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Original Research Article

Phenotypic and genotypic characterization of *Clostridium perfringens* associated with necrotic enteritis in broiler chickens

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ABSTRACT

Necrotic enteritis; caused by *Clostridium perfringens*, is one of the most important enteric diseases in poultry causing severe economic losses and increased mortalities worldwide. The current study was designed to isolate and characterize *C. perfringens* from 115 broiler chicken aged 3-6 weeks were collected from 15 farms in Beni-Suef and El-Menia Governorates during the period from September 2014 to September 2015. A total of 230 samples were aseptically collected form chicken flocks showing signs of depression, decreased feed intake, low conversion rate and diarrhea with variable daily mortalities. Intestinal and liver samples collected from diarrheic chicken were subjected to a bacteriological examination. It has been found that 29 (18.3%) *C. perfringens* isolates was recovered from 21 chickens of which 15 (51.7%) were toxigenic. The later typed using dermonecrotic test and PCR. Thirteen (86.7%) isolates belonged to type A while two (13.3%) isolates belonged to type B. Multiplex PCR was conducted with the primers mixture of alpha, beta, epsilon and iota toxins genes to determine the type of toxin among 15 of *C. perfringens* isolates. The current study revealed that 13 isolates were positive for α-toxin gene, while only two isolates were positive for alpha, beta and epsilon toxins genes.

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1. Introduction

Clostridium perfringens (*C. perfringens*) is the most important clostridial pathogen of poultry. *C. perfringens* is a Gram-positive anaerobic spore-forming, rod-shaped bacterium (Baba*et al.,* 1997). *C. perfringens* is classified into 5 toxinogenic types (A, B, C, D, E), which are differentiated according to the production of four different major toxins (Alpha, Beta, Epsilon, Iota) (Songer and Meer, 1996). Type A produces alpha toxin, type B; beside alpha toxin it produces beta and epsilon, type C produces alpha and beta while both types D and E produce alpha toxin in combination with epsilon and iota, respectively (Quinn et al., 1994).

Necrotic enteritis (NE) in chickens is a globally important welfare and economic problem (Cooper et al., 2009). It was firstly reported by Parish (1961), as an enteric disease caused by *C. perfringens*. NE is a worldwide enterotoxaemic inpoultry disease caused by the alpha toxin-producing *C. perfringen* which can cause both clinical and subclinical disease in poultry (Engström et al., 2003; Williams, 2005) and the toxigenic strains were isolated from both diseased and apparently healthy chickens (Timbermont et al., 2009). The alpha toxin has been implicated in the destruction of mucosal tissue manifested as macroscopic lesions that are usually seen in jejunum and ileum but can also appear in duodenum of chicken (Rood, 1998). NE in poultry is characterized by severe necrosis of the small intestinal mucosa in the proximal jejunum region and it is associated with a high mortality rate (Long, 1973). The disease risk factors include concurrent coccidial infection and the dietary use of cereal grains high in non-starch polysaccharides such as wheat, barley, rye, and oats (Palliyeguru et al., 2010).

The colonies of *C. perfringens* described as smooth, round, glistening colonies, surrounded by an inner zone of complete haemolysis caused by the theta-toxin and an outer zone of incomplete haemolysis caused by the alpha-toxin (Quinn et al., 1994). The polymerase chain reaction assay (PCR) was used for detection of alpha toxigenic strains of *C. perfringens* (Engström et al., 2003; Baums et al., 2004).

The current study was designed to study the prevalence *C. perfringens* in broiler chickens in different poultry farms phenotypically and genotypically.

2. Material and methods

2.1. Chickens and laboratory animals

2.1.1. Broiler chickens

A total of 115 diseased broiler chickens of different ages (3-6weeks) from 15 farms in Beni-Suef and El-Menia Governorates were examined during the period from September 2014 up to September 2015. These diseased chickens showed clinical signs suspected to be NE which characterized by severe depression, decreased appetite, reluctance to move, diarrhea, and ruffled feathers.

2.1.2. Laboratory animals

Sixty white Swiss mice [2 mice for each isolate (*n*=29) and 2 mice as control] with an average weight of 20-25g were used for the detection of toxigenic isolates of *C. perfringens*. Moreover, 15 albino Guinea pigs with an average weight of 350-450g were used for detection of dermonecrotic reaction for typing of *C. perfringes* isolates (*n*=15).

