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Genetic characterization, antibiogram pattern, and pathogenicity of *Clostridium perfringens* isolated from broiler chickens with necrotic enteritis

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ABSTRACT

The aims of this investigation were characterization, demonstration of the antibiogram pattern and detection of the pathogenicity of *Clostridium perfringens* (*C. perfringens*) strains isolated from broiler chickens in Damietta governorate, Egypt. A total of 357 samples representing 202 intestinal contents and 155 liver samples from freshly dead broiler chickens were collected from 18 broiler farms. Isolates of *C. perfringens* were identified morphologically, microscopically, and biochemically. Forty-seven *C. perfringens* isolates were recovered, which represented 20.3% of the intestinal contents and 3.8% of the liver samples. The toxins and virulence genes of *C. perfringens* were investigated using polymerase chain reaction. All of the toxigenic *C. perfringens* strains were type A and carried *netB*, *tpeL*, *cpe*, and *plc* genes. The *in vitro* antibiogram of *C. perfringens* strains revealed 100% sensitivity to gentamycin and levofloxacin and 100% resistance to nalidixic acid and ceftriaxone. The isolated *C. perfringens* strains were highly pathogenic and induced signs and lesions of necrotic enteritis as well as 43.3% mortalities in 20-day-old chicks. In conclusion, *C. perfringens* is an important pathogen that affects broiler chickens due to the presence of virulence genes and the pathogenicity in the inoculated birds.

Keywords: Antibiotics, C. perfringens, PCR, Poultry, Toxins

INTRODUCTION

Enteric diseases are very important in the poultry industry as they lead to production losses, mortalities, and risk of contamination of poultry products (Dahiya *et al*., 2006). Infection with *Clostridium perfringens* (*C. perfringens*) is considered one of the most critical enteric problems in chickens and causes necrotic enteritis (NE) (Cooper *et al*., 2013). The first case of NE in fowl was reported in Australia in 1930 and was fully investigated in England (Parish, 1961). Later, the disease spread rapidly in almost all poultry-producing countries around the world (Finken and Wages, 1997). The disease causes severe economic problems represented by low feed conversion rate, mortalities, and increased treatment costs (Cooper and Songer, 2009). The production losses due to NE outbreaks in the global poultry industry are estimated to be US $6 billion annually (Moore, 2016). The main sources of NE infection are litter and contami-

nated environment (Craven *et al*., 2003; Profeta *et al*., 2020), and transmission of infection occurs through ingestion of contaminated food and water. Husbandry practices like diet and litter types influence the incidence and severity of NE in poultry (Henry *et al*., 1995). Two to six-week-old broiler chickens and 12- to 24-week-old layers are highly susceptible to NE (Lovland *et al*., 2004). Affected birds with acute NE show severe necrosis and damage of the intestinal mucosa, which lead to high mortalities (Wu *et al*., 2010) and poor performance in subclinical cases (Skinner *et al*., 2010).

The causative agent of NE is *C. perfringens*, which is a Gram-positive, anaerobic, and spore-forming bacillus (Timbermont *et al*., 2011). These bacilli are found naturally in the soil, water, sewage, food, and feces as well as in the intestinal tracts of livestock, poultry, and humans (Li *et al*., 2016). *C. perfringens* is considered a normal inhabitant of the birds' intestinal tract as well as a potential pathogen causing NE. Strains of *C. perfringens* are divided into seven extracellular toxin types: A, B, C, D, E, F, and G (Rood *et al*., 2018; Goossens *et al*., 2020). However, *C. perfringens* type A and to a lesser extent type C have been shown to be the major cause of NE in chickens (Cooper and Songer, 2009). Moreover, alpha (α) toxin is primarily responsible for NE in poultry (Keyburn *et al*., 2010). The virulence of *C. perfringens* is attributed to more than 20 toxins and hydrolytic enzymes (Kiu and Hall, 2018; Gu *et al*., 2019), while individual strains only produce a subset of these toxins (Van Immerseel *et al*., 2008). Major extracellular toxins of *C. perfringens* are alpha (α) (*cpa*), beta (β) (*cpb*), epsilon (ε) (*etx*), and iota (ι) (*iap*). However, different strains of *C. perfringens* can also produce other enzymes and toxins, namely, β2, theta (θ) [perfringolysin O (*PFO*)], kappa (κ), delta (δ), mu (μ), sialidase, hyaluronidase, collagenase, neuraminidase, enterotoxin (*cpe*), necrotic enteritis toxin B-like (*netB*), and toxin perfringens large (*tpeL*) (Lukinmaa *et al*., 2002; Li *et al*., 2013; Duff *et al*., 2019; Wei *et al*., 2020).

