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Original Research Article

The incidence of *C. perfringens* in chickens in different seasons and Governorates in Egypt

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ABSTRACT

A total of 247 intestinal samples from freshly dead broiler and layer chickens were collected from 150 farms in Giza, Sharkia, Qalubia, El-Behera, Daqahlia and Cairo governorates in different seasons. These samples showed different degrees of intestinal lesions from apparently normal to sever necrosis with ulcerations. *Clostridium perfringens* was isolated from 138 samples with incidence of 55.9%. The incidence of NE was higher in spring and summer than winter and autumn. According to polymerase chain reaction and intradermal injection of guinea pig all isolates were *Clostridium perfringens* type A. *In vitro* antibiotic sensitivity tests made for 15 isolates and most of the examined isolates were highly sensitive to amoxicillin, ampicillin, florfenicol, penicillin and metronidazole. Three isolates showed resistance to most of antibiotics were used. Effect of piperazine salt on antibiotic resistance of *C. perfringens* isolate was studied in this work.

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1. Introduction

Necrotic enteritis (NE) is one of the most important enteric diseases in poultry and is a high cost to the industry worldwide (Shojadoost et al., 2012). It is caused by *C. perfringens* type A, producing the alpha toxin, and to a lesser extent type C, producing both alpha toxin and beta toxin (Van Immerseel et al. (2004). The key risk factor for development of NE is an intestinal environment that favours the growth of the organism. Birds rose on high energy, protein-rich, wheat or barley based diets experience NE at a rate up to ten times greater than do birds on maize based diets (Cooper and Songer, 2009).

The presence of aflatoxin and/or coccidia vaccine (Hamouda et al., 2011), also act as predisposing factor for NE. Natural outbreaks of NE have been reported in 2 to 5 weeks old broiler chickens raised on litter (Bains, 1968). Mortality can reach 1% per day with a total mortality of 10-40% (McDevit et al., 2006). Necrotic enteritis can be controlled prophylactically with certain antimicrobial growth promoters (e.g. bacitracin, the oligosaccharide avilamycin, the streptogramin virginiamycin and the glycopeptide avoparcin) and therapeutically by treating with antimicrobials such as amoxicillin or lincomycin (Prescott et al., 1978; George et al., 1982; Hamdy et al., 1983; Stutz and Lawton, 1984; Elwinger et al., 1992, 1998; Ficken and Wages, 1997; Bolder et al., 1999; Vissiennon et al., 2000).

The present study was designed for isolation and identification of *C. perfringens* from diseased broiler

and layer chickens obtained from different governorates in Egypt in relation to seasons. Typing the obtained *C. perfringens* isolates using multiplex PCR and the effect of piperazine salt on antibiotic-resistance of *C. perfringens* isolate were assessed.

2. Materials and methods

2.1. Samples

A total of 247 intestinal samples representing 150 flocks were collected from diseased chickens with clinical symptoms of necrotic enteritis (Table 1).

2.2. Isolation and identification of *C. perfringens*

Intestinal tissue samples were inoculated into tubes of cooked meat medium and incubated anaerobically for 24 h at 37° C (Willis, 1977). A loopful of inoculated fluid media was streaked onto 10% sheep blood agar with neomycin sulphate (200 µg /ml) (Smith and Holdeman, 1968; Cruickshank et al., 1975). *C. perfringens* colonies are flat olive color with double zone of haemolysis as shown in Fig. 1 (Vaikosen and Muller, 2001). Microscopic examination showed non motile Gram positive bacilli. Suspected purified isolates were obtained and identified according to the schemes of Koneman et al. (1992) which showed catalase and indole negative, nitrate reduction and lecithinase activity on egg yolk agar positive (Fig. 2) (Cruickshank et al., 1975).

2.3. Polymerase chain reaction (PCR)

Oligonucleotide primers (100 pmol) for the four toxin genes (alpha, beta, epsilon and iota) of *C. perfringens* in a concentration of 20 pmol. (Yoo et al., 1997) (Table 2).

Extraction of DNA was done by Gena JET Viral DNA and RNA Purification Kit. The amplification and cycling protocol were done by using PCR 1.1x Ready-mix TM Master Mix (Thermo Scientific). DNA samples were amplified in a total of 50 µl of the following reaction mixture: 25 µl of PCR master mix, 1µl of forward primer for each toxin gene (alpha, beta, epsilon and iota), 1µl of reverse primer for each toxin gene (alpha, beta, epsilon and iota), 12 µl of PCR grade water, 5 µl of the template. PCR cycling program designed as shown in (Table 3) (Yoo et al., 1997). Detection of PCR products were analyzed by electrophoresis on 1.5% agarose gel in the presence of 100 bp DNA ladder supplied from QIAGEN (Sambrook et al. 1989; Augustynowicz et al., 2000).