2.2. Samples

A total of 230 samples were collected;115from small intestine and 115 from liver of scarified and freshly dead chickens (*n*=115) showed PM lesions of NE, then placed in sterile plastic bags, labeled and transported in ice box to the laboratory of Bacteriology, Mycology and Immunology department, Faculty of Veterinary Medicine, Beni-Suef University for bacteriological examinations.

2.3. Isolation and identification of *C. perfringens*

Collected samples were inoculated into freshly prepared boiled and suddenly cooled cooked meat broth (Oxoid). After an overnight incubation anaerobically (using Gaspak anaerobic jar) at 37°C, a loopful was streaked onto neomycin sulphate (150µg/ml) sheep blood agar plates and incubated anaerobically at 37°C for 24-48h. Suspected *C. perfringens* colonies were cultivated onto 2 plates of 10% sheep blood agar (for purification) and other 2 plates of egg yolk agar for detection of lecithinase activity (Nagler's reaction). One plate of both media was aerobically incubated and the other plate was incubated anaerobically. The colonies that grew only in anaerobic condition showing double zone of

haemolysis on blood agar and give Nagler's reaction on egg yolk agar were picked up and purified for identification tests (Ficken and Berkhoff, 1989). Based on microscopic appearance, colonial morphology, motility, hemolytic activity, gelatin hydrolysis, fermentation of glucose, lactose, sucrose, maltose, salicin and mannitol, indol test, urease production, litmus milk test and lecithinase production, identification of *C. perfringens* was done (Murray et al., 2003).

2.4. Detection and typing of toxigenic *C. perfringens* strains recovered from broiler chickens

The toxigenic isolates of *C. perfringens* were detected *in vitro* by Nagler's test. Meanwhile, they were detected *in vivo* by pathogenicity test in white Swiss mice according to Willis (1964) by intraperitoneal injection of 0.5 ml of the culture supernatant. The mice were observed for seven days for neurological symptoms or death.

The typing of toxigenic isolates of *C. perfringens* was conducted by dermonecrotic test in Guinea pigs (Sterne and Batty, 1975). Furthermore, PCR was used to type toxigenic isolates.

2.5. Molecular typing and characterization of the toxigenic *C. perfringens* by multiplex PCR

Multiplex-PCR was applied on 15 toxigenic isolates of *C. perfringens* using primers mixture of alpha, beta, epsilon and iota toxins genes (Table 1) to investigate genes encoding the four major toxins (Alpha, Beta, Epsilon, Iota) according to Yoo et al. (1997).

Table 1. Primers sequences and amplified products for targeted genes of *C. perfringens* isolates.

Primer		Primer Sequence	Amplified product	Reference
Alpha toxin	F	5'- *GTTGATAGCGGCAGGACATGTTAAG*- 3'	402 bp	Yoo et al. (1997)
	R	5'- *CATGTAGTCATCTGTTCCAGCATC*- 3'		
Beta toxin	F	5'- *ACTATACAGACAGATCATTCAACC*- 3'	236 bp	
	R	5'- *TTAGGAGCAGTTAGAACTACAGAC*- 3'		
Epsilon toxin	F	5'- *ACTGCAACTACTACTCATACTGTG*- 3'	541 bp	
	R	5'- *CTGGTGCCTTAATAGAAAGACTCC*- 3'		
Iota toxin	F	5'- *GCGATGAAAAGCCTACACCACTAC*- 3'	317 bp	
	R	5'- *GGTATATCCTCCACGCATATAGTC*- 3'		

3. Results

3.1. Identification of *C. perfringens* recovered from broiler chickens

Microscopic appearance showed large Gram positive rods with subterminal, oval and bulged spores. All isolates showed double zone of haemolysis on blood agar and gave Nagler's reaction on egg yolk agar, negative catalase, gelatin liquefaction, stormy fermentation as well as glucose, lactose, sucrose, and maltose fermentation.

3.2. Prevalence of *C. perfringens* in the examined broiler chickens

Out of 115 diseased broiler chickens, *C. perfringens* were recovered from 21 birds with a prevalence rate of 18.3%. Out of 230 total collected samples *C. perfringens* were recovered from 29 (12.6%) samples, of which 21 isolates were from intestine (*n*=115) with a prevalence rate of 18.3% while 8 isolates only were recovered from liver samples with a prevalence rate of 7% (Table 2).