All *C. perfringens* type A strains possess phospholipase C (*plc*) or *cpa* gene that produces α toxin in varying amounts (Kumar *et al*., 2019; Helal *et al*., 2019). This gene is present on the chromosome close to the origin of replication of all *C. perfringens* strains (Canard *et al*., 1989). It was found that *netB* and *tpel* toxins play a role in the virulence of some *C. perfringens* strains of avian origin (Rood *et al*., 2016; Elsharkawy *et al*., 2020; Thi *et al*., 2021). Most *C. perfringens* strains that produce a pore-forming toxin (*netB*) belong to toxin type G (Rood *et al*., 2018). In addition, *tpel*, a recently designated novel family member of large clostridial cytotoxins, was detected in some *C. perfringens* type A strains isolated from NE cases (Coursodon *et al*., 2012; Mwangi *et al*., 2019). Enterotoxin gene (*cpe*) coding toxin of *C. perfringens* has been identified by Gao and McClane (2012), and it induces gastroenteritis (Lukinmaa *et al*., 2002).

NE has become a hurdle affecting broiler production especially after the great restrictions on the application of antibiotics in ration under modern high stalking density (Van Immerseel *et al*., 2008). Therefore, there is an urgent need to select the drug of choice to control this critical disease.

Therefore, this study aimed to characterize, investigate the antibiogram pattern and determine the pathogenicity of *C. perfringens* strains isolated from broiler chickens in Damietta governorate, Egypt.

MATERIALS AND METHODS

Sample Collection

A total of 357 samples were taken from 202 intestines and 155 livers of sacrificed diseased and freshly dead chickens (2-8-week-old) representing 18 commercial broiler chicken farms at different locations in Damietta governorate, Egypt, from December 2019 to June 2020 (Table 1). Clinically suspected cases with NE showed anorexia, depression, reluctance to move, diarrhea, and death. Sacrificed and dead chickens showed dehydration, enteritis, ballooned and friable intestines with hemorrhages, and yellow diphtheritic necrotic membranes on the mucosa

Table 1. The number of the examined samples distributed in Damietta governorate, Egypt

Locality	Number of examined farms	Number of examined samples	
		Intestine	Liver
Kafer-Saad	3	18	18
Farskour	3	32	31
Om El-Reda	4	44	40
Kafer El-Batekh	2	19	9
Cinania	1	11	11
Kafer-ElGhabe	2	35	29
Zarka	3	43	17
Total	18	202	155

as well as liver necrosis. The samples were aseptically collected in sterile plastic bags and quickly transported to the laboratory in ice-cooled containers for further microbiological examination.

Conventional Isolation and Identification

Sample processing was done according to a routine protocol as previously described by Willis (1977). For enrichment, one gram of each of the intestinal contents or liver tissue samples was inoculated into tubes of freshly prepared Robertson cooked meat broth (Oxoid, UK) and incubated for 24 h at 37°C in a Gas-Pak anaerobic jar. Aliquots of 0.1 ml were streaked onto a perfringens agar base containing 400 µg/ml of tryptose sulfite cycloserine (TSC) with egg emulsion (Oxoid, UK) and incubated anaerobically. For the proliferation and detection of the hemolytic characteristics of Clostridium isolates, 5% de-fibrinated sheep blood agar with neomycin sulphate (200 µg/ml) was prepared. After 24-48 h incubation at 37°C, typical black colonies were selected and cultured onto de-fibrinated 5% sheep blood agar and egg yolk agar plates and incubated anaerobically for 24 h at 37°C (Cruickshank *et al*., 1975). Typical colonies on blood agar or egg yolk agar were further identified according to the morphological characteristics using Gram staining and different biochemical tests, such as catalase, nitrate reduction, gelatinase, lecithinase, indole, oxidase, urease storm gas production on litmus milk medium, and fermentation of glucose, lactose, fructose, sucrose, and mannitol.

