Clostridium perfringens in Chicken: Relevant to Public Health from Egypt

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A multiplex and duplex PCR procedure was used to identify four toxitypes of *Clostridium perfringens* collected from apparently healthy and diseased chicken as well as characterization of *Clostridium perfringens* for the presence of beta2 and enterotoxin genes. Sixty strains of *Clostridium perfringens* were identified and typed by classical methods. All the strains were analyzed by PCR for the presence alpha, beta, toxin and iota genes as well as beta2 and enterotoxin genes. The results reveal α toxin gene in 45 strains of *Clostridium perfringens*, only 43 (84.31%) strains of them were identified previously as type A by classical method, as well as 8 strains (15.69%) were identified as type C and 3 strains (5.88%) of type A were associated with beta2 toxin gene by multiplex and duplex PCR typing. Also PCR 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 traditional examination. Thus PCR technique can become a first-choice tool for the identification, typing and characterization of the virulence genes encoded for enterotoxin and beta 2 of *Clostridium perfringens* field isolates recovered from poultry which initiate enteric disease in Egypt.

Key words: Clostridium perfringens, PCR typing, necrotic enteritis disease, poultry.

Clostridium perfringens is a wide spread spore-forming, Gram-positive, anaerobic, non motile rod. *Clostridium perfringens* is a causative agent of human and animal foodborne diseases. It is known as a normal inhabitant of the intestinal tract of chickens as well as a potential pathogen causing necrotic enteritis. *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 τ)^{1,2}. Only *C. perfringens* types A and C are pathogenic for poultry. However, the presence of *C. perfringens* does not lead directly to the disease. Although 75 - 95% of birds are colonised by *C. perfringens*, only a small proportion of these ever shows symptoms of the disease.³

The most important causes of food poisoning and non-food-borne diarrheas in humans are *Clostridium perfringens* type A isolates harboring the enterotoxin (cpe) gene, which are usually associated with foods of animal origin (WHO 2004).

Typing of an organism is accomplished with the culture filtrate, type-specific antisera and experimental animals such as mice and guinea

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pigs.⁴The 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.⁵Although molecular toxin typing of C.perfringens in poultry were investigated in Egyptian studies, to the authors knowledge, to detect the presence of entertoxin and beta2 toxin has not been reported in Eygpt. So this study reports a multiplex polymerase chain reaction (PCR) for characterization of the genes encoding Clostridium perfringens toxins among chicken as well as duplex PCR assay to investigate the role of the enterotoxin and beta2 gene in the field isolates of C. perfringens from Giza, Cairo and Al-Fayoum governorates, Egypt.

MATERIALS AND METHODS

Samples

Between 20 June 2010 and 15 September 2012, 140 samples from apparently healthy and diseased chicken. Cecal content of broiler chickens exhibited diarrhea and showed clinical signs of necrotic enteritis as well as apparently healthy birds were collected from different farms at different localities in Giza, Cairo and Al-Fayoum governorates, Egypt. This work was done at Faculty of Veterinary Medicine, Cairo University as well as Center of Excellence in Biotechnology Research, King Saud University.

The samples were collected in plastic bags and were transported refrigerated to the laboratory where they were processed within 4 hours of collection.

Bacteriological identification of C. perfringens

C. perfringens was isolated by the procedure of Quinn *et al.*,⁶ Typical colonies were identified as described by Murray *et al.*,⁷depending 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 was conducted according to Baldassi *et al.*,⁸ and pathogenicity to guinea pigs was according to Quinn *et al.*,⁶

For typing toxigenic isolates of C.

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perfringens isolates, neutralization test in mice was carried on according to Baldassi *et al.*,⁸ and dermonecrotic test in guinea pigs was performed according to Sterne and Balty.⁹

General procedures for animal care and housing were in accordance with the United States Department of Agriculture, through the Animal Welfare Act (7USC 2131) 1985 and Animal Welfare Standards incorporated into Title 9 of the Code of Federal Regulations, Part 3, 1991.

Bacterial strains used for determination of primers specificity

Three strains of *C. perfringens* types A, B, C and D (Animal health Research institute, Dokki, Egypt) 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

DNA was isolated using DNeasy® Tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

PCR design and amplification

PCR primer pairs were designed with reference to sequence published by Yoo *et al.*,¹⁰ were used in the multiplex PCR as well as enterotoxin and beta2 primers for duplex PCR as described by Baums *et al.*,¹¹ Details of the nucleotide sequence and the size of the PCR product for each primer pair are listed in Table 1 and 2.

The extracted DNA of the standard strains and of the bacterial isolates yielded from bacteriological examination was tested by PCR using the primers listed in Table 1 and 2. Concurrently the crude DNA extracted from fecal samples tested by the same primer pairs.

