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IN VIVO EXPERIMENTAL STUDIES ON CLOSTRIDIUM PERFRINGENS Tpel TOXIN

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ABSTRACT

The present study was conducted to evaluate the prevalence of *C. Perfringens* in boiler chickens in El Behiera and Matrouh governorates. Samples (n=362) were collected from intestine (n=162), feed (n=100) and litter (n=100). The prevalence of *C. Perfringens* was 49.1, 82.5, 60 and 50% in apparently healthy birds, diseased birds, feed and litter, respectively. Isolates were confirmed by Matrix-assisted laser desorption/ionization (MALDI). Antimicrobial susceptibility of *C. perfringens* showed sensitivity to Cefotaxime, Chloramphenicol, Bacitracin, Norfloxacin, Ciprofloxacin, Doxycycline, Telmicosin, Amoxicillin + Clavulanic acid and Clindamycin, while resistant to Gentamycin, Erythromycin, Amoxicillin and Neomycin. Eighteen *C. perfringens* isolates were examined by multiplex PCR using specific primers for Alpha, Beta, Epsilon and Iota genes. The alpha gene was detected in 17 of the examined isolates. By PCR, only five isolates were positive for the Tpel toxin gene. Further, the Net-B toxin gene was detected in one isolate. By neutralization test in mice and Dermonecrotic test in Albino guinea pigs, 5 *C. perfringens* isolates were toxigenic (type-A). Furthermore, experimental effect of trypsin on Tpel toxin in guinea pigs showed inhibition of the Tpel pathogenic effect. *Bla* and *Erm*-B genes specific to resistance of *C. perfringens* to beta lactam and macrolides, respectively, were detected by PCR in 100% and 60% of the tested isolates, respectively. These results showed matching between phenotypic and genotypic resistance in *C. Perfringens*. Further, environmental factors (feed and litter) represent an important source of toxigenic *C. perfringens* infection and apparently healthy birds may act as a major source of infection.

KEYWORDS: Broiler chickens, *C. Perfringens*, Tpel gene, PCR, Trypsin, Beta lactams, Macrolides.

INTRODUCTION

Clostridium perfringens is widespread in the environment and is commonly found among normal gut flora. It is the most serious cause of clostridial enteric disease in domestic animals (Johansson et al., 2006).

C. perfringens is a Gram-positive spore-forming anaerobic bacteria 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 5 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). 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 (Cooper 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).

Emerging problems in antimicrobial resistance was because of intensive antimicrobial use in animals as growth promotors (Murray, 1998).

Since TpeL is a recently identified toxin of *C. perfringens*, many of its features remain poorly

understood, including TpeL sensitivity to Trypsin (Gurjar et al., 2010).

This study aimed to study *C. perfringens* prevalence in necrotic enteritis in broilers in El Behaira and Matrouh governorates. Further to understand the role of Tpel in *C. perfringens* pathogenesis.

MATERIALS AND METHODS

Samples

A total of 362 samples were collected from intestinal contents (n=162, 59 apparently healthy birds and 103 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 ug/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 isolated clostridium perfringens

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, (2012). The discs used were Cefotaxime (CTX; 30µg), Amoxicillin + Clavulanic acid (AMC; 30µg), Amoxicillin (AML; 25µg), Bacitracin (B; 10µg), Telmicosin (T; 30µg), 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		
*Erm*B	F	GAA AAG GTA CTC AAC CAA ATA	638 bp	**Soge et al., 2009**
	R	AGT AAC GGT ACT TAA ATT GTT TAC		
Bla	F	ATGAAAGAAGTTCAAAAATATTTAGAG	780 bp	**Catalán et al., 2010**
	R	TTAGTGCCAATTGTTCATGATGG		

Typing of toxigenic *C. perfringens* isolates

Typing of *C. perfringens* isolates was performed using neutralization test in mice (Smith and Holdeman, 1968) and dermonecrotic test in Albino guinea pigs (Stern and Batty, 1975).

Pathological effect of Tpel toxin in guinea pigs

C. perfringens toxin was prepared according to Roberts et al., (1970). Guinea pigs were divided into three groups; three animals each. Group 1 was injected with *C. perfringens* culture supernatant (containing Alpha and Tpel toxins). Group 2 was injected with *C. perfringens* culture supernatant and antiserum against Alpha toxin (containing Tpel toxin only). Group 3 was injected with *C. perfringens* culture supernatant and antiserum against Alpha toxin and Trypsin (containing Tpel toxin only). Animals were monitored daily for 72 hours after intradermal injection. For histopathological examination, animals were sacrificed and subjected to postmortem (PM) examination. Tissue specimens were immediately collected from liver, skin, kidney, spleen, and intestine. The specimens were fixed in 10% neutral buffered formalin. Fixed specimens were processed through dehydration in ascending grades of ethanol and then cleared in xylene and embedded in paraffin blocks. Paraffin sections were prepared and sections were stained with hematoxylin & eosin for examination (Bancroft et al., 1994). All animal experiments were performed and approved by the animal ethical code of Damanhour University, Egypt.