Molecular Detection of the Toxins and Virulence Genes

DNA extraction from suspected samples was performed using the QIAamp DNA Mini Kit (Qiagen, Germany, GmbH) with modifications according to the manufacturer's recommendations. Briefly, 200 µl of the sample suspension was incubated with 10 µl of proteinase K and 200 µl of lysis buffer at 56°C for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. Primers provided by Metabion (Germany) are listed in Table (2). Multiplex polymerase chain reaction (PCR) was used for the detection of α, β, ε, and ι toxins. Primers were utilized in a 50 µl reaction containing 25 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 11 µl of water, and 6 µl of DNA template. For uniplex PCR, primers were utilized in a 25 µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. All the reactions were performed in an Applied Biosystems 2720 thermal cycler.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analy-

Table 2. Primers sequences, target genes, amplicon sizes and cycling conditions for *C. perfringens*.

Target toxin and virulence genes	Primers sequences	Amplified segment (bp)	Amplification (35 cycles)					Reference
			Primary denaturation	Secondary denaturation	Annealing	Extension	Final extension	
α	GTTGATAGCGCAG GACATGTTAAG CATGTAGTCATCT GTTCCAGCATC	402	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Yoo *et al*. (1997)
β	ACTATACAGACAG ATCATTCAACC TTAGGAGCAGTTA GAACTACAGAC	236						
ε	ACTGCAACTACTA CTCATACTGTG CTGGTGCCTTAAT AGAAAGACTCC	541						
ι	GCGATGAAAAGCC TACACCACTAC GGTATATCCTCCA CGCATATAGTC	317						
NetB	GCTGGTGCTGGAA TAAATGC TCGCCATTGAGTA GTTTCCC	560	94˚C 5 min.	94˚C 30 sec.	58˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Datta *et al*. (2014)
TpeL	ATATAGAGTCAAG CAGTGGAG GGAATACCACTTG ATATACCTG	466	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	Bailey *et al*. (2013)
cpe	ACATCTGCAGATA GCTTAGGAAAT CCAGTAGCTGTAA TTGTTAAGTGT	247	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Kaneko *et al*. (2011)
Plc	ATA GAT ACT CCA TAT CAT CCT GCT	283	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	Akhi *et al*. (2015)

sis, 40 µl of the multiplex PCR products and 15 µl of the uniplex PCR products were loaded in each gel slot. A gel pilot 100 bp ladder (Qiagen, Gmbh, Germany) and gene ruler 100 bp ladder (Fermentas, Thermo, Germany) were used to determine the fragment sizes. The gel was photographed using a gel documentation system (Alpha Innotech, Biometra), and the data was analyzed through computer software.

The Antibiogram Pattern

The antimicrobial susceptibility testing of *C. perfringens* strains was done using the disc diffusion method developed by the National Committee for Clinical Laboratory Standards (NCCLS, 2007). The used chemotherapeutic agents discs (Oxoid) and the inhibition zones (susceptible, intermediate susceptibility, and resistant) are shown in Table (3). All *C. perfringens* strains were cultivated in cooked meat broth for 24 h, and then the culture broth was suspended into 0.85% NaCl to obtain an optical density equal to MacFarland 0.5 standards. After that, the strains were inoculated in 5% defibrinated sheep blood agar for 10 minutes and the antibiotic discs were dispersed in the agar plates. The plates were incubated anaerobically at 37°C overnight, and the inhibition zones were

Table 3. The interpretation of *C. perfringens* antibiogram pattern

Antibiotic disc (Code)	Disc content/ µg	Interpretation (Diameter of the zone/ mm)		
		Susceptible ≥	Intermediate susceptibility	Resistant ≤
Amoxycillin/Clavulanic acid (AMC)	20/10	18	14-17	13
Neomycin (NE)	10	17	-	16
Doxycycline (Do)	30	16	13-15	12
Erythromycin (E)	15	21	16-20	15
Nalidixic acid (NA)	30	19	14-18	13
Penicillin (P)	10	22	-	23
Ciprofloxacin (CIP)	5	21	16-20	15
Gentamycin (CN)	10	15	13-14	12
Levofloxacin (LEV)	5	17	14-16	13
Ceftriaxone (CES)	30	28	24-27	23

measured as recommended by the manufacturer.

