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Clostridium perfringens in Broiler Chickens: Isolation, Identification, Typing, and Antimicrobial Susceptibility

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ABSTRACT

Necrotic enteritis (NE) is a common worldwide poultry disease caused by the bacterium of *Clostridium perfringens* (*C. perfringens*) which has significant economic losses in the poultry industry as well as the cost of treatment and preventive measures. The current study was conducted to evaluate the incidence of NetB toxin positive in *C. perfringens* on different farms in Egypt. In the years 2020 and 2021, on industrial broiler farms (15-45 days- old), 100 intestinal samples were collected consisting of 30 healthy Ross broiler chickens and 70 unhealthy Ross broiler chickens. Culture and biochemical characterization (Catalase, urease, sugar fermentation, gelatin liquefaction, nitrate reduction, and lecithinase reaction tests) confirmed that *C. perfringens* was isolated at a rate of 10% (3/30) from apparently healthy broiler chickens and 40% from unhealthy broiler chickens. Thirty-one isolates were tested for toxigenicity and typing by ELISA kits and the results showed that 80% of the isolates from unhealthy broiler chickens were *C perfringens* type A alpha-toxin (toxigenic), 20% were non-toxigenic, and 66.7% isolates from apparently normal broiler chickens were toxigenic. The same thirty-one (44%) *C. perfringens* isolates were detected by PCR to investigate the presence of the NetB toxin gene in apparently healthy and unhealthy broilers and subsequently detect the role of NetB toxin in inducing NE. Of the samples, 82% of the isolates from unhealthy chicks were found to incode NetB gene, while none of the isolates from healthy broiler chickens had NetB. *Clostridium perfringens* showed sensitivity to amoxicillin, amoxicillin with clavulanic acid and ampiclox, intermediate for ofloxacin, and high resistance to cephalexin, streptomycin, colistin sulfate, erythromycin, sulfa trimethoprim, gentamycin, and oxytetracycline. The present study revealed the importance of NetB gen in the appearance of clinical signs of NE in broiler chickens.

Keywords: Alpha toxin, Antibiotic sensitivity test, *Clostridium perfringens*, Necrotic enteritis, NetB

INTRODUCTION

Necrotic enteritis (NE), as a major global threat, is an enteric disease caused by *Clostridium perfringens* (*C.perfrengens*) type A and C, contributing to economic losses (Jesudhasan et al., 2021). Several experimental studies investigate the induction of NE occurred mainly by *C. perfringens* which is Net B positive rather than alpha-toxin (Keyburn et al., 2013). NetB is a crucial virulence determinant in a *C. perfringens* strain isolated from NE. The toxin shared only 38% amino acid sequence identity with two other pore-forming toxins, beta-toxin, and *Staphylococcus aureus alpha-toxin* (Mohiuddin et al., 2021).

Clostridium perfringens is a Gram-positive, anaerobic bacilli bacterium, spore-forming found in nature as a normal flora in animals and humans' gastrointestinal tracts (Mora et al., 2020). Alpha, beta, epsilon, and iota are four typing of *C. perfringens* from A to G based on the ability to produce toxins (Sarmah et al., 2021). More than 15 toxins are produced by *C. perfringens*, including collagenase (κ-toxin), *Clostridium perfringens* enterotoxin

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(CPE), bacteriocin adhesins, proteolytic enzymes, collagenolytic enzymes, and tpel (Uzal et al., 2014). The largest class of bacterial protein toxins is pore-forming toxins, which include perfringolysin O, NetB toxin, beta2 toxin, and enterotoxin (Lee and Lillehoj, 2022). Pore-forming toxins are a common mechanism of cell death as it generates pores to access the enterocytes (Lee and Lillehoj, 2022).

Clinical NE is distinguished in poultry by a significant increase in mortality without warning signs, whereas subclinical NE is distinguished as depressed and having diarrhea with low performance, high feed conversion ratio (FCR), and poor weight gain (Broom, 2017; Tsiouris, 2016). Diarrhea is sometimes associated with acute NE, but not always, although water-to-food ratios may be increased (Calefi et al., 2014). Gross lesions in small intestines were ballooned, friable, and contained brown blood-tinged fluid with a foul odor. Furthermore, the mucosa of infected poultry is covered by a tan-to-yellow pseudo-membrane resembling a "Turkish towel" (Hofacre et al., 2018).