2.4. Pathogenicity and typing of *C. perfringens* isolates to guinea pigs

Albino guinea pigs with an average body weight of about 350-450 g were experimentally inoculated with 0.2 ml production medium intradermally; the medium was inoculated with 5 ml of 24 hours cooked meat cultures of toxigenic strains of *C. perfringens* and incubated in water bath at 37° C for 6 h. After six hours of incubation, half of the culture was syphonized and centrifuged at 3000 rpm for 20 minutes. The clear supernatant fluid was divided into 4 portions; each portion was neutralized with 0.1 ml of type "A" diagnostic antiserum, type "B" diagnostic antiserum, type "C" diagnostic antiserum, respectively. The fourth portion (0.3 ml) was added to 0.1 ml of saline as control. Another half was incubated at 37° C for 48 h anaerobically and centrifuged at 3000 rpm for 20 minutes. The supernatant incubated at 37°C for an hour and neutralized by type D and E diagnostic antisera in the same ratio (3:1) toxin antitoxin respectively (Bullen, 1952; Willis, 1964). The injected guinea pigs were kept under observation for 48-72 hours. The results were interpreted by the colour degree of the dermonecrotic reaction, where, in type "A" an irregular area of yellowish necrosis (alpha toxin) (Fig. 3), type “B” purplish yellow hemorrhagic necrosis (beta toxin), type “C” the reaction was intensively purplish than that produced by type "B" filtrates, type “D” circular white necrosis (epsilon toxin), type “E” a reaction similar to that elicited by type "D" but with irregular outline (iota toxin) (Stern and Batty, 1975).

2.5. Antibiotic sensitivity testing

Antibiotic sensitivity testing of *C. perfringens* isolates was done using disc diffusion test (Oxoid, UK). The antibiotics used were ampicillin, amoxicillin, cefepime, cefoperazon, metronidazole, florfenicol, lincomycin, ceprofloxacin, naldixic, doxcycilin, colstin, gentamycin, penicillin G, bacitracin, neomycin and enrofloxacin. All isolates were incubated overnight in 10% neomycin sheep blood agar, then cultures were suspended in saline to an optical density equivalent to that of McFarland 0.5 standards; each isolate was then inoculated into Mueller Hinton agar medium (Oxoid, UK), after 15 minutes, antimicrobial discs were applied. Plates were incubated anaerobically at 37°C for 24 h and the interpretation was performed according to Martel et al. (2004).

2.6. Effect of piperazine salt on antibiotic resistance of *C. perfringens* isolate

0.2 of overnight broth culture of resistant strain was inoculated into 50 mg/ml of piperazine citrate diluted in 2.0 ml of nutrient broth, then incubated on shaker for 6-8 h at 38ºC anaerobically. 10^6 dilution of each culture were plated on nutrient agar and then the sensitivity plated were floated, after 15-30 min; disks constituting resistance patterns were placed and incubated at 37ºC for 24 h, then record the result.

3. Results and discussion

Necrotic enteritis (NE) is the most clinically dramatic enteric bacterial disease of poultry induced by *C. perfringens* (Tolooe et al., 2011). In this study, 247 intestinal chicken samples were examined for *C. perfringens* by traditional isolation and identification techniques and polymerase chain reaction. After examination was found that 138/247 samples were positive for *C. perfringens* with incidence of 55.9% (Table 4). Such result matched with Abd El-wahab (2002), Mahmoud et al. (2008) and Samir (2013) where they isolated *C. perfringens* in rates of 54.4% and 54.1% and 57.9% respectively. In our work we found that the incidence of *C.perfringens* in layer 70.3% is higher than in broiler 53.3% while Abd-El All and Maysa (2014) found that *C. perfringens* was more frequent in broilers (38.7%) than layers (30.3%), Ahmed, (2010) isolated *C. perfringens* from broiler farms in Egypt with an infection rate of 43.38%.