The Pathogenicity Test in Broiler Chickens

The experiment was done according to the National Regulations on Animal Welfare and Institutional Animal Ethical Committee (IAEC). A total of 105 day-old Cobb chicks were obtained from local hatcheries, and five birds were subjected on arrival to bacteriological examination to confirm the absence of *C. perfringens*. The chicks were reared on thoroughly cleaned and disinfected semi-closed houses and vaccinated using the standard protocol for vaccination. Feed and water were given *ad libitum*. The ration was supplemented with 12% wheat to enhance the experimental induction of infection. The chicks were divided into two equal groups, each containing 50 birds. Group (1) was the negative control non-challenged group and was inoculated with sterile phosphate buffered saline. Each bird in group (2) was orally inoculated with a field mixture of *Eimeria* oocysts in a dose of $1x10^3$ sporulated oocysts/0.1ml of oocysts mixture at the age of 10 days. However, at the age of 20 days, each chick in group (2) was challenged orally with 1 ml of 24 hr broth culture containing 1.7×10^8 viable cells of the toxigenic strain of *C. perfringens* type A for four successive days (Timbermont *et al*., 2009). All chicken groups were kept under observation for two weeks post-challenge (PC) to monitor the clinical picture.

RESULTS AND DISCUSSION

C. perfringens is a widely distributed bacterium in the environment and is mostly found in the intestinal tracts of humans and domestic animals (Kiu and Hall, 2018). The organism is a major enteric pathogen that can lead to both clinical (Long and Truscott, 1976) and subclinical diseases (Lovland and Kaldhusdal, 2001). The pathogen is responsible for causing NE in poul-

Table 4. The incidence rate and the type of *C. perfringens* in Damietta governorate, Egypt

Age of chicken/Week	Intestine			Liver			
	No. of samples	No. of positive	% positive	No. of samples	No. of positive	% positive	No. of examined farms
1-2	62	2	3.2	47	0	0	5
2-3	56	15	26.8	48	3	6.3	10
3-4	45	13	28.9	39	2	5.1	12
4-8	39	11	28.9	21	1	4.8	8
Total	202	41	20.3	155	6	3.8	18

try, especially *C. perfringens* type A, which is the most frequently isolated clostridial type (Opengart, 2008).

Based on the cultural, morphological, and biochemical characteristics of the isolates, 20.3% and 3.8% *C. perfringens* isolates were recovered from 202 intestine and 155 liver samples, respectively, from freshly dead broiler chickens in Damietta governorate (Table 4). Morphologically, *C. perfringens* isolates grew anaerobically and produced double zones of hemolysis (an inner zone of complete hemolysis and an outer zone of discoloration and incomplete hemolysis) on 5% sheep blood agar with neomycin sulfate (Figure 1). However, *C. perfringens* isolates on TSC showed black colonies due to the reduction of sulfite to sulfide, which in turn reacts with iron and forms a black iron sulfide precipitate (Figure 2). A zone of opalescence appeared around the *C. perfringens* colonies on egg yolk agar plates. Microscopically, *C. perfringens* isolates revealed Gram-positive, non-motile, and spore-forming large-sized bacilli. Biochemically, all *C. perfringens* isolates were positive for nitrate reduction and lecithinase activity (Figure 3), but they were negative for catalase, indole production, and oxidase. The isolates produced typical stormy fermentation reaction in litmus milk medium.

Manfreda *et al*. (2006) isolated *C. perfringens* from broiler farms with a rate over 90% and found *C. perfringens* in 87 out of 149 samples (58.40%). However, the lowest frequency of isolated *C. perfringens* was reported by Kalender and Ertas (2005) who showed that only 5% of the intestinal contents were positive for *C. perfringens*. In Egypt, Hussein and Mustfa (1999) demonstrated 30 isolates of *C. perfringens* out of 60 intestinal samples (50%) in 4-6-week-old broiler chickens in Assiut governorate, while Ebtehal (2000) found that out of 470 broiler chicken samples, 231 (71.9%) strains of *C. perfringens* could be isolated in Assiut and El-Minia governorates. This high incidence was not surprising if the spread of the microorganisms in the environment, diet, water, litter, and slaughtering houses was considered. Other Egyptian studies reported isolation of *C. perfringens* from the intestines of both apparently healthy and diseased broiler chickens with high rates of 42.0% and 91.3%, respectively (El-Refaey *et al*., 1999); 30% and 75%, respectively (Rasha, 2009); and 35.4% and 100%, respectively (Osman *et al*., 2012). Moreover, *C. perfringens*

Figure 1. Colonies of *C. perfringens* on 5% sheep blood agar with neomycin sulfate showing double zones of β hemolysis (Inner zone of complete hemolysis and outer zone partial hemolysis).

