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Molecular typing of four major toxins of *Clostridium perfringens* recovered from Egypt

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Abstract

A simple mPCR procedure was used to identify four toxitypes of *Clostridium perfringens* collected from different origins. Eighteen strains of *Clostridium perfringens* were identified and typed by classical methods (dermonecrotic method in guinea pigs and seroneutralization 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 *Clostridium perfringens*, only 11 (61.11%) strains were identified previously as type A by classical method, as well as 3 strains (16.67%) were identified as type C and 1 strain (5.56%) was identified as D by PCR typing. Moreover, mPCR results confirmed the traditional methods in typing one strain as type B (5.56%). Also, mPCR method can detect 2 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 *Clostridium perfringens* strains 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 *Clostridium perfringens* types as an alternative to animal tests.

Key words: *Clostridium perfringens*, major toxins, PCR typing, enteric disease.

Introduction

C. perfringens is a Gram-positive anaerobic spore forming bacterium, able to produce various toxins and enzymes responsible for the associated lesions and symptoms. *C. perfringens* strains are classified into five toxinotypes (A, B, C, D and E), based on the production of four major toxins (α, β, ε and ι) [1, 2]. Type A strains are the most commonly encountered and produce food poisoning and gas gangrene in humans and animals, and necrotizing colitis and enterotoxemia in horses [3]. Types B, C and D primarily occur in the intestine of animals and only 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 [4].

The strains of types B and 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 [3,4]. The major toxins produced by the five types of *C. perfringens* are illustrated in Table 1.

Typing of an organism is accomplished with the culture filtrate, type-specific antisera and experimental animals such as mice and guinea pigs [5]. 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[6].

The present study was an endeavour aimed to for detection of the genes encoding the different *Clostridium perfringens* toxins from different sources in Egypt as an alternative diagnostic method by PCR. This is the first study concerning the molecular typing of *C. perfringens* field isolates recovered from different sources in Egypt.

Materials and Methods

Samples: Samples from feces and intestinal contents were obtained from 35 diarrheic calves (age ranged from 1-4 months) showing signs of enterotoxemia and 24 adult sheep and 22 lambs (age from 1-12 weeks) showing signs of diarrhea and enterotoxemia during the winter of 2008 to winter of 2010. As well as 25 samples from intestine of broiler chickens, exhibiting diarrhea and showing 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 hours of collection. The complete list of sample numbers and sources used in this study is given in Table 2.

Bacteriological identification of C. perfringens from samples: *C. perfringens* was isolated by the procedure of Willis [7]. Typical colonies were identified as described by Murray *et al.* [8] depending

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

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

Table 2. Numbers and sources of examined fecal and intestinal 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

on 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* by Nagler's test by half antitoxin plate according to Smith and Holdeman [9], and pathogenicity to guinea pigs according to Willis [7].

For typing toxigenic isolates of *C. perfringens*, neutralization test in mice was carried out according to Smith and Holdeman [9], and dermonecrotic test in guinea pigs was performed according to Sterne and Balty [10].

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. As well as 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 standard strains and of the other bacterial isolates yielded from bacteriological examination was extracted by hexadecyltrimethyl ammonium bromide (CTAB), according to Sambrook *et al.* [11]. The extractions of DNA from fecal samples were carried out according to Uzal *et al.* [12], 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 5000 x g/5 min., then the sediment was washed five times with sterilized phosphate buffered saline, 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, beta, epsilon and iota toxin by Yoo *et al.* [13]. 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 the bacterial isolates yielded from bacteriological examination was tested by PCR using each primer set. Concurrently the crude DNA extracted from each fecal sample 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 Yoo *et al.* [13] according to Table 3, and the PCR products were stored in thermal cycler at 4ºC until they were collected.

Table 3. PCR primers used for multiplex PCR: target toxin gene, nucleotide sequence, and length of the amplification products according to Yoo *et al.* [13].

Primer (direction)	Nucleotide sequence	Amplicon size (bp)
Alpha-toxin		
Forward	5'-GTTGATAGCGCAGGACATGTTAAG-3'	402
Reverse	5'-CATGTAGTCATCTGTTCCAGCATC-3'.	
Beta-toxin		
Forward	5'-ACTATACAGACAGATCATTCAACC-3'	236
Reverse	5'-TTAGGAGCAGTTAGAACTACAGAC-3'.	
Epsilon toxin		
Forward	5'-ACTGCAACTACTACTCATACTGTG-3'	541
Reverse	5'-CTGGTGCCTTAATAGAAAGACTCC-3'.	
Iota toxin		
Forward	5'-GCGATGAAAAGCCTACACCACTAC-3'	317
Reverse	5'-GGTATATCCTCCACGCATATAGTC-3'.	

Agarose gel electrophoresis: The PCR products were visualized by agarose gel electrophoresis, according to Sambrook *et al.* [11]

Results and Discussion

C. perfringens has been identified as an important agent of different diseases including gas gangrene, food poisoning and diarrhea as well as enteritis and fatal enterotoxemias in domestic animals and humans [14] .The results in Table 4 revealed that the incidence of *C. perfringens* isolated from feces and intestinal contents of diseased and freshly dead animals 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.

