Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

Prevalence of *Clostridium perfringens* infection and virulence genes detection in broiler chickens

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ARTICLE INFO

Keywords

Broiler chickens
C. perfringens
cpa gene
net B gene

Received 07/02/2019
Accepted 19/03/2019
Available On-Line 18/07/2020

ABSTRACT

Clostridium perfringens (C. perfringens) produces toxins that cause necrotic enteritis in broiler chickens resulting in sever losses in poultry industry. This study aimed to investigate the prevalence of *C. perfringens* in broiler chickens in Toukh, Qaliubiya governorate, Egypt. A total of 100 broilers (10-40 days old) were collected from Toukh, Qaliubiya in 2017. The samples were collected from liver and intestine of both diseased and apparently healthy broilers. All samples were subjected to bacteriological examination. *C. perfringens* was isolated from (70%) and (22%) of diseased and apparently healthy broilers, respectively. The isolates were typed by dermo-necrotic test in Guinea pig and PCR. The results of dermonecrotic test from diseased chickens showed that 40 (65.6%) of the isolates from diseased chickens were type A and 8 (13.1%) were type D. But the isolates from the apparently healthy chickens were (9) type A and (6) type D. Typing of the nine *C. perfringens* isolates (from diseased broilers) by PCR for toxin associated genes (*cpa*, *cpb* and *etx*) showed that 8 strains were positive for *cpa* gene (typed as type A). Detection of the virulence genes by PCR showed, *pfo*S and *col*A genes were in eight isolates but *netB* gene was detected in three isolates only. *C. perfringens* showed high prevalence among diseased broiler chickens with high isolation rate from intestinal sample than hepatic samples. Moreover, high percentage of *C. perfringens* is toxigenic type A in case of diseased or apparently healthy broiler chickens. The *net*B gene seems to be unnecessary for inducing necrotic enteritis in broiler chickens.

1. INTRODUCTION

Clostridium perfringens (*C. perfringens*) is a gram positive rod-shaped, anaerobic, spore-forming bacterium of the genus *Clostridium*. Necrotic enteritis (NE) caused by *C. perfringens* type A, C and D. NE is an enterotoxaemia of poultry which lead to an important economic losses in poultry production. This economic effect is due to decrease growth rate, feed conversion and increase condemnation rates in broiler due to hepatitis (Van immerseel et al., 2004). *C. perfringens* present in intestinal tract and produces a toxin leads to necrotic enteritis. Although, small number of organisms are found in the intestinal tract, they don't cause disease under normal condition as it is overcome by a considerable population of "good bacteria" (Vijay and Clerk, 2007).

C. perfringens could cause impaired production performance in chicken. The intestinal function is to achieve optimal feed conversion. There are some factors that reduce feed conversion as the necrotic gut lesion and abnormal *clostridium* dominance in gut micro flora together with *clostridium* toxin all this reduced productivity (Lovland and Kaldhusdal, 2001).

Immunosuppression predisposes animals to NE, immunosuppression likely alter the intestinal environment and the intestinal microbial population (Timberment et al., 2011).

Necrotic enteritis prevention is usually associated with management practices that minimize the effects of the predisposing factors that contribute to disease development. Reducing the inclusion of dietary ingredients that may lead to NE such as fish meal, oats, barley and rye has been acknowledged as natural solution in decreasing NE (Cooper and Songer, 2009).

Clinical necrotic enteritis occurs when *C. perfringens* proliferates to high number in the small intestine and produce extracellular toxins which damage the intestine (Keyburn et al., 2006). The major toxin believed to be involved is the alpha toxin, but its important role in the disease process is not completely understood.

The sings of necrotic enteritis in chicken are inappetence, marked depression with grayish diarrhea, unable to walk, voiding dually brownish to bloody stained diarrhea in well fleshed and nourished older broiler chicken and ruffled feather. On postmortem examination the lesions are restricted enteritis in small intestine of different degree. Intestine is ballooned with thickened wall. Necrosis in intestinal mucosa. The intestinal lumen filled with bloody stained ingest or blood (darkly stained) in severe cases. Liver is congested with areas of whitish to hemorrhagic necrosis and distended gall bladder (Ehab, 2007).

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Clostridium perfringens strains are classified into five types (A-E) on the basis of their ability to produce major lethal toxins. All types of *C. perfringens* produce alpha toxin. In addition, type B produces beta and epsilon toxin, type C produces beta toxin, type D isolates produce epsilon toxin and type E produces iota toxin (Hatheway, 1990).

