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) (ets), 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.
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% defibrinated 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 defibrinated 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 t 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-
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>
<thead>
<tr>
<th>Target toxon and virulence genes</th>
<th>Primers sequences</th>
<th>Amplified segment (bp)</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>GTGGATAGGCAG</td>
<td>402</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
<td>Yoo et al. (1997)</td>
</tr>
<tr>
<td></td>
<td>GACATGTTAAG</td>
<td></td>
<td>94°C</td>
<td>30 sec.</td>
<td>40 sec.</td>
<td>45 sec.</td>
<td>10 min.</td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>CATGTAGTCATCT</td>
<td>236</td>
<td>5 min.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTCCAGCACATC</td>
<td></td>
<td>94°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACTATACAGACAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCATCAACC</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ε</td>
<td>TTAGGACAGTTA</td>
<td>541</td>
<td>94°C</td>
<td>94°C</td>
<td>58°C</td>
<td>72°C</td>
<td>72°C</td>
<td>Datta et al. (2014)</td>
</tr>
<tr>
<td></td>
<td>GAACATACGAC</td>
<td></td>
<td>94°C</td>
<td>30 sec.</td>
<td>40 sec.</td>
<td>45 sec.</td>
<td>10 min.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACTGCAACTAATA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTGACAGTTG</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AGAAAGACTCC</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t</td>
<td>GCGATGAAAAAGCC</td>
<td>317</td>
<td>94°C</td>
<td>94°C</td>
<td>58°C</td>
<td>72°C</td>
<td>72°C</td>
<td>Bailey et al. (2013)</td>
</tr>
<tr>
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<td>94°C</td>
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<td>40 sec.</td>
<td>45 sec.</td>
<td>10 min.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTATATCTCTCCA</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGCATATAGTC</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCTGAGCTGAAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NetB</td>
<td>TAAATGC</td>
<td>560</td>
<td>94°C</td>
<td>94°C</td>
<td>58°C</td>
<td>72°C</td>
<td>72°C</td>
<td>Kaneko et al. (2011)</td>
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<tr>
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<td>30 sec.</td>
<td>40 sec.</td>
<td>45 sec.</td>
<td>10 min.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GTTTCC</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>TpeL</td>
<td>ATATAGGTTCAAG</td>
<td>466</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
<td>Akhi et al. (2015)</td>
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<tr>
<td></td>
<td>CGTGGAG</td>
<td></td>
<td>94°C</td>
<td>30 sec.</td>
<td>40 sec.</td>
<td>45 sec.</td>
<td>10 min.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATATACCTG</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACATCGAGATA</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>GCATTGAAAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCAGTACCTGAAA</td>
<td></td>
<td>55°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TTGTAAAGT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>εpe</td>
<td>ATAGACCTCCTAA</td>
<td>247</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>TATCATCCTG</td>
<td></td>
<td>94°C</td>
<td>30 sec.</td>
<td>30 sec.</td>
<td>30 sec.</td>
<td>7 min.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCTGCTGCTG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Plc</td>
<td>ATA GAT ACT CCA</td>
<td>283</td>
<td>94°C</td>
<td>94°C</td>
<td>55°C</td>
<td>72°C</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TAT CAT CCT GCT</td>
<td></td>
<td>94°C</td>
<td>30 sec.</td>
<td>30 sec.</td>
<td>30 sec.</td>
<td>7 min.</td>
<td></td>
</tr>
</tbody>
</table>
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 $1 \times 10^3$ 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 $1.7 \times 10^8$ 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 pou-

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

<table>
<thead>
<tr>
<th>Antibiotic disc (Code)</th>
<th>Disc content/ µg</th>
<th>Interpretation (Diameter of the zone/ mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Susceptible ≥</td>
</tr>
<tr>
<td>Amoxycillin/Clavulanic acid (AMC)</td>
<td>20/10</td>
<td>18</td>
</tr>
<tr>
<td>Neomycin (NE)</td>
<td>10</td>
<td>17</td>
</tr>
<tr>
<td>Doxycycline (Do)</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>Erythromycin (E)</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Nalidixic acid (NA)</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>Penicillin (P)</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>Ciprofloxacin (CIP)</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td>Gentamycin (CN)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Levofloxacin (LEV)</td>
<td>5</td>
<td>17</td>
</tr>
<tr>
<td>Ceftriaxone (CES)</td>
<td>30</td>
<td>28</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Age of chicken/Week</th>
<th>Intestine</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>No. of positive</td>
</tr>
<tr>
<td>1-2</td>
<td>62</td>
<td>2</td>
</tr>
<tr>
<td>2-3</td>
<td>56</td>
<td>15</td>
</tr>
<tr>
<td>3-4</td>
<td>45</td>
<td>13</td>
</tr>
<tr>
<td>4-8</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>202</td>
<td>41</td>
</tr>
</tbody>
</table>
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 Mustafa (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*
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, El-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.
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
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 amoxicillin/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*. Algamal 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 (Algamal 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 typhilitis) (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 periodic 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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