Figure 2. Colonies of *C. perfringens* on tryptose sulfite cycloserin (TSC) showing black colonies.

Figure 3. Lecithinase activity of *C. perfringens* on egg yolk agar (lecithinase: α toxin phospholipase hydrolyzes phospholipids in egg yolk agar around streaks).

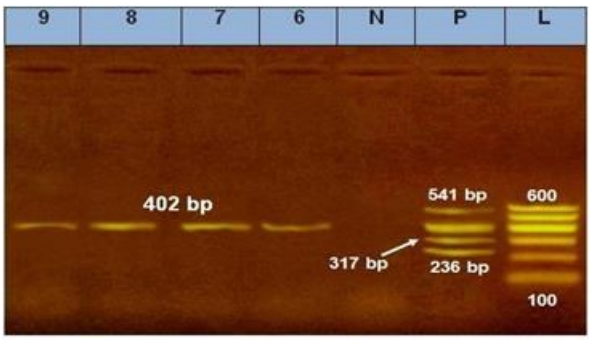


Figure 4. PCR amplification using *Clostridium* genus -specific primers for toxins (α, β, ε and ι), P= Positive control, L= Ladder, Lines 6-9 = *C. perfringens* type A strains, N= Negative control.

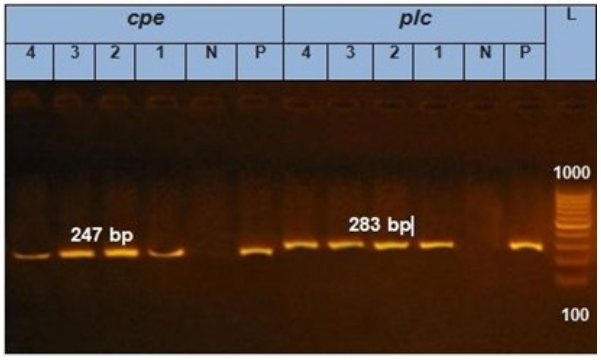


Figure 5. PCR amplification using *C. perfringens* genus-specific primers (*cpe* and *plc* genes), P= Positive control, L= Ladder, lines 1-4 = *cpe* and *plc* genes of *C. perfringens*, N= Negative control.

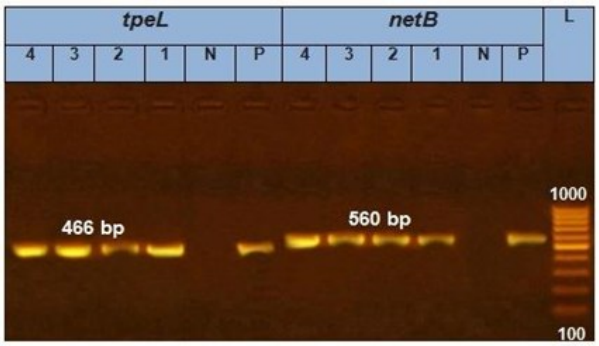


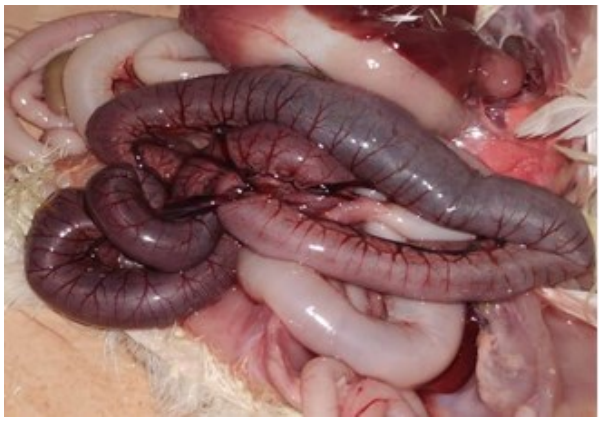
Figure 6. PCR amplification using *C. perfringens* genus-specific primers (*tpeL* and *netB* genes), P= Positive control, L= Ladder, lines 1-4 = *tpeL* and *netB* genes genes of *C. perfringens*, N= Negative control.

was isolated from the intestines of chickens with NE with incidence rates of 47.70% (El-Rash, 2012) and 60% (Eman *et al*., 2013). Out of 120 intestine and liver samples taken from diseased broiler chickens, EI-Jakee *et al*. (2013) isolated 90 (75%) *C. perfringens* with an incidence rate of 53.8%.