The pathogenicity of *C. perfringens* is associated with several toxins; alpha, beta and epsilon toxins are the major toxic substances produced by the organism. These toxins encoded by *cpa, cpb* and *etx* genes, respectively. Genotyping of *C. perfringens* is done by using specific primers in PCR for detection of toxin genes (*cpa, cpb, etx*). This method is a powerful, rapid and confirmatory technique for typing of *C. perfringens* isolates.

Before the net B toxin was identified as the major toxin in necrotic enteritis in broiler, alpha toxin was believed to be crucial and thus multiple studies used alpha toxin derivatives as vaccine antigen.

The aim of the present study was to verify the prevalence of *C. perfringens* causing necrotic enteritis in broiler chickens and to study the toxin type of *C. perfringens* isolates with special reference to the virulence genes.

2. MATERIAL AND METHODS

2.1. Sampling:

A total of 100 broilers (10-40 days old) were collected from Toukh, Qaliubiya in 2017, of which (50) were sufferings from diarrhea and (50) were apparently healthy. The samples were collected from liver and intestine of both diseased and apparently healthy broilers. The collected 200 samples were kept under sterile conditions and were brought to the laboratory in an ice box without delay for bacteriological examination.

2.2. Isolation and identification of C. perfringens

Each sample was inoculated separately in cooked meat broth (Oxoid) and incubated anaerobically at 37 °C for 24 hrs (Quinn et al., 2002). A loopful from the broth culture was streaked directly onto 200 mg/ml neomycin sulphate sheep blood agar plates (Oxoid) and incubated anaerobically (Carter and Cole, 1990). The isolated colonies were identified by the cultural, morphological and biochemical characters (lecithinase, Catalase, indol, gelatin liquefaction and sugar fermentation test (Koneman et al., 1992; Macfaddin, 2002).

2.3. Typing of C. Perfringens isolates:

Clostridium perfringens isolates (n=70) from diseased broiler were typed by two different methods. Sixty-one isolates were typed by the dermonecrotic test in *Guienea pig* (*G. pig*) and nine randomly isolates were typed by PCR (Genotyping).

All the isolates (n= 22) from apparently healthy broiler were typed by the dermo-necrotic test in *G. pig* (Quinn et al., 2002).

2.4. Clostridium perfringens typing with detection of some virulence genes by PCR:

Genomic DNA was extracted from isolated strains using QIAamp DNA Mini Kit (Cat. No. 51304, Qiagen). PCR amplification of the genomic DNA was performed using Emerald Amp GT PCR master-mix (Takara) Code No.RR310A. Briefly, PCR program was done by initial denaturation at 94 °C for 2 min, 35 cycle of denature at 94 °C for 15 sec. annealing (55 °C for *col*A and -52 °C for *pfo*S) for 30 sec. and final extension at 68 °C for 1 min. The primers used in the PCR are shown in table (1). PCR products were separated by electrophoresis in 1.5% agarose gel. Bands were visualized by ethidium bromide staining (Sambrook et al., 1989)

2.5. Antibiotic sensitivity test:

C. perfringens isolates (from diseased broilers) were tested for their resistance to the following 15 antibiotics (Oxoid); amoxicillin (AMC) 30 µg, ampicillin (AMP) 10 µg, ampiclox (AX) 30 µg, penicillin (P)10 µg, cefadroxil (CDX) 30 µg, streptomycin (S) 10 µg, tetracycline (TE) 30 µg, cloxacillin (CX) 1 µg, mupirocin (MUP) 5 µg, cefprime (FEP) 30 µg, cefrodoxim (CRD) 30 µg, erythrocine (E) 15 µg,oxytetracycline (OT) 30 µg, max fur (EFT) 30 µg and epicoflocin (OFX) 5 µg. The degree of sensitivity was interpreted according to NCCLS (2002) and NCCLS (2004).

Table 1 The PCR primers used for detection of virulence genes and toxin genes of *C. perfringens*

Target genes		Primer Sequence5'-3'	Reference	Amplicon size bp
Virulence genes Uniplex PCR	*col*A	F-TAG GAA CAA AGG CGC AAG AT R-TTC TCC TTG TCC CCA CAT TC	Alejandro et al., 2006	924
	*pfo*S	F-CGG GTA TAG GCA TAC AAA AGG A R-GTG CAG TTG CAA CCA CTG TT		1090
	*net*B	F-GCTGGTGCTGGAATAAATGC RTCGCCATTGAGTAGTTTCCC	Anthony et al., 2010	383
Genotyping genes Multi-plex PCR	*cpa*	F-GCTAATGTTACTGCCGTTGA R-CCTCTGATACATCGTGAAG	Yoo et al.,1997	324
	cpb	F-GCGAATATGCTGAATCATCTA R-GCAGGAACATTAGTATATCTTC	Yoo et al., 1997	196
	etx	F-GCGGTGATATCCATCTATTC R-CCACTTACTTGTCCTACTAAC	Yoo et al., 1997	665

3. RESULTS

3.1. Isolation and identification of *C. perfringens*

The isolated colonies showed a gram-positive anaerobic rods, sporulated, catalase negative, characterized by double zone of haemolysis on blood agar surrounding the colonies and cause stormy fermentation reaction in litmus milk media indicating *C. perfringens*.

