringens* were identified and typed by classical methods (dermonecrotic method in guinea pigs and sero-neutralization test in mice). All the strains were analyzed by PCR using gene of toxin alpha, gene of toxin beta, gene of toxin epsilon and gene of toxin iota. The results reveal a toxin gene in 13 (72.22%) strains of *C. perfringens*; only 11 (61.11%) strains of them were identified previously as type A by classical method, three strains (16.67%) were identified as type C and one strain (5.56%) was identified as type D by PCR. Moreover, PCR results confirmed the traditional methods in typing one strain as type B (5.56%). Also, PCR method could detect two other strains of type A directly in the feces and intestinal contents of the examined chicken which gave negative results in bacteriological examination. Thus, PCR technique can become a first-choice tool for the identification and typing of the *C. perfringens* which initiate enteric disease. In turn, this would simplify the development of vaccines adapted to the epidemiological situation. Taken all together, PCR method is easy, time saving and applicable to differentiate *C. perfringens* types as an alternative to animal tests.

Key words: *Clostridium perfringens*, major toxins, polymerase chain reaction (PCR) typing, enteric disease.

INTRODUCTION

Clostridium perfringens is an important pathogenic agent that cause among other diseases, enteritis in humans and enterotoxemia in domestic animals (Smith and Williams, 1984; Songer, 1996; Miwa et al., 1999; Tallis et al., 1999; Netherwood et al., 1998). *C. perfringens*, the third most common cause of foodborne illness in the United States (Scallan et al., 2011), most often causes a self-limited, diarrheal disease that lasts for 12-24 hours. Fatalities are very rare. Death usually is caused by dehydration and occurs among very young and old persons as well as persons debilitated by illness. The

pathogenicity of this organism is associated with several toxins. Alpha, beta, epsilon and iota toxins are the major lethal toxins produced by the organism and are closely related to its virulence. Usually, *C. perfringens* has been classified into five toxigenic types (A through E) on the basis of its ability to produce the major lethal toxins (Cato et al., 1986; Hatheway, 1990; Badagliacca et al., 2010). The major toxins produced by the five types of *C. perfringens* are illustrated in Table 1.

Type A strains are the most commonly encountered and produce food poisoning and gas gangrene in

Table 1. Major toxins produced by the five types of *C. perfringens*.

Types of <i>C. perfringens</i>	Major toxins produced by <i>Clostridium perfringens</i>			
	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Table 2. Numbers and sources of examined fecal and intestinal contents samples.

Species	Number of samples	Clinical symptoms
Calves	35	Enterotoxaemia
Lambs	22	Enterotoxaemia and rapid death
Adult sheep	24	Enterotoxaemia and rapid death
Chickens	25	Necrotic enteritis

humans and animals, and necrotizing colitis and enterotoxemia in horses (Canard et al., 1992; Songer, 2004; Immerseel et al., 2004). Types B, C and D primarily occur in the intestine of animals and only occur occasionally in humans. The strains of these types have also been isolated from soil in areas where enteritis by the organisms was affecting a significant number of animals and humans (Smith, 1975).

The strains of types B, D are the causative agents of enterotoxemia in domestic animals such as calves, lambs and piglets. Type C strains cause enteritis necroticans in humans and enterotoxemia in animals. The pathogenicity of E strains is not clear and has seldom been isolated (Canard et al., 1992; Smith, 1975).

Typing of an organism is accomplished with the culture filtrate, type-specific antisera and experimental animals such as mice and guinea pigs (Takayoshi et al., 1997). The most commonly used test to detect the toxin in clinical specimens is the mouse neutralization test. However, it requires large number of mice, is time consuming and non specific toxicity caused by other substances can falsify the interpretation (Buogo et al., 1995). This study reports a polymerase chain reaction (PCR) for the detection of the genes encoding the different *C. perfringens* toxins as an alternative diagnostic method for molecular typing.