RESULTS

Prevalence of *C. perfringens* in different samples

C. Perfringens was detected in 224 (61.8%) of the samples as shown in Table.2.

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

Samples		No. of examined samples	Positive samples	
			No.	%
Intestinal Contents	Apparently healthy	59	29	49.1%
	Diseased	103	85	82.5%
Environment	Feed	100	60	60%
	Litter	100	50	50%
Total		**362**	**224**	**61.8%**

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
Intestinal content	Apparently healthy	6	2	-	2
	Diseased	8			3
Feed		9	-	1	-
Litter		8	-	1	1
Total		**31**	**2**	**2**	**6**

Antimicrobial susceptibility of *C. perfringens* isolates

Antimicrobial sensitivity of isolated *C. perfringens* showed sensitivity to Cefotaxime, Chloramphenicol, Bacitracin, Norfloxacin, Ciprofloxacin, Doxycycline, Telmicosin, Amoxicillin + Clavulanic acid and Clindamycin. The isolates were resistant to Gentamycin, Erythromycin, Amoxicillin and Neomycin 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)	97%
Amoxicillin - clavulanic acid (AMC)	42%
Amoxicillin (AML)	17%
Bacitracin(B)	84%
Telmicosin(T)	48%
Erythromycin(E)	9%
Norflocxacin (NOR)	66%
Ciprofloxacin(CIP)	64%
Clindamycin(DA)	37%
Doxycycline (DO)	59%
Chloramphenicol (C)	97%
Gentamycin (CN)	0%
Neomycin (N)	0%

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

MAR	No. of isolates			Total
	Intestinal content	Feed	Litter	
0.4	0	2	2	4
0.6	2	2	2	6
0.7	0	2	2	4
0.8	6	0	0	6

Polymerase chain reaction for the detection of *C. perfringens* toxin and antimicrobial resistance genes.

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

Further, antimicrobial resistance gene *B-lactam; bla*, was detected in 100% of tested isolates while resistance to macrolides' gene; *erm*B was detected in 60% of tested isolates.

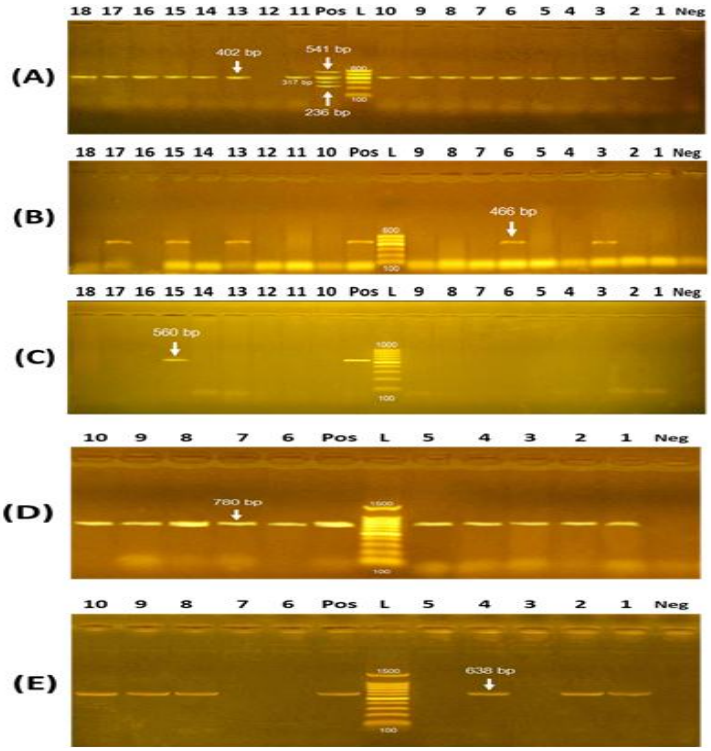

Figure 1: Polymerase chain reaction for the detection of *C. perfringens* toxin and antimicrobial resistance genes. Multiplex PCR for the detection of (A) Only Alpha toxin gene, (B) Tpel toxin gene, (C) Net-B toxin gene. Uniplex PCR for the detection of (D) *bla* gene and (E) *erm*B gene. L; DNA ladder, Pos; control positive, Neg; control negative, 1-10 corresponds to the tested isolates.

Typing of the toxigenic *C. perfringens* isolates

By Neutralization test in mice, five isolates were selected containing Alpha and Tpel genes.

Group 1 that was injected with culture supernatant only died within 24 hr. Group 2 that was injected with culture supernatant pre-incubated with type-A antiserum remained alive along the experiment indicating that the five isolates are typed as type-A *C. perfringens*.