3.2. Prevalence of *C. perfringens* in broiler chickens:

Out of 100 samples from diseased broiler (50 intestines and 50 livers), 70 C. *perfringens* (42 from intestine, 28 from liver) were isolated with prevalence (70%). Out of 100 samples from apparently healthy broilers (50 intestines and 50 livers), 22 *C. perfringens* (12 from intestine, 10 from liver) were isolated with prevalence (22%).

3.3. Lecithinase activity of *C. perfringens* isolates from diseased and apparently healthy broiler:
Lecithinase activity was detected in 56 out of 70 *C. perfringens* (80%) isolates from diseased broiler characterized by an opalescence surrounding the colonies which inhibited *by C. perfringens* type A antitoxic sera. Lecithinase activity was detected in 14 out of 22 *C. perfringens* isolates (63.6%) from apparently healthy broiler.

3.4. Typing by the dermonecrotic test:
Typing of (61) isolates from diseased broiler using intradermal inoculation test in G. pig showed that 40 isolates (65.6%) were of type A and 8 isolates (13.1%) were of type D. On the other hand, typing of (22) *C. perfringens* isolates from apparently healthy broiler showed that 9 isolates were of type A and 6 isolates were type D (40.9% and 21.3%, respectively). Non-toxigenic strain were 13 (21.3 %) and 7(21.8%) for diseased and apparently healthy broiler, respectively.

3.5. Typing of *C. perfringens* isolates using PCR (Genotyping):
Typing of nine *C. perfringens* isolates (diseased broilers) by multiplex PCR for alpha, beta and epsilon toxin genes (*cpa* ,*cpb*, *etx*) showed that eight isolates were positive only for *cpa* gene. This indicated that eight isolates were *C. perfringens* type A (fig.1).

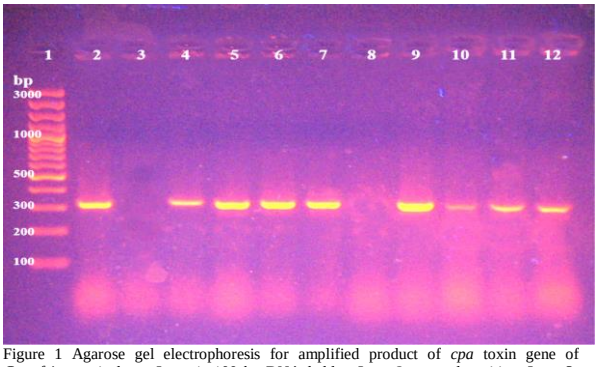

Figure 1 Agarose gel electrophoresis for amplified product of *cpa* toxin gene of *C.perfringens* isolates. Lane 1: 100 bp DNA ladder. Lane 2: control positive. Lane 3: control negative. Lane (4-7 & 9-12) : positive isolates for *cpa* gene which amplified at 324 bp. Lane 8: negative for *cpa* gene.

3.6. Detection of the virulence genes in *C. perfringens* isolates by PCR :
Out of the nine isolates of *C. perfringens* (diseased broilers), *pfo*S and *col*A genes were detected in eight isolates while *net*B virulence gene was positive in three isolates only as shown in Fig. (2, 3, 4)

3.7. Antibiotic sensitivity test:
Antibiotic sensitivity test revealed *C. perfringens* isolates were highly sensitivity to amoxicillin (89.6%) followed by ampicillin and cefadroxyl (87.5%) for each and ampiclox (81.3%). Moderate sensitivity was noticed to penicillin and mupirocin (77.1%) for each and cefeprim (72.9%).The lowest sensitivity of the isolates was to maxfur (63.5%) and epicoflocin (58.3%). On the other hand, all isolates were (100%) resistant to cloxacillin, streptomycin, tetracycline, oxy tetracycline, erythrocine and cefrodoxim