Currently, samples were collected in different seasons. After isolation, a high incidence in spring (73.75%) rather than in summer and winter (59.2% and 50% respectively), and a low incidence in autumn (25.6%) was found (Table 5). This result is closed to long (1973) who detected a high incidence in summer and autumn. Oppositely, Kaldhusdal and Skjerve (1996) and Ahmed (2010) said that the highest incidence of *C. perfringens* was recorded in winter and a lower incidence in summer and autumn.

In the present work, samples were collected from six governorates (Giza, Sharkia, Qalubia, El-Behira, Daqhlia and Cairo), each governorate represented with 25 chicken flocks (Table 6). The high rate of isolation of *C.perfringens* was found in El-Behera governorate (65.3%) which is similar to those obtained by Abd El-Hamid et al. (2015) who said that the isolation rate of *C.perfringens* in El-Behera governorate was 65.1%. Sharkia governorate came after El-Behera with an isolation rate of 63.2%. This result was corresponding to what given by Ahmed (2010) and Sally (2010) who detected that the higher incidence of *C.perfringens* was recorded in Sharkia governorate. The other governorates reported incidence rates of 61.7, 55.2, 45.9 and 34.4% in Qalubia, Giza, Cairo and Daqhlia, respectively.

Multiplex polymerase chain reaction detected that 28 of 47 samples were *C. perfringens* type A (Alpha toxin) with band at 402 bp (Fig. 4) and all were negative for Beta, Iota and Epsilon toxins. This result was in relation with data that recorded by Crespo et al. (2007), Das et al. (2008), Ahmed (2010) and Sarker et al. (2013) who detected only *C. perfringens* type A in the recovered isolates. Samir (2013) proved that the main predominant type in the obtained isolates in chickens farms in Egypt was type A with lesser extent to type C. Dermo necrotic test was done to the rest samples in albino guinea pigs and also all samples were *C. perfringens* type A only, with irregular area of yellowish necrosis and the lesion tends to spread downward (alpha toxin) (Table 5) (Stern and Batty, 1975).

In vitro antibiotic sensitivity of *C. perfringens* isolates to 16 different antibiotics showed that most of isolates were highly sensitive to ampicillin, metronidazole, amoxicillin, cefoperazon, penicillin G and florfenicol (Table 7). These results were in sync with Abd-El Gwad and Abd El-Kader (2001), Llanco et al. (2012) who showed that the examined isolates were highly sensitive to ampicillin, ciprofloxacin, amoxicillin, enrofloxacin, colstin and lincomycin. Abd El-Hamid et al. (2015) showed that isolates were sensitive to amoxicillin, metronidazole, and florfenicol, and with intermediate sensitivity to doxycycline, cefepime, bacitracin, enrofloxacin and ciprofloxacin. A low sensitivity to naldixic, lincomycin, colstin and gentamycin was found. This agreed with Osman and Elhariri (2013) who determined that isolates were resistant to gentamicin, streptomycin, oxolinic acid, lincomycin, erythromycin and spiramycin. Neomycin showed resistant to *C. perfringens* so it was used in *C. perfringens* isolation where Abd-El Gwad and Abd El-Kader (2001) said that isolates didn't affect with neomycin, gentamycin and streptomycin.

Currently, three isolate showed a resistance to most of antimicrobials, and were treated with

piperazine citrate (50 mg) to study the effect of piperazine in changing resistant isolates to sensitive isolates (Table 8). After treatment with piperazine citrate, the study reported that isolate A became sensitive to penicillin G, bacitracin and enrofloxacin, and the resistance of other antibiotics had no effect. Isolate B became highly sensitive to doxycycline, amoxicillin, colstin, gentamycin, cefepime, ceprofloxacin and enrofloxacin and less sensitive to ciprofloxacin, enrofloxacin while naldixic, cefoperazon, penicillin, bacitracin and florfenicol not affected. Isolate C showed an intermediate sensitivity to metronidazole, doxycycline, colstin, gentamycin, cefepime, penicillin, bacitracin and ceprofloxacin while isolate C remained resistant to ampicillin, naldixic, amoxicillin, lincomycin, cefoperazon, enrofloxacin and florfenicol and did not affected by piperazine citrate.

From this trial we found that piperazine citrate had an effect on resistant *C. perfringens* isolates and made it sensitive to some antibiotics but also resistance to some antibiotics didn't affected.

Table 1. Number of examined samples from different flocks.

Chickens	No. of samples	No. of flocks
Broilers	210	128
Layers	37	22
Total	247	150

Table 2. Primers for the four toxin genes of *C. perfringens* used in multiplex PCR.

