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## Molecular characterization of *Clostridium perfringens* isolated from broiler chickens in Egypt

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The present study was carried out to investigate the role of *C. perfringens* in necrotic enteritis in broiler chickens from various farms in Giza Governorate. Samples were collected from apparently healthy and diseased chickens showing diarrhea before death. A total of 200 samples representing the intestinal content of broiler chickens showing enteric disorder symptoms and lesions suspected to be due to necrotic enteritis were analyzed by conventional methods and polymerase chain reaction (PCR). *C. perfringens* was isolated at the rate of 10% (10/100) from apparently healthy chickens, and at the rate of 25% (25/100) from diseased chickens, and its presence was confirmed by cultural & biochemical characterization. Among the recovered isolates, 10% (20/200) of *C. perfringens* isolates were toxigenic and 7.5% (15/200) were non toxigenic. Multiplex PCR was performed to toxinotyping the 20 toxigenic isolates. The result revealed that all isolates were positive for the alpha toxin gene.

**Keywords:** *C. perfringens*, in broiler chickens, gastrointestinal tract, haemolysis, Primer sequences

### INTRODUCTION

*Clostridium perfringens* is the main causative agent of avian necrotic enteritis (NE), an enteric disease of chickens that was first described in 1961 (Parish, 1961) and has since been found in all poultry producing countries. NE in chickens manifests as an acute or chronic enterotoxaemia (Songer, 1996). The acute disease results in significant levels of mortality, whereas the chronic disease lead to loss of productivity and welfare concerns. It has been estimated that the disease cost the international poultry industry (Lovland and Kaldhusdal 2001) and (Vander sluis, 2000). NE is primarily caused by *C. perfringens* type A and to lesser extent type C strains. Clinical NE is thought to occur when *C. perfringens* proliferates to large number in the small intestine and produce extracellular toxins that damage the gastrointestinal tract. *C. perfringens* is a gram-

positive, anaerobic, fermentative spore forming bacillus, which classified into five types (A, B, C, D and E) according to the production of four major toxins (alpha  $\alpha$ , Beta  $\beta$ , epsilon  $\epsilon$  and iota $\iota$ ). In addition, *C. perfringens* can produce additional virulence factors, including enterotoxin, necrotic enteritis like B (Net B) toxin and beta-2 toxin (Popoff and Bouvet, 2013)

### MATERIALS AND METHODS

#### Sampling

Two hundred gastrointestinal tract samples were collected from broiler chickens (100 apparently healthy and 100 diseased). They were acquired from separate commercial poultry facilities in Egypt in which birds had recently experienced clinical signs of necrotic enteritis associated with *C. perfringens*, including depression, ruffled

feathers, diarrhea and macroscopically evident lesions in the small intestines. Samples were transported to the laboratory in an ice box as soon as possible.

#### Isolation and Identification of *Clostridium perfringens*

To isolate sporulating strains, luminal material from the intestines was enriched for 24 h in cooked meat broth (Oxoid) under anaerobic conditions at 37°C, and subjected to heat and alcohol shock. A loopful of culture was plated onto a blood agar base (Oxoid) with 10% sheep blood and 70 µg/ml neomycin sulphate. The plates were incubated at 37°C for 24 h under anaerobic conditions (gas-generating kit, B 36, Oxoid). Typical colonies showing a double zone of beta haemolysis were picked up and subcultured. The sub-cultured colonies were identified by colony morphology, staining, Nagler reaction, urease test, lecithinase test, aero-tolerance in chocolate agar plate (at 37°C in 5–10% CO<sub>2</sub> for 24 h), reverse CAMP (Christie Atkins and Munch-Peterson) reaction, catalase test, lactose fermentation, gelatinase production, nitrate reduction, motility test, acid phosphatase reaction, and other biochemical tests (Quinn *et al.*, 2002).

#### Toxinotyping of *Clostridium perfringens*

##### Biological assay typing for *C. perfringens* toxins

Typing of *C. perfringens* isolates was performed traditionally using neutralization test. The culture supernatants were collected by centrifugation at 3000 rpm for 15 minutes and 3 white swiss mice (20 g weight) were injected with 0.3 ml I/V in coccygeal vein. If the mice died within 24 hours, this indicated the presence of toxin. Identification of the type of the toxin was carried

out by the serum neutralization test according to (Smith and Holdman 1968). The serum neutralization test in mice was applied using the commercial antitoxins for typing as follow: the clear supernatant fluid was divided into five portions;

-The first portion (0.3 ml) was neutralized with 0.1 ml of type antiserum.

-The second portion (0.3 ml) was neutralized with 0.1 ml of type B antiserum.

-The third portion (0.3 ml) was neutralized with 0.1 ml of type C antiserum.

-The fourth portion (0.3 ml) was neutralized with 0.1 ml of type D antiserum.

-The fifth portion (0.3 ml) was added to 0.1 ml of saline as control.