By Dermonecrotic test in Albino guinea pigs, culture supernatant of *C. perfringens* from the above used five isolates was injected intradermally in the right side of the abdomen of guinea pigs and culture supernatant neutralized with type-A antiserum on the other side.

All guinea pigs injected showed an irregular area of yellowish necrosis on the right side. With tendency to spread downwards, which is the characteristic lesion of alpha toxin, type-A *C. perfringens* (Fig.2).

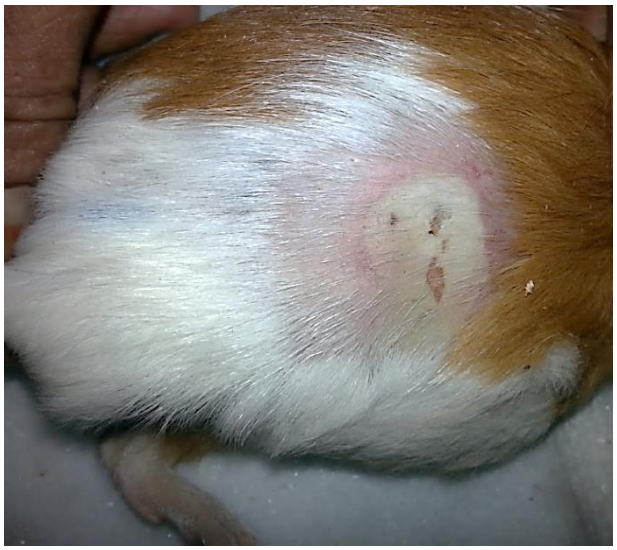

Figure 2: Dermonecrotic test in Albino guinea pigs. Guinea pig showing an irregular area of yellowish necrosis following injection with *C. perfringens* culture supernatant.

Pathological effects of the Tpel toxin in guinea pig

a. Clinical signs and gross pathological findings in toxin-injected guinea pigs

Most animals in the three injected groups seemed to be normal for about one day and then refused to eat or ate poorly until death. The first group showed yellow pus accumulation in the site of injection, off food, restricted movement then no movement on the second day, slow reaction and death after two days. The second group showed Red-hot inflammation at site of injection, low feed intake, stiff movement, weak reaction during straining for anesthesia before scarification. The 3rd group showed slight inflammation at the site of injection firstly then it became normal, normal feed intake and movement, aggressive reaction during straining for anesthesia before scarification. Gross pathological lesions were seen as minor haemorrhagic patches in the caecum in severe cases and small gut was also involved. The first group showed subcutaneous yellow pus accumulation and profuse ascites (reddish ascetic fluid) in internal organs. The second group showed redness of subcutaneous tissue at the site of injection and yellowish small amount of ascetic fluid. The 3rd group showed normal PM lesions.

a. Histopathological Findings

Histopathological examination of specimens from animals scarified at 72 hr post-injection showed necrosis of hepatocytes with activation of Kupffer cells lining the walls of the sinusoids (Fig.3A and B). The lung showed lymphocytic and suppurative inflammation with sever hemorrhage and edema (Fig.3C and D). Skin showed severe suppurative dermatitis of subcutaneous tissue (Fig.4A and B). Kidney showed mild tubular degeneration and necrosis (Fig.4C). Intestinal necrosis, sloughing of mucosal epithelium and involvement of the muscle coats with leukocyte cell infiltration was also detected (Fig.4D).

