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DETECTION OF CLOSTRIDIUM PERFRINGENS AND THEIR VIRULENCE TOXINS IN BROILER CHICKEN

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ABSTRACT

The present study was conducted to evaluate the prevalence of *C. Perfringens* in broiler chickens in El Behiera, Alexandria and Matrouh governorates. Samples (n= 400) were collected from intestine (n=200; 100 apparently healthy and 100 diseased birds), feed (n=100) and litter (n=100). The prevalence of *C. Perfringens* was 57, 88, 52 and 60% in apparently healthy birds, diseased birds, feed and litter, respectively. Isolates were confirmed by Matrix-assisted laser desorption/ionization (MALDI). Antimicrobial sensitivity of isolated *C. perfringens* showed sensitivity to Cefotaxime, Chloramphenicol, Bacitracin, Norfloxacin, Ciprofloxacin, Doxycycline, Amoxicillin + Clavulanic acid and Clindamycin. The isolates were resistant to Gentamycin, Erythromycin, Amoxicillin, Ampicillin and Neomycin. Eighteen *C. perfringens* isolates from different samples were examined by PCR for Alpha, Beta, Epsilon and Iota genes. The alpha gene was detected in 17 of the examined isolates. Only five isolates were positive for the Tpel toxin gene. Further, the Net-B toxin gene was detected in one isolate. Environmental factors (feed & litter) in poultry farms represent an important source of *C. perfringens* infection (toxigenic types) and apparently healthy birds may act as a major source of infection. Alpha toxin was the predominant major toxin in our investigation and the Tpel gene was detected more than the Net-B gene.

KEYWORDS: Broiler chickens, *C. Perfringens*, Tpel gene, PCR.

INTRODUCTION

The most common clostridial enteric disease in poultry is necrotic enteritis (NE) caused by *Clostridium perfringens*, which typically occurs in broiler chickens 2-6 weeks of age **(Kerry et al., 2013)**. Avian necrotic enteritis costs the world poultry industry an estimated $2 billion annually, largely due to the costs of antimicrobial prophylaxis and inefficient feed conversion **(Cooper et al., 2009; Van Immerseel et al., 2009)**.

C. perfringens is a Gram-positive spore-forming anaerobic bacterium present in the intestinal flora of humans and animals as well as in soil and feed, where its presence might be indicative of fecal contamination **(Florence et al., 2011)**. *C. perfringens* is classified into five toxinotypes (A, B, C, D, and E) according to the production of 4 toxins, namely alpha, beta, epsilon and iota. Several other toxins (e.g. enterotoxin, beta 2 and perfringolysin O) can also be produced by some strains of all types of *C. perfringens* **(Songer and Uzal, 2005)**.

C. perfringens type A produces only alpha toxin, type B produces alpha, beta and epsilon toxins, type C produces alpha and beta toxins, type D produces alpha and epsilon toxins, while type E produces alpha and iota toxins **(Kalender et al., 2005)**. In addition to the major toxins, there are other minor toxins produced by some strains of *C. perfringens*, which may play a role in pathogenicity, including NetB and TpeL. While the roles of alpha, beta, iota, and epsilon toxins in the pathogenesis of enteritis among animals are well documented, the roles of other toxins such as TpeL in necrotic enteritis pathogenesis and its cytotoxic effect are still unclear **(Popoff et al., 2009)**.

The most recently identified toxin in the *C. Perfringens* armory is NetB, which is produced by many avian isolates of *C. perfringens* type A **(Keyburn et al., 2010)**.

Diagnosis of *C. perfringens* is challenging, because many clostridial species can be normal inhabitants of the gut. So diagnosis is based on clinical and pathological findings, negative culture and toxin detection **(Kerry et al., 2013)**. Various PCR protocols including multiplex PCR assays have been established to genotype *C. perfringens* isolates with respect to cpa, cpb, etx, itx genes encoding the alpha, beta, epsilon and iota toxins, respectively **(Garmory et al., 2000)**.

C. perfringens is the primary causative agent of Our understanding of the pathogenesis of avian necrotic enteritis, an economically important disease, has been enhanced by the discovery of *C. perfringens* NetB toxin, which belongs to the α-haemolysin family of β-pore-forming toxins **(Rood et al., 2016)**.

TpeL-positive strains are associated with avian necrotic enteritis, although NetB toxin is considered to play a major role in pathogenesis **(Keyburn et al., 2010)**.