These bacteria require predisposing factors as the most well-known risk infectious factor is coccidiosis which induces mucosal damage to the gut epithelium (Moore, 2016), facilitating *C. perfringens* colonization and proliferation (Kaldhusdal et al., 2021). Concurrently, the damage can cause ruptured epithelial cells to release plasma proteins into the lumen of the gut, which serves as a rich nutrient as it includes more than 11 amino acids that represent growth factors and vitamins for *C. perfringens*. (Mora et al., 2020). Moreover, *Eimeria* infection causes a mucogenic response in the host, resulting in the production of mucous, leading to *C. perfringens* growth (Moore, 2016). In addition to that, poultry infection by coccidia may cause immunological stress, making them more susceptible to *C. perfringens* infection (Boulianne et al., 2020).

Furthermore, as a nutritional factor, the diet was rich in indigestible, water-soluble non-starch polysaccharides (NSP), including rye, wheat, and barley, known to increase intestinal viscosity (Boulianne et al., 2020). Indigestible water-soluble non-starch polysaccharides could also leave undigested nutrients available for microbial proliferation and engage with glycoproteins on the intestinal epithelium to increase mucin production, thus also encouraging *C. perfringens* overgrowth. High protein of animal sources diets, particularly those based on fishmeal, provide an abundance of nutrients, including specific amino acids that *C. perfringens* cannot synthesize, causing an increase in bacterial growth (Yang et al., 2019). Moreover, gizzerozine, a biogenic amine observed in fishmeal, has been linked to broiler chicken alimentary tract erosion (Wu et al., 2014). In addition, adding fishmeal to the diet has an adverse effect as it destabilizes and alters the underlying gut microbial population, which predisposes to NE in broiler chickens (Moore, 2016).

Furthermore, management factors, such as acute diet changes, high-density broiler chickens housing, and extreme environmental temperatures, are also important risk factors for NE (Antonissen et al., 2014).

According to new research on the NetB toxin, immune responses to the toxin can provide some protection against NE (Prescott et al., 2016). NetB toxin is fully accountable for necrotizing tissues, causing perforations in epithelial cell membranes, destruction, and bowel leakage (Adhikari et al., 2020). The present study was conducted to investigate whether NetB is an essential factor for inducing NE in broiler chickens.

MATERIALS AND METHODS

Ethical approval

The animal use protocol in this study was approved by the Institutional Animal Care and Use Committee (022-374).

Sampling methods

From the broiler chicken farms of Egypt that are reared in a deep litter system for meat production, and had a high mortality rate of above 15%, at the age of 2-4 weeks, a hundred intestinal samples were collected (70 from unhealthy Ross broiler chickens, and 30 from apparently healthy Ross broiler chickens). Broiler chickens were clinically examined for observation of clinical signs and gross lesions related to NE under the supervision of the farms` veterinarians. Unhealthy broiler chickens suffered from depression, diarrhea, and a low growth rate. Samples were collected after a post-mortem examination of the intestine and taken by sterilized forceps. Samples were transported to the laboratory in an ice box immediately.

Isolation and identification of *Clostridium perfringens*

Samples were inoculated in cooked meat media and incubated in a Gas pack anaerobic gar at 37°C with anaerobe kits (Oxoid, India) for 24 hours. The cultures were then cultivated onto 10% sheep blood agar supplemented with 200 µg/ml neomycin sulfate and then incubated anaerobically at 37°C for 24 hours. Colonies

with a transparent double zone of hemolysis were presumptive and identified by biochemical methods. Catalase, urease, sugar fermentation, gelatin liquefaction, nitrate reduction, and lecithinase reaction tests were used (Rana et al., 2023).

Typing of *Clostridium perfringens*

Sandwich the enzyme-linked immunosorbent assay (ELISA) kits (Bio-X Diagnostics, Belgium) were used to detect *C. perfringens* typing (Alpha, Beta, and Epsilon toxins) in 31 isolates from culture supernatants (28 isolates from dead broiler chickens and 3 from apparently heavy ones) according to the manufacturer's instructions as a new alternative method for detection typing and toxinogenicity of *C. perfringens*. Specific monoclonal and polyclonal antibodies against *C. perfringens* (Alpha, Beta, and Epsilon toxins), as well as a monoclonal antibody specific for a structural protein of this bacterium, were immobilized. These antibodies could capture specific toxins or bacteria that may be present in the sample culture.