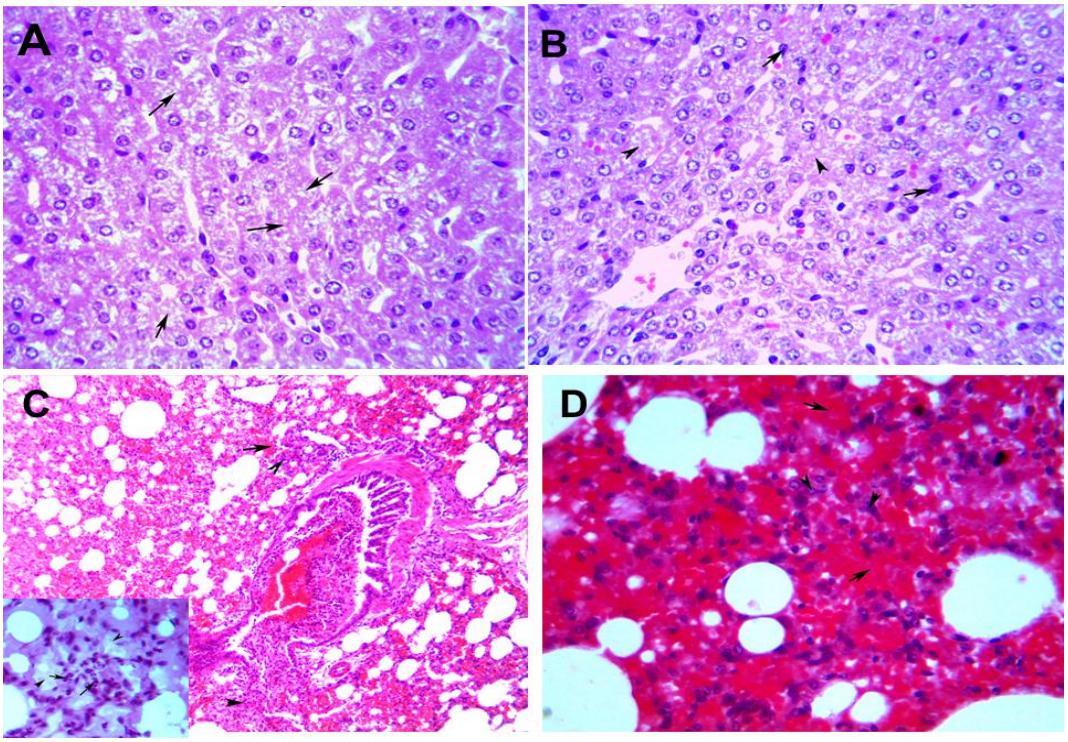

Figure 3: Histopathological Findings of Tpel toxin in guinea pig. (A) Mild necrosis of hepatocytes (arrows) of guinea big group 2 (H&E) x400. (B) Coagulative necrosis of hepatocytes (arrows) with activation of kuffer cell lining the sinusoids (arrowhead) guinea big group 1. (H&E) x400. (C) Suppurative pneumonia of lung (arrows) of guinea pig guinea big group 1 (H&E) x100, the inset picture showing neutrophils cells (arrows) with edema (arrowhead) (H&E) x400. (D) Hemorrhagic pneumonia (arrows) with leukocytic cell infiltration (arrowhead) guinea big group 2 (H&E) x400.

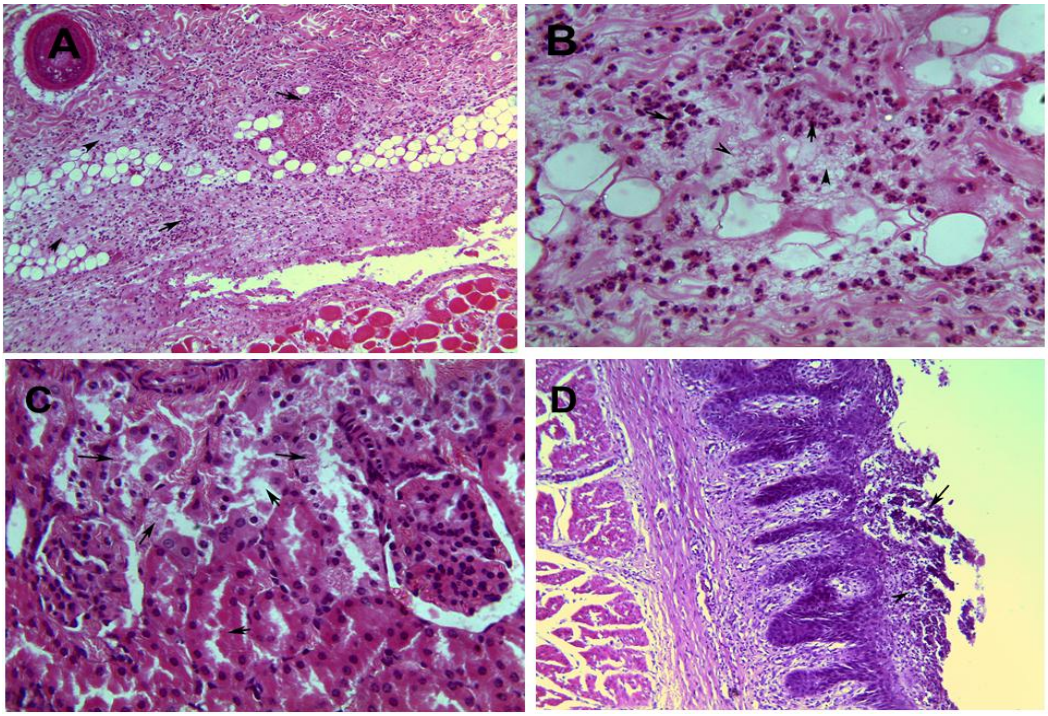

Figure 4: Histopathological Findings of Tpel toxin in guinea pig. (A) Severe diffuse suppurative dermatitis (arrows) of subcutaneous tissue of skin with edema of guinea pig group 1 (H&E) x100. (B) Higher magnification of photo (A) showing severe subcutaneous infiltration with neutrophils (arrows) with edema (arrowhead) (H&E) x400. (C) Coagulative necrosis of renal tubular epithelium (arrows) of kidney of guinea pig group 1 (H&E) x400. (D) Mild necrotic enteritis (arrows) of intestine of guinea pig group 1 with minimal sloughing of mucosa (H&E) x100.