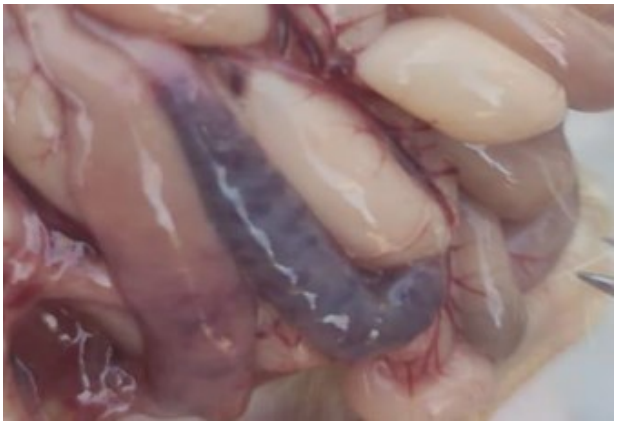
Multiplex PCR showed that *C. perfringens* strains belonged to type A as they contained the *cpa* gene (402 bp) that coded for α toxin and the *cpb* (236 bp), *etx* (541 bp), and *iA* (317 bp) genes that coded for β, ε, and ι. toxins, respectively (Figure 4). Molecular detection of the virulence genes of *C. perfringens* type A strains showed the presence of the *netB*, *tpeL*, *cpe*, and *plc* genes in all isolated strains (Figures 5 and 6). The PCR-based detection of α toxin is essential for the typical identification of α toxigenic *C. perfringens* strains (Baums *et al*., 2004). Several *Clostridia* enteric diseases occur in poultry, but probably the most common and severe one is NE, caused by *C. perfringens* type A (Moore, 2015). In Sweden, Engstrom *et al*. (2003) demonstrated that all *C. perfringens* strains were classified as type A without enterotoxin genes. Furthermore, in Finland, Heikinheimo and Korkeala (2005) showed that 118 poultry isolates of *C. perfringens* were classified as type A strains using multiplex PCR. In a Belgian study, five out of 63 *C. perfringens* isolates were β2 toxin-positive, and the authors indicated that this type of toxin is not an essential virulence factor in the development of NE in poultry (Gholamiandekhordi *et al*., 2006).

It is well known that *C. perfringens* type A induces intestinal mucosal damage in chickens (Moore, 2015). The α toxin producing *C. perfringens* is phospholipase C sphingomyelinase that hydrolyzes lecithin into phosphorylcholine and diglyceride and as a consequence induces the production of inflammatory mediators causing blood vessel contraction, platelet aggregation, myocardial dysfunction, and finally acute death (Matsuda *et al*., 2019).

Detection of *C. perfringens* toxin types and subtypes is critical for a better understanding of the epidemiology of *C. perfringens* infection and may be helpful in the implementation of effective preventive measures (Fancher *et al*., 2021). In this study, the presence of eight toxin genes (*cpa*, *cpb*, *etx*, *iA*, *netB*, *tpeL*, *plc*, and *cpe*) of *C. perfringens* type A isolates has been investigated.



A



B

Figure 7. A: The intestine is filled with blood (hemorrhagic enteritis) and distended with gases. B: The caecum is filled with blood (hemorrhagic typhlitis).

The results revealed the presence of *netB*, *tpeL*, *plc*, and *cpe* genes. This result confirms high production of toxins that lead to the destruction of the intestinal mucosa and consequently the development of NE (Mwangi *et al*., 2019). Similar findings were reported by Ebtehal (2000) who indicated the role of toxigenic *C. perfringens* in the production of toxins that lead to NE in poultry.

In addition, *C. perfringens* strains possess other common virulence genes (*netB*) producing β toxin (Yang *et al*., 2018). Since the discovery of this new virulence factor, the presence of the *netB* gene in *C. perfringens* strains has been investigated in different regions of the world. The results indicated the existence of this gene in *C. perfringens* type A strains. Johansson *et al*. (2010) reported that more than 90% of all isolates from cases of NE carried the *netB* gene. Through the examination of 36 isolates of *C. perfringens*, 19 (52.8%) isolates showed presence of the *netB* gene (Tolooe *et al*., 2011). A previous study of Miwa *et al*. (1998) demonstrated that strains of *C. perfringens* that were *netB*-negative failed to cause disease in an experimental model, whereas all *netB*-positive strains produced typical lesions of NE. In addition, it has been found that *netB*, a pore-forming toxin, plays a role in the pathogenesis of NE in poultry as a strongly necrotizing and lethal toxin (Keyburn *et al*., 2010; Wade *et al*., 2020). Native and recombinant *netB* were cytotoxic for chicken hepatocytes. The *netB* gene is mostly found in outbreaks of NE but is relatively uncommon in healthy birds (Tolooe *et al*., 2011). However, several studies demonstrated the absence of the *netB* gene in *C. perfringens* isolates (Datta *et al*., 2014; Li *et al*., 2018; Zhang *et al*., 2019).