Each portion was injected into a group of three white mice and animals were noticed for 24 hours. The surviving group of mice indicated the type of the toxin.

##### Toxinotyping of recovered *Clostridium perfringens* isolates using multiplex PCR

Pure and young cultures of the isolated were harvested from agar plates. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The toxin gene primers were used in multiplex PCR amplification conditions; The PCR amplification reaction on all of the isolates was performed according to the following thermal profile:

One cycle of denaturation at 94°C for 3 min followed by 37 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. PCR results were assessed by electrophoresis in 2% agarose gels stained with ethidium bromide (Van Asten *et al.*, 2008).

**Table 1: Primer sequences and amplicon sizes used in toxinotyping of *C. perfringens* isolates**

Toxinotyping primers	Nucleotide sequence (5' - 3')	Amplicon (bp)
<i>cpa</i>	GCTAATGTTACTGCCGTTGA CCTCTGATACATCGTGTAAG	324
<i>cpb</i>	GCGAATATGCTGAATCATCTA GCAGGAACATTAGTATATCTTC	196
<i>etx</i>	GCGGTGATATCCATCTATTC CCACTTACTTGTCTACTAAC	655
<i>iap</i>	ACTACTCTCAGACAAGACAG ACTACTCTCAGACAAGACAG	446
<i>cpe</i>	GGAGATGGTTGGATATTAGG GGACCAGCAGTTGTAGATA	233*

\*:Meer and Songer (1997)

## RESULTS

In the present study, *C. perfringens* was isolated in both NE diseased and apparently healthy broiler 17.5% (35/200). It was recovered from the intestine of diseased broilers in 25% (25/100) and in 10% (10/100) of apparently healthy broilers as shown in (Table 2).

**Table (2): Incidence of *C. perfringens* recovered from apparently healthy and diseased broiler chickens:**

Positive samples				Total positive	
Apparently healthy		Diseased			
No.	%	No.	%	No.	%
10/100	10	25/100	25	35/200	17.5

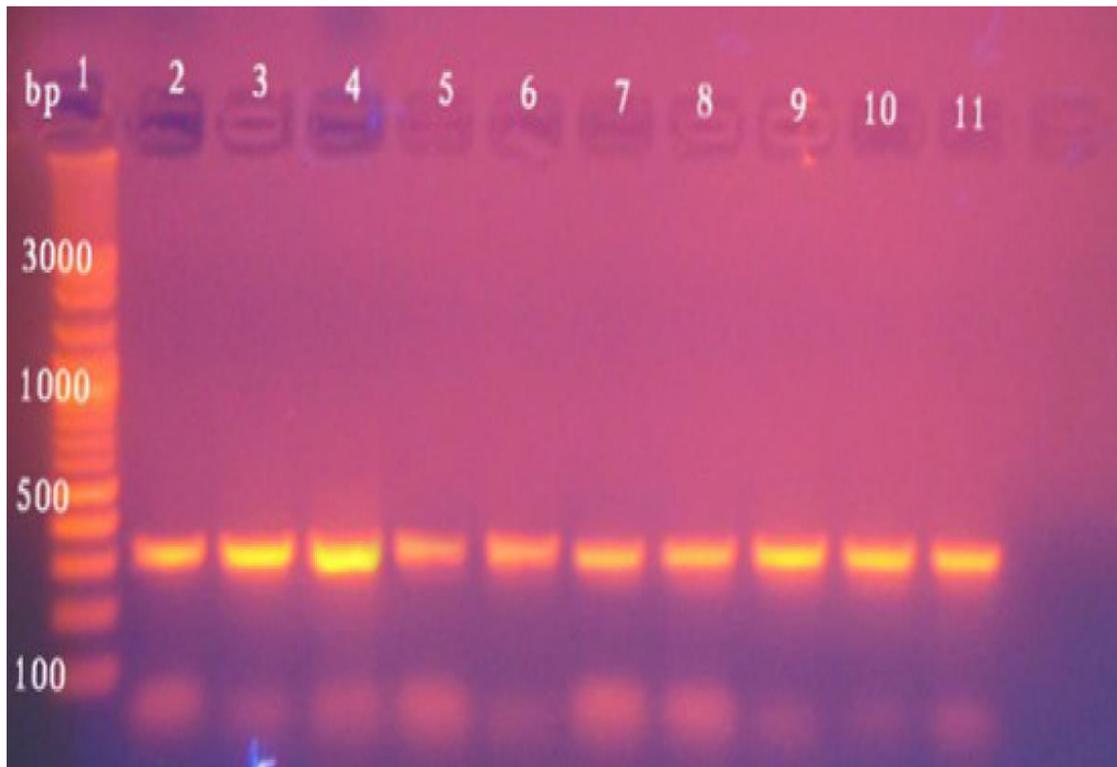
Toxiogenicity assay of *C. perfringens* isolates from apparently healthy and diseased broiler chickens revealed that, 20 strains out of examined *C. perfringens* isolates were toxigenic. All isolates were of type A due to the presence of alpha toxin only (Table 3).