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 (49%) was nearly similar as reported by El-Refaey et al., (1999), who isolated clostridium species from 42.0% of apparently normal poultry but 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.

The incidence of *C. perfringens* in diseased birds was 82.5%. These results were close to Rasha (2009) who isolated *C. perfingens* from intestine of diseased broiler chickens with an incidence of 75%. These results were lower than El-Refaey et al., (1999) who isolated clostridium species from diseased chickens with an incidence of 91.3%. Osman et al., (2012) detected *C. perfringens* in 100% of broiler chickens with clinical signs but higher than Amal (2012) who isolated *C. perfringens* from intestine of infected 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 A. Abd-Elall et al., (2014) isolated *C. Perfringens* from 14/25 (56%) of caecal contents of diseased birds.

The incidence of *C. perfringens* in feed samples (60%) 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 A. Abd-Elall et al., (2014) who isolated *C. perfringens* from poultry feed with an incidence of 33.3%.

The incidence of *C. perfringens* in litter samples (50%) was similar to Sarkar et al., (2013) who isolated *C. perfringens* from poultry litter with an incidence of 53%, also A. Abd-Elall et al., (2014) isolated *C. perfringens* from poultry litter with an incidence of 46.7%.

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 41 isolates subjected to MALDI TOF, 31 were confirmed as *C. perfringens* as shown in table (3). This could be applied for identification of clostridia, however, in combination with other identification methods for accurate diagnosis.

Antimicrobial susceptibility showed that *C. perfringens* isolates were sensitive to Cefotaxime, Chloramphenicol, Bacitracin, Norfloxacin, Ciprofloxacin and Doxycycline. On other hand, the isolates showed moderate sensitivity to Telmicosin, Amoxicillin + Clavulanic acid and Clindamycin. This agreed with Silva et al. (2009) who reported that susceptibility of *C. perfringens* to bacitracin was 52.7% 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 while, 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. Marcin et al., (2015) recorded that antibiotic resistance-determinants are frequently associated with plasmids and other mobile genetic elements. Several groups of plasmids were found to play an important role in the dissemination of resistance genes encoding *β*-lactamases. Yunoki et al., (2016) recorded that antimicrobial susceptibility of the isolates was also evaluated and more than 90% of the isolates of Clostridium species were susceptible to beta-lactam/beta-lactamase inhibitor combinations. On the other hand, Roberts et al., (1999) stated that the most common mechanism of macrolide resistance is target-site modification due to a methylase encoded by the *erm*B gene. The ermB genotype display high-level resistance to all macrolides.

By PCR, Alpha gene was detected in 17 out of 18 samples in isolates from apparently health 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 the 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 strains isolated from dead chickens was significantly higher (8 of 17) than the rate among healthy chickens (2 of 50). Fan et al., (2016) reported that all the isolates were *C. perfringens* type A, only possessing 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.

Traditionally, in vivo serum neutralization tests in mice or dermonecrotic test in guinea pigs was performed to classify *C. perfringens* according to toxin produced by each type. In addition, Ashgan et al., (2013) used dermonecrotic test in guinea pigs for typing of *C. perfringens* isolates from different origins. Rahman et al., (1999) used guinea pigs for studying the clinical and histopathological effect of *C. perfringens* toxins by intraperitoneal (I.P) injection of the crude toxins. The I.P injection of 0.3 ml of the toxin produced typical clinical signs and pathological changes. Moreover, Chen et al., (2015) identified that TpeL, which is likely produced in the intestine during disease caused by TpeL-positive type B and C strains, as a toxin whose cytotoxicity decreases after treatment with Trypsin. This finding may have pathophysiologic relevance by suggesting that, like beta toxin, TpeL contributes to type B and C infections in hosts with decreased trypsin levels due to disease, diet, or age.

Postmortem examination of Guinea pigs infected experimentally with *C. perfringens* revealed hydrothorax, pulmonary edema, and congestion of the lung. Liver was enlarged and friable. Similar results were recorded by Gardner (1973), EL Bardisy et al. (1995), Uzal et al. (2002) and Adamson et al. (2005). Microscopical examination of guinea pigs infected experimentally with *C. perfringens* showed pulmonary edema, lymphocytic and suppurative pneumonia and mild to severe vacuolar degeneration, necrosis of some hepatocytes with activation of Kupffer cells, Necrotic enteritis with sloughing of epithelial lining mucosa, Degeneration and necrosis in renal tubules, atrophy in some glomeruli. The pervious findings also were obtained by EL Bardisy et al., (1995), Uzal et al., (2002); Uzal et al., (2004) and Mariano et al., (2005). Injection of toxic filtrates into animals caused edema, necrosis in addition to capillary and venous thrombosis and these findings also were obtained by Topley and Wilsons (1998).