Nucleotide sequence	Primer name and direction
CpA(402bp):	
Forward	**5`**GTT GAT AGC GCA GGA CAT GTT AAG **3`**
Reverse	**5`** CAT GTA GTC ATC TGT TCC AGC ATC **3`**
CpB(236bp):	
Forward	**5`**ACTATACAGACAGATCATTCAACC **3`**
Reverse	**5`**TTAGGAGCAGTTAGAACTACAGAC **3`**
CpE(541bp):	
Forward	**5`** ACT GCA ACT ACT ACT CAT ACT GTG **3`**
Reverse	**5`** CTG GTG CCT TAA TAG AAA GAC TCC **3`**
CpI (317bp):	
Forward	**5`**GCGATGAAAAGCCTACACCACTAC **3`**
Reverse	**5`**GGTATATCCTCCACGCATATAGTC **3`**

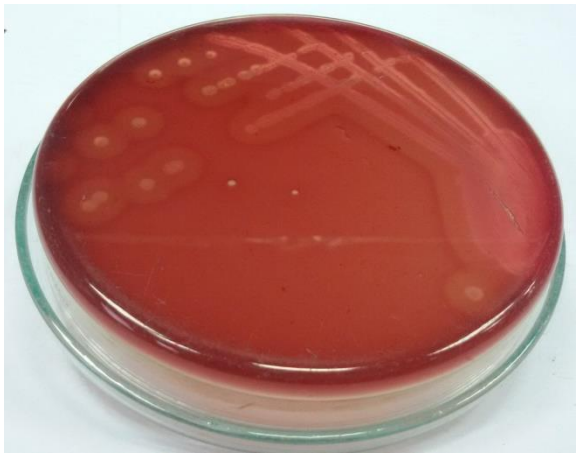

Fig 1. *C. perfringens* on 5% sheep blood agar with neomycin showing a double zone of hemolysis.

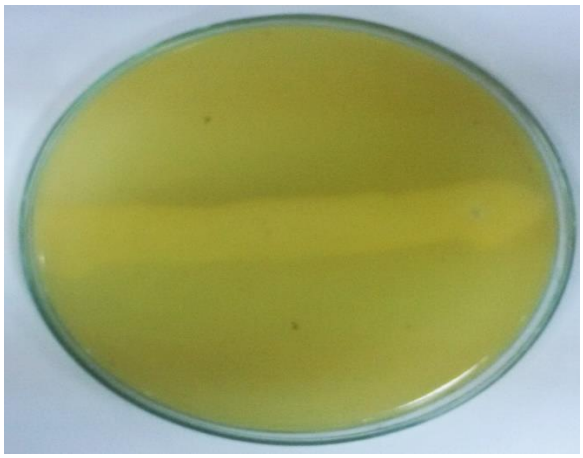

Fig. 2. Toxigenic *C. perfringens* isolate on egg yolk agar medium.

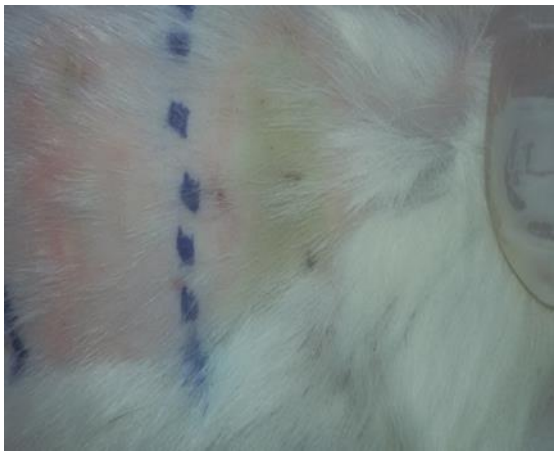

Fig. 3. Necrotic dermatitis in guinea pig showed *C. perfringens* type A.

Table 3. PCR cycling protocol for the four toxin genes of *C. perfringens* using a multiplex PCR

Amplified DNA	PCR condition
C. perfringens toxin genes (alpha, beta, epsilon and iota)	A) Initial denaturation: 95°c for 5 min B) Actual cycles: 30cycles of 1- 94°c for 1 min 2- 55°c for 1 min 3- 72°c for 1 min C) final extension: 72 °c for 3 min.

Table 4. Incidence of *C.perfringens* in examined samples chicken.