3.2. Detection and typing of toxigenic *C. perfringens* strains recovered from broiler chickens

Out of 29 *C. perfringens* isolates, 15 (51.7%) isolates were toxigenic. White Swiss mice subjected to toxicity and pathogenicity tests showed nervous signs and death after 3 days post injection (Table 3).

Table 2. Prevalence *C. perfringens* in examined broiler chickens.

Source of sample	No. of examined samples	Positive isolation	
		No.	%
Intestine	115	21	18.3
Liver	115	8	7.0
Total	230	29	12.6

% was calculated according to No. of examined samples

C. perfringens typing revealed that *C. perfringens* type A was the most prevalent representing 13 isolates (86.7%) as they produced irregular areas of yellowish necrosis, lesion tend spread downwards (alpha toxin). Meanwhile the other 2 isolates (13.3%) belonged to *C. perfringens* type B as they produced purplish yellow hemorrhagic necrosis (beta toxin).

3.3. Molecular typing and characterization of the toxigenic *C. perfringens* by multiplex PCR:

Multiplex-PCR was applied on 15 toxigenic isolates of *C. perfringens* to investigate genes encoding the four major toxins (Alpha, Beta, Epsilon, Iota). All *C. perfringens* type A (*n*=13) were found to contain alpha toxin gene only while the 2 *C. perfringens* type B isolates contained alpha, beta and epsilon toxins genes. On the other hand, none of them contained iota toxin gene (Fig. 1).

Table 3. The prevalence and typing of toxigenic *C. perfringens* recovered from broiler chickens.

Number of *C. perfringens* isolates	Toxigenic						Non toxigenic	
	Type A		Type B		Total toxigenic			
	No.	%	No.	%	No.	%	No.	%
29	13	86.7	2	13.3	15	51.7	14	48.3

% was calculated according to No. of examined samples

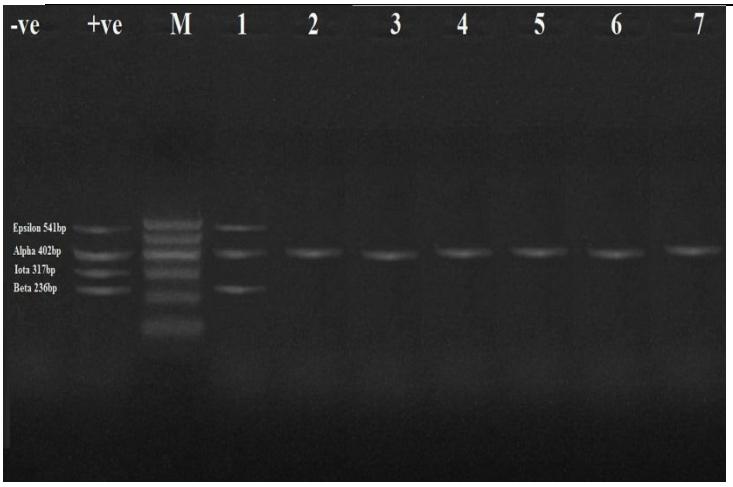

Fig. 1. The multiplex-PCR showing the detection of toxins encoding genes of *C. perfringens* isolates. M: DNA size marker (100-600 bp). Lane 1. *C. perfringens* type B. Lane 3-7. *C. perfringens* type A.

4. Discussion

NE is considered the most clinically dramatic bacterial enteric disease of chickens characterized by sudden onset, high mortality, loss of growth efficiency and negatively impact profitability so, NE are estimated to cost the poultry industry more than couple of billion annually worldwide. NE affects broiler chickens aged between 2-6 weeks (Cooper and Songer, 2010). Mortality may reach 1% per day and total mortalities reach 10-40% (McDevit et al., 2006). Two forms of the disease are described, clinical and subclinical (Kaldhusdal and Hofshagen, 1992). Clinical signs include depression, dehydration, diarrhea, ruffled feathers and lower feed intake (Songer, 1996). The gross lesions of the small intestine range from thin and friable walls to frank and extensive necrotic lesions (Cooper and Songer, 2010). The subclinical form presents as poor performance (reduced growth, reduced feed efficiency) without mortality. This form of the disease can be diagnosed by reduced feed conversion, by gross lesions in the small intestine and by bacteriology (Kaldhusdal and Hofshagen, 1992). Most of the economic losses due to NE are related to the subclinical form and the high cost of preventing the disease with antibiotics.