To detect the effect of Trypsin enzyme on Tpel toxin, the toxin was injected in guinea pigs with or without treatment with Trypsin enzyme. Treatment with Trypsin inhibited the pathogenic effect of Tpel toxin. This finding agreed with Chen et al., (2015) who identified that TpeL cytotoxicity decreases after treatment with trypsin.

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 Net-B gene.

REFERENCES

1. Johansson A., Aspan A., Bagge E., Baverud V., Engström B.E., Johansson K.E. (2006). Genetic diversity of Clostridium perfringens type A isolates from animals, food poisoning outbreaks and sludge. BMC Microbiol., 6: 47.
2. Florence, L.C.H., Hakim, S.L., Kamaluddin, M.A., Thong, K.L., (2011). Determination of toxinotypes of environmental Clostridium perfringens by polymerase chain reaction. Trop. Biomed, 28(1): 171–174.
3. Songer, J.G., Uzal, F.A. (2005). Clostridial enteric infections in pigs. J. Vet. Diagn. Invest., 17: 528–536.
4. Kalender, H., Erats, H.B., Cetinkaya, B., Muz, A., Arsan, N., Kilie, A. (2005). Typing of isolates of C. perfringens from healthy and diseased sheep by multiplex PCR. Vet. Med. Czech, 50(10): 439-422.
5. Popoff MR, Bouvet P. (2009). Clostridial Future Microbiol; toxins., 4: 1021-1064.
6. Rahman, M.S., Rahman, M.M., Khan M.A.H.N.A. (1999). Clinic pathological observation of *C.perfringens* toxins in experimental guinea pigs. Indian. Vet. J., 6(6): 497-500.
7. Cooper, K. K., Songer, J. G., Uzal, F. A. (2013). Diagnosing clostridial enteric disease in poultry. J Vet Diagn Invest., 2013 May; 25(3): 314-27.
8. Garmory, H.S., Chanter, N., French, N.P., Bueschel, D., Songer, J.G., Titball, R.W. (2000). Occurrence of Clostridium perfringens beta2-toxin amongst