Detection of NetB

Bacterial DNA was extracted from bacterial cultures overnight using the QIAamp DNA extraction Mini Kit (Indian) according to the manufacturer's instructions. Oligonucleotide primers (F: GCTGGTGCTGGAATAAATGC and R: TCGCCATTGAGTAGTTTCCC) (Metabion AG, Germany) targeting the NetB gene of CP were used. The PCR of 20 µl consisted of 10 µl of 2X PCR Mix (Thermo Scientific™, USA.), 1 µl of each forward and reverse primer, 5 µl of template DNA, and 3 µl of PCR grade water. The first denaturation at 95°C for 5 minuts was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. Amplicons (383bp) were separated on 1.5 percent agarose gel (Sigma, USA) and photographed using an ultraviolet (UV) Tran illuminator (Keyburn et al., 2010).

Antimicrobial susceptibility testing

The disc diffusion technique was used on Muller Hinton agar plate to test commercially prepared, fixed concentration paper antibiotic disc (Ampiclox, amoxicillin with clavulanic acid, amoxicillin, and tetracycline, streptomycin, colistin sulfate, chloramphenicol, sulfa-trimethoprim, gentamycin, ofloxacin, and oxytetracycline Oxoid Corporation, UK). Plates were incubated anaerobically at 37°C in a Gas Park anaerobic jar for 24 hours (Algammal and Elfeil, 2015). The diameter of the zone is closely linked to the sensitivity of the isolate and the rate of drug diffusion through the agar medium, and the zone of the diameter of each drug is interpreted using the clinical laboratory standard NCCLS criteria (Reller et al., 2009) or those included in the United State Food and Drug Administration.

RESULTS

Isolation and identification of *Clostridium perfringens*

Microbiological and molecular methods were used to screen 100 broiler chickens (70 unhealthy broiler chickens and 30 randomly selected from a healthy flock of broilers). Isolation of *C. perfringens* on blood agar forms a double zone of hemolysis (Figure 1).

Figure 1. Cultivation of *Clostridium perfringens* on blood agar

Prevalence of *Clostridium perfringens*

An investigation of 100 intestinal samples (30 from healthy broiler chickens and 70 from freshly dead broiler chickens suffered from severe dehydration, dilatation of the small intestine, and necrosis showed that the prevalence of *C. perfringens* was 10% (3/30) in extremely healthy broiler chicken and 40% (28/70) in dead cases according to culture and biochemical tests as in Table 1.

Typing of *Clostridium perfringens*

According to culture and biochemical identification of *C. perfringens*, 31 isolates were typed by sandwich ELISA kits (28 isolates were from dead broiler chickens and 3 from apparently healthy ones). It was revealed that 22 isolates were positive for *C. perfringens* type A alpha-toxin from dead broiler chickens, while 2 healthy broiler chickens were positive for alpha toxin (Table 2).

Detection of NetB toxin

NetB gene 82% (23/28) isolates from unhealthy broiler chicken had been NetB positive and 3 apparently normal isolates were NetB negative, as shown in figures 2 and 3, respectively, and Table 3. Typing of 31 *C. perfringens* isolates using ELISA test revealed that 80% of isolates from unhealthy broiler chicken were type A (positive for the alpha toxin). Moreover, 66.7% of healthy chickens were positive for alpha toxin type A.

Table 1. Incidence of *Clostridium perfringens* in healthy and unhealthy broiler chickens in Egypt

Groups of broiler chicken	Incidence (Number)	Percent (%)
Unhealthy (70)	28	40
Healthy (30)	3	10
Total (100)	31	31

Table 2. Typing of *Clostridium perfringens* according to the collected samples from the small intestines of broiler chickens in Egypt

Number of *Clostridium perfringens* isolates	ELISA	
	Alpha positive	Percentage (%)
Unhealthy broiler chicken (28)	22	80
Healthy broiler chicken (3)	2	66.7

Table 3. Detection of NetB from broiler chickens in Egypt

Groups	NetB gene	
	Number	Percentage (%)
Unhealthy broiler chicken (28)	23	82
Healthy broiler chicken (3)	0	0

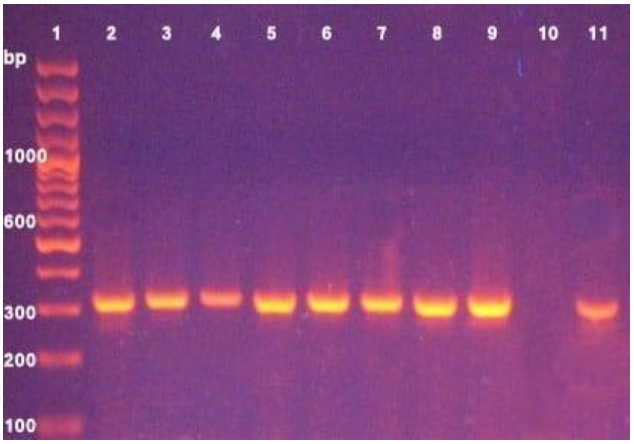

Figure 2. Detection of NetB from unhealthy broiler chickens in Egypt using PCR. Lane 1: 100bp pair DNA ladder, Lane 2 to 9 isolates are *C. perfringens* NetB-positive, Lane 10: control negative, Lane 11: control positive.