**Table (3):Toxinotypes of *C. perfringens* isolates from apparently healthy and diseased birds:**

Toxin type	Toxigenic strains			
	Healthy		Diseased	
	No.	%	No.	%
A	2/10	20	18/25	72
B	--	-	-	
C	-	-	-	
D	-	-	-	
Total	10	20	25	72

### Confirmation of toxotyping of *C. perfringens* isolates using PCR

In vitro typing method of *C. perfringens* based on the amplification of toxin genes by polymerase chain reaction (PCR) is an accurate and rapid method. Multiplex PCR showed that the characteristic clear amplicons at 324 bp (Figure 1) for  $\alpha$  toxin (*cpa*) in the examined *C. perfringens* isolates; however, no bands were detected for other toxin genes.



**Figure 1: Agarose gel electrophoresis showing amplification of a 324 base pair fragment of Alpha-toxin gene from the extracted DNA of *C. perfringens* isolates, Lane 1 DNA marker GeneRuler 100 bp plus (Thermo Fisher)**

## DISCUSSION

*Clostridium* species cause considerable economic losses to the poultry industry, especially in broiler chickens, all over the world and is associated with gastrointestinal disorders, poor performance and embryo mortality (Cortes et al., 2004 and Soad et al., 2017). *C. perfringens* is a normal commensal bacterium present in the chicken gut (Cooper et al., 2013), and consequently the farm litter, which containing a mortality in rapidly growing broilers with an adverse effect on performance (El-winger et al., 1998). In this study, The overall recovery rate of *C. perfringens* from broiler chickens was (17.5%), where it recorded (10%) from apparently healthy and (25%) from the diseased birds. Comparatively, higher incidence (70%) of *C. perfringens* from apparently healthy chickens were recorded by El-Ged and Hegazy (1985) and (50%) by Hussein and Mustafa (1999) as there is a relationship between number of *C. perfringens* in the intestine and the incidence and the severity of NE (Droual et al., 1995). It was clear that the incidence of *C. perfringens* recovered from diseased chickens with diarrhea before death is relatively higher than those recovered from apparently healthy birds (25 % and 10% respectively). However, these results were much lower than those obtained by Abd El-Salam (2000) and Abd El-Wahab (2002) who isolated *C. perfringens* from intestines of diseased chickens with NE in an incidence of 49.7%. (Ibrahim et al., 2017) whereas, as lower incidence was recorded (16.3 %) (Das et al., 1997). *C. perfringens* produce a variety of fatal extracellular toxins, designated as alpha ( $\alpha$ ), beta ( $\beta$ ), epsilon ( $\epsilon$ ) and iota (i), which are considered to be the major toxins and are used to group the bacteria into five types A, B, C, D and E (Songer, 1996). Alpha toxin is commonly produced by all five types and is a phospholipase C that can hydrolyze lecithin into phosphocholine and diglyceride and is believed to be a major factor responsible for the organism tissue pathology (Awad et al., 1995). *C. perfringens* isolates from both normal and NE afflicted chickens were screened for various toxin types. All the tested strains were Type A CP, which produce alpha toxin depending on neutralization test, as shown in table (3) as previously reported (Li et al., 2013). Molecular approaches serve as a rapid and accurate detection tool for most of microbial pathogens either by direct application on clinical samples (Hossam et al., 2016, Elhariri et al., 2017a,b) or genotyping (Khalifa et al., 2014) and toxinotyping tool (Mansour et al., 2017). A

high percent of *C. perfringens* acting as a major infecting source to healthy birds (Ahmed et al., 2012). *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).

NE is a disease with variable frequency in commercial poultry. The occurrence of necrotic lesions in the intestinal tract associated with *C. perfringens* infection causes significant multiplex PCR assay has been established to toxinotype *C. perfringens* isolates with respect to the genes *cpa*, *cpb*, *etx*, *cpe* and *iap* (Crespo et al., 2007). All *C. perfringens* isolates recovered from apparently healthy and diseased chickens with NE were genotyped and all were positive for  $\alpha$  toxin gene with an amplified product of 324 bp. So, the recorded results revealed that the tested strains were identified as *C. perfringens* type A. These results are in line with the results reported by several authors, which they mentioned that *C. perfringens* type A is the most predominant type of toxin isolated from chickens with necrotic enteritis (Das et al., 1997; Crespo et al., 2007 and Archambault, 2009).

## CONCLUSION

The present study provided confirmatory data about that *C. perfringens* type A, is the major type associated with necrotic enteritis from broiler chickens in Egypt

## CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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## AUTHOR CONTRIBUTIONS

HA & ME designed and supervised the experiments protocol and also wrote the manuscript. AN, RE and EF performed samples collection, Molecular and data analysis. RS and reviewed the manuscript. All authors read and approved the final version.

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