animals, determined using genotyping and subtyping PCR assays. Epidemiol Infect, 124: 61–67.
9. Gurjar A, Li J, McClane BA. 2010. Characterization of toxin plasmids in *Clostridium perfringens* type Cisolates. Infect Immun, 78: 4860–4869.
10. Smith, L.D.S., Holdman, L. (1968). The pathogenic anaerobic bacteria. 1st Ed., pp. 201-255.
11. Koneman, E.W., Allen, S.D, Dowell, V.R., Summers, H.W. (1992). Colour atlas and text book of diagnostic microbiology. 4th Ed. J.B. Lippin Cott, New York, London.
12. Macfaddin, J.F. (2000): Biochemical test for identification of medical bacteria. 3 Ed. Lippincott Williams and Willikons, Washington, Philadelphia, USA.
13. Chean R, Kotsanas D, Francis MJ, Palombo EA, Jadhav SR, Awad MM, Lyras D, Korman TM, Jenkin GA (2014). Comparing the identification of Clostridium spp. by two Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) mass spectrometry platforms to 16S rRNA PCR sequencing as a reference standard: a detailed analysis of age of culture and sample preparation. Anaerobe, 2014 Dec.; 30: 85-9.
14. Clinical and laboratory standers institute (2012). suggested groupings of antimicrobial agents that should be considered for routine testing and reporting on anerobic organism, 32: 42-43.
15. Yoo, H.s., Lee, S.U., Park, K.Y., Park, Y.H. (1997). Molecular Typing and Epidemiological Survey of Prevalence of *Clostridium perfringens* Types by Multiplex PCR. JOURNAL OF CLINICAL MICROBIOLOGY, 35(1): 228-232.
16. Datta, S., Rakha, N.K., Narang, G., Arora, D., Mahajan, N.K. (2014). Prevalence oF α, ß, Epsilon, Iota and NetB toxin producing strains of *Clostridium perfringens* in broiler chickens in Haryana. Haryana Vet., June, 2014; 53(1): 39-42.
17. Bailey, M.A., Macklin, K.S., Krehling, J.T. (2013). Use of a Multiplex PCR for the Detection of Toxin-Encoding Genes *netB* and *tpeL* in Strains of *Clostridium perfringens.* ISRN Veterinary Science, Volume 2013, Article ID 865702, 4 pages.
18. Soge, O.O., Tivoli, L.D., Meschke, J.S., Roberts, M.C. (2009). A conjugative macrolide resistance gene, mef(A), in environmental Clostridium perfringens carrying multiple macrolide and⁄or tetracycline resistance genes. Journal of Applied Microbiology, 106: 34–40.
19. Catalán, A., Espoz, M.C., Cortés, W., Sagua, H., González, J., Araya, J.E. (2010). Tetracycline and penicillin resistant Clostridium perfringens isolated from the fangs and venom glands of Loxosceles laeta: Its implications in loxoscelism treatment. Toxicon, 56: 890–896.
20. Sterne, M. and Batty, I. (1975). Pathogenic clostridia. Butter Worth, London, Boston.
21. Roberts, R.S., Guven, S., Worrall, E.E. (1970). Response of Turkish breeds of sheep to Clostridium antigen. Pendik Vet. Kontrol Ara. Inst. Derg, 2(2): 39-43.
22. Bancroft, D. J., Cook, C.H., Stirting, R.W., Turner, D.R. (1994). Manual of histopathological Techniques and their diagnostic application. Churchill, Livingstone, Edinburgh.
23. El-Refaey, T.M. (1999). Bacteriological studies on Clostridium microorganisms in poultry. M.V.Sc. Thesis, Microbiology, Fac. of Vet. Med., Cairo University.
24. Rasha, M. (2009). Studies on *Clostridial* microorganisms infection in poultry and trials for vaccination. Department of Bacteriology, Immunology and Mycology, Faculty of Veterinary Medicine, Minufiya University.
25. Osman K.M., Soliman Y.A., Amin Z.M., Aly M.A. (2012). Prevalence of Clostridium perfringens type A isolates in commercial broiler chickens and parent broiler breeder hens in Egypt. Rev Sci Tech., Dec; 31(3): 931-41. PubMed PMID: 23520746.
26. Fan Y.C., Wang C.L., Wang C., Chen T.C., Chou C.H., Tsai H.J. (2016). Incidence and Antimicrobial Susceptibility to Clostridium perfringens in Premarket Broilers in Taiwan. Avian Dis., 60(2): 444-9.
27. Eman A. K., Sohad M. D., Bakry. M.A., Hakim A.S. (2013). Molecular Diversity of Alpha Toxin Produced by Clostridium perfringens Strains Causing Avian Necrotic Enteritis. World Appl. Sci. J., 21(1): 15-20.
28. Abd-ELALL, M.M., Maysa A.I.A. (2014). Toxin genotyping of Clostridium perfringens isolated from broiler and layer farms and their workers in Egypt. Revue Méd. Vét., 2014; 165: 9-10, 272-279.
29. Sarkar M., Ray J. P., Mukhopadhaya S. K., Niyogi, D., Ganguly S. (2013). Study on *Clostridium perfringens* type A infection in broilers of West Bengal, India. *IIOABJ*, 4: 1-3.
30. La Scola B., Fournier P.E., Raoult, D. (2011). Burden of emerging anaerobes in the MALDI–TOF and 16S rRNA gene sequencingera. *Anaerobe*., 17(3): 106–112.
31. Alam S.I., Kumar B., Kamboj D.V. (2012). Multiplex detection of protein toxins using MALDI-TOF-TOF tandem mass spectrometry application in unambiguous toxin detection from bioaerosol. Anal Chem., 4; 84(23): 10500-7.
32. AlMogbel M.S. (2016). Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry for identification of Clostridium species isolated from Saudi Arabia.
33. Braz J Microbiol, 2016 Apr-Jun; 47(2): 410-3. doi: 10.1016/j.bjm.
34. Liu H., Ray W.K., Helm R.F., Popham D.L., Melville S.B. (2016). Analysis of the Spore Membrane Proteome in Clostridium perfringens Implicates Cyanophycin in Spore Assembly. J Bacteriol, 27; 198(12): 1773-82.
35. Silva, R.O.S., Salvarani, F.M., Assis, R.A., Martins, N.R.S., Piers, P.S., Lobato, F.C.F. (2009).