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

Sources of samples	Number of Samples	Bacteriologicaly positive samples		Toxigenic isolates		Non-Toxigenic 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

These results go hand in hand with Hosli *et al.* [15], Popoff [16] and Sasaki *et al.* [17], that *C. perfringens* was the most prevalent isolate 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 of chicken have been reported as ranging from 0 to 22% [18-20].

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 specimens are frequently contaminated with other anaerobic bacteria which outgrow more than the pathogenic clostridia [17]. Therefore, rapid

and direct detection systems for pathogenic *C. perfringens*, without the need for culture, are desirable. The differentiation between toxigenic and non toxigenic. *C. perfringens* isolates depending on Nagler's reaction and pathogenicity in guinea pigs as shown in Table 4, indicates that out of the 21 tested *C. perfringens* isolates 18 (85.71%) were toxigenic and 3 (14.29%) non toxigenic. Typing of toxigenic *C. perfringens* isolates recovered from calves, lambs, adult sheep and chickens depending on neutralization test in mice and dermonecrotic test in guinea pigs is shown in 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 one as 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 type in calves, lambs and broiler chicken. The *C. perfringens* prevalence in calves, lambs, sheep and chickens were similar to those found in other studies [13, 15, 16, 21, 22]. *C. perfringens* type A and to a lesser extent type C in broiler chicken have been reported as a cause of necrotic enteritis worldwide [13, 23-26]. However, Hunter *et al.* [27] 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 among literatures could be explained on the basis of epidemiological predisposing factors that could affect the animals farms.

Characterization of *C. perfringens* and its toxins is well established, although few data are available in Egyptian literature about its prevalence related to animal diseases with special reference to enterotoxemia in lambs and calves. Our study is the first one that used PCR for typing *C. 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 [10, 22].

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 [28, 29] and disfavor on humanitarian grounds. In addition, this method may not detect the non- or poorly-toxigenic variants found within all types on *C. perfringens* [12].

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 [2, 22].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 adapted the epidemiological situation.

Compared with the classical technique, 18 fecal and intestinal content samples which revealed *C. perfringens* by bacteriological examination from different animals were examined using PCR for the presence of α, β, ε and ι toxins genes as well as l0 fecal and intestinal content samples were selected to be tested with the same primers did not reveal any *C. perfringens* after bacteriological examination.

Table 6 shows positive genotyping of 18 fecal and intestinal content samples which proved to be infected with *C. perfringens* as confirmed bacteriologically (100%). From the 10 fecal and intestinal content 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%) were observed (Fig. 1). However, direct testing of fecal samples by PCR may be hampered due to inhibition of DNA polymerase by substances present in specimens [30, 31], a procedure to extract the DNA in order to overcome these hindrances was used according to Uzal *et al.* [12] in this investigation.

In order to compare between the 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 ι toxins genes. Table 7 and Fig. 1 revealed 13 (72.22%) strains showing positive amplification of 402 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 showing positive amplification of 236 bp fragment of beta toxin, 541 bp fragment of epsilon toxin gene and 402 bp fragment of alpha toxin gene by PCR typing, which were consistent with conventional typing by animal test as shown in Fig. 1. 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.* [22].

The study showed that the PCR is a rapid and useful method for genotyping of *C. perfringens*

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

Sources of isolates	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

and suggested as a diagnostic method for confirmation of *C. perfringens* species, on the other hand, toxin gene typing by PCR has advantage to be practicable directly from primary culture colonies and hence is able to detect toxin genes which are unstable maintained, such as beta toxin gene and iota gene, which might be lost during the cultivation process needed for the biological method[22]. Moreover, toxin gene detection also 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. The mPCR 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. The two isolates of *C. perfringenes* which recovered by mPCR were from fecal intestinal contents of examined chickens.

The study showed that the PCR is a rapid and useful method for genotyping of *C. perfringens* and suggested as a diagnostic method for confirmation of *C. perfringens* species, on the other hand, toxin gene typing by PCR has advantage to be practicable directly from primary culture colonies and hence is able to detect toxin genes which are unstable maintained, such as beta toxin gene and iota gene, which might be lost during the cultivation process needed for the biological method [22]. Moreover, toxin gene detection also 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.

Conclusions

Our study is the first molecular study to demonstrate toxin gene typing of *C. perfringens* from different sources in Egypt. The mPCR 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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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	*C. perfringens*	18	100	0	0
10	*C. perfringens*	2 (type A)	20	8	80

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

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

Figure 1. Multiplex PCR showing amplification of 402 bp fragment of alpha toxin gene from the extracted DNA of *C. perfringens type A* isolates. Amplification of 541 bp,236 bp and 402 bp fragments of epislon, beta and alpha toxin gene, respectively, from the extracted DNA of *C. perfringens type B* isolates. Amplification of 236 bp and 402 bp fragments of alpha and beta toxin gene from the extracted DNA of *C. perfringens type c* isolates. Type D showed amplification of 402 and 541 bp.

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