This study aimed to study *C. perfringens* prevalence in necrotic enteritis in broilers, in El Behiera, Alexandria and Matrouh governorates. Further to demonstrate the prevalence of Net-B and Tpel toxins in *C. perfringens* isolates.

MATERIALS AND METHODS

Samples

A total of 400 samples were collected from intestinal contents (n= 200; 100 apparently healthy birds and 100 diseased birds), feed (n= 100) and litter (n= 100).

Isolation of *C. Perfringens*

Samples were enriched and isolated according to **Smith and Holdman (1968)**. Each sample was selectively enriched by transferring 1 ml of the processed sample into a tube of freshly prepared cooked meat medium (Oxoid, CM0081B) and incubated anaerobically at 37˚C for 24 hr using anaerogen atmosphere generation system (Oxoid). Isolates were cultured on blood agar (Oxoid, CM965) with 5% defibrinated sheep blood containing neomycin sulphate 200 µg/ml and incubated anaerobically at 37˚C for 24 hr. Isolates yielding double zone of hemolysis (beta-hemolysis) were confirmed as *C. perfringens*. The suspected colonies were picked up and examined for their morphological and cultural characters. All isolated strains were stored in a cooked meat medium at -70˚C for subsequent experiments. Suspected colonies were streaked in duplicate onto tryptose sulfite cycloserine agar (TSC) (Oxoid, CM0587B) with perfringens selective supplement without egg yolk emulsion (Oxoid, SR0088E) and then incubated anaerobically at 37˚C for 24h. Typical black colonies with creamy zone around the colony were used for further characterization.

Identification of *Clostridium perfringens* isolates

C. perfringens isolates were identified by colonial appearance, microscopical appearance and biochemical identification according to **Koneman *et al.*, (1992)** and **Macfaddin (2000)**.

Matrix-assisted laser desorption/ionization (MALDI)

The isolates were identified by MALDI–TOF-MS (Bruker Daltonics, Bremen, Germany) **(Chean et al., 2014)** according to manufacturer's instructions, cut off scores of ≥ 2.000 identifies the species, scores between 1.700 and 1.999 identifies the genus, and scores of < 1.700 indicates no identification. The isolates producing scores of < 1.700 were retested, and the highest score was used for the final analysis.

Antimicrobial susceptibility of *C. perfringens* isolates

Antimicrobial susceptibility was detected by the disc diffusion method according to **CLSI, (2019)**. The discs used were Cefotaxime (CTX; 30µg), Amoxicillin + Clavulanic acid (AMC; 30µg), Amoxicillin (AML; 25µg), Bacitracin (B; 10µg), Erythromycin (E; 15µg), Norfloxacin (NOR; 10µg), Ciprofloxacin (CIP; 5µg), Clindamycin (DA; 2IU), Doxycycline (DO; 30µg), Chloramphenicol (C; 30µg), Gentamycin (CN; 10µg) and Neomycin (N; 30µg).

Detection of *C. perfringens* by polymerase chain reaction (PCR)

DNA was extracted using QIAamp DNA Mini Kit according to manufacturer's instructions. Primer sequences are listed in **Table.1**. Preparation of PCR Master Mix and PCR conditions were done according to Emerald Amp GT PCR master mix kit (Takara Code No. RR310A). PCR products were separated and visualized by gel electrophoresis in 1.5% agarose.

Table (1): PCR primers for amplification of virulence genes.

Toxin	Primer	Sequence	Amplified product	Reference
Alpha toxin	*F*	GTTGATAGCGCAGGACATGTTAAG	402 bp	**Yoo et al., 1997**
	R	CATGTAGTCATCTGTTCCAGCATC		
Beta toxin	*F*	ACTATACAGACAGATCATTCAACC	236 bp	
	R	TTAGGAGCAGTTAGAACTACAGAC		
Epsilon toxin	*F*	ACTGCAACTACTACTCATACTGTG	541 bp	
	R	CTGGTGCCTTAATAGAAAGACTCC		
Iota toxin	*F*	GCGATGAAAAGCCTACACCACTAC	317 bp	
	R	GGTATATCCTCCACGCATATAGTC		
NetB	*F*	GCTGGTGCTGGAATAAATGC	560 bp	**Datta et al., 2014**
	R	TCGCCATTGAGTAGTTTCCC		
TpeL	*F*	ATATAGAGTCAAGCAGTGGAG	466 bp	**Bailey et al., 2013**
	R	GGAATACCACTTGATATACCTG		

RESULTS

Prevalence of *C. perfringens* in different samples

C. Perfringens was detected in 257 (64.25%) of the samples as shown in Table.2.

Table (2): Prevalence of *C. perfringens* in different samples.