Antimicrobial susceptibility of *Clostridium perfringens* strains isolated from broiler chickens. Braz. J. Microbiol., 40: 262-264.
36. Osman K.M., Elhariri M. (2013). Antibiotic resistance of Clostridium perfringens isolates from broiler chickens in Egypt. Rev Sci Tech., Dec.; 32(3): 841-50.
37. Martel, A., Devvriese, L.A., Cauwerts, K., De Gussem, K., Decostere A., Haesebrouck, F. (2004). Susceptibility of *Clostridiuin perfringens* strains from broiler chickens to antibiotics and anticoccidials. Avian Pathol. 2004 Feb.; 33(1): 3-7.
38. Marcin A., Piotr Z., Lukasz D., Renata W., Marta N., Pawel W., Piotr K., Andrzej P., Dariusz B. (2015). Diversity and Global Distribution of IncL/M Plasmids Enabling Horizontal Dissemination of β-Lactam Resistance Genes. BioMed Research International. Volume 2015, Article ID 414681, 12 pages. http://dx.doi.org/10.1155/2015/414681
39. Yunoki T., Matsumura Y., Nakano S., Kato K., Hotta G., Noguchi T., Yamamoto M., Nagao M., Takakura S., Ichiyama, S. (2016). Genetic, phenotypic and matrix-assisted laser desorption ionization time-of-flight mass spectrometry-based identification of anaerobic bacteria and determination of their antimicrobial susceptibility at a University Hospital in Japan. J Infect Chemother, 2016 May; 22(5): 303-7.
40. Roberts, M. C., Sutcliffe, J., Courvalin, P., Jensen, L. B., Rood, R., Seppa la., H. 1999. Nomenclature for macrolide and macrolide-lincosamidestreptogramin B resistance determinants. Antimicrob. Agents Chemother, 43: 2823–2830.
41. Cooper K.K., Trinh H.T., Songer J.G. (2009). Immunization with recombinant alpha toxin partially protects broiler chicks against experimental challenge with Clostridium perfringens. Vet. Microbiol., 33: 92–97.
42. Younes, A.M. (2005). Use of Polymerase Chain Reaction (PCR) For Diagnosis of *Clostridium Perfringens* in Chicken. MVSC. Thesis, Fac. Vet. Med., Cairo Univ.
43. Siragusa, G.R., Danyluk, M.D., Hiett, K.L., Wise, M.G., Craven, S.E. (2006). Molecular subtyping of poultry- associated type "A" *Clostridium perfringens* isolated by repetitive element PCR. J. Clin. Microbiol., 44(3): 1065-1073.
44. Coursodon C.F., Glock R.D., Moore K.L., Cooper K.K., Songer J.G. 2012. TpeL-producing strains of *Clostridium perfringens* type A are highly virulent for broiler chicks. Anaerobe, 18: 117–121.
45. Park J.Y., Kim S., Oh J.Y., Kim H.R., Jang I., Lee H.S., Kwon Y.K. (2015). Characterization of Clostridium perfringens isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. Poult Sci., 94(6): 1158-64. doi: 10.3382/ps/pev037. PubMed PMID: 25840962.
46. Keyburn A.L., Bannam T.L., Moore R.J., Rood J.I. 2010. NetB, a poreforming toxin from necrotic enteritis strains of *Clostridium perfringens*. Toxins, 2: 1913–1927.
47. Ashgan M. H., Abdullah A. A., Moussa I. M. (2013). Molecular typing of four major toxins of Clostridium perfringens recovered from Egypt. Journal of Food, Agriculture & Environment, 11(1): 151-155.
48. Rahman, M.S., Rahman, M.M., Khan, M.A.H.N.A. (1999). Clinic pathological observation of *C.perfringens* toxins in experimental guinea pigs. Indian. Vet. J., 76(6): 497-500.
49. Chen J., McClane B.A. 2015. Characterization of *Clostridium perfringens* TpeL toxin gene carriage, production, cytotoxic contributions, and trypsin sensitivity. Infect Immun, 83:2369 –2381.
50. Gardner, D.E. (1973): Pathology of Clostridium welchii type D enterotoxemia. I. Biochemical and haematological alterations in lambs. J. Comp. Pathol., 83: 499-507. II. Structural and ultrastructural alterations in tissue of lamb and mice, 509-524.
51. El-Bardisy, M. M., Mervat, A. R., Gehan, A. H., Gehan, J. S. (1995). Bacteriological and pathological studies on guinea pigs microorganisms. Egypt. J. Comp. Pathol. and Clinical Pathol., Vol. 8, No. 2 (October), 1-14.
52. Uzal, F. A., Kelly, W. R., Moriss, W. E., Assis, R. A. (2002). Effects of intravenous injection of Clostridium perfringens type D epsilontoxin in calves. J. Comp. Pathol., 126(1): 71-75.
53. Adamson, R.H., Lyee, S., Ternande, Z., Miyakawa, M., Ochi, S. (2005). Clostridium perferingens epsilon toxin increases permeability of single perfused microvessels of rat mesentry. Infect. Immun., 73(8): 4879 – 4887.
54. Uzal, F. A., Kelly, W. R., Moriss, W. E., Bermude, Z. J., Baison, M. (2004). The pathology of peracute experimental Clostridium perfringens type D enterotoxemia in sheep. J. Vet. Diagnostic Investigation, 16(5): 403-410.
55. Mariano, E., Miyakawa, F., Uzal, F. A. (2005). Morphologic and physiologic changes induced by Clostridium perfringens type A and toxin in the intestine of sheep. Am. J. Vet. Res., 66(2): 251-255.
56. Topley, Y., Wilsons, T. (1998). Microbiological and Microbial Infections. 9th ed., Vol. 3., Systemic bacteriology Oxford University Press, USA.