MATERIALS AND METHODS

Samples

Samples from feces and intestinal contents obtained from 35 diarrheic calves (age ranged from one to four months) showed signs of enterotoxemia; that from 24 adult sheep and 22 lambs (age from 1-12 weeks) showed signs of diarrhea and enterotoxemia

during the winter of 2008 to winter of 2010. Also 25 samples from the intestine of broiler chickens that exhibited diarrhea and showed clinical signs of necrotic enteritis were collected from different farms at different governorates in Egypt.

The samples from sick and freshly dead animals and chickens were collected in plastic bags and transported refrigerated to the laboratory where they were processed within 4 h of collection. The complete list of samples' number and their sources used in this study are given in Table 2.

Bacteriological identification of *C. perfringens* from the various samples

C. perfringens was isolated using the procedure of Willis (1977). Typical colonies were identified as described by Murray et al. (2003) depending on their characteristic colonial morphology, hemolysis activity, Gram staining and biochemical test.

Determination and typing of toxigenic isolates of *C. perfringens* isolates by conventional method

Determination of toxigenic isolates of *C. perfringens* was done by Nagler's test using half antitoxin plate according to the study of Smith and Holdeman (1968). *C. perfringens* antitoxin serum was spread on half of the egg yolk agar plate and allowed to dry in the incubator for half an hour. The culture was then streaked across the plate, starting from the half of the egg yolk agar plate without antitoxin, and then incubated anaerobically at 37°C for 24 h. Pathogenicity of the isolates to guinea pigs was determined according to the study of Willis (1964). Two guinea pigs were experimentally inoculated with 0.5 ml of an overnight cooked meat broth of *C. perfringens* mixed with 0.5 ml of calcium chloride solution. Albino guinea pigs were under observation for 3 days. Post mortem examination of the dead animals was carried out and cultures from heart blood, liver and exudates were made onto neomycin sulphate sheep blood agar plates; and meat broth was cooked to re-isolate the inoculated organism.

For typing toxigenic isolates of *C. perfringens*, neutralization test in mice was carried out according to the study of Smith and

Table 3. Primers used for typing of major toxins of *C. perfringens* by PCR.

Amplicon size (bp)	Nucleotide sequences	Annealing temperature	Primer (direction)
1167	5` - AAG ATT TGT AAG GCG CTT-3` 5` - ATT TCC TGA AAT CCA CTC-3`	56	Alpha-toxin Forward Reverse
1025	5` - AGG AGG TTT TTT TAT GAA G-3` 5` - TCT AAA TAG CTG TTA C TT- TG TG-3`	39	Beta-toxin Forward Reverse
961	5` - AAG TTT AGC AAT CGC ATC-3` 5` - TAT TCC TGG TGC CTT AAT-3`	46	Episoin toxin Forward Reverse
298	5` - TTT TAA CTA GTT CAT TTC CTA GTTA-3` 5` - TTT TTG TAT TCT TTT TCT CTA GATT-3`	45	Iota-toxin Forward Reverse

Holdeman (1968) by adding 0.1 ml of specific antisera to 0.3 ml of the centrifuged (3000 rpm for 15 min) supernatant fluid of cooked meat culture for each type of *C. perfringens*. The culture supernatant of only type and E were treated by 0.1% trypsin for 45 min at 37°C, before adding its specific antiserum. The mixture was left for 30 min at 37°C before infection as 0.5 ml intraperitoneal into 2 mice kept under observation for 3 days and the supernatant fluid of the cooked meat culture without adding any antiserum was used as the control. Dermonecrotic test in guinea pigs was performed according to the study of Sterne and Balty (1975).

Bacterial strains used for determination of primers specificity

Three strains of *C. perfringens* types A, B and D (Animal health Research institute, Dokki) were used in this study as a positive control. Reference strains of enteric bacteria including *Salmonella* Typhimurium ATCC 11511, *Staphylococcus aureus* ATCC 29737, *Salmonella* Enteritidis ATCC 13076 and *Escherichia coli* serotype 0157:H7 ATCC 35150 were used as negative controls.