Figure 3. Detection of NetB from healthy broiler chickens in Egypt using PCR. Lane 1 to 3 isolates from apparently healthy broiler chicken are *C. perfringens* NetB- negative.

Antimicrobial susceptibility testing

Clostridium perfringens showed sensitivity at 100% to amoxicillin, amoxicillin with clavulanic acid and ampiclox, intermediate at 100% for ofloxacin, and high resistance to cephalexin (82%), streptomycin (100%), colistin sulfate (100%), erythromycin at (100%), sulfa trimethoprim (100%), gentamycin (100%), and oxytetracycline (89%; Oxoid, UK) according to the diameter of zone of inhibition as shown in Table 4.

Table 4. Antibiogram profile of the *Clostridium perfringens* isolates

isolates	Antibiotics and concentarion										
	AML 25 µg	AMC 30 µg	AX 30 µg	OFX 5 µg	CL 30 µg	S 10 µg	CT 10 µg	E 15 µg	SXT 25 µg	CN 30µg	OT 30µg
CP.EGY4	S	S	S	I	S	R	R	R	R	R	R
CP.EGY5	S	S	S	I	S	R	R	R	R	R	I
CP.EGY9	S	S	S	I	I	R	R	R	R	R	R
CP.EGY12	S	S	S	I	R	R	R	R	R	R	R
CP.EGY18	S	S	S	I	R	R	R	R	R	R	R
CP.EGY22	S	S	S	I	R	R	R	R	R	R	R
CP.EGY24	S	S	S	I	R	R	R	R	R	R	R
CP.EGY27	S	S	S	I	R	R	R	R	R	R	R
CP.EGY29	S	S	S	I	R	R	R	R	R	R	I
CP.EGY31	S	S	S	I	R	R	R	R	R	R	R
CP.EGY34	S	S	S	I	R	R	R	R	R	R	R
CP.EGY35	S	S	S	I	R	R	R	R	R	R	R
CP.EGY53	S	S	S	I	R	R	R	R	R	R	R
CP.EGY57	S	S	S	I	R	R	R	R	R	R	R
CP.EGY62	S	S	S	I	R	R	R	R	R	R	R
CP.EGY64	S	S	S	I	R	R	R	R	R	R	R
CP.EGY69	S	S	S	I	R	R	R	R	R	R	R

AML: Amoxicillin, AMC: Amoxicillin with clavulanic acid, AX: Ampiclox, OFX: Ofloxacin, CL: Cephalexin, S: Streptomycin, CT: Colistin sulfate, E: Erythromycin, CN: Erythromycin Gentamycin, OT: Ox tetracycline, SXT: Sulfa trimethoprim. S: Sensitive, I: Intermediate, R: Resistance

DISCUSSION

Necrotic enteritis is an acute enterotoxemia affecting poultry caused by *C. perfringens* and characterized by severe depression followed quickly by an asudden increase in flock mortality. Unhealthy broiler chickens showed ruffled feathers and diarrhea before death. The gross lesions were found in the small intestine, which was ballooned, friable, and contained brown fluid. Furthermore, the mortality rate may reach 50%. Presumptive diagnosis in the current study is based on gross lesions, culture characters, microscopic examination, and biochemical tests. More importantly, *C. perfringens* is responsible for subclinical infections associated with chronic intestinal mucosa damage, resulting in reduced weight gain and low-performance and consequently significant economic losses (Hofacre et al., 2018). The present study confirmed the importance of NetB as a major virulence factor in the appearance of symptoms in NE as the rate of isolation of *C. perfringens* was higher in unhealthy broiler chickens than that of healthy ones (40 percent and 10%, respectively), which agrees with Rizk et al. (2020), indicating a high occurrence 70% of *C. perfringens* in unhealthy broiler chickens of NE but a lower incidence in healthy broiler chickens (22%).