Samples	No. of examined samples	Positive samples	
		No.	(%)
Apparently healthy	100	57	(57%)
Diseased	100	88	(88%)
Feed	100	52	(52%)
Litter	100	60	(60%)
Total	**400**	**257**	**(64.25%)**

Detection of *C. perfringens* isolates by MALDI

Table.3 shows the Matrix-assisted laser desorption/ionization (MALDI) confirmation of the isolated *C. perfringens*.

Table (3): Confirmation of the isolated *C. perfringens* by MALDI–TOF.

Sample		MALDI–TOF MS at log (score)			
		≥2.000	1.700-1.999	0.000-1.699	Others
Intestine	Apparently healthy	7	-	-	1
	Diseased	9	1	-	1
Feed		6	-	-	-
Litter		8	-	1	1
Total		**30**	**1**	**2**	**3**

(>2.000): highly probable species identification, secure genus identification.
(1.700 ~1.999): probable genus identification.
(0.000~ 1.699): not reliable identification.
(Misidentification): bacteria other than *C. perfringens* on species level or genus level.

Antimicrobial susceptibility of *C. perfringens* isolates

Antimicrobial sensitivity of isolated *C. perfringens* showed high sensitivity to Cefotaxime, Chloramphenicol and Bacitracin. While moderate sensitivity to Norfloxacin, Ciprofloxacin, Doxycycline, Amoxicillin + Clavulanic acid and Clindamycin. The isolates were resistant to Gentamycin, Neomycin, Erythromycin and Amoxicillin as shown in **Table.4**. Multiple antimicrobial resistance (MAR) was also detected as shown in **Table.5**.

Table (4): Antimicrobial susceptibility of *C. perfringens isolates.*

Antimicrobial	Sensitivity %
Cefotaxime (CTX)	93.3%
Amoxicillin - clavulanic acid (AMC)	46.7%
Amoxicillin (AML)	20%
Bacitracin(B)	86.7%
Ampicillin (AMP)	13.3%
Erythromycin(E)	6.7%
Norflocxacin (NOR)	60%
Ciprofloxacin (CIP)	60%
Clindamycin (DA)	40%
Doxycycline (DO)	53.4%
Chloramphenicol (C)	93.3%
Gentamycin (CN)	0%
Neomycin (N)	0%

Table (5): Multiple antimicrobial resistance (MAR) index of *C. perfringens isolates.*

MAR	No. of isolates			Total
	Intestine	Feed	Litter	
0.4	2	0	2	4
0.6	3	2	1	6
0.7	0	2	2	4
0.8	5	1	0	6

Polymerase chain reaction for the detection of *C. perfringens* toxins.

As shown in **Figure.1**, the Alpha toxin was detected in 17/18 (94.5%) of the isolates. Tpel toxin gene was detected in five isolates 5/18 (27.8%), Net-B toxin gene was detected in only one isolate 1/18 (5.6%).