Extraction of DNA

The DNA of the standards strains and of the other bacterial isolates yielded from bacteriological examination was extracted by hexadecyltrimethyl ammonium bromide (CTAB), according to the study of Sambrook et al. (1989). Meanwhile, the extractions of DNA from fecal samples were carried out according to the study of Uzal et al. (1996) with few modifications as follows. Broth enrichment of fecal samples was carried out on thioglycolate broth at 37°C under anaerobic conditions. After overnight incubation, one of each culture was centrifuged at 5000x g/5 min; then the sediment was washed five times with sterilized phosphate buffered saline at pH 7.2 (PBS) and finally suspended in 500 µl of sterilized PBS. The suspension was kept at 95°C for 15 min, and after centrifugation at 15,000 rpm for 5 min, 10 µl of the supernatant was directly used for PCR.

PCR design and amplification

PCR primer pairs were designed with reference to sequence published for alpha toxin by Saint Joanis et al. (1989), beta toxin by Hunter et al. (1993), epsilon toxin by Hunter et al. (1992) and iota

toxin by Perelle et al. (1993). Details of the nucleotide sequence, the size of the PCR product for each primer pair and the annealing temperature are listed in Table 3. The extracted DNA of the standard strains and of the bacterial isolates recovered from bacteriological examination were tested by PCR using each primer set. Concurrently, the crude DNA extracted from each fecal sample was tested by the same primer pairs. All reactions were carried out in a final volume of 50 µl in micro- amplification tube (PCR tubes). The reaction mixture was adjusted according to the study of Buogo et al. (1995). The samples were subjected to 25 PCR cycles, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at temperature (Table 3) and 30 s of extension at 72°C. Final extension was carried out at 72°C for 10 min, and the PCR products were stored in thermal cycler at 4°C until they were collected. The PCR products were visualized by agarose gel electrophoresis, according to the study of Sambrook et al. (1989).

RESULTS AND DISCUSSION

C. perfringens has been identified as an important agent of different diseases such as gas gangrene, food poisoning and diarrhea as well as enteritis and fatal enterotoxemias in domestic animals and humans (Songer and Meer, 1996). The obtained results in Table 4 revealed that the incidence of *C. perfringens* isolated from feces and intestinal contents samples of diseases and freshly dead animals and chickens was 19.81%. The prevalence rate reached 22.86%, 13.64%, 20.83% and 20% in feces and intestinal contents of diseased and freshly dead calves, lambs, adult sheep and broiler chickens, respectively.

These results go hand in hand with that recorded by Hosli et al. (1980), Popoff (1984) and Sasaki et al. (2000) who found that *C. perfringens* was the most prevalent isolates in cases of gas gangrene and enterotoxaemia in sheep and lambs with similar incidence rates. Furthermore, the incidence of *C. perfringens* in fecal and intestinal contents samples of chicken has been reported as ranging from 0 to 22% (Tschirdewann et al., 1991; Saito, 1990; Miwa et al., 1997).

Table 4. Bacteriological examination and differentiation between toxigenic and non toxigenic isolates of *C. perfringens*.

Sources of samples	Number of samples	Bacteriologically positive samples		Toxigenic isolates		Non-toxicogenic isolates	
		No	%	No	%	No	%
Calves	35	8	22.86	6	75	2	25
Lambs	22	3	13.64	3	100	0	0
Adult sheep	24	5	20.83	5	100	0	0
Broiler chickens	25	5	20	4	80	1	20
Total	106	21	19.8	18	85.71	3	14.26

Table 5. Typing of toxigenic *C. perfringens* isolates by using mice neutralization test and dermonecrotic test in guinea pigs.

Sources of isolate	Toxigenic isolates	Types of toxigenic isolates									
		Type A		Type B		Type C		Type D		Type E	
		No	%	No	%	No	%	No	%	No	%
calves	6	6	100	0	0	0	0	0	0	0	0
Lambs	3	2	66.66	1	33.33	0	0	0	0	0	0
Adult sheep	5	1	20	0	0	3	60	1	20	0	0
Broiler chickens	4	2	50	0	0	1	25	1	25	0	0