C. perfringens is a ubiquitous bacterium were found in the poultry farms and its surrounding places, Craven et al. (2001) also, it is often found in the intestinal tract of healthy birds but it can cause outbreaks of disease in many species of poultry and especially in broiler and turkey flocks, (Engström et al., 2003). *C. perfringens* in poultry constitutes a risk for transmission to humans through the food chain. Colonization of poultry by clostridia is a very early event in the animals' life and can be transmitted within the broiler chicken operation.

Many authors reported that gross lesions are typically restricted to small intestine (Bernier et al., 1974; Porter, 1998) and the small intestine is usually distended with gas and contains a foul-smelling dark brown, bile-stained fluid (Ficken and Wages, 1997) but other lesions can also occur in other organs such as liver (Timbermont et al., 2011). Intestinal damage may allow *C. perfringens* and their toxins access to portal circulation and biliary ducts, and colonization in the liver by high numbers of resulting in cholangiohepatitis (Lovland and Kaldhusdal, 1999). Liver lesions associated with NE usually manifest as pale enlarged livers with small, focal red or white foci (Ficken and Wages, 1997; Sasaki et al., 2000). The development of liver lesions is poorly understood.

In the current study, the prevalence rate of *C. perfringens* isolation from diseased broiler chickens was 18.3%. The recovery rate from the total collected samples was 12.6%. The recovery rates from both intestine and liver samples were 18.3% and 7%, respectively (Table 2). The present recovery rate was lower than that obtained by EI-Jakee et al. (2013) who found that the prevalence rate of *C. perfringens* recovery from diseased chickens was 75% of samples and the recovery rates from intestine and liver samples were 100% and 50%, respectively.

As *C. perfringens* produces 4 major toxins (Alpha, Beta, Epsilon, Iota) according which *C. perfringens* is classified and differentiated into 5 toxinogenic types (A, B, C, D, E) (Songer and Meer, 1996). Toxigenic characters and type of *C. perfringens* could be detected either *in vivo* by using pathogenicity tests in mice (Willis, 1964) or *in vitro* by Nagler's test (Smith and Holdeman, 1968). Also, dermonecrotic test in Guinea pigs (Sterne and Batty 1975) and PCR (Yoo et al., 1997) have been used for typing of toxigenic isolates of *C. perfringens*.

It has been fund that 15 (51.7%) out of 29 *C. perfringens* isolates were toxigenic (Table 3). Such findings were slightly lower than those obtained by EI-Jakee et al. (2013) who found that 68.2% of recovered *C. perfringens* isolates were toxin-producing. The majority of toxigenic isolates were type A with a prevalence of 86.7%, while only 13.3% belonged to type B. This finding run hand to hand with those obtained by El-Seedy (1990), Songer and Meer (1996), Xiao et al. (2012), and EI-Jakee et al. (2013). Moreover, Hatakka and Pakkala (2003) reported that *C. perfringens* type A is the most prevalent cause of food poisonings in the industrialized world.

PCR was established to replace animal testing and to reduce cost and time (EI-Jakee et al., 2010). A multiplex-PCR was conducted on the toxigenic isolates of *C. perfringens* (n=15) using 4 primers for genes encoding major toxins alpha, beta, epsilon and iota. The current investigation detected that type A isolates (n=13) contained alpha toxin gene only while type B isolates contained alpha, beta and epsilon toxins genes (Fig. 1). Such findings confirmed those obtained by previous literature (Yoo et al., 1997; EI-Jakee et al., 2013).

5. Conclusion

NE caused by *C. perfringens* is considered the most important enteric diseases in poultry causing severe economic losses and mortalities worldwide. The pathogenicity of *C. perfringens* is closely related to the production of major lethal toxins (alpha, beta, epsilon and iota). *C. perfringens* type A is the most prevalent. PCR was established to replace animal testing and to reduce cost and time.

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