Type of chicken	No. of samples	Positive samples		Negative samples	
		No.	%	No.	%
Broiler	210	112	53.3	98	46.7
layer	37	26	70.3	11	29.7
Total	247	138	55.9	109	44.1

Table 5. Incidence of *C.perfringens* in different seasons.

Season	No. of samples	Positive samples		Negative samples	
		No.	%	No.	%
Winter	48	24	50	24	50
Spring	80	59	73.75	21	26.25
Summer	76	45	59.2	31	40.8
Autumn	43	11	25.6	32	74.4
Total	247	138	55.9	109	44.1

Table 6. Incidence of *C.perfringens* in different governorates.

Governorate	No. of farm	No. of samples	No. of positive samples	Percentage
Giza	25	38	21	55.2
Sharkia	25	57	36	63.2
Qalubia	25	34	21	61.7
El-Behera	25	49	32	65.3
Daqhlia	25	32	11	34.4
Cairo	25	37	17	45.9
Total	150	247	138	55.9

Table 7. *In vitro* antibiotic sensitivity of *C. perfringens* isolates.

Antibiotic	Concentration	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ampicillin	10 µg	+++	+++	R	+	R	+++	+	+	R	++	+++	+	+	+++	+++
Metronidazole	5 µg	+++	++	R	R	R	++	++	++	R	++	+++	+	++	++	++
Naldixic	30 µg	+	R	R	R	R	R	++	++	R	++	+	+	++	R	R
Doxycycline	30 µg	++	++	+	+	+	++	+	++	R	++	++	+	+	++	++
Amoxicillin	10 µg	+++	+++	R	+	R	+++	+	+	R	+	+++	++	+	+++	+++
Colstin	10 µg	+	+	R	+	+	R	R	+	R	+	R	+	+	+	++
Lincomycin	2 µg	++	+	R	R	R	R	R	R	R	++	++	++	++	+	+
Gentamycin	10 µg	++	R	R	R	+	R	R	R	R	+	++	+	+	R	+
Cefoperazon	75 µg	+++	+	R	++	R	+	+	R	R	++	+++	++	+	+	+
Cefepime	30 µg	++	++	R	++	R	++	++	R	R	+++	++	++	++	++	+
Penicillin G	10 µg	++	+++	+++	R	R	+++	+++	++	R	++	+++	+	++	+++	+++
Bacitracin	10 µg	++	++	++	R	R	++	R	++	R	++	++	+	+	+++	++
Enrofloxacin	5 µg	+	++	+	R	R	++	++	+	R	++	+	+	++	++	++
Florfenicol	10 µg	+++	++	+++	+	R	++	R	++	+	++	+++	+	+	++	++
Ciprofloxacin	5 µg	++	++	+	R	R	++	++	++	R	+++	+	++	++	++	++
Neomycin	30 µg	+	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Antibiotic sensitivity for C. Perfringens type A isolates. (+), Low sensitivity; (++), Moderate sensitivity; (+++), High sensitivity, (R) resistant.

Table 8. Effect of piprazine salts in different concentration on antibiotic resistance for *Clostridium* sp.

Sample	A		B		C	
AB	Control	Treated	Control	Treated	Control	Treated
Ampicillin 10 (Amp)	R	R	R	+++	R	R
Metronidazole 5 (MTZ)	R	R	R	++	R	++
Naldixic 30 (Na)	R	R	R	R	R	R
Doxycycline 30 (DO)	R	R	+	+++	+	++
Amoxicillin 10 (Aml)	R	R	R	+++	R	R
Colstin 10 (CT)	R	R	R	+++	+	+
Lincomycin 2 (L)	R	R	R	+	R	R
Gentamycin 10 (CN)	R	R	R	++	+	+
Cefoperazon 75 (CFP)	R	R	R	R	R	R
Cefepime 30 (FEP)	R	R	R	++	R	++
Penicillin G 10 (P)	+	++	+++	+++	R	++
Bacitracin (B)	R	+++	++	++	R	+
Enrofloxacin 5 (ENR)	R	+++	+	+++	R	R
Florfenicol 10 (F)	+	++	+++	+++	R	R
Ceprofloxacin 5 (CIP)	R	+++	+	+++	R	++

(+), Low sensitivity; (++), Moderate sensitivity; (+++), High sensitivity, (R) resistant.

Fig. 4. Five examined field isolates were identified as *C. perfringens* type A (alpha toxin) giving a characteristic band at 402 bp

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