The isolation of pathogenic *C. perfringens* in gas gangrene and enterotoxiemia is very difficult, since the clostridia must be cultured under strict anaerobic conditions, and affected specimen are frequently contaminated with other anaerobic bacteria which outgrow more than the pathogenic clostridia (Sasaki et al., 2000). Therefore, rapid and direct detection systems for pathogenic *C. perfringens*, without the need for culture, are desirable. The differentiation between toxigenic and non-toxicogenic *C. perfringens* isolates depending on Nagler's reaction and pathogenicity in guinea pigs (Table 4) indicates that out of the 21 tested *C. perfringens* isolates, 18 (85.71%) were toxigenic and 3 (14.29%) were non toxigenic. While, typing of toxigenic *C. perfringens* isolates were recovered from calves, lambs, adult sheep and chickens depending on neutralization test in mice and dermonecrotic test in guinea pigs (Table 5).

Depending on the conventional phenotyping methods, it was noticed that 6 *C. perfringens* isolates which were recovered from calves were identified as type A (100%), while two isolates recovered from lambs were identified as type A (66.66%) and type B (33.33%).

In case of isolates, which were recovered from adult sheep, 3 were identified as type C (60%), one as type D (20%) and one as A (20%). Meanwhile, 2 *C. perfringens* isolates which were recovered from chickens were identified as type A (50%), one as type C (25%) and one as type D (25%).

The results of the present study indicate that *C.*

perfringens type A is the most prevalent in calves, lambs and broiler chicken. The *C. perfringens* prevalence in calves, lambs, sheep and chickens was similar to those found in other studies (Holsi et al., 1980; Popoff, 1984; Cho et al., 1990; Yoo et al., 1997; Kadra et al., 1999).

C. perfringens type A and to a lesser extent type C in broiler chicken have been reported as the cause of necrotic enteritis worldwide (Shane et al., 1985; Yoo et al., 1997; Ficken and Wages, 1997; Engstrom et al., 2003; Effat et al., 2007). However, the monitoring of *C. perfringens* incidence in intensive and extensive broiler farms was also done in Italy (Manfreda et al., 2006). In this study, 22 intensive farms (total of 99 samples examined) and 11 extensive broiler farms (total of 50 samples), were tested and the authors reported an overall prevalence of over 90%. The pathogen was detected in 87 out of the total of 149 samples (58.40%).

However, Hunter et al. (1993) and Sayeed et al. (2010) reported that type B of this organism was identified as a causative agent of enterotoxaemia in foals, lambs, sheep and goats.

The variations in the prevalence of diseased cases in literatures could be explained on the basis of epidemiological predisposing factors that could affect farm animals.

Characterization of *C. perfringens* and its toxins is well established, although few data are available in Egyptian literature on its prevalence related to animal diseases with special reference to enterotoxiemia in lambs and calves. Our study is the first to use PCR for typing *C.*

Table 6. Comparison between bacteriological examination and PCR of the examined samples (feces and intestinal contents).

No. of examined samples	Results of bacteriological examination	Results of PCR			
		Positive		Negative	
		No	%	No	%
18	<i>C. perfringens</i>	18	100	0	0
10	<i>C. perfringens</i>	2 (type A)	20	8	80

*The two isolates of *C. perfringens* which recovered by PCR were from fecal intestinal contents of examined chickens

perfringens from different sources.

In traditional procedures, *C. perfringens* was first isolated from the samples under investigation and then the toxigenicity of the isolates was tested for the detection of toxigenic *C. perfringens*. Up till now, the toxin has been identified by seroneutralization in laboratory animals (mouse or guinea pig) using specific antisera. This toxino-typing requires a continuous supply of laboratory animals and the use of monovalent diagnostic sera which are increasingly difficult to find and are extremely expensive. Moreover, the result of the toxino-typing cannot be obtained until 24 or even 48 h observation (Sterne and Batty, 1975; Kadra et al., 1999).

It also has the inaccuracy of biological assays, such as variation in individual animal sensitivity, non-specific toxicity from other substances that may be present in intestinal contents (Kozaki et al., 1979; Henderson, 1984) and disfavor on humanitarian grounds. In addition, this method may not detect the non- or poorly-toxigenic variants found within all types of *C. perfringens* (Uzal et al., 1996).