Furthermore, all *C. perfringens* type A strains of avian origin possess phospholipase C (*plc*) or the *cpa* gene that produces α toxin (Abildgaard *et al*., 2009). This gene has also been discovered in strains of human origin (Matsuda *et al*., 2019). Moreover, Kimy *et al*. (2017) classified *C. perfringens* as a toxin type A based on the presence of the α toxin gene (*plc*).

Isolates of *C. perfringens* that have α toxins as well as enterotoxin (*cpe*) are regarded as type F. Enterotoxin (*cpe*) is produced by about 1%-5% of *C. perfringens* type A. This toxin is a single polypeptide chain of about 35 KDa and, unlike other toxins, is released upon lysis of the mother cell in the sporulation stage (Abildgaard *et al*., 2010). Previous studies showed that there is a relationship between *C. perfringens* type A isolates that carry the *cpe* gene and foodborne infection (Miyamoto *et al*., 2012) as well as non-foodborne gastrointestinal diseases (Azimirad *et*

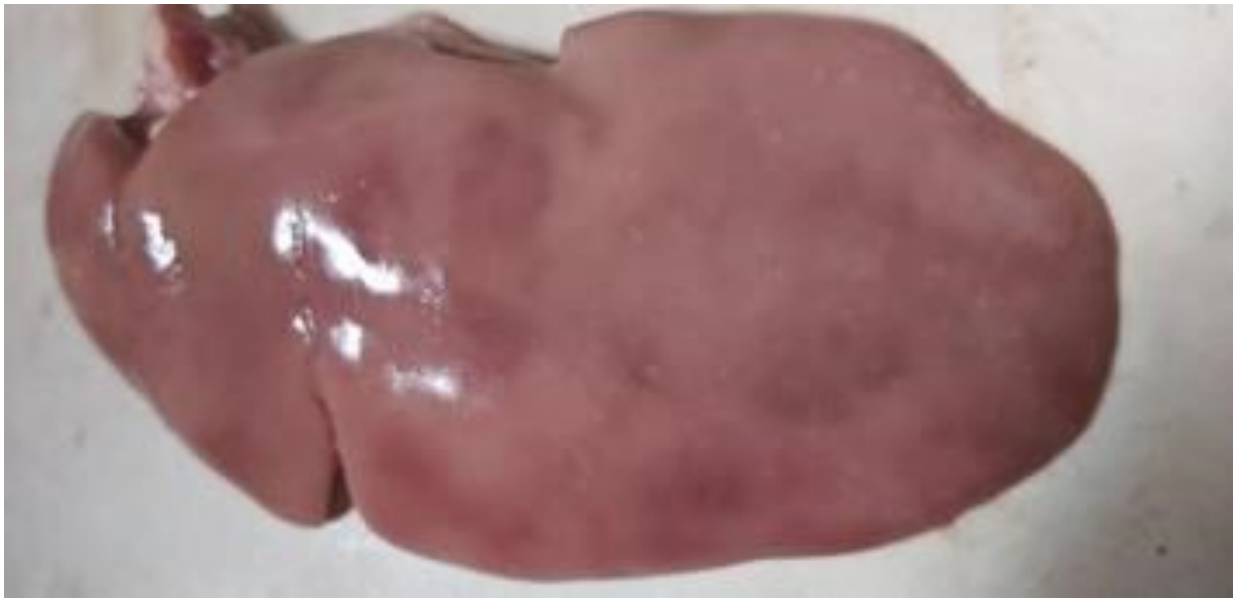


Figure 8. Enlarged and pale liver with necrotic foci.

al., 2019). The findings of this study showed that *C. perfringens* isolates carry the *cpe* gene which is similar to the findings of other studies (Asaoka *et al*., 2004**)**. The authors suggested that *cpe* plays a role in intestinal necrosis with minor intestinal damage, allowing the multiplication of *C. perfringens* and consequently development of the disease.