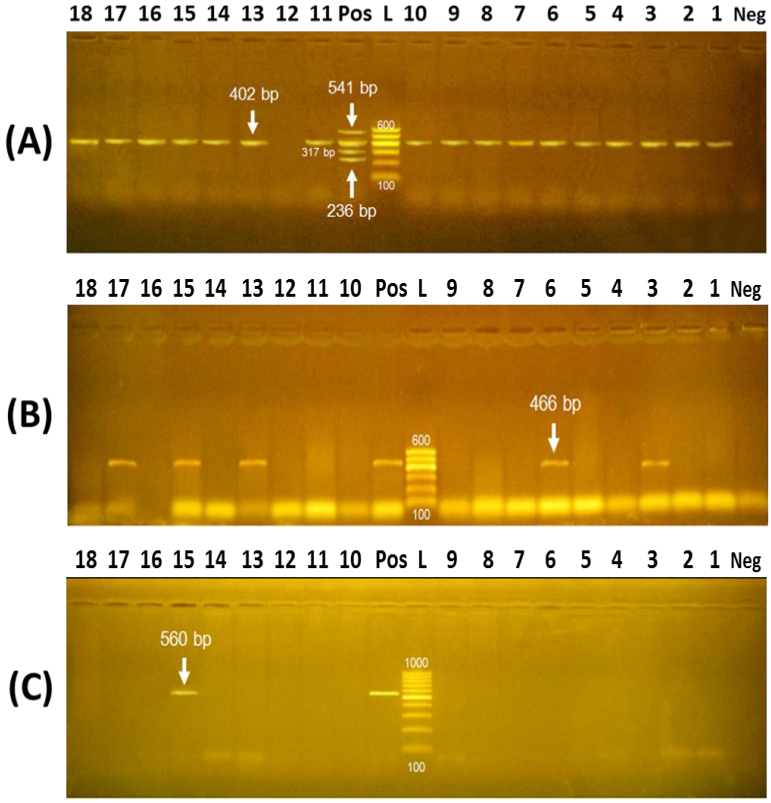

Figure 1: Polymerase chain reaction for the detection of *C. perfringens* toxin genes. PCR for the detection of (A) Alpha toxin gene, (B) Tpel toxin gene and (C) Net-B toxin gene. L; DNA ladder, Pos; control positive, Neg; control negative, 1-10 number of the tested isolates.

DISCUSSION

Necrotic enteritis is one of the most common and financially disturbing diseases affecting global poultry flocks. *C. perfringens* is the most serious cause of clostridial enteric disease in domestic animals **(Johansson et al., 2006).** In the present study, the incidence of *C. perfringens* in samples of apparently healthy birds was higher (57%) than that reported by **El-Refaey et al., (1999)**, who isolated clostridium species from 42.0% of apparently normal poultry and higher than **Rasha (2009)** who isolated *C. perfringens* from intestine of apparently normal chickens with an incidence of 30%. **Osman et al., (2012)** detected *C. perfringens* in 35.4% of asymptomatic broiler chickens and **Fan et al., (2016)** isolated *C. Perfringens* from premarket, 5-wk-old, clinically healthy broiler chickens in Taiwan, with isolation rate of 9.9% of total samples. Various management, dietary and flock health practices are thought to be responsible for this variability. Husbandry factors including diet and litter type have been shown to influence the incidence and severity of necrotic enteritis in chickens **(Henry et al., 1995)**.

The incidence of *C. perfringens* in diseased birds was 88%. These results were similar to **El-Refaey et al., (1999)** who isolated clostridium species from diseased chickens with an incidence of 91.3%, but lower than **Osman et al., (2012)** who detected *C. perfringens* in 100% of broiler chickens with clinical signs. While higher than **Rasha (2009)** who isolated *C. perfingens* from intestine of diseased broiler chickens with an incidence of 75%. **Amal (2012)**, isolated *C. perfringens* from intestine of chicken with necrotic enteritis with an incidence of 47.70%, **Eman et al., (2013)** isolated *C. perfringens* from intestinal samples of chicken with necrotic enteritis with incidence of 60 % and **Abd-Elall et al., (2014)** isolated *C. Perfringens* from 14/25 (56%) of caecal contents of diseased birds. The increased occurrence of *C. perfringens* in diseased than apparently healthy broilers birds in this study might be attributed to that, disturbance in normal intestinal microflora may cause rapid proliferation of *C. perfringens*, increasing their numbers with subsequent toxin production and damage of intestinal mucosa **(KONDOF 1988)**.

The incidence of *C. perfringens* in feed samples (52%) was similar to **Sarkar et al., (2013)** who isolated *C. perfringens* from poultry feed with an incidence of 59.33% and the result of this study was higher than **Abd-Elall et al., (2014)** who isolated *C. perfringens* from

poultry feed with an incidence of 33.3%. The most important source of infection in poultry appears to be contaminated feed, litter, water and the environment **(CRAVEN, 2001)**.

The incidence of *C. perfringens* in litter samples (60%) was similar to **Sarkar et al., (2013)** who isolated *C. perfringens* from poultry litter with an incidence of 53%, but higher than **Abd-Elall et al., (2014)** isolated *C. perfringens* from poultry litter with an incidence of 46.7%. *C. perfringens* is a normal inhabitant of the healthy broiler chicken gut microflora, and frequently found in the feces of livestock and poultry at high levels **(Tschirdewahn et al., 1992)**.