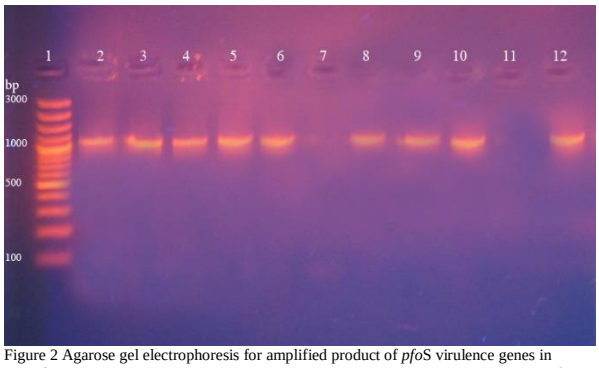

Figure 2 Agarose gel electrophoresis for amplified product of *pfo*S virulence genes in *C.perfringens* isolates. Lane1:100 bp DNA ladder. Lane (2-6& 8-10) positive isolates for *pfo*S gene which amplified at 1090 bp.. Lane 7: negative for *pfo*S gene. Lane 11: control negative. Lane 12: control positive.

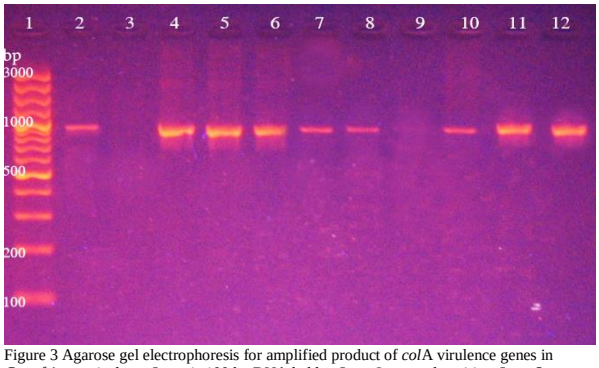

Figure 3 Agarose gel electrophoresis for amplified product of *col*A virulence genes in *C.perfringens* isolates. Lane 1: 100 bp DNA ladder. Lane 2: control positive. Lane 3: control negative. Lane (4-8&10-12): positive isolates for *col*A gene which amplified at 924 bp. Lane 9: negative for *col*A gene.

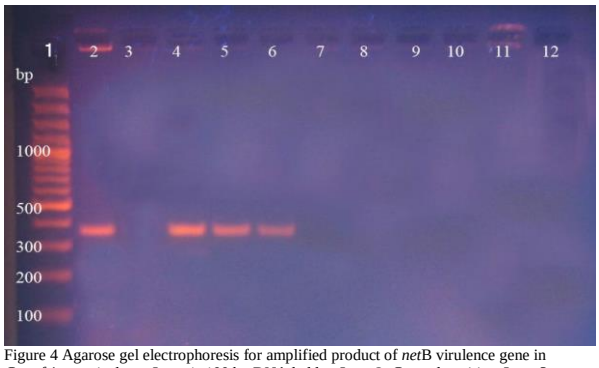

Figure 4 Agarose gel electrophoresis for amplified product of *net*B virulence gene in *C.perfringens* isolates. Lane 1: 100 bp DNA ladder. Lane 2: Control positive. Lane 3: Control negative. Lane (4-6): positive isolates for *net*B gene which amplified at 383 bp. Lane (7-12): negative for *net*B gene.

4. DISCUSSION

Worldwide, NE is a serious disease affects poultry caused by *C. perfringen*s and leads to production losses through increasing feed consumption and mortality rates and reducing broiler chicken welfare (Adhikari et al., 2020). Acute NE is characterized by a sudden increase in mortality without clinical signs. Meanwhile, the subclinical form is becoming more prevalent and is characterized by intestinal mucosal damage, decreasing nutrient digestion, absorption and feed conversion rate.In the current study, the *C. perfringens* prevalence among diseased broiler chickens was 70%.

This result is in accordance with previous results showed prevalence of 78% in NE affected broilers (Shane et al., 1984), 77.3% in broiler aged 1-15 week (Das et al., 2001) and 80% in poultry farms suffered from enteritis (Tschir et al., 1992). However, a lower *C. perfringens* prevalence of (48.5%) has been reported by Abd El-salam (2000).