Periodic evaluation of *C. perfringens* antimicrobial susceptibility testing is important to avoid the losses resulting from this infection (Finken and Wages, 1997). In 47 *C. perfringens* strains, the *in vitro* sensitivity test revealed high susceptibility to levofloxacin and gentamycin (100%) as well as ciprofloxacin (85.1%). Low degree of susceptibility to doxycycline and erythromycin (25.5%), in addition to neomycin (23.4%), was reported. Resistance to penicillin, nalidixic acid, and ceftriaxone was 100%, while resistance to amoxycillin/clavulanic acid was 72.3% (Table 5). Nearly similar antibiotic sensitive patterns were observed by Mehtaz *et al*. (2013) who found that *C. perfringens* isolates were sensitive to some fluoroquinolones, such as ciprofloxacin and ofloxacin. However, these results are inconsistent with those reported by Hussein and Mostfa (1999) who stated that neomycin was highly effective but enrofloxacin was not effective against *C. perfringens*. Algammal and Elfeil (2015) reported 100% resistance of *C. perfringens* to neomycin, which is commonly used as an antimicrobial drug to treat bacterial enteritis in poultry. In this study, *C. perfringens* isolates showed resistance to nalidixic acid and amoxicillin, similar to the results reported by another study (Camacho *et al*., 2008). Nevertheless, another study demonstrated a high level of sensitivity to penicillin (Algammal and Elfeil, 2015).

Clinical signs observed among *C. perfringens*-challenged chicks in the challenged group were depression, ruffled feathers, decreased appetite, and diarrhea. Mortalities were observed at 48 hr PC at a rate of 43.3%. No clinical signs or mortalities were observed in control birds that were inoculated with phosphate buffered saline. The intestines of dead and sacrificed chickens at the end of the observation period were filled with blood (hemorrhagic enteritis) and distended with gases (Figure 7 A), and the caecum was filled with blood (hemorrhagic typhlitis) (Figure 7 B). Enlargement, paleness, and necrosis of the liver were also observed (Figure 8). The pathogenesis of *C. perfringens* infection

involves the colonization of the tissue's host, acquisition of nutrients to allow more multiplication, dodging of the immune system of the host, and finally transmission of toxins with tissue damage (Prescott *et al*., 2016). The presence of some risk factors associated with *C. perfringens* challenge enhances the development of NE clinical infection. Some predisposing factors, such as *Eimeria* species and the use of wheat and barley, are important for the induction of NE (Kocher, 2003). Moreover, *C. perfringens* infection was significantly higher in the presence of stress factors, such as worm infestation or coccidiosis (Mateos *et al*., 2002). It has been found that *Eimeria* species colonize the bird's intestinal tract, causing damage and releasing plasma proteins which is the minimal requirements for growth of *C. perfringens* include more than 11 amino acids, besides many growth factors and vitamins (Hofacre *et al*., 2003). Moreover, Lovland *et al*. (2004) reported that *C. perfringens* type A causes mucosal damage in the intestines of chickens. Regarding the pathogenicity test in broiler chicks using *C. perfringens* strains, the results revealed general signs with variable degrees of diarrhea, mortalities (43.3%), and intestinal and liver lesions. Similar observations were reported in previous studies (Freedman *et al*., 2015; Thi *et al*., 2021). Lovland and Kaldhusdal (2001) found that NE can present as an acute clinical disease characterized by sudden high mortality rates that can reach 50% in flocks. Moreover, Ebtehal (2000) found that *C. perfringens* given orally to chicks caused 80% mortality. Similar intestinal lesions were also reported in previous studies (Park *et al*., 2015; To *et al*., 2017; Abdul-Aziz and Barnes, 2018). They mentioned that infected chickens with NE showed intestinal lesions ranging from thin and friable walls to frank hemorrhagic enteritis along with gas distension. In addition, necrotic lesions present on the liver of chickens after *C. perfringens* challenge were the same as the lesions reported by Lovland and Kaldhusdal (2001), Sasaki *et al*. (2003), and Thi *et al*. (2021).

CONCLUSION

Continuous and periodical surveillance studies should be conducted to alleviate the severe economic losses caused by *C. perfringens* infection in broiler chicken flocks. Detection of the sensitivity of the bacterium to different antibiotics is a must before developing successful control and treatment strategies. Future studies on the preparation of bacterin to prevent such infection are needed.

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