La Scola et al., (2011), **Alam et al., (2012)**, **AlMogbel (2016)** and **Liu et al., (2016)** recognized *C. perfringens* by MALDI TOF and reported that it is a useful, rapid, accurate and simple technique for the correct identification of micro-organisms. Out of 36 isolates subjected to MALDI TOF, 30 were confirmed as *C. perfringens* as shown in Table (3). Thus, such analysis could be applied for identification of clostridia, however, in combination with other identification methods for accurate confirmed diagnosis.

Antimicrobial susceptibility showed that *C. perfringens* isolates were sensitive to Cefotaxime, Chloramphenicol, Bacitracin, Norfloxacin, Ciprofloxacin and Doxycycline. On other hand, the isolates showed low sensitivity to Ampicillin, Amoxicillin + Clavulanic acid and Clindamycin. This agreed with **Silva et al. (2009)** who reported 52.7% susceptibility of *C. perfringens* to bacitracin and **Fan et al., (2016)** found that most of the *C. perfringens* isolates were susceptible to bacitracin. On the contrary, **Osman et al., (2013)** reported that the prevalence of resistance to antibiotics was high; 46%, 58%, 67% and 98% to chloramphenicol, ciprofloxacin, norfloxacin and doxycycline, respectively. However, *C. perfringens* isolates here were mainly resistant to Gentamycin, Erythromycin, Amoxicillin and Neomycin. This agreed with Osman **et al., (2013)** who reported that all tested isolates were resistant to gentamicin and erythromycin. The prevalence of resistance to neomycin was also high (93%). **Fan et al., (2016)** found that most of the *C. perfringens* isolates were resistant to erythromycin, but differ from **Martel et al. (2004)** who reported that all isolates were sensitive to amoxicillin. **Fan et al., (2016)** found that most of the *C. perfringens* isolates were susceptible to amoxicillin. Antimicrobial susceptibilities could differ as a result of diverse purposes for usage of antimicrobials either for treatment or as growth promoters. There are many factors affecting *C. perfringens* sensitivity to antimicrobials but the main factor is the genotypic resistance.

By PCR, Alpha gene was detected in 17 out of 18 samples in isolates from apparently healthy and diseased birds, feed and litter, while Beta, Epsilon, Iota genes were not detected. **Cooper and Songer (2009)** reported that Alpha toxin (CPA) has been considered a critical virulence factor in the pathogenesis of necrotic enteritis. CPA is the only "major" toxin produced by type A strains and higher levels have been detected in birds with necrotic enteritis than in normal birds.

Others found other genes with the Alpha one as **Younes (2005)** who typed 60 toxigenic strains of *C. perfringens* and found that the most prevalent type was type-A (93.3%) followed by type-D with an incidence of 6.7%. **Siragusa et al., (2006),** reported that 48 isolates of *C. Perfringens* were alpha-toxin gene positive and 46 of 48 were negative for beta and epsilon-toxin genes.

The PCR detection of tpel and net-B genes in *C. perfringens* isolates showed that tpel gene detection in healthy birds, diseased birds, feed and litter was 0%, 40%, 25% and 50%, respectively. On the other hand, net-B gene was detected only in isolates from diseased birds (20%). This may indicate that tpel gene could be more frequently expressed than Net B gene. **Coursodon et al., (2012)** reported that TpeL, a recently described novel member of the family of large clostridial cytotoxins, was found in *C. perfringens* type C. Others have since reported TpeL in type A isolates from necrotic enteritis outbreaks, suggesting that it may contribute to the pathogenesis of necrotic enteritis. **Park et al., (2015)** found that of 17 chickens that died from necrotic enteritis, the rate of netB-positive isolates was significantly higher (8 of 17) than the rate among healthy chickens (2 of 50). **Fan et al., (2016)** reported that the *C. perfringens* type A isolates expressed only the cpa gene encoding for alpha toxin. No netB gene encoding NetB toxin was associated with necrotic enteritis. **Keyburn et al., (2010)** detected the tpeL gene in two type A avian necrotic enteritis strains, both netB positive. This finding is consistent with previous reports suggesting that TpeL-positive strains often are associated with avian necrotic enteritis.

CONCLUSION

Necrotic enteritis is a major problem facing the poultry industry. Further, environmental factors (feed & litter) in poultry farms represent an important source of *C. perfringens* infection (toxigenic types) and apparently healthy birds may act as a major source of infection. Moreover, Alpha toxin was the predominant major toxin in our investigation others couldn't be detected and the Tpel gene was detected more than the Net-B gene.

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