In the present study, the intestinal samples from diseased broiler showed a higher (84%) isolation rate than hepatic samples with (56%) isolation rate. This higher isolation rate from intestinal sample than that from hepatic samples is in line with previous results showed that *C. perfringens* isolation rate from intestine and liver was 47.4% and 12.3%, respectively (Amal, 2012). Previous study reported lower *C. perfringens* isolation rate of 56% from balady chicken aged 4-8 weeks suffering from enteritis (Ahmed and Abd El-latif, 2004), 50% of intestinal samples from broiler chickens with sever haemorrhagic enteritis (Ismail, 2002). This lower isolation rate compared to our results may be attributed to difference in the management practice and/or the use of different feed mixture. Indeed, the addition of fishmeal in the diet was described to alter the gut microbiota profile (Stanley et al., 2014), providing high nutrient levels for *C. perfringens* growth (Drew et al., 2004).

The current results showed that apparently healthy broiler harbored *C. perfringens* much lower than diseased broiler as isolation rate of *C. perfringens* was 24% and 20% from apparently healthy intestinal sample and liver sample, respectively. The tendency of higher isolation rate from intestinal sample than that from hepatic sample is similar albeit much lower than that recovered from diseased broiler.

In the current results, typing of (70) *C. perfringens* isolates from diseased broiler by the intradermal inoculation test in Guinea Pig and PCR revealed that 80 % were toxigenic. Out of this toxigenic isolates 85.7% belonged to type A while 14.3 % belonged to type D. On the other hand of the toxigenic 15 isolates recovered from apparently healthy broilers were 60% belonged to type A and 40% belong to type D. These results are in accordance with that reported by Abd-El-Tawab (2002), who isolated *C. perfringens* type "A", type "C" and type "D" from diseased geese with incidence of (73%), (6.7%) and (20%), respectively. However, the current results are higher than that of a previous study reported that about 20% were Type A, 5% of each of Type D and C (Latino Vic, 1983).

C. perfringens type C was not detected in the present study in a disagreement with the finding of Sarakbi (1991), who reported that *C. perfringens* types C and A being the main cause of necrotic enteritis in broiler. A recent study reported that *C. perfringens* types A and C were the main etiological agents of necrotic enteritis in chicken (Diego and Andrey, 2014). However, *C. perfringens* type A was the majority (96%) in isolates from chickens and turkey (Archambanlt, 2009). Moreover, *C. perfringens* type A was the only type of isolates from different sources including poultry (Johansson, 2006). Furthermore, *C. perfringens* types A was the only type identified in 75% of the isolates from chicken slaughtered for human consumption (Hakan and Hasan, 2005). This result augment our results that *C. perfringens* type A being the major cause of NE.

In the present study the presence of *net*B in only 3 isolates out of 9 type A isolates form diseased broiler may indicate the unnecessary presence of *net*B for induction of the NE in broiler chickens. This assumption may contradict a previous proposal suggesting the presence of *net*B as a main factor for *C. perfringens* to induce NE in chickens (Keyburn et al., 2008 & 2010; Smyth and Martin, 2010). However, in accordance with the results of this study, a minority of *C. perfringens* strains isolated from clear cases of necrotic enteritis in chicken were reported to lack the *net*B gene (Chalmers, 2008; Keyburn, 2008).

In this study, Antibiotic sensitivity test of isolated *C. perfringens* showed high sensitivity to amoxicillin, ampiclox, ampicillin, pencillin and cefadroxyl. These results are in agreement with a previous results showed that *C. perfringens* were highly sensitive to ampicillin, cepelothin and penicillin (Park et al.,1994). More recently, *C. perfringens* strains isolated from intestine of broiler chickens were reported to be highly sensitive to penicillin (Silva et al., 2009).

All *C. perfringens* strains obtained in the present study were resistant to streptomycin, tetracycline, cloxacillin, erythrocin and oxytetracycline. Indeed, isolates from chickens with necrotic enteritis were reported to be completely resistant to streptomycin. (Park et al., 2015). Moreover, *C. perfringens* isolates were highly resistant to streptomycin (100%), gentamicin (93.8%) and neomycin (93.8%) (Jun-Ho Kim et al., 2018).

5. CONCULOSION

In the current study, *C. perfringens* showed high prevalence among diseased broiler chickens with high isolation rate from intestinal sample than hepatic samples. Apparently healthy broiler harbored toxigenic *C. perfringens* and should be considered a potential source of dissemination and spreading of the microorganism to the environment. High percentage of *C. perfringens* were toxigenic type A in case of diseased or apparently healthy broiler chickens. *C. perfringens* virulence genes varies from locality to another with the *net*B gene seems to be unnecessary for inducing necrotic enteritis in broiler chickens.

ACKNOWLEDGMENT

The authors thank the members of the Animal Health Research Institute, Dokki Giza, Egypt for their technical support during the working and analysis of the samples.

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