In the present investigation, the types of *C. perfringens* isolates recovered from feces and intestinal contents of different sources by PCR using alpha, beta, epsilon and iota were undertaken. Also, attempts to use this technique to detect these genes in intestinal contents and feces directly were described.

Firstly, the specificity of the oligonucleotide primers was confirmed by the positive amplification of only toxin genes from the extracted DNA of *C. perfringens* without non specific amplification of other standard enteric bacterial strains.

Enterotoxemia, a disease which mainly affects sheep, is a toxi-infection originating in the digestive system. It can lead to serious losses if prophylactic measures are not strictly applied (Kadra et al., 1999; Petit et al., 1999).

The prophylaxis of enterotoxemia in animals is achieved by vaccination: the PCR technique can thus become a first-choice tool for the identification and typing of the *C. perfringens* strains which initiate these diseases. In turn, this would simplify the development of vaccines which can adapt to the epidemiological situation.

Compared with the classical technique, 18 fecal and intestinal contents samples which revealed *C. perfringens* by bacteriological examination from different animals

were examined using PCR for the presence of α , β , ϵ and i toxins genes; 10 fecal and intestinal contents samples selected to be tested with the same primers did not reveal any *C. perfringens* after bacteriological examination.

The results observed in Table 6 shows positive genotyping of 18 fecal and intestinal contents samples which proved to be infected with *C. perfringens* as confirmed bacteriologically (100%).

From the 10 fecal and intestinal contents samples which did not reveal any *C. perfringens* after bacteriological examination, positive amplification of the 1167 bp fragment of alpha toxin gene from the extracted DNA of 2 samples (20%) was observed. However, direct testing of fecal samples by PCR may be hampered due to inhibition of DNA polymerase by substances present in specimens (Saiki et al., 1988; Fach and Guillou, 1993). A procedure to extract the DNA in order to overcome these hindrances was used according to the study of Uzal et al. (1993).

In order to compare between traditional typing and PCR, all the 18 *C. perfringens* isolates recovered from bacteriological examination of feces and intestinal contents of different isolated were typed by PCR for the presence of α , β , ϵ and i toxins genes.

The results observed in Table 7 reveal 13 (72.22%) strains showing positive amplification of 1167 bp fragment of alpha toxin gene and identified as type A by the PCR; however, 11 strains only were previously identified as type A by classical tests. None of the isolates were found to be iota producers; one strain (5.56%) was identified as B and showed positive amplification of 1025 bp fragment of beta toxin, 961 bp fragment of epsilon toxin gene and 1167 bp fragment of alpha toxin gene by PCR typing, consistent with conventional typing by animal test. Moreover, only 3 strains (16.67%) were identified as type C and one strain (5.56%) was identified as type D by PCR typing. These results confirm observations made by Kadra et al. (1999).

The study showed that the PCR is a rapid and useful method for genotyping of *C. perfringens* and is suggested as a diagnostic method for confirmation of *C. perfringens* species. Typing of toxin gene by PCR has advantage of being practicable directly from primary culture colonies and hence is able to detect toxin genes (beta toxin gene

Table 7. Comparison between the traditional methods and PCR for typing of *C. perfringens* isolates.

Methods for typing	No. of tested isolates	Types of toxigenic isolates									
		Type A		Type B		Type C		Type D		Type E	
		No	%	No	%	No	%	No	%	No	%
Traditional methods	18	11	61.11	1	5.56	4	22.22	2	11.11	0	0
PCR	18	13	72.22	1	5.56	3	16.67	1	5.56	0	0

and iota gene) which are unstably maintained and might be lost during the cultivation process needed for the biological method (Kadra et al., 1999). Moreover, toxin gene detection is able to measure the presence of virulence factors that are tightly regulated and specifically expressed during infection and hence remain undetected by phenotypic methods in culture (Gholamiandekhordi et al., 2006).

The PCR toxin gene typing method is well applicable to the analysis of large numbers of bacterial strains and has shown to be a rapid and efficient method for epidemiological investigations of clostridial disease of animals.

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