The PCR reaction mixture (25 μ l) contained 5 μ l of bacterial lysate as template DNA, 2.5 μ l of 2 mM dNTP's, 2.5 μ l 10× PCR buffer, 0.25 μ l of 5U/ μ l Taq DNA polymerase (Vivantis, Malaysia), 1 μ l of each of the primers (10 pmol/ μ l) and 6.75 μ l distilled water. The PCR reaction mixtures were placed in a Biometra PCR thermal cycler. Following initial denaturation for 5 min at 94°C, the samples were subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. After the last cycle, a final extension for 10 min at 72°C was performed in multiplex PCR as described by Yoo *et al.*, ¹⁰ and 54°C in duplex PCR as, 1min 20 sec at 72°C and a final step of 2 min at 72°C described by Baums *et al.*, ¹⁰ The PCR reaction mixtures (10µ1) were analyzed by electrophoresis on a 1% (w/v) agarose gel in the presence of 100-bp DNA ladder (Fermentas Life Science, EU). The agarose gel was supplemented with ethidium bromide in order to visualize the DNA on an UV transilliminator. The PCR products were visualized by agarose gel electrophoresis according to Sambrook *et al.*, ¹²

RESULTS AND DISCUSSION

C. perfringens is a pathogenic Gram positive bacterium which can cause outbreaks of serious diseases like myonecrosis, enterotoxemia, cholangio-hepatitis, and necrotic enteritis in human as well as in animals¹³, which costs the worldwide poultry industry \$2 billion annually¹⁴

During the summer of 2010 to 2012, a total of 140 fecal content samples from apparently healthy broiler and diseased chickens from different farms were examined for the presence of *C. perfringens*. The diseased birds exhibited general signs in the form of depression, reluctance to move, pronounced apathy, ruffled feather and watery diarrhoea as well as high mortality rate. Moreover, the pathological lesions of dead birds' revealed friable small intestine (jejunum and ilieum) distended with gas. The intestinal mucosa as were covered by yellowish or green pseudo membrane. The obtained results in Table 3 revealed that *C. perfringens* isolated from intestinal contents was 45 out of 70 and 15 of 70 samples of diseases and apparently healthy chickens with incidence 64.29% and 21.43%, respectively.

The monitoring of *C. perfringens* incidence on intensive and extensive broiler farms was also conducted in Italy¹⁵. In his 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 of the total of 149 samples (58.40%). While in our study on *C. perfringens* isolated from broiler chickens in Saudi Arabia, the incidence in the diseased chicken was $17.1\%^{16}$. The incidence in Egypt seems to be higher than in KSA.

 Table 1. PCR primers used for multiplex PCR: target toxin gene, nucleotide sequence, and length of the amplification products according to Yoo *et al.*,(1997).

Primer (direction)	Nucleotide sequence	Amplicon size (bp)
Alpha-toxinForward Reverse Beta-toxinForward	5'-GTTGATAGCGCAGGACATGTTAAG-3' '5'-CATGTAGTCATCTGTTCCAGCATC-3'. 5'-ACTATACAGACAGATCATTCAACC-3'	402
Reverse	5'-TTAGGAGCAGTTAGAACTACAGAC-3' '	230
Epsilon toxinForward Reverse	5'-ACTGCAACTACTACTCATACTGTG-3' 5'-CTGGTGCCTTAATAGAAAGACTCC-3'	541
Iota toxinForward Reverse	5'-GCGATGAAAAGCCTACACCACTAC-3' 5'-GGTATATCCTCCACGCATATAGTC-3'.	317

Table 2. PCR primers used for duplex PCR: target toxin gene, nucleotide sequence, and length of the amplification products according to Baums *et al.*,2004

Primer (direction)	Nucleotide sequence	Amplicon size (bp)
Entero-toxinForward Reverse Beta-2toxinForward Reverse	5'-TGG GAA GTT CGA AAG CA-3' ' 5'-TTA ACT CAT CTC CCA TAA CTG CAC-3'. 5'-CAA GCA ATT GGG GGA GTT TA-3' 5'-GCA GAA TCA GGA TTT TGA CCA-3' '	396 200

The incidence of *C. perfringens* in the intestinal tract and in processed meat of poultry is high. When the intestinal contents of broiler chickens are analyzed for the presence of *C. perfringens*, approximately 75% to 95% of the animals are found positive.¹⁷When poultry meat is analyzed for *C. perfringens*, high percentages of positive meat samples are reported, in some cases up to 84%.¹⁷

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

The differentiation between toxigenic and non toxigenic. *C. perfringens* isolates depending on Nagler's reaction and pathogenicity in guinea pigs as shown in Table (3), indicates that out of the 60 tested *C. perfringens* isolates 51 (85%) were toxigenic and 9 (15%) were non toxigenic. Out of the 51 toxigenic isolates, 11 isolates were from the apparently healthy chickens and 40 isolates were from the diseased chickens. While typing of toxigenic C. perfringens isolates recovered apparently healthy and diseased chickens depending on neutralization test in mice and dermonecrotic test in guinea pigs is conducted in Table (4). Depending on the conventional phenotyping methods, it was noticed that 32 out of 40 C. perfringens isolates which were recovered from diseased chickens were identified as type A (80%) and 8 as type C (20%), while 11 isolates recovered from apparently healthy chickens were identified as type A (100%). The results of the present study indicate that C. perfringens type A is the most prevalent type among broiler chicken. The C. perfringens prevalence in chickens was similar to those found in other studies.^{10,16,18,19} C. perfringens type A and to a lesser extent type C in broiler chicken have been reported as a cause of necrotic enteritis worldwide.10,18,20