In the present study, detection of the NetB gene using PCR revealed that out of 28 isolates of unhealthy ones, 23 were found to encode the NetB toxin gene 82% meanwhile. None of the isolates from healthy broiler chickens had the NetB toxin gene. These may reflect the importance of the presence of NetB toxin in inducing NE, which may agree with previous studies (Keyburn et al., 2013; Keyburn et al., 2010), indicating that 70% (31/44) of *C. perfringens* isolates from poultry affected by NE had been positive for the NetB gene, suggested that NetB toxin is important in inducting NE. The NetB gene was found in the majority of necrotic enteritis-infected broiler chickens but not in healthy broiler chickens. In the same vein, Mwangi et al. (2019) detected NetB toxin in 81% of unhealthy broiler chickens. However, their finding for healthy broiler chickens where NetB toxin was detected (68%) was too high and did not reflect the role of NetB. The isolation of NetB positive from normal healthy broiler chickens does not change the fact that these isolates are highly pathogenic (Smyth and Martin, 2010). As healthy broiler chickens get a diverse *C. perfringens* population, there may be a limited number of NetB-positive isolates (Abildgaard et al., 2010). However, another study confirmed that *C. perfringens* NetB negative was detected in NE in broiler chickens (Chalmers et al., 2008). These findings could imply that NE is a multifactorial disease (Williams, 2005). Coccidiosis mucosal damage, poor sanitation, unbalanced nutrition, and poor housing are all stressors that contribute to the rapid growth of *C. perfringens* and the massive production of toxins (Jia et al., 2009). This suggests that these virulent strains may require additional predisposing conditions to proliferate to the point where they can cause NE.

The sensitivity of *C. perfringens* strains to penicillin, amoxicillin, and amoxicillin with clavulanic acid are 100%. These findings agree with Gharaibeh et al. (2010) as the combination of amoxicillin and clavulanic acid was effective against *C. perfringens* strains. On the other hand, the isolates were resistant at 100% to streptomycin. Similarly, Silva et al. (2009) found the resistance to streptomycin could be due to the absence of quinones in *C. perfringens*. In addition, the resistance of isolates to colistin sulfate and gentamycin was 100% except for oxytetracycline at 89% which agrees with Park et al. (2010), reporting presence of the tetP gene as the primary cause for resistance to oxytetracycline. Moreover, the resistance of isolates to sulfa-trimethoprim was 100%, and cephalexin was 82%. These results were almost identical to those obtained by Llanco et al. (2012), who detected the resistance to sulfa-trimethoprim and cephalexin. Gad et al. (2011) found that most *C. perfringens* strains in turkey flocks were sensitive to Sulfa trimethoprim, and the tested isolates were resistant to erythromycin (macroloide group). According to Anju et al. (2021), the resistance is due to the presence of the *ermQ* and *ermB* genes, which code for the production of enzymes for the di methylation of the 23S rRNA. This results in the inhibition of antibacterial drug action on bacteria, which is primarily responsible for *C. perfringens* resistance to the Macrolides group (erythromycin). Johansson et al. (2004) reported the isolates of *C. perfringens* were susceptible to erythromycin, meaning that a pattern of increased resistance against antimicrobial agents is commonly used in the control and treatment of NE.

CONCLUSION

Type A is the most predominant type in inducing NE in poultry as NetB is essential, especially with alpha toxins. It may act as a synergistic factor in inducing the NE in poultry. It is recommended to vaccinate broiler chickens with *C. perfringens* strain positive for NetB toxin to protect flocks from NE.

DECLARATIONS

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Authors' contributions

Nashwa Mohamed. Eid contributed to the conceptualization, methodology, data curation, formal analysis, visualization, writing, original draft preparation, review, and editing of the manuscript. Eman Fathy. Ahmed is responsible for data curation, formal analysis, visualization, writing, review, and editing of the manuscript. Salama Abohamra Shany contributed to data curation, formal analysis, visualization, writing, review, and editing of the manuscript. Al-Hussien Momamed Dahshan contributed by writing, reviewing, and editing the manuscript. Ahmed Ali Ahmed contributed through conceptualization, methodology, data curation, formal analysis, visualization, writing, original draft preparation, review and editing of the manuscript, and supervision of the present study. All authors have read and approved the final version of the manuscript for publishing in the present journal.

Competing interests

The authors have no competing interests.

Ethical consideration

All relevant ethical issues have been checked by all the authors.

Availability of data and materials

The authors confirm that the data showing the findings of this study are available within the article.

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