Table 3. Bacteriological examination and differentiation between toxigenic and non toxigenic isolates of C. perfringens

Sources of samples	Number of Samples	Bacteriologicaly positive samples		Toxiger	Toxigenic isolates		Non-Toxigenic isolates	
		No.	%	No.	%	No.	%	
Apparently health chickens	70	15	21.43	11	18.33	4	6.66	
Diseased chickens	70	45	64.29	40	66.66	5	8.33	
Total	140	60	42.86	51	85	9	15	

 Table 4. Typing of toxigenic C. perfringens isolates by using mice neutralization test and dermonecrotic test in guinea pigs

Sources of	Toxigeni	с	Types of toxigenic isolates									
isolates	isolates	Тур	Type A		Туре В		Type C		Type D		Туре Е	
		No.	%	No.	%	No.	%	No.	%	No.	%	
Apparently health chickens	11	11	100	0	0	0	0	0	0	0	0	
Diseased chickens	40	32	80	0	0	8	20	0	0	0	0	
Total	51	43	84.31	0	0	8	15.69	0	0	0	0	

Table 5. Characterization	C. perfringens	isolates by multiplex	and duplex PCR
	1 0 0	2 1	1

No. of tested	Types of toxigenic isolates							
isolates	Туре А	Type B	Type C	Type D	Туре Е	Beta2	enterotoxin	
51 89	43(84.31%) 2(2.24%)	0(0%) 0(0%)	8(15.69%) 0(0%)	0(0%) 0(0%)	0(0%) 0(0%)	3(5.88%) 0(0%)	0(0%) 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.²¹Therefore, rapid and direct detection systems for pathogenic C. perfringens, without the need for culture, are desirable.

Characterization of C. perfringens and its toxins is well established, although few data are available in Egyptian literature about its prevalence related to the presence of beta 2 gene and enterotoxin gene. So this study is the first study that use PCR for characterization C. perfringens recovered from broiler chicken for the presence of beta 2 toxin and enterotoxin.

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 48h observation.^{9,22} 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 ^{23,24} 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.²⁵

In the present investigation, the types of



Fig. 1. Multiplex PCR showing amplification of 402 bp fragment of alpha toxin gene from the extracted DNA of C. perfringens type A isolates and 236 bp and 402bp fragments of alpha and beta toxin gene from the extracted DNA of C. perfringens type C isolates



Fig. 2. Agarose gel electrophoresis showing amplification of 200 bp fragment of beta2 toxin gene from the extracted DNA of C. perfringens type A isolates

C. perfringens isolates recovered from feces and intestinal contents of apparently healthy and diseased chicken by PCR using alpha, beta, epsilon and iota were undertaken as well as characterization of these isolates to detect the presence of beta 2 and enterotoxin genes. 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.

Fecal and intestinal contents samples were examined using PCR for the presence of alpha, beta, epsilon and iota toxins genes. Polymerase chain reaction methods may detect all the bacteriologically positive samples for toxigenic *C. perfringens* (n=51 [100%]). Moreover, this methods may also detect the alpha toxin gene in other 2 samples (2.24%) previously revealed negative for the presences of toxigenic *C. perfringens* by traditional methods. All *C. perfringens* isolates were recovered from the bacteriological examination of feces and intestinal contents were typed using multiplex PCR for the presence of alpha, beta, epsilon and iota toxins genes.

The results observed in Table 5 and Fig.1 revealed 45 strains showing positive amplification of 402 bp fragment of alpha toxin gene and identified as type A by the PCR, however, 43 strains only were previously identify as type A by classical tests and 2 strains (2.24%) out of 89 fecal and intestinal content were identified as negative for the presence of toxigenic C. perfringens strains by traditional methods. None of the isolates were found to be iota or epsilon producers, 8 strain (15.96 %) was identified as C and showing positive amplification of 236 bp fragment of beta toxin and 402 bp fragment of alpha toxin gene by PCR typing, which were consistent with conventional typing by animal test. Moreover, only 3 strains (5.88%) types A were associated with beta2 gene as shown in Fig. 2. The enterotoxin gene was detected in none of the samples tested. Recently, the newly discovered beta 2 toxin has been associated with porcine, equine and bovine gastro-enteritis.²⁶⁻²⁸ This toxin has also been demonstrated in avian C. perfringens type A strains.²⁶

Engström *et al.*, ¹⁸ from Sweden reported the presence of cpb2 gene in only 2 of the total of 53 strains tested. All of the strains were classified as type A with no enterotoxin gene present. The most recent study from Belgium²⁹ reports the presence of beta 2 genes in 4 strains, and the presence of the enterotoxin gene in 2 strains of a total of 27 healthy broiler chickens examined.

In all these studies, a significantly higher incidence of beta 2 toxin positive isolates was detected in animals having intestinal disease. This seems to be in contrast with *C. perfringens* isolates from poultry.^{1,29}

The study showed that the PCR is a rapid and useful method for genotyping and characterization 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 other toxin genes, such as beta2 toxin gene and enterotoxin gene.²²Moreover, 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.

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